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Original Research

Comparison the biodegradation of herbicide pyroxasulfone by some soil bacteria

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Abstract: The biodegradation of pyroxasulfone ($C_{12}H_{14}F_5N_3O_4S$), which is the selective herbicide in wheat farming in Turkey is compared with some soil bacteria. These microorganisms were isolated in collected soil samples in Thrace region of Turkey from an agricultural area previously unexposed to pyroxasulfone. The microbial biodegradation of pyroxasulfone was investigated using liquid experiments with identified cultures to determine which of the microorganisms shows best removal performance under agitated culture conditions. The experiments continued about two weeks. Five different apparatuses, were set up and pyroxasulfone in 750 ppm concentration (advised concentration of wheat farmers) was added to each Erlenmayer flasks. Approximately 10^7CFU/ml of each bacteria added to these flasks. These flasks were shaken at 130rpm at 27 ^{0}C in sterile conditions for 8 days. Every day, each sample was collected by filtering from flasks and Chemical Oxygen Demand (COD), biochemical oxygen demand (BOD_5), total organic carbon (TOC) was determined. As a result of the study, best removal performance observed in *Bacillus thuringiensis* and *Fusarium fujikuroi* as 91 and 93% at 7 days in COD, 88 and 83% in BOD_5, 90 and 86% in TOC parameters. The lowest performance was seen on *Clostridium tetani* species for COD, BOD_5 and TOC as 55%, 61% and 60% respectively on 7 days. The performance for *Bacillus simplex* and *Bacillus megaterium* species occurred between 70% and 80% for these three parameters.

Key words: Biodegradation; Pyroxasulfone; Chemical oxygen demand; Biochemical oxygen demand; Total organic carbon.

Introduction

During the last half decades, pesticides have become an important part of the agricultural activities. Altough the purpose of pesticide using is to increase the production capacity; with the increase in services and produced due to the advance in agricultural industry, important risks were potentiated as a result of excessive use of pesticides (1).

Pesticides are widely applied on field crops or soil and could penetrate into contaminating the environment easily (2). The potential environmental hazard from pesticides is raising concerns for the regulatory agencies and public (3). These might be one of the most hazardous groups of contaminants for human health, and the environment (4).

Microbial biodegradation is environment-friendly treatment technique for detoxification of persistent organic pollutants, compared with conventional methods. Some biotechnological applications such as biodegradation of organic pollutants could be given as examples of biodegradation (5). Bacteria and fungi are both responsible for biodegradation of polyaromatic hydrocarbons (PAHs) and other petroleum hydrocarbons (6). Microbial populations have high biodegradation capacity for organic material (7).

Pyroxasulfone is a selective herbicide for controlling annual grases, annual broadleaf weeds and sedges. This herbicide can only be applied to surface of the agricultural field. This pyrazole herbicide kills weeds by inhibiting plant shoot growth. Pyroxasulfone is an isoxazoline,a new class of chemicals that delivers higher intrinsic activity and a broader spectrum of control of small-seeded broadleaf weeds and grasses.

Materials and Methods

Field study, isolation of microorganisms and molecular characterization studies

Bacteria, were isolated from collected soil samples obtained at 0 – 20 cm depth in Karaagac village, located at the Thrace region in Turkey (Figure 1). The agricultural field was not previously exposed to pyroxasulfone before and there were no agricultural crops when these samples were taken. These collected samples were sent

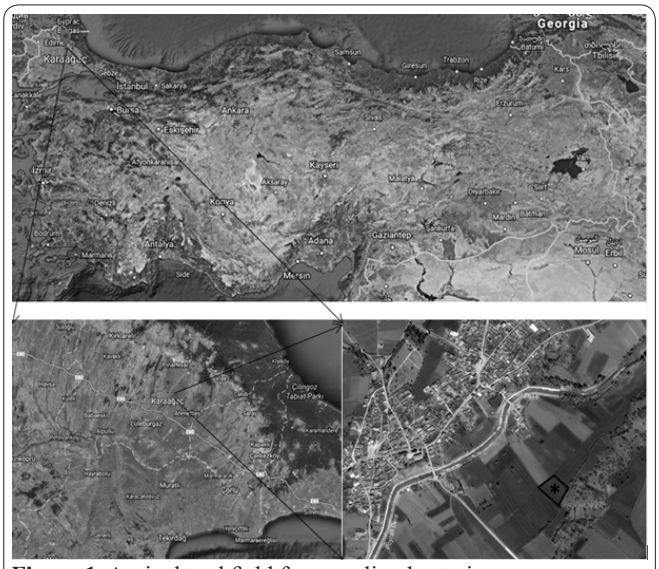

Figure 1. Agricultural field for sampling bacteria.

Table 1. Physical and chemical properties of the soil sample.

Soil properties	Unit	Methods	Value (N=10)
pH	%	(11)	7.2
Salt			0.08
Saturity (*WHC_{max})	%	(12)	59
Organic matter (**TOC)	%	(13)	1.3
Total nitrogen	%	(14)	0.15
Cation change capacity	%		27.6
Clay	%	(15)	28
Silt	%		48
Sand	%		24
Moisture	%	(16)	29.7
Phosphorus	kg/ha	(17)	0.76
Potassium		(18)	2.58

*: Water holding capacity **:Total Organic Carbon.

to Tunceli Directorate of Provincial Food Agriculture and Livestock Soil Analyses Laboratory for physical and chemical analyses, in accordance with the regulations of the Turkey Ministry of Food, Agriculture and Livestock according to method T.S 9923. Properties of the soil sample is given in Table 1. According to world reference base for soil resources (WRB) classification (8), the soil type is silty clay loam. For microorganism isolation, about 10 grams of the collected soil sample was placed in sterile glass jars initially and stored at +4 C^0 in plastic containers and taken to the laboratory while kept at 4 C^0 (9). 10 g of the soil sample was diluted in 0.8% sodium chloride isotonic water up to 10^{-4} to gain an isolated bacteria. To isolate the bacteria; plate count agar was prepared according to manufacturer's instructions. After inoculation, petri dishes were taken into a 27^0C incubator for storage and the bacteria completed their development in three days. Bacteria were selected by visualy differences from others and separated. Then, they were coded as B1-B5 All experiments were performed triplicate (10). Bacteria colonies developed in petri dishes were taken separately into the sabouraud dextrose broth and stayed to reproduce under 27^0C incubation in 130 rpm.

In petri dishes, streak plate seeding was conducted for homogenous marked colonies and they decreased to a single colony. Phire Hot Start II DNA Polymerase was utilized and 1000 – 3000 bp PCR strips were obtained in different lengths with bacteria 16S ribosomal general primers.

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) 1492R 5' TACGGYTACCTTGTTACGACTT 3' positions 1492–1512) (19). Bacteria species, isolated and identified in agricultural soil are presented in Table 2.

Preparation of the pyroxasulfone solution

The pyroxasulfone was prepared from Kelt WG 85 (trade name of the herbicide) in the same concentration as used in the field (750ppm). pH of the herbicide solution was 7.0 and temperature was 27^0C. This herbicide contains 85% of pyroxasulfone active ingredient.

Microbial biodegradation studies

The growing media used in these experiments were the previously isolated and the enriched bacteria types, with 1 ml of the solutions obtained from the separately enriched solution used in the experiments. 98 ml of 0.8% isotonic sodium chloride solution, 1 ml of the pyroxasulfone and 1 ml of growing bacteria (app. 10^7CFU/ml) taken from the broth media were added to the erlenmayer flasks. Totally 6 different apparatus (5 of them includes each 5 types of bacteria and 1 of them is blank (with no microorganism) were used.

In order to assess the degradation ratio of the pyroxosulfane, firstly; from the 5 species of each bacterial cultures grown with shaker and blank solution controlled at 27°C through the broth enrichment techniques, COD measurement was carried out in 24-hour intervals according to the Standard Methods 5220C closed reflux titrimetric method according to (25) and decreasing of the substrate followed. After the end of the sixth day (at the end of the log phase on the growth curve) the COD value occurs nearly COD of the pyroxasulfone solution(nearly 18.000mg/l), 1 ml of culture (app. 10^7 CFU/ml) was taken at sterile conditions from these culture media, added into liquid media with isotonic pyroxasulfone solution, and bioremediation studies were started and continued to 8 days.

In this phase, these solutions were shaken continuously at Gallenkamp orbital incubator at 27°C. Solution samples were monitored at 24-hour intervals on the basis of the COD, BOD_5 and TOC parameter. BOD_5 measurement was carried out in 24-hour intervals according to the Standard Method 5210B (5 day BOD5 test) and for total organic carbon analyses, standard method 5310B High temperature combustion method was used according to (25) and decreasing of the pyroxasulfone followed.

Results and Discussion

The experiments were carried out using a medium inoculated with five different species of bacteria in a liquid medium, which pyroxasulfone was added, and these medium have been monitored using COD, BOD_5 and TOC analysis. The reduction rates of pyroxasulfone solution on COD, BOD_5 and TOC parameters by *Bacillus thuringiensis, Bacillus simplex, Fusarium fujikuroi, Clostridium tetani* and *Bacillus megaterium* are presented between Figure 2-6. These reduction rates in the media have showed different results depend on differences in bacterial species in the liquid medium.

COD removal rate of pyroxasulfone was observed between 60% and 91%. According to these results,

Table 2. Types of the bacteria identified and references.

Accession Number	Approximate Species Identity	Identity	Reference
KF317874.1	*Bacillus thuringiensis*	99%	(20)
KF831394.1	*Bacillus simplex*	99%	(21)
Hf679029.1	*Fusarium fujikuroi*	82%	(22)
HG530135.1	*Clostridium tetani*	93%	(23)
KC246043.1	*Bacillus megaterium*	99%	(24)

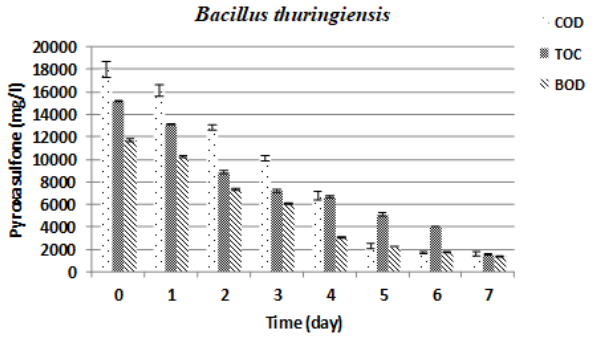

Figure 2. Reduction of Pyroxasulfone via *Bacillus thuringiensis.*

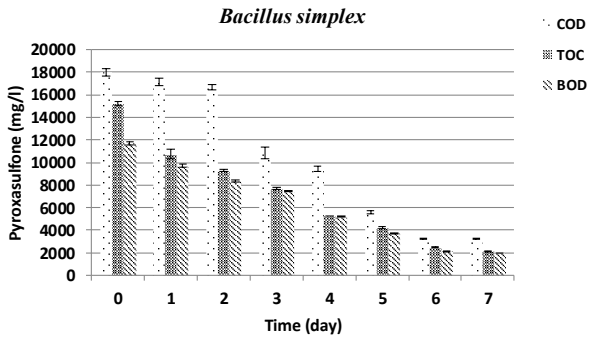

Figure 3. Reduction of Pyroxasulfone via *Bacillus simplex.*

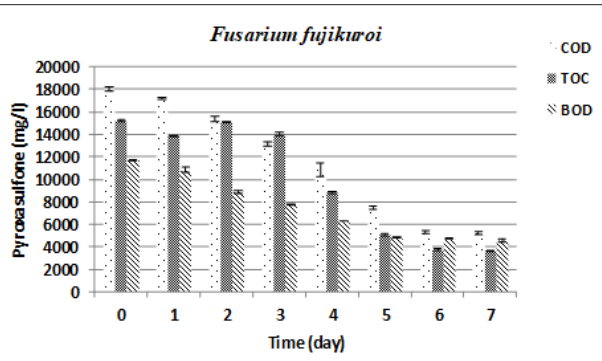

Figure 4. Reduction of Pyroxasulfone via *Fusarium fujikuroi.*

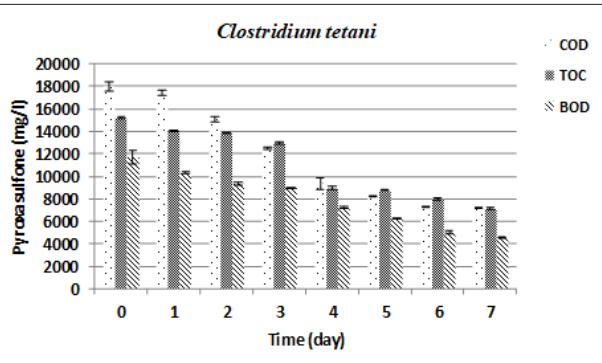

Figure 5. Reduction of Pyroxasulfone via *Clostridium tetani.*

bacillus thurigiensis has the best removal performance about 91%. At the end of day 7, The COD which was calculated as 18000mg/l decreased to 1620 mg/l on *bacillus thurigiensis.* The worst removal performance was observed with *Clostridium tetani*, decreasing the COD from 18000 mg/l to 7200 mg/l. *Bacillus simplex, Fusarium fujikuroi* and *Bacillus megaterium* showed 82%, 71% and 66% reduction rate respectively in same time period.

In the TOC studies, reduction rates were for *Bacillus thuringiensis, Bacillus simplex, Fusarium fujikuroi, Clostridium tetani* and *Bacillus megaterium* was

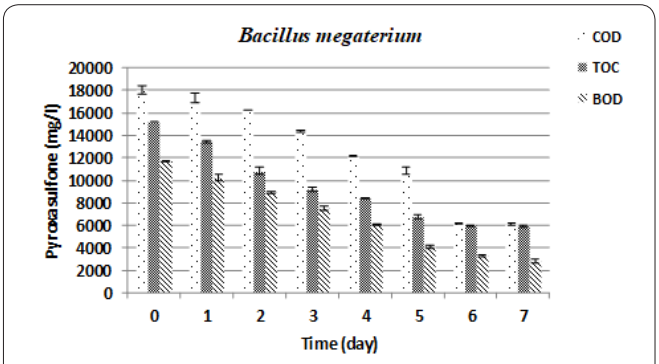

Figure 6. Reduction of Pyroxasulfone via *Bacillus megaterium.*

90%, 86%, 60%, 53% and 71% respectively in seven day period. This means the 15200mg/l of TOC value decreased to between 1520 and 7140 mg/l.

The removal performance for pyroxasulfone on BOD_5 via *Bacillus thuringiensis, Bacillus simplex, Fusarium fujikuroi, Clostridium tetani and Bacillus megaterium* was 88%, 83%, 72%, 61% and 76% respectively on 1 week. According to these results; the best performance for BOD_5 parameter seen on *Bacillus thurigiensis.* This type of bacteria decreased BOD_5 value from 11700mg/l to 1400mg/l in 7 days.

To cater their carbon requirements, soil microorganisms removed pesticide residues (26). Maya et al. (27) used *Pseudomonas, Agrobacterium* and *Bacillus* species on biodegrading chlorpyrifos by soil bacteria, As a result; they conducted with *Bacillus subtilis, Brucella melitensis, Bacillus cereus, Klebsiella species, Pseudomonas fluorescence, Pseudomonas aeroginosa* and *Serratia marcescens* species, as 46 – 72% yield at the end of the day 20, and identified that chlorpyrifos was used as a carbon source in aqueous media.

Erguven and Yildirim (28) studied to find out the efficiency of certain soil bacteria for chemical oxygen demand reduction of synthetic chlorsulfuron solutions under agitated culture conditions. They found that biodegradation rate by *B. simplex, B. muralis, M. yunnanensis, C. tetani* and *M. luteus* were 94, 78, 79, 74 and 70%, respectively.

Mixed cultures provided a more effective degradation yield compared to single cultures There are examples where herbicides were degraded in aqueous media using species that were isolated from soil polluted with trifluralin (29). Previous studies on microbial degradation of herbicide 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* (30).

Bellinaso et al. (31) studied the removal process of herbicide trifluralin in liquid media. Results of the studies conducted with cultures enriched in yeast extract media and grafted in 50 mg/L trifluralin medium demonstrated that a removal yield of 25% in *Klebsiella oxytoca*; 16% 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. Belala and Mohamed (32) 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 1 month; they observed that all 100 µg/mL concentration of pendimethalin was removed by that bacteria species. Similarly, Belal and Negwa (33), in their biodegradation study conducted with *Phanerochaete chrysosporium* species in a liquid media of pendimethalin, demonstrated that 100 ppm concentration pendimethalin was 56% removed at the end of 7 days, and 75%, 85% and 95% removed at the end of 14, 21 and 28 days, respectively.

Erguven et al. (34) investigated the microbial biodegradation of aclonifen (another type of herbicide) using liquid and soil experiments with identified cultures and mixed consortia. According to their results; isolated fungi and bacteria consortia showed the highest degradation at 93% of the Chemical Oxygen Demand (COD) over five days. For Total Organic Carbon (TOC) experimental results, bacteria mix, fungi mix, and bacteria and fungi mix, showed 86%, 88% and 88% respectively. 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.

In their another study with herbicide aclonifen performed using bacteria and fungi isolated from an agricultural area. In these laboratory experiments, five soil units were prepared with the soil samples obtained from the Thrace region and 1900µg/L aclonifen was added to each of sample. They used 1, 2, 5 and 10 ml mixed cultures (approximately 10^9CFU/ml) for biodegradation process in the same system. According to their 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 (35).

Erguven (36) studied to find out the removal performance of some soil microorganism on ethalfluralin degradation. It was observed that; where no medium was added, 48% ethalfluralin removal occured 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 fifth week, COD, TOC and BOD5 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.

One of the method of reduction of herbicides from liquid media is bioremediation and this is a natural process. In this process, microorganisms can survive by degrading a herbicides. Most of the bacteria live in agricultural fields and this situation is possible to change them to degrade herbicides more rapidly. This properties of bacteria can be used as a cheap and useful technology for biodegradation of herbicides. In addition, because using physical and chemical methods to degrade pesticide compounds are very expensive and hard. Using especially soil microorganisms is an economical and easy method so many researches have focused on biodegradation of herbicides. In recent years, most of studies regarding on biodegradation process of herbicides has been realized in fields where these herbicides are applied. Researches have been conducted around the world to determine pollutants and microorganisms in soil that have the ability to degrade pesticides. This research suggests biodegradation of herbicide pyroxasulfone with isolated some soil bacteria from agricultural field so these bacteria can improve the treatment. As a conclusion, biodegradation was high in the experiments and the results suggest that agricultural soils have bacteria for removing pesticide compounds. But the mechanisms of pesticide removal are no entirely clear. For further insight into the results, the kinetic of pesticide removal and its toxic effect on bacteria cultures need to be studied. It can be said that the results of the experiments showed important results in the development of in-field treatment systems for pesticide-contaminated waters. In addition, it was observed that *Bacillus thuringiensis* had the highest pyroxasulfone removal efficiency and it was a suitable bacteria species for bioremediation of pyroxasulfone contaminated liquid medium.

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