REMOVAL RATE OF HERBICIDE ACLONIFEN WITH ISOLATED BACTERIA AND FUNGI

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Abstract. In this research the microbial biodegradation of aclonifen was investigated using liquid and soil experiments with identified cultures and mixed consortia. Isolated fungi and bacteria consortia showed the highest degradation at 93% of the Chemical Oxygen Demand (COD) parameter over five days. Bacteria mix and fungi mix performed 90% and 91% degradation in five days, as COD, while 71% and 91% were active ingredients. For Total Organic Carbon (TOC) experimental results, bacteria mix, fungi mix, and bacteria and fungi mix, showed 86%, 88% and 88% respectively. Soil studies with mixed cultures of bacteria and fungi performed the most efficient degradation, at 97% after five weeks. The degradation of aclonifen by 2 ml mixed cultures showed about 63% of degradation in five weeks and 5 ml of mixed cultures showed about 90% in six weeks.
Keywords: *microbial biodegradation, aclonifen, mixed consortia, chemical oxygen demand, total organic carbon*

Introduction

One of the main factors of environmental pollution is the excessive use of chemicals and pesticides, used on a global scale, to increase production and for the protection of crops. In addition to the main problems with soil, ground water, and surface water pollution, there are also many risks to human health from these chemicals, such as direct poisoning and their residues contaminating drinking water (Nemeth et al., 2002). As a consequence, these environmental problems, particularly regarding the presence and accumulation of pesticides in surface and ground water as well as soil, are becoming increasingly importance with every passing day (Carrizosa et al., 2001).

Studies relating to soil degredation focus on degrading residual pesticides and their components. Degradation amounts of pesticides are becoming critical and their rate of spread is an important environmental risk.

Meanwhile, microbial degradation is not only an important mechanism for controlling pesticides in soils it is also an environmentally friendly method. Temperature and humidity are the main parameters for controlling microbial degradation of pesticides in soils. Pesticide degradation in dry soils is slower than in wet soils (Masutti, 2003).

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Aclonifen is a diphenylether herbicide in which one ring is unsubstituted and the other, NH_2^-, Cl_2^-, NO_2^-, is substituted. This substitution, especially NH_2^-, is a particular feature in the diphenylether herbicidal family. In contrast with other herbicides of the same family (acifluorfen, oxyfluorfen, bifenox), this compound is not only acting through a phytotoxic protoporphyrin IX accumulation but also through an inhibition of carotenoid biosynthesis (Kilinc et al., 2001).

Nitrodiphenyl ether herbicides are potent herbicides. Some metabolites and parent compounds are considered as possible mutagens and endocrine disruptors. Both properties pose serious health and environmental risks (Keum et al., 2001).

Several diphenyl ether degrading strains have been reported, such as *Coriolus versicolor* (Hiratsuka et al., 2001), *Azotobacter chroococcum* (Chakraborty et al., 2002) and *Sphingomonas wittichii* RW1 (Keum et al., 2001). Moreover, as one of the same kind of herbicides, only a few reports on fomesafen degrading strains are available with only two such strains, namely, *Aspergillus niger* S7, having been reported (Li et al., 2009).

The metabolism pathways of the microbial degradation of several diphenyl ethers have been studied. It is reported that the main degradation pathway of oxyfluorfen by *A. chroococcum* was the reduction of nitro group to amino compound, further acetylation of amino derivative, O-dealkylation and dechlorination (Chakraborty et al., 2002). The experiments were performed to study the degradation of chlornitrofen (Kamei and Kondo, 2006), and diphenyl ethers (Federici et al., 2011), using *Phlebia brevispora* and *Lentinus tigrinus*, respectively.

In this study, the microbial degradation of aclonifen was studied using bacteria and fungi isolated from an agricultural area previously unexposed to aclonifen. Soil samples, from a sunflower field with a known history of extensive pesticide usage, located in Kirklareli City, Turkey, were also collected as a source of pesticide degrading microbes. In the experiments, aclonifen degradation was investigated using five species of bacteria and six species of fungus that were isolated from the soil samples, using different media plates.

Biological degradation is the most frequently used method for the remediation of pesticides in soil and water. The results of experiments have shown that levels of biodegradation depend on the removal of pesticide residuals. Biodegradation/bioremediation is a low cost and theoretically alternative process that does not result in toxic final products (Massiha et al., 2011).

Materials and Methods

Chemicals and reagents

An agricultural products shop supplied the aclonifen herbicide under the trade name Chekic 600. This herbicide contains 600 gr L^{-1} of aclonifen active ingredient. Aclonifen standard was supplied as a yellow powder with 99.9% purity from Dr. Ehrenstorfer GmbH Co. All media for the isolation and enrichment of bacteria and fungi were obtained from Sigma Aldrich. Acetone and hexane were obtained from Merck Company. All the chemicals used were of HPLC grade. Analytical standards for calibration were in the range of 0.1-100 mg L^{-1}. Methanol was chosen as the diluting solvent.

Instruments

The quantification of aclonifen was performed by LC-MS-MS (Dionex Ultimate 3000) equipped with a C18 Thermo Accucore column 100mmx2.1mm 2.6 mic. Owen

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temperature of the column was 400°C and Auto Sampler temperature was 50°C . Retention time was 9.0 min. Mass (Thermo Access Max) HESI ion source was 3500 volt and Ion Transfer Tube temperature was 270°C . Sheath and Aux gas were 50 Arb and 15 Arb, and the quantification limit was 15 ppb. Collisure gas pressure was 1.5 mTorr. LOD and LOQ values were 4 ppt and 15 ppt. Ion transmissions were; for primary ion 265.1, second ions 182.1 and 247.9. The quantification (method detection) limit was 0.1-1 mg kg^{-1}. The average value of determination coefficient (R^2) of the calibration curve was 0.999.

All samples were spiked with surrogate and internal standards in order to determine the recovery rate, with tetrachloro-m-xylene (TCMX) 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 rate was 86 percent. The limit of detection (LOD) values was 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

The experiments were conducted on soil samples obtained from agricultural areas in Turgutbey Village, Kirklareli City. The majority of the farms selected in the area have been cultivating sunflowers and wheat for several years. Soil with no background of aclonifen concentration was collected from a field. All the samples were collected from five selected points (*Figure 1*), from the uppermost 0–20 cm of soil following the standard procedure and stored in glass vessels at 4°C temperature in a thermos (Carter and Gregorich, 2006). The soil samples were analyzed at the Trakya Agricultural Research Institute and the results of the analyses are shown in *Table 1*.

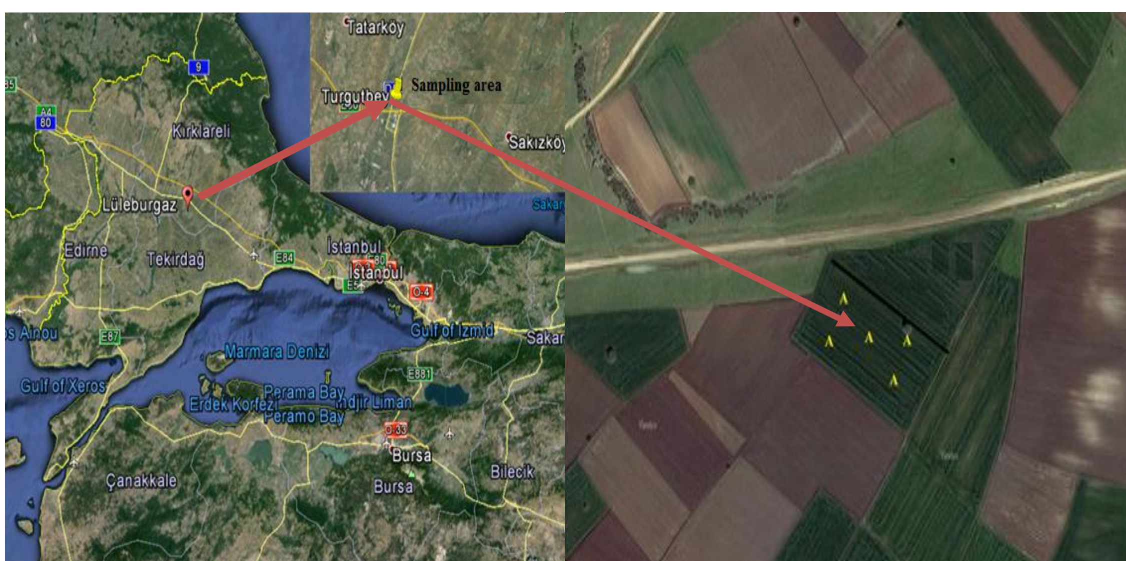

Figure 1. *Turgutbey Village and soil sampling area*

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***Table 1.** Analyses results of the soil sample used for isolation of cultures*

PARAMETER	VALUE
Depth, cm	0-20
pH	6.5
Organic carbon, %	2.1
Clay, %	62
Sand, %	32
Silt, %	5
Moisture Content, %	21

Culture media preparation

The plate count, dextrose casein peptone, potato dextrose, dichloran rose bengal chlorinated, sabouraud dextrose as media agar and malt extract, sabouraud dextrose, were prepared as media broth, and used according to manufacturer's instructions (Sigma Aldrich-USA). After cooling, diluted agricultural soil (containing no trace of aclonifen) in a sterile isotonic solution (0.08% NaCl) was added into petri dishes. The medium pH was adjusted to 6.5.

Isolation and enrichmet of bacteria and fungi

For the isolation of bacteria and fungi, soil samples taken from a depth of 0 to 20 cm before herbicide application were placed in sterile glass jars (Zelles et al., 1991). Approximately 10 g of soil sample was diluted to 10^{-4} in 0.8% sodium chlorate isotonic water. 0.1 ml of this diluted sample was taken and sown into plate count agar, dextrose casein peptone agar, potato dextrose agar, dichloran rose bengal chlorinated agar, malt extract agar, sabouraud dextrose agar and yeast extract agar which was prepared by being sterilized in an autoclave at 121°C for 15 min at 1 atm pressure in a sterile cabin. After preparation, the petri dishes were put in a 20°C incubator; the growing phase for the bacteria was about three days, and ten days for the fungi. Growing bacteria in petri dishes were marked as B1-B5 and fungi as F1-F6. These bacteria were then added to sabouraud dextrose broth and fungi were added into malt extract and incubated at 20°C for enrichment.

Identification of fungi and bacteria species

Molecular characterization procedures were applied to Fungi, isolating the Genomic DNA from Yeast; and to bacteria, isolating the Genomic DNA from gram positive and Gram Negative Bacteria (Beutler et al., 1990).

Fungi studies

The fungi marked on the petri dishes were sown in PDA (Peptone Dextrose Agar) petri dishes by streak plates to ensure the reproduction from sport fungi. The fungi that were grown at room temperature and from single colony isolation were transferred to other PDA petri dishes and were kept at room temperature until they reached the appropriate size for DNA isolation. The growing fungi were scratched using a sterile blade and crushed with liquid nitrogen in sterile conditions, after which, DNA was isolated from the fungal hyphae.

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An ordinary taq polymerase was conducted for PCR (Polymerase Chain Reaction) using many combinations of ITS (Internal Transcribed Spacer) region primers, which are often used in the definition of DNA. The PCR conditions are given below.

Final concentrations (total 25 µL reaction volumes): 1X Taq polymerase buffer / 1.5 µM MgCl2 / 0.4 µM forward primer / 0.4 µM reverse primer / 0.5 µM dNTP / 1 U(unit) 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°C -3 min / 35 cycles: 94°C - 15 s, 55°C - 30 s, 72°C - 30 s / 1 cycle: 72°C 1 - 5 min.

In the PCR, the expected length of the bands was obtained only for F1 (*Penicillium thrichoderma*), F4 (*Metacordyceps chlamydosporia*) and F6 (*Alternaria 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 (personal communication, 2014) gave two results. These tapes, which were cut from the agarose gel and cleaned (in the case of multiple bands) or as a single band, PCR reaction were sent directly for sequence analysis. A Thermo-Scientific Gene JET 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).

Bacteria studies

Phire Hot Start II DNA Polymerase was used for PCR, because 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 are given below

For 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°C – 5 min / 40 cycles: 98°C – 5 s, 72°C – 20 s / 1 cycle 72°C – 4 min/4°C-∞

Bacteria isolated and denoted from B1 to B5 were identified using 16sRNA Universal Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8–27) 16S rRNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8–27) (Edwards et al., 1989). 1492R 5' TACGGYTACCTTGTTACGACTT 3' positions 1492–1512) (Weisberg et al., 1991; Park et al., 1995).

Microbial biodegradation studies

In order to assess the degradation ratio of the aclonifen from the fungi and bacteria, 5 species of each bacterial cultures and their mixtures, 6 species of each fungi cultures and their mixtures, and the mixtures of 5 bacteria and 6 fungi cultures were grown with shaker controlled at 20°C through the broth enrichment techniques. In this enriched media, Chemical Oxygen Demand (COD) measurement was carried out in 24-hour intervals (according to the Standard Methods 5220C closed reflux titrimetric method, SMC, 2009b) and decreasing of the substrate followed. After the end of the seventh day (at the end of the log phase on the growth curve) 1 ml of culture was taken at sterile

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conditions from these culture media, added into liquid media with isotonic pesticide solution, and bioremediation studies were started.

Studies in liquid media

In the liquid medium study, in 98 ml of 0.8% isotonic sodium chloride solution, 1 ml of the Chekic 600 (includes 0.6 gr aclonifen active ingredient) and 1 ml of growing cultures from the broth media (approximately 10^9 unit/1ml) were added. The aclonifen was prepared from Chekic 600 (trade name of the herbicide) in the same concentration as used in the field (200 ml/1000 m^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, these enriched solutions were shaken continuously at Gallenkamp orbital incubator at 20°C. Solution samples were monitored at 24-hour intervals on the basis of the COD, the active ingredient, and TOC parameter. For COD studies, standard method 5220 C (SMC, 2009b) and for TOC studies, standard method 5310 A (SMC, 2009a) were used. For determination of the active ingredient; firstly EPA 3510 C Seperatory Funnel Liquid-Liquid extraction method (EPA, 1996) was used, followed by the EPA 1614 method (EPA, 2007a).

Studies in soil media

According to the results of bioremediation studies in liquid media, the best removal efficiency was observed in a mixture of bacteria and fungi cultures; therefore soil media studies were also conducted in these mixed culture media. For this study, sterilized glass jars measuring 10 x 10 x 10 cm were filled with soil samples obtained before herbicide application. These soil samples were blended 3-4 times a day and kept in a drying oven at 105°C for four days. At the end of day four, soils were cooled at room temperature and made ready for experimental studies. The amount of soil in each jar was about 700 g. To provide suitable soil humidity, a total of 350 ml microbial culture in various volumes (2, 5 and 10 ml) and aclonifen containing culture media were added as defined in Dileep (2008). Four different solutions, including a replicate sample, were used to add to soil samples in jars. 2400 µg of aclonifen was added into three of these solutions of 350 ml. In this experimental setting, the humidity of the soil was maintained at about 50-60% with stabilized tap water for 12 weeks. Each week (every seven days), 20 g soil was taken from each soil medium, 10 g of which was used for analytical studies and 10 g of which was used for determining humidity.

Over 12 weeks, 10-gram samples were mixed with anhydrous sodium sulphate to form a free-flowing powder. The samples were extracted with solvent once using ultrasonic extraction (EPA, 2007b), and a portion of the extract was collected for cleanup and was analyzed. In order to determine the percent dry weight, a separate portion of the sample was weighed out at the same time as the portion used for analytical determination. Immediately after weighing the sample aliquot to be extracted, 10 g aliquot of the sample was measured out into a tared crucible. This aliquot was dried overnight at 105°C and allowed to cool in a desiccator before weighing, although this oven-dried aliquot was not used for the extraction. The percent dry weight was calculated as follows: approximately 10 g of the sample was

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weighed into a 200 ml beaker, and 1.0 ml of matrix spiking and surrogate spiking solutions were added to each sample. The sample was scanned ultrasonically twice for 30 min with 50 ml of the extraction solvent mixture (50% acetone and 50% Hexane for LC-MS-MS analyses). The extract decant was filtered through Whatman No.41 filter paper using a Buchner funnel. In agricultural soils there are many organic substances that can interfere with the target analyte, so clean-up procedures are essential prior to chromatographic measurement. In order to eliminate possible interfering organics such as PAHs, and PCBs, alumina-silicic acid column was prepared. These two chemicals were baked at 450°C for six hours and then cooled down to room temperature in a desiccator. Separation column was formed by 3 g silicic acid (3% water), 2 g neutral alumina (6% water), and 2 g Na_2SO_4 (Jantunen, et al., 2000). Next, column was pre-washed with 20 mL of Dichloromethane and 20 mL of PE, respectively. Sample was evaporated to 1-2 ml and then poured to column. Finally, 20 ml dichloromethane was added to elute the pesticides (Cindoruk, 2011). The aliquot of the sample was placed into a concentrator tube in a warm bath and evaporated to 1 ml volume using a gentle stream of clean, dry nitrogen, after which it was analyzed. The internal wall of the concentrator tube was rinsed several times with solvent during concentration. Then, the extract was analyzed for the target analytes using the EPA 1614 method (EPA, 2007a) over eight weeks.

Results

Results of identified bacteria and fungi

The results of identification of fungi and bacteria isolated from sunflower growing field are given for fungi species in *Table 2*, and for bacteria species in *Table 3*.

Table 2. *First and second primers, sequences and references used to identify fungi (Erguven, 2015)*

Fungi Code and Approximate species identity	**First Primer 5'-3' sequence and reference**	**Second Primer 5'-3'sequance and reference**
Penicillium thrichoderma	ITS* GCATCGATGAAGAACGCAGC (White et al., 1990)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)
Penicillium simplicissimum	ITS GAAGGTGAAGTCGTAACAAGG (Cooke et al., 2000)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)
Penicillium talaromyces	ITS TCCTCCGCTTATTGATATGC (White et al., 1990)	ITS GAAGGTGAAGTCGTAACAAGG (Cooke et al., 2000)
Metacordyceps chlamydosporia	ITS GAGACCGCCACTGTATTTCG (Avcioglu-Dundar, 2014)	ITS GCATCGATGAAGAACGCAGC (White *et al.,* 1990)
Stachybotrys chartarum	ITS TCCGTAGGTGAACCTGCGG (White et al., 1990)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)
Alternaria alternata	ITS GCATCGATGAAGAACGCAGC (White et al., 1990)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)

*ITS:Internal Transcribed Spacer

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Table 3. *Identified bacterial codes and their species*

Accession Number	Bacterial Code and Approximate Species Identity	Identity	Reference
KF831394.1	*Bacillus simplex*	99%	(Heyrman et al., 2005)
HE646789.1	*Bacillus muralis*	99%	(Li et al., 2014)
KF555623.1	*Micrococcus luteus*	99%	(Bahig et al., 2008)
KC634108.1	*Micrococcus yunnannesis*	99%	(Chitra et al., 2014)
HG530135.1	*Clostridium tetani*	99%	(Ortega et al., 2012)

Results of removal rate of aclonifen in liquid media

COD and active agent results of aclonifen with identified fungi and bacteria are given in *Table 4*. The removal rate of aclonifen with fungi at the end of day five is given in *Figure 2*, for bacteria are given in *Figure 3*. The removal rate of the active agent for the bacteria mixture, fungi mixture and mixture of bacteria + fungi are given in *Table 5*. Removal rates of COD for mixture cultures are given in *Figure 4* and TOC removal values are given in *Figure 5*.

Table 4. *Removal rate of aclonifen with bacteria and fungi species*

Fungi	Day	COD Aclonifen (mg/l)	COD Aclonifen (%)	Aclonifen (mg/ml)	Aclonifen (%)	Bacteria	COD Aclonifen (mg/l)	COD Aclonifen (%)	Aclonifen (mg/ml)	Aclonifen (%)
Penicillium taloremyces	0	15600	53	2,45	49	Bacillus simplex	15600	91	1,68	72
	1	14720					13600			
	2	13920					6800			
	3	12480					3360			
	4	8250					1680			
	5	7330					1360			
Penicillium thrichoderma	0	15600	77	1,49	69	*Bacillus muralis*	15600	78	2,28	62
	1	13920					14720			
	2	7330					13600			
	3	6880					7700			
	4	5160					4620			
	5	3590					3430			
Metacordceps chlamydosporia	0	15600	91	1,39	71	*Micrococcus luteus*	15600	70	2,7	55
	1	14720					14720			
	2	13920					13600			
	3	8600					7700			
	4	3480					4680			
	5	1040					4680			
Penicillium simplicissimum	0	15600	68	1,97	59	*Micrococcus yuannesis*	15600	93	1,62	73
	1	14720					7360			
	2	13920					5460			
	3	8600					3600			
	4	5440					1700			
	5	4990					1090			
Stachybotrys chartarum	0	15600	82	1,25	74	*Clostridium tetani*	15600	80	2,22	63
	1	14720					14040			
	2	12480					7700			
	3	4680					6160			
	4	4620					4620			
	5	2800					3120			
Alternaria alternata	0	15600	69	1,92	60					
	1	14720								
	2	13920								
	3	10290								
	4	6880								
	5	4840								

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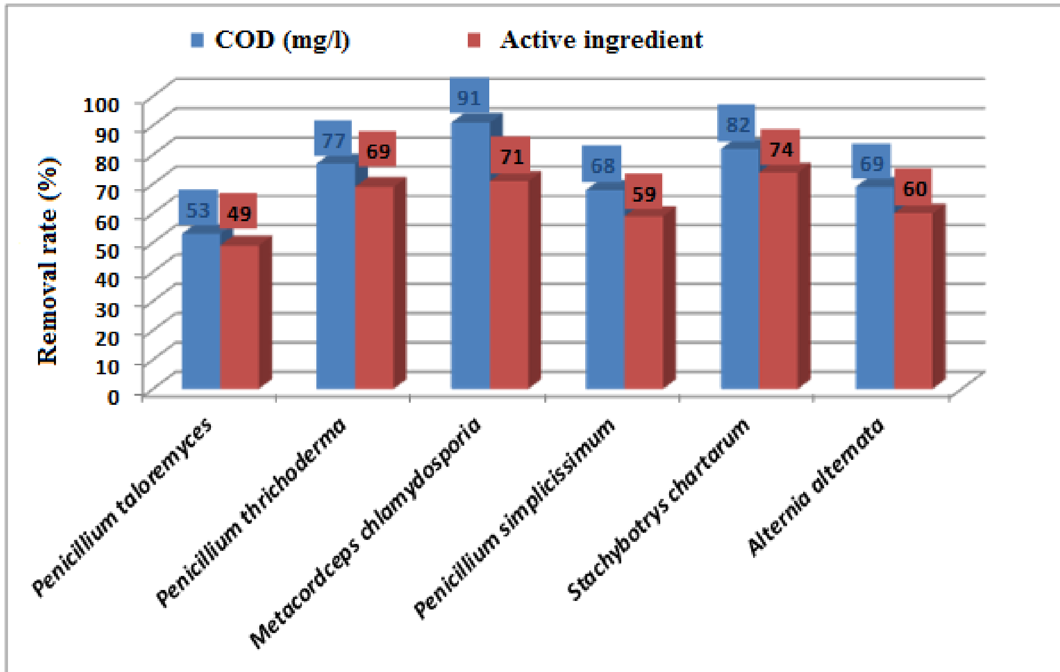

Figure 2. *Removal rate of aclonifen with all isolated fungi*

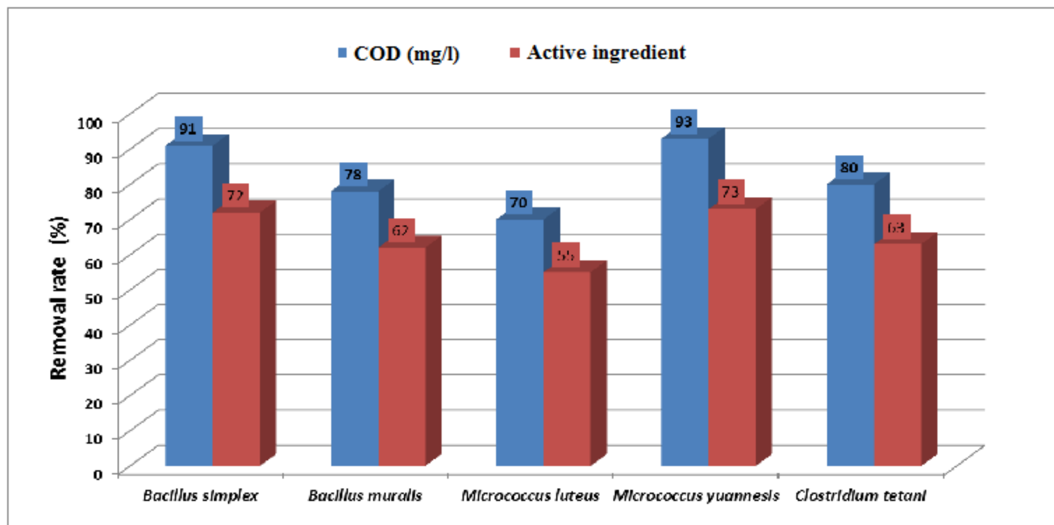

Figure 3. *Removal rate of aclonifen with all isolated bacteria*

Table 5. *Removal rate of aclonifen with mixed cultures*

Sample Type	Day	Aclonifen* mg/ml	Aclonifen Removal Efficiency (%)
Mixture of bacteria	1	5,46	9
	2	5,16	14
	3	5,04	16
	4	3,00	50
	5	1,74	71

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Mixture of fungi	1	5,58	7
	2	5,04	16
	3	4,14	31
	4	2,28	62
	5	0,54	91
Mixture of bacteria + fungi	1	4,5	25
	2	3,48	42
	3	2,64	56
	4	1,44	76
	5	0,06	99

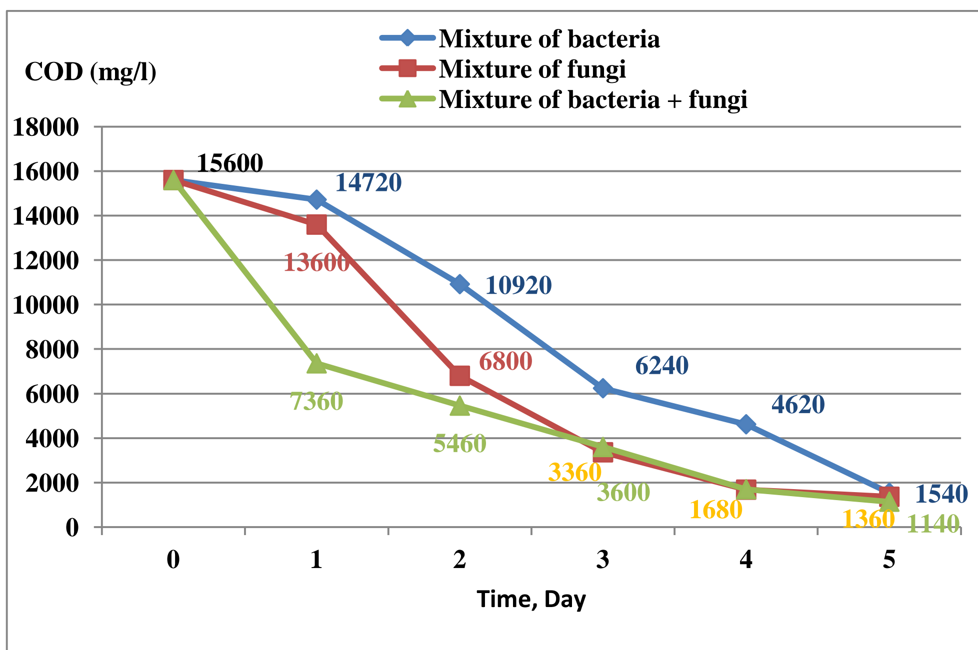

***Figure 4.** Removal rate of aclonifen as COD with mixed cultures*

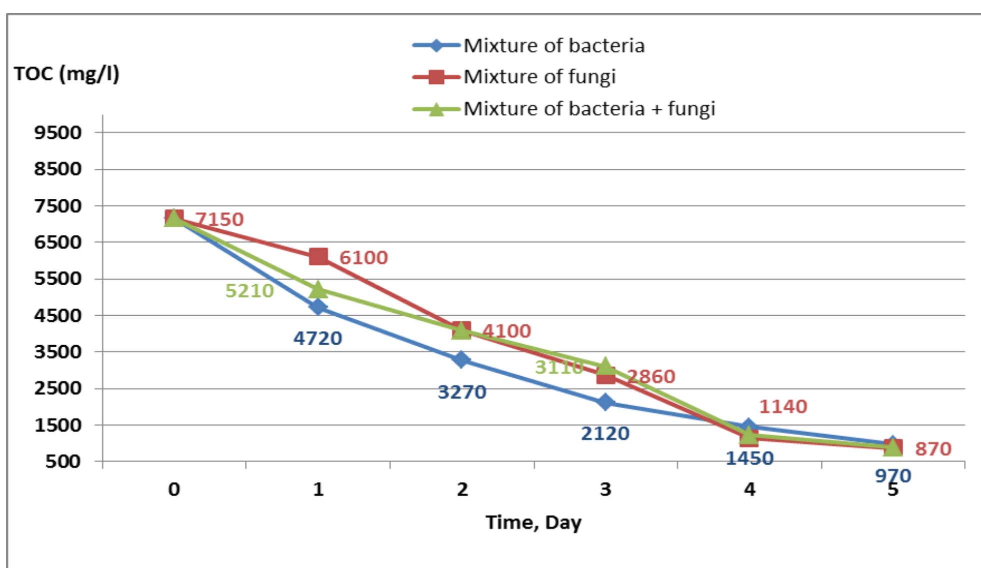

***Figure 5.** Removal rate of aclonifen as TOC with mixed cultures*

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Results of removal rate of aclonifen in soil media

Bioremediation results obtained from mixed cultures in soil with aclonifen are given in *Table 6*, and removal rates are given in *Figure 6*.

Table 6. *Removal rate of aclonifen in soil media with mixed cultures*

Week	Mixture of bacteria + fungi				Mixture of bacteria + fungi			
	Blank	2ml	5 ml	10 ml	Blank	2ml	5 ml	10 ml
	Removal rate (%)				Concantration (ng/g)			
1	0,9	9,2	19,0	52,0	3399,1	3114,4	2778,3	1646,4
2	5,8	7,1	18,8	63,7	3231,1	3186,5	2785,2	1245,1
3	10,7	19,2	28,4	67,0	3063,0	2771,4	2455,9	1131,9
4	18,0	35,8	45,8	72,5	2812,6	2202,1	1859,1	943,3
5	25,8	48,7	55,5	82,9	2545,1	1759,6	1526,4	586,5
6	31,1	61,8	62,1	96,7	2363,3	1310,3	1300,0	113,2
7	37,4	62,5	68,7	96,9	2147,2	1286,3	1073,6	106,3
8	42,3	60,6	70,5	97,2	1979,1	1351,4	1011,9	96,0
9	49,3	61,1	71,1	99,4	1739,0	1334,3	991,3	20,6
10	53,4	64,9	69,5		1598,4	1203,9		
11	59,1				1402,9			
12	69,1				1059,9			

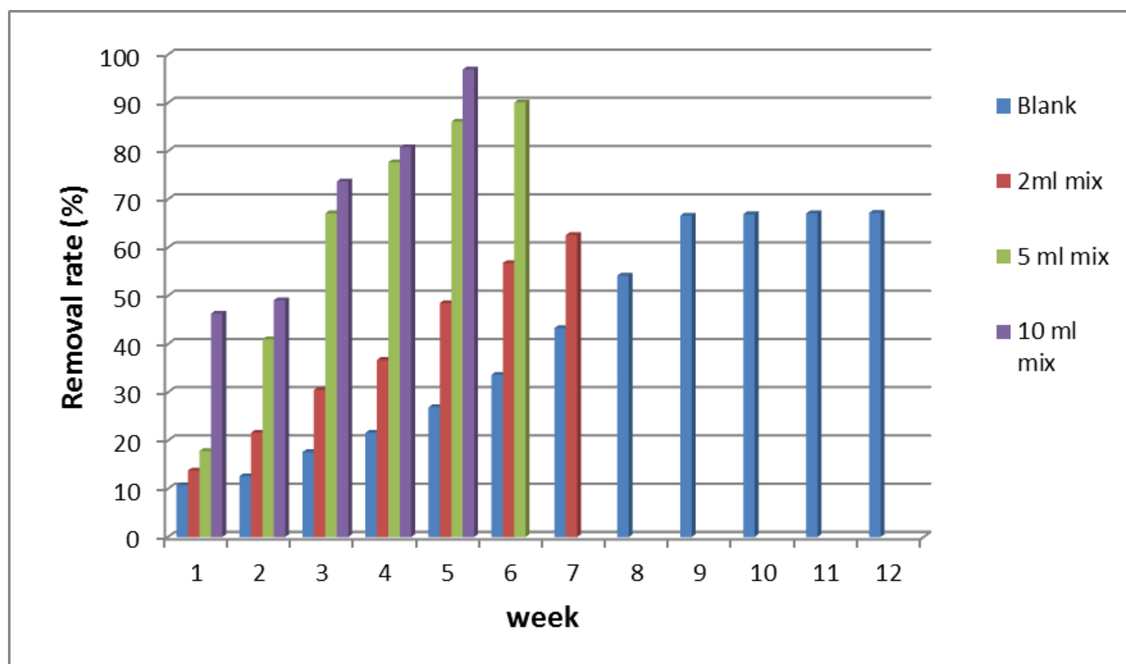

Figure 6. *Removal rate of aclonifen with mixture cultures*

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In the studies conducted on aclonifen-added experimental media, the best removal rate of the active agent within five days was achieved with *Stachybotrys chartarum* at a rate of 74%. Within this time period, the lowest removal efficiency was observed with *Penicillium talaromyces* at a rate of 49%. As for the results of the bioremediation of this herbicide by fungi, 6.00 mg/l of active agent decreased to 1.25mg/l with *Stachybotrys chartarum*. This value is 2.45 mg/l for *Penicillium talaromyces*. COD removal increased between 91% and 53% for the same herbicide. According to these results, *Metacordyceps chlamydosporia* has the best removal performance. 15600mg/l of COD decreased to 1040 mg/l after the end of day five. The lowest removal performance was seen with *Penicillum talaromyces* was 15600mg/l to 7330 mg/l as COD. Results for the other four species occur between these values.

In the studies conducted in liquid media administered with bacteria and the addition of aclonifen, the best removal rate based on the active agent within five days was achieved with *Micrococcus yuannesis*, at a rate of 73%. Within this time period, the lowest removal was with *Micrococcus luteus* at 55%. Regarding the active agent concentration, if we consider the bioremediation of the same herbicide by bacteria, 6.00 mg/l of active agent decreased to 1.62 mg/l with *Micrococcus yuannesis* by the end of day five, and to 2,70 mg/l with *Micrococcus luteus*. COD removal rate was observed to be between 93% and 70% with same herbicide. According to these results, *Micrococcus yuannesis* has the best removal performance. The COD, which was calculated as 15600mg/l decreased to 1090 mg/l at the end of day five. The poorest removal performance was observed with *Micrococcus luteus*, decreasing the COD from 15,600mg/l to 4,680 mg/l. Results for the other four species varied between these values.

In the studies conducted in liquid media administered with bacteria, fungi, and bacteria + fungi mixtures the best removal rate of aclonifen based on the active agent within five days was achieved with the bacteria + fungi mixture at a rate of 99%. Within this time period, the lowest removal rate was observed with the mixture of bacteria at a rate of 71%. Regarding the active agent concentration, if we consider the bioremediation of the same herbicide by bacteria, fungi, and bacteria+ fungi, the active agent of nearly 6.00 mg/l was decreased to 0.06 mg/l with the mixture of bacteria + fungi at the end of day five, to 1.74 mg/l with the mixture of bacteria, and to 0.54 mg/l with the mixture of fungi (*Table 5*). The COD removal was observed between 93% and 90% with the same herbicide. According to these results, the mixture of bacteria + fungi had the best removal performance. The COD calculated 15,600mg/l decreased to 1,140 g/l at the end of day five. The poorest removal performance was observed with the mixture of bacteria, decreasing the COD of 15,600mg/l to 1,540 mg/l. Other removal efficiencies varied between these values. In the study, the removal efficiency of TOC was observed to be between 86% and 88%. According to these results, the best removal was achieved with the fungi mixture and the mixture of bacteria + fungi. The TOC, which was calculated to be 7,150 mg/l, decreased to 870 mg/l at the end of day five with the mixture of fungi, and to 880 mg/l with the mixture of bacteria + fungi. The lowest removal efficiency was observed with the mixture of bacteria, decreasing the TOC to 970 mg/l at the end of day five.

According to the bioremediation studies on soil media with aclonifen, removal rate with a mixture of 10 ml culture solution was 99%, which is higher than the values obtained with the mixture of 2 ml and 5 ml culture solutions. The next best removal efficiency was observed with 5 ml culture solutions with a rate of 70% at the end of

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week nine, however, the 2 ml. solution reached 65% at the end of week 10. When the results of the study were evaluated based on the active agent, nearly 3,420 ng/g of aclonifen, which was added to the soil, was observed to decrease to 21 ng/g at the end of week nine, in 10 ml solution mixture, while the amount decreased to 1,204 ng/g at the end of week 10 in the 2 ml mixture solution, and to 991 ng/g at the end of week nine in the 5 ml mixture. According to these results, it was understood that increasing the amount of mixed cultures in soil shortens the removal time of aclonifen and increases the rate of removal.

Discussion

Herbicides and surfactants differ in chemical composition and react differently when incorporated into the soil system due to differences in chemical properties and interactions with soil components and environmental factors (Smith et al., 1982; Ray et al., 1985). Microbial community structure, often used as an indicator in monitoring soil quality, is affected by various environmental and growth factors, such as moisture, temperature, nutrient availability, and management practices (Petersen et al., 2002).

In aerobic laboratory soil degradation studies, the overall geometric mean DT50 value of aclonifen at 20°C is 62.3. Degradation of aclonifen in soil under anaerobic conditions was investigated in one of the studies presented in the dossier but it was not considered reliable (EFSA, 2008). Aclonifen may be considered moderate to highly persistent (DT50 = 32.2 – 134 d) in soil, under aerobic conditions, at 20°C. Based on the findings from the screening test on ready biodegradability, water/sediment simulation test and soil aclonifen appears to be susceptible to primary degradation (a degradation of >70% within 28 days) and not ultimate mineralization (Humburg, 1989).

Conclusion

The route of degradation in soil under dark aerobic conditions was investigated in two studies in a total of five soils. In our study, it was observed in the weekly active ingredient soil studies that the removal efficiencies were higher when the concentrations of mixed microorganism culture were increased. The values were 63% for 2 ml culture in 7 weeks, 90% for 5 ml culture in 6 weeks, and increased to 97% for the 10 ml culture in 5 weeks.

This study showed that biodegradation of aclonifen was found sufficiently high, especially for the mixed cultures. Isolated fungi and bacteria consortia showed the highest degradation of 93% as COD parameter in 5 days. Bacteria mix and fungi mix performed 90% and 91% degradation in 5 days as COD while 71% and 91% as active ingredient. Soil studies with mixed cultures of bacteria and fungi performed the most efficient degradation as 97% after 5 weeks. The degradation of aclonifen by 2 ml mixed cultures showed about 63% of degradation in 7 weeks and 5 ml of mixed cultures showed about 90% in 6 weeks. As a conclusion it can be said that the results of the experiments showed important implication potential in the development of in-field treatment systems for pesticide-contaminated soils.

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