Removal of Aclonifen with Some Soil Microorganism as Chemical Oxygen Demand and Investigation Population-Time Relationship

Hurrem Bayhan  
Department of Environmental Engineering,  
Faculty of Civil Engineering,  
Yildiz Technical University, 34220, Istanbul, TURKEY  
Email: hurremabayhan@gmail.com

Bahar İkizoğlu  
Department of Environmental Engineering,  
Faculty of Civil Engineering,  
Yildiz Technical University, 34220, Istanbul, TURKEY  
Email: ikizoglibahar@gmail.com

Gökhan Önder Ergüven*  
Department of Environmental Engineering,  
Faculty of Civil Engineering,  
Yildiz Technical University, 34220, Istanbul, TURKEY & Tunceli University, 62000, Tunceli, TURKEY  
Email: gokhanondererguven@gmail.com

Gûrdal Kanat  
Department of Environmental Engineering,  
Faculty of Civil Engineering,  
Yildiz Technical University, 34220, Istanbul, TURKEY  
Email: gkanat@gmail.com

Abstract — The study is about the removal of Aclonifen as COD parameter and monitoring the population dynamic of isolated bacteria and fungi. Some bacteria and fungi were isolated from soil samples for their identifications before the use of herbicide. Removal rates of Aclonifen was determined by microbiological degradation with these enriched bacteria and fungi in liquid media. With these microbial cultures, removal rate by bioremediation method studies were carried out. In addition to microbial degradation, with the measurements of the turbidity and the numerical value (colony number) is monitored with isolated bacteria and fungi species. The removal efficiency for Micrococcus yuannesis was 93% and Micrococcus luteus was 70%, by the fungi Penicillium talaromyces, and Metacordyceps chlamydospora species, were 53% and 91% respectively in five days. Bioremediation of Aclonifen results have shown differences with respect to species in liquid media. Degradation time of Aclonifen in liquid culture media with isolated bacteria and fungi varied between 10 min. and 120 min.

Keywords — Aclonifen, Bioremediation, Colony Number, Herbicide, Population Dynamic.

I. INTRODUCTION

Inadequate mineral nutrient, especially nitrogen, and phosphorus, often limits the growth of hydrocarbon utilizing bacteria in water and soil. Addition of nitrogen and phosphorus to a soil has been shown to accelerate the biodegradation of the pesticides [1]. Addition of a carbon source as a nutrient in contaminated soil is known to enhance the rate of pollutant degradation by stimulating the growth of microorganisms responsible for biodegradation of the pollutant. It has been suggested that the addition of carbon in the form of pyruvate stimulates the microbial growth and enhances the rate of degradation [2]. It is observed that utilization of organic waste in the bioremediation of soil seems a highly potential area.

Pesticide biodegradation is a ubiquitous process. It has been documented in a wide range of habitats, including soils, ground water and sewage sludge, surface, sediments, etc. The ubiquity of pesticide degradation suggests that bioremediation strategies can play a significant role in the treatment of pesticide wastes. The microbial degradation of pesticides offers a promising strategy by which toxic chemicals may safely to be converted to non-toxic chemicals. For that reason there is a need to isolate and identify the microorganisms that subsist interact in contaminated fields [3].

The aerobic degradation of nitrobenzene by M. luteus Z3. Strain Z3 was able to utilize nitrobenzene as a sole source of carbon, nitrogen, and energy. Z3 tolerated nitrobenzene up to the concentration of 250mg/l-1 [4]. The biochemical and molecular modes of pesticide degradation by microorganisms have been well documented [5,6].

One of the principle mechanisms which prevents the accumulation of these chemicals in the environment is microbial degradation. But, when pesticide degradation is too rapid, pest control may be less effective. One way to increase the rate of microbial degradation of pesticides in soil is one or more previous applications of the same type of pesticide with a similar chemical structure. This phenomenon is known as accelerated or enhanced degradation and can result in economic losses to farmers [7].

Persistence of pesticides makes bioremediation of such soils challenging. It is regarded that it requires a sequence of anaerobic–aerobic conditions and a presence of a co-substrate [8]. During anaerobic phase, the pesticides are dechlorinated into more susceptible metabolites to further degradation. Sequestered fractions of these compounds show reduced toxicity, reduced mobility and are resistant to degradation [9]. For soil contaminated with chlorinated pesticides the research focuses mainly on ecotoxicological aspects. There is little studies have done to recognize its significance for their bioremediation [10].

The side effects of pesticides on the soil microflora were studied by several authors [11]. Pesticides may affect the microbial population by controlling the survival and reproduction of individual species. On the other hand, several microorganisms were reported to degrade some pesticides [12]. Population size, enzymatic activity and biodiversity of certain systematic and physiological groups of microorganisms may serve as bioindicators of changes.
taking place in the soil following herbicide application [13,14].

Bioremediation mediated by fungi and other organisms is considered to be a more environmentally approach for the detoxification of persistent organic substances in comparison with traditional chemical and physical methods, and there are several efficient potential biotechnological applications, e.g. organic pollutant biodegradation [15]. Fungi possess extracellular enzymes, many of which are highly potent and relatively non-specific to the chemical composition of the substrate, and may be induced by nutrient-limiting conditions [16].

Microorganisms are thought to play an important role in the removal of pesticides from the environment. Many bacteria that are able to degrade pesticides have been isolated from soil around the World [17]. Many members of different groups of soil microorganisms (Bacteria, Fungi, Actinomycetes and Algae) isolated from the soil are capable to degrade pesticides [18]. The goal of this work was to monitoring the removal of Aclonifen with isolated soil bacteria and fungi as COD parameter day by day and investigate the colony number related with Aclonifen and non Aclonifen media in time.

**II. MATERIALS AND METHOD**

**A. Chemicals**

The Aclonifen herbicide active ingredient, sold under the trade name “Chexic 600”, was supplied by an agricultural products shop. The physical and chemical properties of Aclonifen is given in Table I. pH of Aclonifen was 6.5 and temperature was 250C. This herbicide contains 600 gr L-1 of Aclonifen. All media for the isolation and enrichment of bacteria and fungi were obtained from Sigma Aldrich.

**B. Media Preparation**

Plate Count Agar (PCA), Malt Extract Agar (MEA), 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 1210C for 15 min to ensure a sterilized solution. After cooling, diluted agricultural soil (containing no trace of Aclonifen) in an isotonic solution was added to petri dishes. The medium pH was adjusted to 6.5 and temperature was 250C.

**C. Isolation and Enrichment of Bacteria and Fungi**

Bacteria and fungi were isolated from the soil samples using serial dilution on different media plates. Bacteria incubation took about three days, while fungi took about one week at 250C. Other isolation studies were also done at 40C and 350C, but best growing seen at 250C. After growing, the plates were screened for any colonies that were visually different from the others. 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 Aclonifen at 250C, and were shaken continuously.

**D. Isolation 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 [19].

**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 a 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. Growing fungi were scratched using a sterile blade and crushed with liquid nitrogen in sterile conditions, after which, DNA was isolated from the powder hyphaes.

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 MgCl2 / 0.4 μM forward

---

**Table 1: Physical and Chemical Properties of Aclonifen**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Unit</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>-</td>
<td>C12H9ClNO3</td>
<td>[25]</td>
</tr>
<tr>
<td>Molecular structure</td>
<td>-</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td></td>
</tr>
<tr>
<td>Molecular mass</td>
<td>g/mol</td>
<td>264.7</td>
<td></td>
</tr>
<tr>
<td>Visual at standard temperature and pressure</td>
<td>Pa</td>
<td>1.6 x 10^5 Pa 20 °C (%99.3 pure)</td>
<td></td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>Pa</td>
<td>1.6 x 10^5 Pa 20 °C (%99.3 pure)</td>
<td></td>
</tr>
<tr>
<td>Water solubility</td>
<td>g/l</td>
<td>1.4 mg/l 20 °C pH 5, 7 ve 9 (%97pure)</td>
<td></td>
</tr>
<tr>
<td>Melting point</td>
<td>°C</td>
<td>81.2 °C (%96 pure)</td>
<td></td>
</tr>
</tbody>
</table>
primer /0.4 μM reverse primer / 0.5 mM dNTP / 1 U Taq polymerase (Metacordyceps chlamydosporia) or 1.25 U Taq polymerase (Penicillium simplicissimum) and 200ng DNA.

Heat cycle conditions: 1 cycle: 940C - 3 min / 35 cycles: 940C - 15 s, 550C - 30 s, 720C - 30 s / 1 cycle: 720C 1 - 5 min.

In PCR, the expected length of bands were obtained for Metacordyceps chlamydosporia. For the Penicillium Talaromyces, One-Taq polymerase was used. 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:

For final concentrations: (total 20μl reaction volume); 1X Phire Animal Tissue PCR Buffer (includes dNTPs and MgCl2) / 0.5μM forward primer / 0.5μM reverse primer / Phire Hot Start II DNA polymeraz and H2O.

Heat cycle conditions: 1 cycle: 980C – 5 min / 40 cycles: 980C – 5 s, 720C – 20 s / 1 cycle 720C – 4 min/40C–∞

Bacteria were identified using 16S rRNA Universal Primers 27F (5'-AGAGTTTGATCCTGGCTGAG-3'; Escherichia coli positions 8-27) [20], 16S rRNA universal primers 27F (5'-AGAGTTTGATCCTGGCTGAG-3'; Escherichia coli positions 8–27) [20]. 1492 R 5'TACGGYTACCTTG GTTACGACTT 3' positions 1492–1512) [21, 22].

**Microbial Biodegradation Studies**

In order to assess the degradation ratio of the pesticide from the fungi and bacteria, 4 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 chekic 600 and 1 ml of enriched cultures (approximately 2 x 107 microorganism/ml) were added to 98 ml of 0.8 % isotonic saline water. Aclonifen was prepared in the same media was placed in a shaker incubator (Gallenkamp Orbital Incubator) controlled 250C. Turbidity measurements were done at 650 nm on Photolab 6600 UV-VIS spectrophotometer. Inoculation of bacteria and fungi were done with serial dilution method (10-7,10-8,10-9) to PCA and MEA culture media for determination of colony counts. Incubation time was carried on for 7 days, for fungi, took about 7 days. In blank media there was no Aclonifen. For carry out experiment studies in aerobic media and gaining oxygen from air, caps of the styrene bottles were limited open. Prepared culture media was placed in a shaker incubator (Gallenkamp Orbital Incubator) controlled 250C. Turbidity measurements were done at 650 nm on Photolab 6600 UV-VIS spectrophotometer. Inoculation of bacteria and fungi were done with serial dilution method (10-7,10-8,10-9) to PCA and MEA culture media for generation a colony. Inoculated petri dishes were put into an incubator controlled at 1500C. Incubation time was carried on for bacteria in media with Aclonifen 1 day, in media without Aclonifen 3 days; for fungi, in media without Aclonifen 7 days, in media with Aclonifen 10 days.

**III. RESULTS**

**A. Identification of Fungi and Bacterial Studies**

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

<table>
<thead>
<tr>
<th>Fungi Code and Approximate species identity</th>
<th>First Primer 5'-3' sequence and reference</th>
<th>Second Primer 5'-3'sequence and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Penicilliumtalaromyces</strong></td>
<td>ITS4 TCCTCGGCTTATTGATATGC [26]</td>
<td>GAAGGTTAGTCTGGTAAACAG [27]</td>
</tr>
<tr>
<td><strong>Metacordycepschlamydosporia</strong></td>
<td>ITS3 GCATCGATGAAAGAACGCAG [26]</td>
<td>ITS3 GAAGGTTAGTCTGGTAAACAG [26]</td>
</tr>
</tbody>
</table>

Table II: Primers, sequence and references used to identify the fungi

Copyright © 2015 IJAIR. All right reserved
When the results were examined, it was observed that as the turbidity increased in all the cultures, the population of microorganisms also increased. In studies using culture media with and without herbicide, although the differences between the values of the turbidity caused by the cultures and the number of population were identified, it was impossible to interpret these differences in terms of turbidity, and the number of microorganisms showing the best and the worst removal, according to the results obtained from COD studies. However, the increase in the value of turbidity and the number of population in the media with herbicide starts later than those in the blank media without herbicide. This period may extend from minutes to hours. This refers to the adjustment periods of the microorganisms (Lag phase) to the media with herbicide.

When the results were examined, it was observed that as the turbidity increased in all the cultures, the population of microorganisms also increased. In studies using culture media with and without herbicide, although the differences between the values of the turbidity caused by the cultures and the number of population were identified, it was impossible to interpret these differences in terms of turbidity, and the number of microorganisms showing the best and the worst removal, according to the results obtained from COD studies. However, the increase in the value of turbidity and the number of population in the media with herbicide starts later than those in the blank media without herbicide. This period may extend from minutes to hours. This refers to the adjustment periods of the microorganisms (Lag phase) to the media with herbicide. It also leads to the increase in the number of microorganisms using carbon and phosphorus and the value of turbidity caused by the microorganisms, along with the decomposition of herbicide.

In media with Aclonifen, results of COD by Metacordyceps chlamydosporia and Penicillium talaromycetes is given in Figure 1, results by Micrococcus yunnanensis and Micrococcus luteus is given in Figure II.

### Table III: Identified bacterial codes and their species

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Bacterial Code and Approximate Species Identity</th>
<th>Identity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC634108.1</td>
<td>Micrococcus yunnanensis</td>
<td>99%</td>
<td>[28]</td>
</tr>
<tr>
<td>KF555623.1</td>
<td>Micrococcus luteus</td>
<td>99%</td>
<td>[29]</td>
</tr>
</tbody>
</table>

### Table IV: Results of increasing of the bacteria and fungi population number in media with and without Aclonifen as Turbidity Parameter

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Media with Aclonifen</th>
<th>Media without Aclonifen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (min)</td>
<td>Turbidity (NTU)</td>
</tr>
<tr>
<td>Micrococcus yunnanensis</td>
<td>0</td>
<td>0,116</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0,135</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0,139</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0,288</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1,2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1,304</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1,369</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>1,503</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>1,726</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>0</td>
<td>0,002</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0,003</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0,004</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0,005</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0,007</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0,01</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0,016</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0,018</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0,019</td>
</tr>
<tr>
<td>Metacordyceps chlamydosporia</td>
<td>0</td>
<td>0,081</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0,124</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0,371</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0,522</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>0,641</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0,773</td>
</tr>
<tr>
<td>Penicillium talaromycetes</td>
<td>0</td>
<td>0,001</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0,002</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0,003</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0,004</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0,007</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0,017</td>
</tr>
</tbody>
</table>
**C. Microbial Activity and Colony Number Results**

Experiment results of media with and without Aclonifen by bacteria and fungi species as Turbidity and colony number is given in Table IV. According to the obtained results, graphic related with *Micrococcus yuannesis* and *Micrococcus luteus* bacteria is given in Figure III and Figure IV, related with *Metacordyceps chlamydosporia* and *Penicillium talaromyces* fungi given in Figure V and Figure VI respectively.

Fig. 1. Removal Rate of Aclonifen by *Metacordyceps chlamydosporia* and *Penicillium talaromyces*

Fig. 2. Removal Rate of Aclonifen by *Micrococcus yuannesis* and *Micrococcus luteus*

Fig. 3. Monitoring Increasing of population number of *Micrococcus yuannesis* in media with and without Aclonifen by Turbidity (NTU: National Turbidity Unit)

Fig. 4. Monitoring Increasing of population number of *Micrococcus luteus* in media with and without Aclonifen by Turbidity

Fig. 5. Monitoring Increasing of population number of *Metacordyceps chlamydosporia* in media with and without Aclonifen by Turbidity

Fig. 6. Monitoring Increasing of population number of *Penicillium talaromyces* in media with and without Aclonifen by Turbidity
IV. CONCLUSION

In the current study, the rate of COD removal was observed to occur between 91% and 53%. According to these results, *Micrococcus chlamydomphila* was observed to have the highest removal rate in terms of this parameter. By the end of the 5th day, the COD calculated as 15,600 mg/L was reduced to 1,040 mg/L by *Micrococcus chlamydomphila*, and to 7350 mg/L by *Penicillium talaromyces*, having the lowest removal rate according to this rate. The data of the other four removal rates were observed to have varied between these two values.

When the experimental results of the monitoring of the microbial activity in the Aclonifen culture medium throughout turbidity and population increase were examined, the increase in turbidity was observed to be dependent on *Micrococcus yuannesis* having the best COD removal rate in the Aclonifen medium, and *Micrococcus chlamydomphila* increasing in the population of microorganisms, particularly after the 25th and 40th minutes, respectively. It is understood from this that the adjustment period (lag phase) of *Micrococcus yuannesis* occurs within a shorter period than that of *Micrococcus chlamydomphila*. It is also understood that the same microorganisms behave differently in the medium without Aclonifen. It can be said that *Micrococcus yuannesis* propagates from the 10th minute, whereas *Micrococcus chlamydomphila* is activated from the 40th minute. There is no substrate, nutrient or Aclonifen in the medium. *Micrococcus yuannesis* started to propagate in the medium without Aclonifen from the 10th minute, and barely reached the number of population obtained in the medium without Aclonifen, approximately from the 20th minute in the Aclonifen medium. While the value of turbidity in *Micrococcus luteus* barely reached 0.019 NTU from the 120th minute, this value was observed in the 15th minute in the medium without Aclonifen.

While the increase in turbidity and the number of microorganisms in *Micrococcus chlamydomphila* had the best removal rate in the Aclonifen medium, as observed in the 40th minute, it was also observed in the 40th minute in the medium without Aclonifen that the population was four times larger in the medium without Aclonifen. This fungus was observed not to have reached the number of microorganisms that it had reached in the Aclonifen medium, that it had in the medium without Aclonifen. The lack of nutrients in the medium caused this to happen.

While the increase in turbidity and the size of the population in *Penicillium talaromyces* having the worst removal rate in the Aclonifen was observed from the fifth minute in the Aclonifen medium, as it was also observed from the fifth minute in the medium without Aclonifen, the number of individuals was approximately two times higher than that in the medium without Aclonifen.

ACKNOWLEDGMENT

This research has been supported by Yildiz Technical University Scientific Research Projects Coordination Department. Number: 2012-05-02-KAP07

REFERENCES


**AUTHOR'S PROFILE**

**Hurrem Bayhan**

received his license degree in Ataturk University Faculty of Science, Biology Department. Bayhan received his Msc and Phd. Degree in Istanbul University Institute of Marine Sciences and Management in 1984 and 1997. He still works as assistant professor at Yildiz Technical University Environmental Engineering Department.

**Bahar Iküzoğlu**

received her Bsc in Environmental Engineering In 2009 from Suleyman Demirel University and her Msc in Environmental Engineering in 2011 from Suleyman Demirel University. She is Still Phd Student At Yildiz Technical University Environmental Engineering Department.

**Gokhan Onder Erguven**

works as a research assistant at Yildiz Technical University and Tunceli University Environmental Engineering Department. Erguven received his Bsc degree In Thrace University Environmental Engineering Department in 2008. He received his Msc. Degree in Agricultural Structures and Irrigatin in Namık Kernal University in 2010 and Phd. degree in Yildiz Technical Univercity Environmental Engineering Department in 2015.