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Monitoring the ethalfluralin biodegradation with certain bactofungi mixed culture

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ABSTRACT

In this study, the biodegradation of ethalfluralin ($C_{13}H_{14}F_3N_3O_4$), which is the most preferred herbicide in sunflower farming in Thrace region in Turkey, is investigated. Bacteria and fungi were isolated in soil samples obtained in Thrace region, and a mixed culture was prepared using equal volume of microorganism cultures. Five different apparatuses, including sterile agricultural soil were setup and ethalfluralin in 1,500 μg/L concentration was added to each apparatus, and they were watered for 6 weeks with sterilized tap water. Drained water was then collected, and the filtrates were measured for chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total organic carbon (TOC) and ethalfluralin. Furthermore, pH and dissolved oxygen (DO) concentration were measured. Study results demonstrated that the highest biodegradation was observed in the soil sample that contained 10 mL mixed microorganism culture and ethalfluralin. In this filtrate, ethalfluralin, COD, BOD_5 and TOC removals were measured as 92%, 85%, 82% and 97% respectively. The DO level remained between 3.00 and 4.50 mg/L in the first 3 weeks for all cultures. After the third week, it increased to 5.8 mg/L in the 5 mL culture mixture and to 7.1 mg/L in the 2 mL culture mixture. pH results demonstrated, an increase during the first 3 weeks and pH values decreased for all culture mixture media due to the formation of carbonic acid.

Keywords: Biodegradation; Ethalfluralin; Chemical oxygen demand; Biochemical oxygen demand; Total organic carbon

1. Introduction

During the last 50 years, pesticides have become a significant part of the agricultural science. Although the purpose of pesticide use is basically to increase the produce yield, with the increase in services and produced due to the advance in agricultural industry, important risks were potentiated as a result of unnecessary excessive use of pesticides [1]. In addition to their benefits, toxic adverse effects of pesticides also create environmental risks [1,2].

Pesticides are widely applied on soil or over field crops and hence could penetrate into contaminating the environment easily [3]. Furthermore, the potential environmental hazard from pesticides is raising concerns for the public and regulatory agencies [4]. These might be one of the most hazardous groups of contaminants for human health, fauna and the environment [5]. Pesticides disintegrate after application, and concern has been expressed for the possible effects of pesticides and degradation products on human health and the environment [6].

Biodegradation by microorganisms is a more environment-friendly treatment approach for detoxification of persistent organic pollutants, compared with conventional chemical and physical methods. Furthermore, certain biotechnological applications such as biodegradation of organic pollutants could be given as examples of biodegradation [7]. Bacteria and fungi are both responsible for biodegradation of polyaromatic hydrocarbons (PAHs) and other petroleum hydrocarbons [8–11]. Microbial populations have high biodegradation capacity for organic material [12].

Since soil is a complex system, degradation of herbicides could be affected by several factors such as temperature, pH, moisture, differences in plowing and plants [13]. Several researchers identified parameters such as soil pH, pesticide application speed and time, organic substance content in laboratory studies; however, major factors on pesticide degradation rate are soil moisture and temperature in field conditions [14]. The behavior of pesticides in soil is governed by a variety of complex factors such as physical, chemical and

dynamic biological processes, including chemical and biological degradation. Soil characteristics vary continuously based on the climate, parent materials, living organisms and crop management [15].

Biodegradation of herbicides is affected by significant permanent and transient properties of the soil such as moisture, temperature, retention time, intensity, nutrients, oxygen level, microbial population and pesticide solubility [16]. Several microorganisms inhabit the natural environment. Recent studies have focused on removal of pesticides using microorganisms. Biodegradation of pesticides could be directly observed in areas exposed to pesticides [17].

Dinitroanilines are special herbicides used for the pre-emergent control of a wide spectrum of grasses and broad-leafed weeds in a wide variety of crops with agro-economic significance. Ethalfluraline, one of the dinitroaniline herbicides has been employed widely for the protection of some crops and sunflowers [18]. The fate of the pesticides in the ecosystem is very important since they are degraded by various factors in the soil, water, etc. Since it is mandatory to monitor the degradation of pesticides in soil, and their persistence, it is also necessary to study the effect of the pesticides on soil health and the soil quality and soil microbial activity. Today, the primary concern for the environmentalists is; the removal of metabolites from the soil. Toxicity of pesticides could be reduced by the bioremediation process which involves the use of plants or microbes, either though degradation or using the pesticides in various co-metabolic processes [19]. Several studies conducted worldwide have focused on identification of soil pollutants, while several others on the determination of microorganisms that could degrade the pesticides in polluted areas. The biodegradation of herbicides by bacterial isolate has been widely studied [20–22]. The biodegradation of pesticides by a bacteria or fungi mixed culture, especially that of ethalfluralin requires further studies and the use of a mixed culture, which is a community of microorganisms, is effective in degrading other organic compounds in the environment [9,10,23,24].

In the present study, initially; different types of bacteria and fungi obtained from agricultural soil samples in Thrace region in Turkey, were isolated and determined in certain selective agar medium. Then, these cultures were grown in enrichment media and taken into the soil units for biodegradation activities. Every week, the filtrate water was obtained from these soil units and the filtrates were examined weekly with ethalfluralin active ingredient, chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total organic carbon (TOC), dissolved oxygen (DO) and pH. Based on the results, the highest removal was observed where 10 mL (approximately 10^9 CFU/mL) mixed culture was present. At the end of the fifth week, it was observed that ethalfluralin, COD, TOC and BOD_5 yielded 92%, 85%, 97% and 82% removal rates, respectively.

2. Materials and methods

2.1. Isolation of microorganisms and molecular characterization studies

Soil samples were obtained at 0–20 cm soil depth in Turgutbey village, located at the Thrace region in Turkey (Fig. 1), for isolation of bacteria and fungi. There were

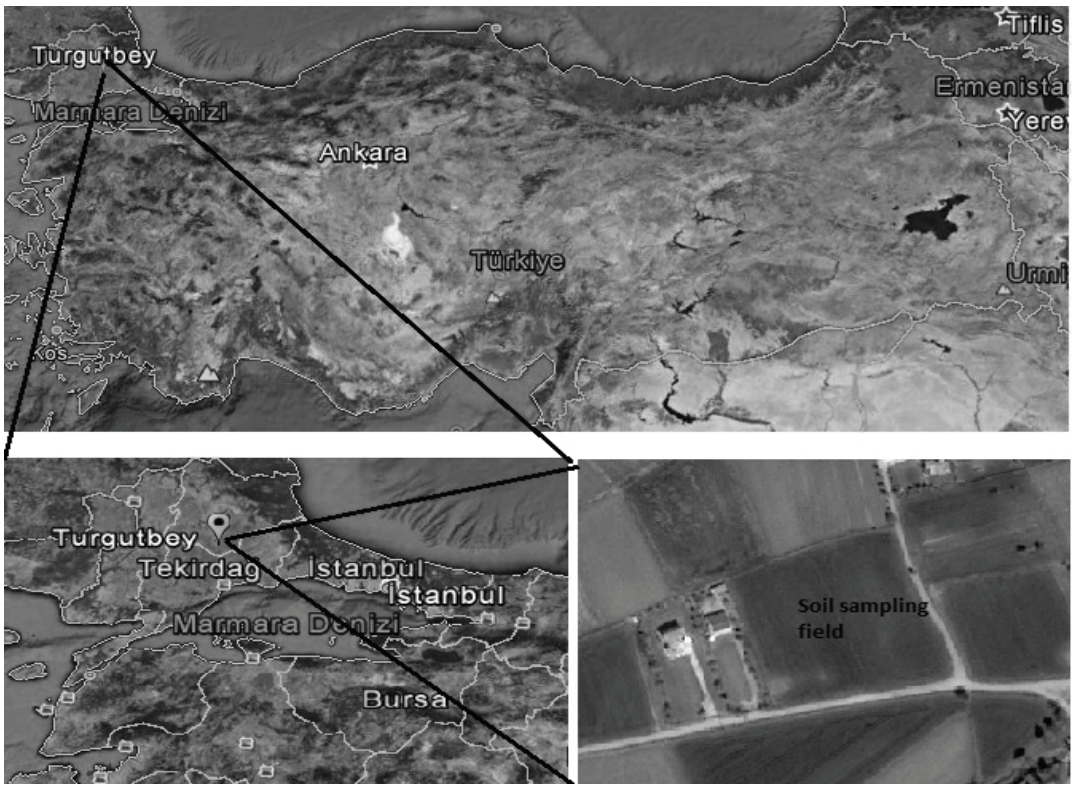

Fig. 1. Soil sampling field.

no agricultural crops present, when these samples were obtained and the area was not previously exposed to herbicidal treatment. Properties of the soil sample used in the current study are given in Table 1. Soil samples were collected and sent to Tunceli Directorate of Provincial Food Agriculture and Livestock Soil Analyses Laboratory for chemical and physical analyses, in accordance with the regulations of the Turkey Ministry of Food, Agriculture and Livestock according to method TS 9923 [25]. According to world reference base for soil resources classification [26], the soil type is silty or clay loam. For isolation of the bacteria and fungi, one portion of the collected soil samples was placed in sterile glass jars initially and stored at 4°C in plastic containers placed in ice bags and taken to the laboratory while kept at 4°C [27]. Approximately 10 g of the soil sample was diluted in 0.8% sodium chloride isotonic water up to 10^{-4} to prepare a bacteria–fungi solution. To isolate the bacteria; plate count agar, to isolate the fungi; dextrose casein peptone agar, potato dextrose agar, dichloran rose bengal chlorinated agar, malt extract agar, sabouraud dextrose agar and yeast extract agar were prepared according to manufacturer's instructions and sterilized at 121°C. 0.1 mL of this solution was poured into plates, 0.1 mL for each feedlot under aseptic conditions [28]. Rose bengal was added at a concentration of 30 mg/L to easily count the fungi. After inoculation, Petri dishes were taken into a 20°C incubator for storage and the bacteria completed their development in 3 d, while for fungi it took 5 d to develop. Bacteria and fungi colonies developed in Petri dishes were taken separately into the enrichment media (sabouraud dextrose broth for bacteria, malt extract for fungi) and reproduced under 20°C incubation. They were selected visually and separated. Then, they were coded for bacteria as B1–B5 and fungi as F1–F6. All experiments were performed in triplicate [29].

Fungi prepared for molecular studies were taken into malt extract agar and bacteria were taken into standard plate count agar media. For polymerase chain reaction (PCR) processes, genetic analysis system (Beckman Coulter CEQ 8000) and MyCycler thermal cycler system, electrophoresis device and gel imaging system (ORTE) were used. Characterization studies were conducted as instructed in Wizard Genomic DNA Purification kit manual. Isolating "Genomic DNA from Gram Positive and Gram Negative Bacteria" method was utilized for bacteria, while "Isolating Genomic DNA from Yeast" [38] method was used for fungi. Initially, nucleic acid extraction was conducted on the samples. Obtained DNA was stored at –20°C. 16S rRNA genes in these DNA mixtures were multiplied with thermal cycler device using PCR method and then methanogen specific diversity was determined with DNA and denaturing gradient gel electrophoresis cycle analysis.

Table 1
Physical and chemical properties of soil sample

Soil properties	Methods	Value (N = 10)
pH	[30]	7.6
Salt (%)		0.05
Saturity (*WHC_{max}) (%)	[31]	64
Organic matter (**TOC) (%)	[32]	1.5
Total nitrogen (%)	[33]	0.11
Cation change capacity (%)		33.2
Clay (%)	[34]	24
Silt (%)		56
Sand (%)		20
Moisture (%)	[35]	34.6
Phosphorus (kg/ha)	[36]	0.97
Potassium (kg/ha)	[37]	4.63

*: water holding capacity, **: total organic carbon.

2.2. Bacteria characterization studies

Streak plate seeding was conducted for homogenous marked colonies in Petri dishes and to lower them to a single colony. Phire Hot Start II DNA Polymerase was utilized and long (1,000–3,000 bp) PCR strips were obtained in different lengths with bacteria 16S ribosomal general primers. Cycle and pipetting conditions were as follows.

- Temperature 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–∞.
- Identification of isolated and coded bacteria (B1–B5) were conducted based on 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) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′ positions 1492–1512) [39].
- Final concentrations (total 20 μL reaction volume): 1× Phire animal tissue PCR buffer (including deoxynucleotide triphosphate and $MgCl_2$)/0.5 μm forward primer/0.5 μm reverse primer/Phire Hot Start II NDA polymerase and H_2O.

2.3. Fungus characterization studies

To enable single spore reproduction, fungi were streak plated in Petri dishes with peptone dextrose agar (PDA). They were grown in room temperature and fungus colonies grown from one spore were taken into another Petri dish with PDA and grown under room temperature until the volume is sufficient for DNA isolation. Grown fungi were scraped from the Petri dish using a scalpel and pulverized in a mortar with liquid nitrogen. Later on, DNA isolation was conducted on pulverized hypha using Promega Wizard® Genomic DNA Purification Kit method (3.E. Isolating Genomic DNA from Plant Tissue).

DNAs were applied PCR with ordinary Taq polymerase using several combinations of internal transcribed spacer region primers, which are generally used for fungus identification. PCR conditions were as described below.

- Temperature 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–5 min.
- Final concentration (total 25 μL reaction volume): 1× 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 and 200 ng DNA.

As a result of this PCR, only M1 (*Metacordyceps owariensis*), M4 (*Verticillium chlamydosporium*) and M6 (*Cordyceps cicadae*) fungi produced expected strip lengths in agarose electrophoresis. For the remaining fungi, a better polymerase, OneTaq polymerase was utilized.

Under OneTaq polymerase conditions, 25 μL reaction was implemented. Temperature was used between 53°C and 57°C (various temperatures were tested with each combination based on primer sintering points). Three primers were designed by Avcioglu-Dundar [40] and two produced results. These strips were sent directly to the PCR reaction cycle analysis, in case they were single strip or cut from agarose gel (in case when there was more than one strip) and cleaned. In case sequence reaction did not produce results based on strips cut from agarose gel, OneTaq polymerase re-amplification was implemented with the strips. Strips cut from agarose gel were cleaned with thermo scientific gene JET gel extraction kit.

2.4. Identified soil bacteria and fungi used in the study

Bacteria and fungi species, isolated and identified in agricultural soil are presented in Tables 2 and 3, respectively.

2.5. Preparation of soil test units

In Fig. 2, holes with a diameter of 0.2 cm were drilled in the bottom of five clean plastic bottles with a diameter of 10 cm. Drilled bottom parts were then cut out and placed inside the bottles near their openings so that they functioned as filters. 7 cm of agricultural soil (approximately 350 g), that had not been exposed to herbicides or other PAHs previously (this has been confirmed with gas chromatography with electron capture detector [GC-ECD] analyses) and was sterilized at 105°C for 4 d, was placed in each setup.

Table 2
Types of the bacteria identified and references

Accession number	Approximate species identity	Identity (%)	References
KF317874.1	*Bacillus thuringiensis*	99	[41]
KF831394.1	*Bacillus simplex*	99	[42]
Hf679029.1	*Fusarium fujikuroi*	82	[43]
HG530135.1	*Clostridium tetani*	93	[44]
KC246043.1	*Bacillus megaterium*	99	[45]

Table 3
Types of the identified fungi

Fungi code and approximate species	Identity (%)	Accession no
Metacordyceps owariensis	88	HQ165699.1
Metarhizium cylindrosporae	88	HQ165693.1
Tolypocladium geodes	88	FJ973059.1
Verticillium chlamydosporium	99	AJ291804.1
Cordyceps sp.	88	KC007337.1
Cordyceps cicadae	88	AJ536574.1

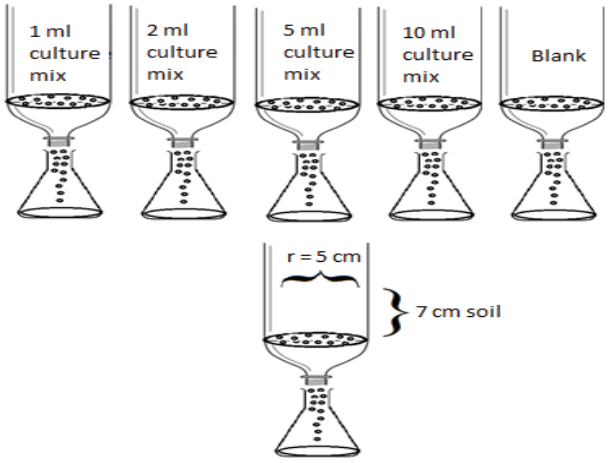

Fig. 2. The soil units.

Surface area of the five soil units was 7.85 × 10^{-3} m^2. Approximately 1,500 μg/L ethalfluralin was added to the soil in each apparatus (twice the amount recommended for use in agricultural fields). The soil in each unit was mixed with 100 mL of sterilized tap water, included 1, 2, 5 and 10 mL of bactofungi mixed culture containing viable cells (approximately 10^9 CFU/mL for each five different species of bacteria and six different species of fungi). They were homogenized and transferred to these soil units with a sterile pipette to these soil units. The microorganisms used in the present study were reproduced in enriched media, incubated for a week and had started to seek nutrients after consuming the existing substrate. The soil in the blank unit did not contain mixed culture. For negative control group (as a blank), one unit with sterile soil (without microorganism) and 1,500 μg/L ethalfluralin was used to determine the decrease in COD, BOD_5, TOC, ethalfluralin, pH and DO parameters for 5 weeks. Each experiment was performed in triplicate for each parameter and total of 15 soil units were used in the study (three replicates for each). Average results were calculated for each experiment.

2.6. Preparation of the herbicide solution

The herbicide ethalfluralin, sold under the trade name "Izolan", was supplied by an agricultural products store. pH of ethalfluralin was 7.0, and temperature was 20°C. This herbicide contains 333 g/L of ethalfluralin. Ethalfluralin standard (CAS Number: 55283-68-6) was supplied by Sigma-Aldrich (Darmstadt, Germany) in 99.9% purity from Dr. Ehrenstorfer GmbH Co. (Augsburg, Germany). Ethalfluralin standard was dissolved in GC-grade methanol used for calibration of the GC. All media for the isolation and enrichment of bacteria and fungi were obtained from Sigma-Aldrich. Acetone and hexane were produced from Merck (Augsburg, Germany). All utilized chemicals were of GC grade. Analytical standards for GC calibration in the range of 0.1–100 mg/L were prepared with the working standard solution of methanol.

2.7. Instrumental analyses

For ethalfluralin analysis procedures in GC-ECD, Perkin Elmer Clarus 500GC was conducted based on the methods specified in EPA 8081 [46]. All samples were spiked with surrogate and internal standards in order to determine the recovery rate. High purity helium (99.99%) was used for the carrier gas at a constant flow rate of 1.5 mL/min. Tetrachloro-*m*-xylene was used as the surrogate standard. The surrogate standard was spiked to the sample prior to extraction. For internal standard, quintozene was used and was spiked just before capping the chromatography vials. Average recovery rate was 91%. The limits of detection (LOD) values were calculated for each congener as average blank concentrations plus three times the standard deviations. In case of weight losses, the new concentration was recalculated accordingly [47]. Any sample concentrations that fall below the LOD value were ignored. For each set of analysis, blank samples were corrected and all results were blank corrected.

2.8. Removal of ethalfluralin in the soil

250 mL distilled water was added to each unit to obtain filtrates and these filtrates were collected in clean bottles displayed in figures. The filtrates were initially passed through a 0.45 μm membrane filter paper and then, COD, BOD_5, TOC, ethalfluralin, pH and DO values were determined for each week. All experiments were performed in triplicate. COD test was conducted with closed reflux titrimetric method as described in standard methods 5220B-BOD_5 test was conducted with standard method 5210B (5 d BOD_5 test), for total organic carbon analyses, standard method 5310B high temperature combustion method was used according to reference [48]. pH and DO parameters were determined with WTW Inolab Multi 9310 device and ethalfluralin amount was determined with EPA 8081B [46].

3. Results

3.1. Filtrate test results obtained in soil units

COD–BOD_5, TOC, ethalfluralin active ingredient, DO–pH test results are presented in Figs. 3–6, respectively.

When there is no ethalfluralin in the medium, there is an existing COD value intrinsic in the soil. COD and TOC values of 50 and 40 mg/L in the filtrate obtained from the soil were the same every week, and there were no differences between

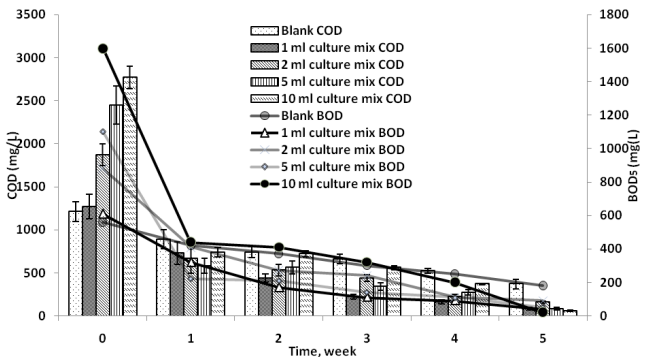

Fig. 3. Reduction of COD and BOD_5 in the filtrate.

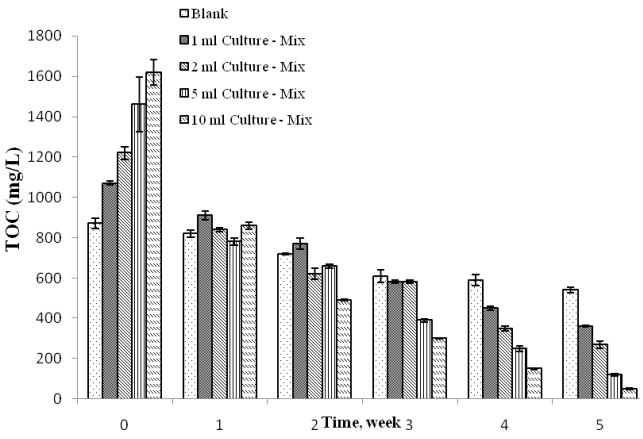

Fig. 4. Reduction of total organic carbon in the filtrate.

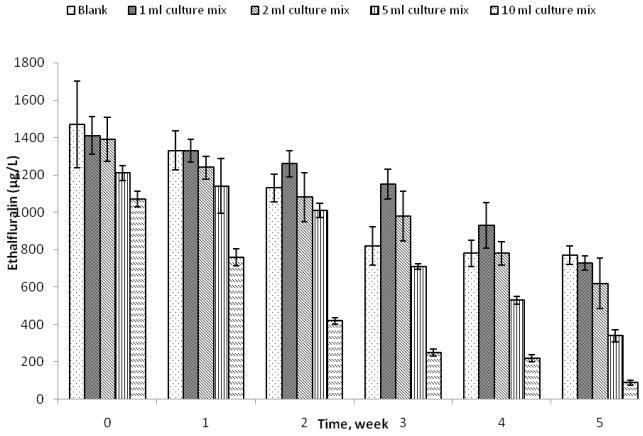

Fig. 5. Reduction of ethalfluralin in the filtrate.

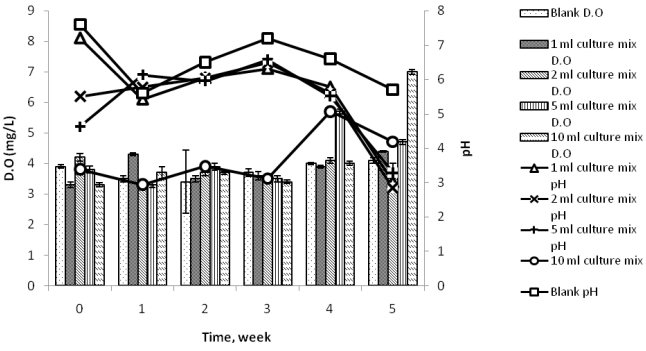

Fig. 6. pH and dissolved oxygen results in the filtrate.

the removal rates when these values were subtracted from COD values when ethalfluralin was present.

Based on the COD and BOD_5 data (Fig. 3), best COD removal was obtained in 10 mL mixed culture as 85%. Removal yield for 5 mL mixed culture was obtained as 80% in COD experiments. COD removal was 69% in arbitration medium at the end of week 5. As shown in Fig. 4, BOD_5 removal was 82% in 10 mL mixed culture soil medium during the same period. In the same period, BOD_5 removal in arbitration soil sample was 73%. Although ethalfluralin concentrations used

in all media were in equal amounts, COD and BOD_5 results were different, which demonstrated that the difference was carbon related. In the beginning of the experimental study, as the nutrient in arbitration (without microorganisms), 1, 2, 5, and 10 mL mixed cultures increased proportionally, COD and BOD_5 values increased as well. It was observed that as the medium concentration grafted to the soil increased, COD, BOD_5 and ethalfluralin removal rates increased significantly as well.

The best degradation performance was 97% for TOC in 10 mL mixed culture. At the end of the fifth week, TOC remediation was 38% in the blank soil unit. Since the ethalfluralin concentration was the same in all environments, different results for TOC tests suggest that there was a change in the culture environment. As the nutrients in the witness culture (without microorganisms) and the 1, 2, 5 and 10 mL mixed cultures increased proportionally, the TOC values increased as well. As the microorganism concentrations grafted in the soil increased, the TOC remediation performances also increased significantly (Fig. 4).

As seen in Fig. 5, ethalfluralin removal results in 1, 2, 5 and 10 mL mixed cultures in soil media were 48%, 55%, 72% and 92%, respectively, at the end of week 5. The reason for the fact that 48% removal yield in the arbitration at the end of the week 5 was close to the yield of 1 mL mixed culture in soil was insufficient microorganism population in the medium for biodegradation.

The DO results suggested a regular increase in the first 3 weeks and a decrease during last 3 weeks. This showed that the soil units were ventilated and microbial degradation occurred in aerobic conditions, which meant that these soil microorganisms perform biodegradation abilities in aerobic conditions, so most of microbial communities in the soil are facultative anaerobe (Fig. 6).

pH measurements showed a decline as the weeks passed, and thus, the filtrate obtained from the soil became acidic. The decrease in pH with bacterial degradation of pollutants is common and mainly due to the formation of acidic metabolites [8]. This was due to CO_2 accumulation induced by carbon dioxide gas emitted to the system as a result of bacteria and fungus activity and creation of carbonic acid as a result of the reaction of CO_2 with water that penetrated the system and the carbon in the pesticide. In addition, CO_2 reacted with the water added to the medium, forming carbonic acid. This was an evidence that the medium had entered the acidic phase (Fig. 6).

4. Discussion

Soil microorganisms removed pesticide residues to cater their carbon requirements [49]. Similarly, it was determined that *Penicillium chrysosporium* fungus species enzymes could metabolize micropollutants such as pesticides and could remove the toxic effects of the chemicals that pesticides contain by 76% in 15 d, and 94% in 30 d by degrading the lignin [50]. In studies conducted in aerobic and anaerobic facilities where mixed bacteria media were used, the literature demonstrated that endosulfan was degraded up to 96% biologically [51].

In a study conducted on biodegrading chlorpyrifos by soil bacteria, Maya et al. [52] used *Pseudomonas, Agrobacterium* and *Bacillus* species. As a result of the studies they conducted with *Bacillus subtilis, Brucella melitensis, Bacillus cereus, Klebsiella species, Pseudomonas fluorescens, Pseudomonas aeruginosa* and *Serratia marcescens* species, they obtained 46%–72% yield at the end of day 20, and identified that chlorpyrifos was used as a carbon source in aqueous media [53]. Erguven and Yildirim [54] studied the efficiency of certain soil bacteria for COD reduction of synthetic chlorsulfuron solutions under agitated culture conditions. They found that biodegradation rate by *Bacillus simplex*, *Bacillus muralis*, *Myristica yunnanensis*, *Clostridium tetani* and *Micrococcus luteus* were 94%, 78%, 79%, 74% and 70%, respectively.

There are examples where dinitroaniline group herbicide was degraded in aqueous media using species that were isolated from soil polluted with trifluralin. However, mixed cultures provided a more effective degradation yield compared with single cultures [16]. Previous studies on microbial degradation of trifluralin demonstrated that a few species of bacteria could manage to accomplish this. Initial examples of bacteria in this group are *Aspergillus carneus, Fusarium oxysporum* and *Trichoderma* [55].

Bellinaso et al. [56] investigated the removal process of trifluralin in aqueous media. Results of the studies that were conducted with cultures enriched in yeast extract liquid feedlot and grafted in 50 mg/L trifluralin medium demonstrated that a removal yield of 24.6% in *Klebsiella oxytoca*; 16.4% in *Herbaspirillum seropedicae*; 25% and 16% in *Bacillus megaterium* I and II species were identified and it was stated that these species could be used in future other dinitroaniline biodegradation processes [56]. Belal and Mohamed [57] conducted a study on bioremediation of pendimethalin, another dinitroaniline group herbicide, and isolated *Pseudomonas putida* species bacteria from pendimethalin polluted soil with 16S rDNA method and at the end of 4 weeks; they observed that all 100 μg/mL concentration of pendimethalin was removed by that bacteria species. Similarly, Belal and Negwa [58], in their biodegradation study conducted with *Phanerochaete chrysosporium* species in aqueous media of pendimethalin, demonstrated that 100 ppm concentration pendimethalin was 56% removed at the end of 7 d, and 75%, 85% and 95% removed at the end of 14, 21 and 28 d, respectively.

In our previous study, microbial degradation of aclonifen using bacteria and fungi isolated from an agricultural area previously unexposed to aclonifen was investigated. In these laboratory experiments, five soil units were prepared with the soil samples obtained from the Thrace region and 1,900 μg/L aclonifen was added to each of sample. We used 1, 2, 5 and 10 mL mixed cultures (approximately 10^9 CFU/mL) for biodegradation process in the same system. According to our results, the highest biodegradation was observed in the soil sample, to which 10 mL of mixed culture was added, and aclonifen, COD, BOD and TOC reduction was observed as 93.2%, 97.8%, 98.8% and 98.7%, respectively [59].

As a result of this study where no medium was added, 48% ethalfluralin removal was observed at the end of 5 weeks, which was explained with the effect of the half-life of the pesticide, especially with the adsorption mechanism. The highest degradation conducted with the medium in different concentrations was observed in the soil system, where 10 mL (approximately 10^9 CFU/mL) mixed culture was present, as 92%. At the end of the week 5, COD, TOC and BOD_5 removal

yields were observed in the same medium as 85%, 97% and 82%, respectively. Same changes in COD, TOC and BOD_5 values were observed in the microorganism activities. This was similar to change in ethalfluralin. The soil pH affects the degradation of a given pesticide based on whether a compound is susceptible to alkaline or acid catalyzed hydrolysis [60].

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