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Original Research

The capacity of some newly bacteria and fungi for biodegradation of herbicide trifluralin under agiated culture media

G. O. Erguven[1,2*], H. Bayhan[2], B. Ikizoglu[2], G. Kanat[2], Y. Nuhoglu[2]

[1] Tunceli University, Faculty of Engineering, Department of Environmental Engineering, 62100, Tunceli, Turkey
[2] Yildiz Technical University, Faculty of Civil Engineering, Department of Environmental Engineering, 34220, Esenler, Istanbul, Turkey

Abstract: Bioremediation is the use of microorganisms to degrade environmental contaminants (pesticides, polyaromatic hydrocarbons etc.) into less toxic forms or compounds. In this study microbial biodegradation of trifluralin was performed in liquid media with 11 different types of identified fungi and bacteria cultures and their mixtures in agiated culture media. The isolated fungi and bacteria mixtures showed the highest degradation, reaching 93% in the chemical oxygen demand (COD) parameter in four days and 82% as trifluralin active ingredient in five days. Bacteria and fungi mixtures achieved 69% and 66% degradations of trifluralin active ingredient respectively. In the fungi studies, the best removal was achieved by *M.Chlamydosporia* at 80%, in the bacteria studies, the best removal was achieved by *Bacillus simplex about* 95% in five days. These different removal rates were due to the microbial differencies.

Key words: Bioremediation, bacteria, fungi, trifluralin, removal efficiency.

Introduction

In Turkey, the average consumption of pesticides is between about 33 000-55 000 tons/year. This amount constitute 47% of the insecticides, 24% of herbicides, 16% fungicides and 13% of the other pest groups. Turkey has about 4100 licensed pesticides and the 420 effective ingredients. The 49 of pesticides has been halted in Turkey (1).

Approximately 600 hectares of sunflowers are planted in Turkey (2). Herbicides are used intensively in Turkish sunflower agriculture, with trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine) being used on almost all sunflower fields in Turkey since the 1970s (3). Trifularin keeps the crops free from weeds in the early stages by controlling annual grass weeds and certain small-seeded broad-leaved weeds. The average half-life of trifluralin in the field is 45 days in most soils, and less than 10% of applied trifluralin remains one year after application (4). A study of Trifluralin degradation in various types of agricultural soil under aerobic and anaerobic conditions found that breakdown was highly dependent on soil type. In a soil used for the disposal of various pesticides (Diuron, Trifluralin, Carbofuran), only trifluralin could still be detected after many years (5)

Microbial degradation is an important mechanism controlling the fate of pesticides in soils, and is generally considered to be desirable both from an environmental as well agricultural perspective. Pesticides in agriculture acquired great importance in the past due to their pest control features, but now the focus is on their potential impact to human health and the environment. Degradation studies in soil are essential an evaluation of the persistence of pesticides and their breakdown components. Data on the rate of pesticide degradation is extremely important, in that they allow the potential risk associated with exposure to be predicted.

Trifluralin has been used for many years to remove the weeds that have an adverse effect on sunflower growth. In general, the degradation of pesticides is facilitated by both biotic and abiotic factors, including chemical, sunlight and microbial agents. Among these factors, biodegradation is the most commonly used method for converting synthetic chemicals into inorganic products (6).

There are only a few studies into the degradation of trifluralin using mixed cultures of bacteria and fungi, the majority of which identified low-efficiency degradation, and identified that the degradation of metabolites is more persistent and toxic than that of the parent compound (7). The bioremediation of trifluralin is currently receiving much attention as an alternative to the existing methods of removing trifluralin from the environment (7). Many microorganisms which are able to degrade trifluralin, including both bacteria and fungi, have been isolated from different sources (8, 9). In this study, bacteria and fungi which have been isolated from an agricultural area with no previous exposure to trifluralin were used to measure microbial degradation. Soil samples were also collected from the sunflower field with a known history of extensive pesticide usage, located in the city of Kirklareli in Turkey, serving as a source of pesticide degrading microbes. In the experiments, five species of bacteria and six species of fungus were isolated from the soil samples using different media plates, and trifluralin degradation was investigated under agiated culture conditions.

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*** Corresponding author:** G.O. Erguven, Tunceli University, Faculty of Engineering, Department of Environmental Engineering, 62100, Tunceli, Turkey. Email: gokhanondererguven@gmail.com

Materials and Methods

Chemicals and reagents

The trifluralin herbicide active ingredient, sold under the trade name "Tefralin", was supplied by an agricultural products shop. pH of Tefralin was 6.5 and temperature vas 25^0C. This herbicide contains 480 gr L^{-1} of trifluralin. Trifluralin standard was supplied by Sigma Aldrich as a yellow powder with 99.9% purity from Dr. Ehrenstorfer GmbH Co. Trifluralin standard was dissolved in GC-grade methanol used for calibration of GC. All media for the isolation and enrichment of bacteria and fungi were obtained from Sigma Aldrich. Acetone and hexane were obtained from Merck company, Germany. All used chemicals were of GC grade. Analytical standards for GC calibration in the range of 0.1-100 mg/l were prepared from methanol of the working standard solution.

Instruments

The quantification of trifluralin was carried out using a Perkin Elmer Clarus 500 gas chromatograph, and a capillary column of HP-5MS, 30 m x 0.25 mm x 0.25 µm, was used in the equipment. Trifluralin was detected using an electron capture detector (ECD), and the oven temperature program used during the analysis was 70°C (2 min), 25°C/min to 145°C, 3°C/min to 190°C, and finally 5 min hold at 190°C. The total oven program was 25 minutes. The inlet temperature was kept at 250°C and the detector temperature at 320°C. The carrier gas was ultrapure helium (He) at 1.2 ml/min flow, and the make-up gas was ultrapure nitrogen at 30 ml/min flow. For the calibration process, five standards were prepared with concentrations of between 1 and 50 ng/µl. After each 10 sample injections, stability was checked with the medium standard. The average determination coefficient value of the calibration curve (R^2) was 0.999.

All samples were spiked with surrogate and internal standards in order to determine the recovery efficiency, with tetrachloro-m-xylene (TMCX) used as the surrogate standard. The surrogate standard was spiked to the sample prior to extraction. Quintozene was used as the internal standard, and was spiked just before capping the chromatography vials. Average recovery efficiency was 86 %. The limit of detection (LOD) values were calculated for each congener as average blank concentrations plus three times the standard deviations. Any sample concentrations falling below the LOD value were ignored. Blank samples were corrected for each set of analysis, and all results were blank corrected.

Soil sample collection

Experiments conducted on soil samples obtained from farming areas around the city of Kirklareli and the majority of farms selected from the area have been cultivating sunflower and wheat for several years. Soil with no background of trifluralin concentration was collected from an organic farm. All samples were collected from the top 0–20 cm of soil following the standard procedure and stored in glass vessels at an ambient temperature (10). The soil samples taken before application of trifluralin were analysed in Thrace Agricultural Research Institute. Properties of the soil samples that were used for laboratory studies and isolation of bacteria and fungi are listed in Table 1.

Table 1. Characteristics of soil samples used for isolation of bacteria and fungi (11).

Parameter	Value
Depth, cm	0-20
pH in water	6.5
Organic carbon, %	2.1
Clay, %	62
Sand, %	32
Silt, %	5
Moisture Content, %	21

Media preparation

Plate count agar, dextrose casein peptone agar, potato dextrose agar, dichloran rose bengal chlorinated agar, sabouraud dextrose agar, malt extract and sabouraud dextrose broth media were prepared according to manufacturer's instructions (Sigma Aldrich-USA) and were autoclaved at 121^0C for 15 min to ensure a sterilized solution. After cooling, diluted agricultural soil (containing no trace of trifluralin) in an isotonic solution was added to petri dishes. The medium pH was adjusted to 6.5 and temperature was 20^0C.

Isolation and enrichment of bacteria and fungi

Bacteria and fungi were isolated from the soil samples using serial dilution on different media plates. Following the inoculation, the petri dishes (3 replicates each of them) were placed in incubators at 20ºC for three days (bacteria) and for five days (fungi). Other isolation studies were also done at 4^0C and 35^0C, but best growing seen at 20^0C. After growing, the plates were screened for any colonies that were visually different from the others. The colonies were selected at random and denoted B1 to B5 for bacteria and F1 to F6 for fungi. After incubation, the cultures were placed carefully in an enrichment media for seven days to grow with the same temperature of taken soil samples before application of trifluralin at 20^0C, and were shaken continuously.

Fungi-bacteria molecular characterization studies

Molecular characterization studies were implemented according to the Wizard Genomic DNA Purification Kit. For fungi; "Isolating Genomic DNA from Yeast", for bacteria, "Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria" methods used (12).

Fungi Studies

The fungi marked on the petri dishes were shown in PDA petri dishes by streak plate to ensure the reproduction from sport fungi. The fungi that were grown at room temperature and from asingle colony isolation were transferred to other PDA petri dishes and were kept at room temperature until they reached the appropriate size for DNA isolation. Growing fungi were scratched using a sterile blade and crushed with liquid nitrogen in sterile conditions, after which, DNA was isolated from the powder hypes.

An ordinary Taq polymerase was conducted for PCR using many combinations of ITS (Internal transcribed

spacer) region primers, which are often used in the definition of DNA. The PCR conditions were:
Final concentrations (total 25 µL reaction volumes):1X Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.4 µM forward primer, 0.4 µM reverse primer, 0.5 mM dNTP, 1U Taq polymerase (F1, F4, and F6) or 1.25 U Taq polymerase (F2, F3, and F5) and 200ng DNA.
Heat cycle conditions:1 cycle: 94^0C -3 min, 35 cycles: 94^0C - 15 s, 55^0C - 30 s, 72^0C - 30 s, 1 cycle: 72^0C 1 - 5 min.

In PCR, the expected length of bands were obtained only for F1 (*Penicillium thrichoderma*), F4 (*Metacordyceps chlamydosporia*) and F6 (*Alternia alternata*). For the other fungi, F2 (*Penicillium simplicissimum*), F3 (*Penicillium Talaromyces*) and F5 (*Stachybotrys chartarum*), One-Taq polymerase was used. The three primers designed by Avcioglu-Dundar (13) gave two results. These tapes, which were cut from the agarose gel and cleaned (in the case of multiple bands) or as single band, PCR reaction were sent directly to the sequence analysis. A Thermo-Scientific GeneJET Gel Extraction Kit was used in the cleaning of the bands cut from the agarose gel. In cases of a sequence reaction on the bands (cut from the agarose gel) not performing well, re-amplification was made (by One Taq polymerase).

Bacterial Studies

Phire Hot Start II DNA Polymerase was used for PCR, given that it allows making no DNA isolation. Then, longer PCR bands of various lengths (1000–3000 bp) were obtained through bacterial 16S ribosomal general primers. The pipette instructions and cycling protocols were:
Final concentrations (total 20µL reaction volume): 1X Phire Animal Tissue PCR Buffer (includes dNTPs and $MgCl_2$), 0.5µM forward primer, 0.5µM reverse primer, Phire Hot Start II DNA polimeraz and H2O.
Heat cycle conditions: 1 cycle: 98^oC – 5 min, 40 cycles: 98^oC – 5 s, 72^oC – 20 s, 1 cycle 72^oC – 4 min, 4^oC-∞
Bacteria isolated and denoted from B1 to B5 were identified using 16sRNA Universal Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8–27) (12). 16S rRNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8–27) (14), 1492R 5' TACGGYTACCTTGTTACGACTT 3' positions 1492–1512) (15, 16).

Microbial biodegradation studies

In order to assess the degradation ratio of the pesticide from the fungi and bacteria, 14 different aerobic consortia (1ml each of them) were developed through the enrichment technique.

Studies in liquid media

In the liquid media study, 1 ml of tefralin 480 (including 0,48gr of Trifluralin active ingredient) and 1 ml of enriched cultures (approximately 2 x 10^7 CFU/ml) were added to 98 ml of 0.8 % isotonic saline water. The trifluralin was prepared from tefralin in the same concentration as used in the field (200ml/1000m^2). The growing media used in the experiments were the previously isolated and the enriched bacteria and fungi mixtures, with 1 ml of the solutions obtained from mixtures of all kinds or from the separately enriched solution (only fungus or bacterium) used in the experiments.

In this phase, solution samples were monitored at 24-hour intervals on the basis of the chemical oxygen demand (COD) parameter with the standard method 5220C Closed reflux titrimetric method (17). According to this method, 1.5 ml of standard potassium dichromate digestion solution ($K_2Cr_2O_7$) and 3.5 ml of 0.0176M Ag_2SO_4 solution were added to sample of 2.5 ml volume. After that, these samples heated at Velp WTW CR3200 thermoreactor for 2 hours at 150^0C. After cooling, samples were taken to flasks and 2-3 drops of Ferroin indicator ($FeSO_4.7H_2O$) added to samples. Then, samples were titrated with 0.25M standard ferrous ammonium sulfate (FAS) titrant and COD results were calculated. The solution was used in separatory funnel liquid-liquid extraction experiments to measure the removal efficiency of the active ingredient. Following the EPA 3510C Seperatory Funnel Liquid-Liquid extraction method (18), 100 ml of sample (includes 98 ml of 0.08% NaCl and 1 ml of enriched culture solution and 1 ml of herbicide) was used in the experiments. The extract was analyzed for the target analyses using the EPA 8081B method for the determination of organochlorine herbicides through gas chromatography (19).

Identified fungi and bacteria species used in the study

The species of fungi obtained according to the results of primers, sequence and references used to identify the fungi are given in Table 2. The identified bacterial codes and their species are given in Table 3.

Table 2. Primers, sequence and references used to identify the fungi (20).

Fungi Code and Approximate species identity	First Primer 5'-3' sequence and reference	Second Primer 5'-3'sequance and reference
Penicillium thrichoderma (F1)	ITS3 GCATCGATGAAGAACGCAGC (21)	ITS ATCCCTACCTGATCCGAGGTC (13)
Penicillium simplicissimum(F2)	ITS6 GAAGGTGAAGTCGTAACAAGG (22)	ITS ATCCCTACCTGATCCGAGGTC (13)
Penicillium talaromyces (F3)	ITS4 TCCTCCGCTTATTGATATGC (20)	ITS6 GAAGGTGAAGTCGTAACAAGG (22)
Metacordyceps chlamydosporia(F4)	ITSTRfw GAGACCGCCACTGTATTTCG (13)	ITS3 GCATCGATGAAGAACGCAGC (21)
Stachybotrys chartarum (F5)	ITS1 TCCGTAGGTGAACCTGCGG (21)	ITS ATCCCTACCTGATCCGAGGTC (13)
Alternaria alternata (F6)	ITS3 GCATCGATGAAGAACGCAGC (21)	ITS ATCCCTACCTGATCCGAGGTC (13)

ITS: Internal transcribed spacer, TRfw: Thrichoderma forward primer.

Table 3. Identified bacterial codes and their species (20).

Accession Number	Bacterial Code and Approximate Species Identity	Identity	Reference
KF831394.1	*Bacillus simplex* (B1)	99%	(23)
HE646789.1	*Bacillus muralis* (B2)	99%	(24)
KF555623.1	*Micrococcus luteus* (B3)	99%	(25)
KC634108.1	*Micrococcus yunnanensis* (B4)	99%	(26)
HG530135.1	*Clostridium tetani* (B5)	99%	(27)

Results

The reduction of trifluralin as COD with identified fungi and bacteria is given in figure 1 and figure 2, additionally, reduction of TOC and trifluralin active ingredient with mixed consortia is given in figure 3 and figure 4 respectively.

According to the results, it is clear that the best performance was obtained from the mixed cultures. The daily removal efficiency of the trifluralin as COD and active ingredient is given in Figure 4 and 5 respectively. From this figures it was observed that, isolated fungi and bacteria consortia showed the highest degradation of 93% in the COD parameter in five days. The initial COD was about 17,400 mg/l. The bacteria mix and fungi mix achieved 91% and 84% degradation respectively in the COD parameter in five days. The reduction of trifluralin for bacteria mix, fungi mix and bacteria + fungi mix was 69%, 66% and 82% respectively in 5 days. In the TOC studies, removal efficiency were for bacteria mix, fungi mix and bacteria + fungi mix was 90%, 82% and 93% respectively in the same time period.

It was observed that the COD-removal efficiency of the systems varied according to the microbial differences in the liquid medium. The removal efficiency by the bacteria, separately, for *Bacillus simplex* was 95%, *Bacillus muralis, Micrococcus luteus, Micrococcus yunnanensis* were 91% and *Clostridium tetani* species

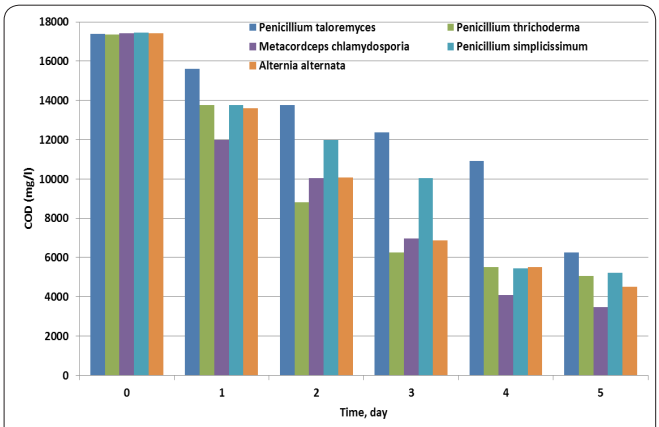

Figure 1. COD reduction of Trifluralin with isolated fungi.

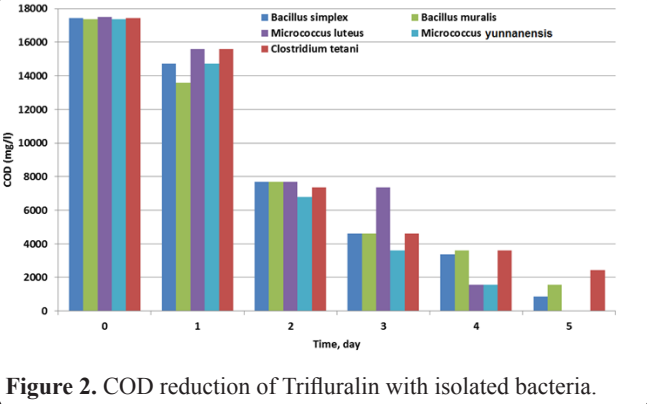

Figure 2. COD reduction of Trifluralin with isolated bacteria.

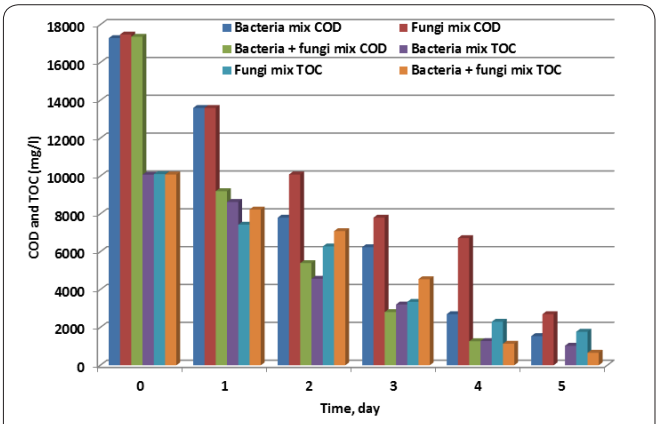

Figure 3. COD and TOC reduction of trifluralin with mixed consortia.

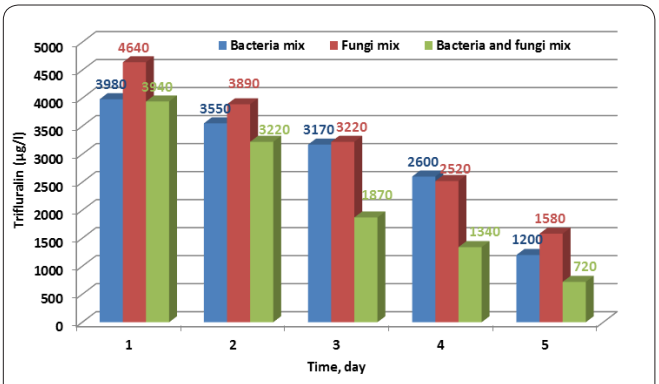

Figure 4. Reduction of trifluralin with mixed consortia.

was 86%. For trifluralin removal experiment, it is reached 69% with bacteria mix. The ratio was 67% for the trifluralin during the same period with mix of fungal culture. The highest reduction seen on bacteria+fungi mix duriing the same time as 82%.

The removal efficiency by the fungi, separately for *Penicillium thrichoderma, Penicillium simplicissimum, Penicillium talaromyces, Metacordyceps chlamydosporia, Stachybotrys chartarum* and *Alternia alternata* species, were 71, 59, 64, 80, 70 and 74% respectively in five days.

Discussion

Bacterial and fungal mixed cultures achieved 93% COD-removal efficiency, and 82% in the trifluralin active ingredient studies. The removal rate for TOC is about 93% with this microbial consortia. As a result, it can be seen that higher removal efficiencies were obtained both for trifluralin active ingredient and COD studies for mixture of bacteria and fungi.

Biodegradation is one of the most viable options for the remediation of pesticides in soil and water, and the results of the experiments show that the biodegradation level can lead to a sufficient removal of pesticides. Biodegradation/bioremediation is a low-cost alternative

that theoretically produces no toxic end products. There have been several researches into microbial degradation, which has been reported as a primary mechanism in pesticide dissipation from soil and water (28, 29).

In biodegradation, generally, three different degradation pathways operate after the application of pesticides, being; chemical e.g. hydrolysis; biological e.g, microorganism activity and physical e.g., photolysis and temperature (30). Transformation resulting from microorganism activity can occur in one of five ways: co-metabolic degradation; detoxification; polymerization; binding to naturally occurring compounds; and finally mineralization. Once pesticides enter the soil, there are several factors affecting their soil behavior (31, 32). The metabolic activities of bacteria, fungi and actinomycetes play a significant role in the degradation of pesticides (33). In our experiments, the biodegradation ratios were sufficiently high, especially for the mixed fungi and bacteria cultures. There have been several studies in literature claiming the ability of many, or at least some, microorganisms to degrade different pesticides efficiently (34, 35, 36).

Previous investigations into the microbial degradation of trifluralin have revealed that relatively few species of bacteria are actually able to degrade this compound, although the degradation of trifluralin by isolated microorganisms *Aspergillus carneus, Fusarium oxysporum* and *Trichoderma viride* was one of the first studies in this regard (37). In another study, bacteria species were isolated from agricultural soil contaminated with trifluralin to decompose the herbicide in a liquid medium (5, 38).

This study has shown that biodegradation ratios of trifluralin were found to be sufficiently high, especially in mixed fungi and bacteria cultures. In the liquid medium, the COD-removal efficiency of the systems varies according to microbial differences. The best removal was achieved with *Micrococcus luteus* and *Micrococcus yunnanensis* in four days, up to 91 %, and by *Bacillus simplex* and *Bacillus muralis* in five days. The results of the experiments showed important implication potential in the development of in-field treatment systems for pesticide-contaminated aquatic environments.

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