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Determination of Herbicide Resistance in Aquatic Cyanobacteria by Probit Analysis

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Abstract

Cyanobacteria are a group of bacteria that obtain their energy by photosynthesis and play a significant role in the breakdown of herbicides in nature. In this study, experiments were performed for a period of 15 days with 12 cyanobacteria obtained from different aquatic environments and three herbicides commonly used in agriculture {2,4-Dichlorophenoxyacetic acid (2,4-D), Trifluralin and Linuron}. The herbicides were pipetted at certain concentrations into BG11 broth. The herbicide resistance of the 12 cyanobacteria was determined by using Chlorophyll-a measurement, rate of growth assessment, the probit analysis and the results of the lethal concentration test ($LC_{10;50}$). According to the LC_{10} values at day 15; the *Synechocystis sp.* strain number 6 in the 2,4-D environment, the *Synechococcus sp.* strain number 41 in the Trifluralin environment, and the *Chroococcus sp.* strain number 4 in the Linuron environment, were determined to be resistant to the concentrations of herbicide. According to the day 15 values, the *Synechocystis sp.* strain number 6 was determined to be resistant to three different herbicide concentrations in the 2,4-D, Trifluralin and Linuron environments. Since it is a study that contributes to the protection of the environment, we intended for this study to set a new precedent for other studies.

Keywords: Herbicide, lethal concentration and probit analysis

INTRODUCTION

Algae are botanically defined as organisms without roots, stems and leaves that have bodies known as "thallus" and chlorophyll-a pigments. Even though cyanobacteria match this definition, they are actually microscopic prokaryotes. Similar to bacteria, cyanobacteria lack a nucleus, and their DNA is spread across the cytoplasm instead of being confined to a single location within the cell. However, unlike other bacteria, they are capable of photosynthesis by using light energy from the sun thanks to their chlorophyll pigments. They were first named as blue-green algae by Sachs in 1874. This name was given to them because of their water-soluble phycocyanin and phycoerythrin pigments. Another important pigment found in cyanobacteria is β-carotene, the precursor of vitamin A. Cyanobacteria are common in lake, pond, river, ocean coast and ocean surface habitats. Terrestrial forms of cyanobacteria can be found both on the surface and at a few cm depth of humid soils. Due to their high physiology and complex metabolism, cyanobacteria are biologically interesting and economically important organisms. There are nearly 2000 species belonging to 150 genus. Due to the sequence analyses of 16S rRNA and the similarity of their cell wall to gram negative bacteria, these organisms are considered as a phylum within the bacteria domain. There are five orders within the cyanobacteria phylum. These order are further divided into sub-categories, first according to whether they are single-celled or filamentous, and then according to their cell division morphology [1].

As a word, herbicide means "herb/weed killer." Chemical substances used as herbicides in agriculture can spread into the soil, and from the soil into lakes, streams, rainwater, underground water, plant and animal products, eventually reaching living organisms in the process. These highly toxic substances can have different half-lives and agricultural uses [2].

Numerous systematic studies have been conducted to date in Turkey's waters, and the majority of our country's water flora has been described [3, 4, 5]. In a study conducted by Cadirici, a thermophilic cyanobacterium in Balcova, İzmir, was isolated and molecularly identified [6]. A study assessing the effects of 2,4-D on the growth, the proteins and chlorophyll-a of *Chlorella vulgaris* and *Spirulina platensis* was performed in 2008 [7]. In a study conducted in Thailand with microcosm, the ecological effects of the Linuron herbicide were investigated [8]. A study performed in the irrigation channels across the Greek, Bulgarian and Turkish borders assessed the risk posed by traces of 15 pesticides including Trifluralin in aquatic environments [9]. In another ecosystem study conducted in the coasts of Florida, the environmental risks associated with four triazine, two triazinone, two urea and one pyridazinone pesticides were evaluated [10]. Comprehensive studies have been conducted to date regarding the widespread use, effects and costs of 2,4-D, Trifluralin and Linuron herbicides in Turkey [11, 12, 13].

In this study, we planned to use cyanobacteria that can reproduce in aquatic environments (blue-green algae) to breakdown herbicides {2,4-Dichlorophenoxyacetic acid (2,4-D), Trifluralin and Linuron} as a source of nitrogen and carbon. Thus, by providing optimum conditions for herbicide breakdown, we aimed to contribute economically to the prevention of pollution.

MATERIALS AND METHODS

Materials

Water samples obtained from the Kurtboğazı Dam, the Mogan Lake, the Eryaman Lake, the Uncalı Stream, the Kızılırmak River and the Bafa Lake were used for the isolation of aquatic cyanobacteria.

Isolation and Cultivation Conditions

The retrieved water samples were rapidly sent to the laboratory, and 5 ml of these samples were separately pipetted into 250 ml Erlenmeyer flasks with BG11 broth [14]. A Minitron brand shaking incubator providing a temperature of 25 °C and 12 hour light/12 hour dark conditions was used. Isolation was performed under an inverted microscope with the aid of Pasteur pipettes specially thinned by heat, and the culture collection was prepared. The isolates were kept in a 4 °C refrigerator. The identification of the cultures was performed according to morphological characteristics [1]. The experiments were performed at the laboratory of the Gazi University Faculty of Science (Table 1).

Table 1. The Cyanobacteria Isolates, and their sources of isolation

CODE NUMBER*		SOURCE
17	*Microcystis sp.*	Bafa Lake, Aydın
31	*Microcystis sp.*	Eryaman Lake, Ankara
80	*Microcystis sp.*	Bafa Lake, Aydın
44	*Microcystis sp.*	Bafa Lake, Aydın
6	*Synechocystis sp.*	Kızılırmak River, Kırıkkale
63	*Synechocystis sp.*	Uncalı Stream, Antalya
8	*Chrococcus sp.*	Kurtboğazı Dam, Ankara
4	*Chrococcus sp.*	Mogan Lake, Ankara
27	*Chrococcus sp.*	Kurtboğazı Dam, Ankara
58	*Chrococcus sp.*	Uncalı Stream, Antalya
41	*Synechococcus sp.*	Uncalı Stream, Antalya
24	*Synechococcus sp.*	Uncalı Stream, Antalya

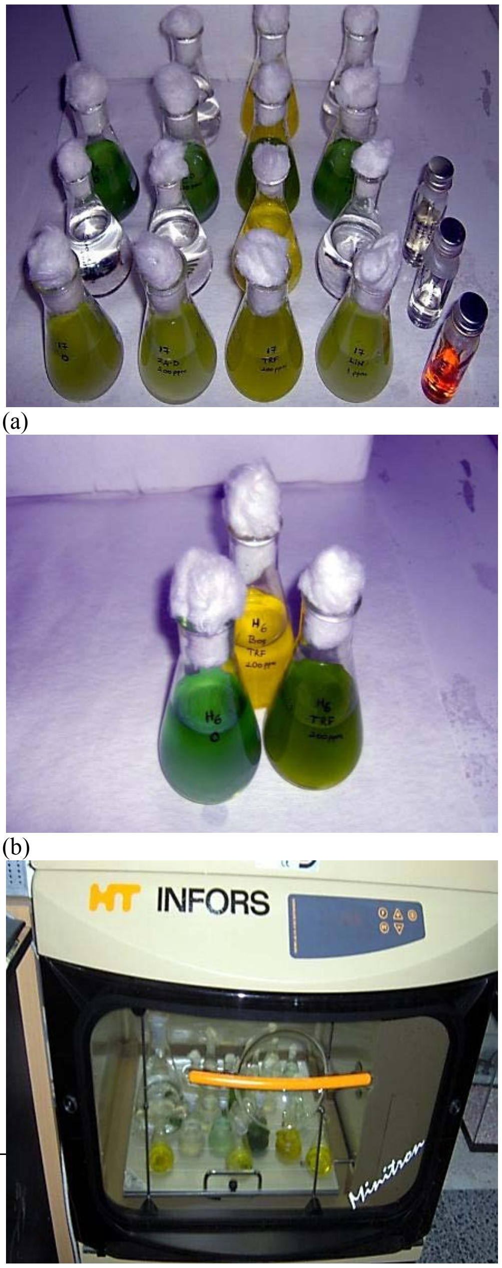
*=Gazi University Faculty of Science, Culture Collection, TURKEY

Source of Herbicide

All three herbicides were purchased as a commercial pure powder from a factory in the Afyon province of Turkey. The herbicides were dissolved in acetone and prepared for the experiment.

Chlorophyll-a Measurement

In this study, 12 cyanobacteria isolated from different aquatic environments were adjusted to 0.5 McFarland and pipetted into BG11 broth [14]. The 2,4-D, Trifluralin ve Linuron herbicides were then pipetted into the BG11 broth at certain concentrations (for 2,4-D and Trifluralin: between 50-200 mg/l, for Linuron: between 0.05-1 mg/l). The trials were performed for 15 days in a Minitron brand incubator (Figure 2). Chlorophyll-a values were measured spectrophotometrically by using samples that were taken every 3 days [15]. The trial for each cyanobacteria was performed for 15 days in two parallels. From the samples that were taken every 3 days, readings were performed with a spectrophotometer at the 630, 647 and 664 nm wavelengths and recorded.



(a) (b) (c)

Figure 2. Pictures taken with a HP M525 6x digital camera

Percent Inhibition

Percent inhibition was calculated with Abbott's formula according to the chlorophyll-a values [16]. The percent inhibition rates were separated for use in the probit analysis [17].

$$PI = \frac{(Control - Bacteria\ Chlorophyll - a)}{(Control x 100)}$$

Growth Rate

The growth rate was calculated with Abbott's formula according to the chlorophyll-a values [16]. For aquatic cyanobacteria, the data for the day 15 growth rates at a concentration of 100-200 mg/l 2,4-D and Trifluralin and at a concentration of 0.1-1 mg/l Linuron were compared with one another.

$$\text{Growth Rate} = \frac{\ln(\text{Control}) - \ln(\text{Bacteria Chlorophyll}-a\ \text{Value})}{\text{Day}}$$

Probit Analysis

It is a method that estimates biological activities by regression analysis. For the SPSS program to function, data must be provided as test parameters such as percent inhibition, optical density and fluorescence [17]. In this study, the chlorophyll-a measurements of the cyanobacteria were calculated, and the results were converted into percent inhibition rates with Abbott's formula [16]. Probit analysis was performed by using the percent inhibition results obtained on day 15 of the experiment. The lethal concentration values (LC_{10} and LC_{50}) of the three herbicides for the 12 aquatic cyanobacteria were calculated. Resistant strains were compared with one another by using column charts, and the strain with the highest resistance was identified.

RESULTS AND DISCUSSION

LC_{10} Assessment

For the herbicide 2,4-D, the LC_{10} value at day 15 was between 43.8 mg/l at the lowest and 166,8 mg/l at the highest (*Chroococcus sp.* number 4 and *Synechocystis sp.* number 8).

For the herbicide Trifluralin, the LC_{10} value at day 15 was between 3.32 mg/l at the lowest and 181 mg/l at the highest (*Chroococcus sp.* number 4 and *Chroococcus sp.* number 8).

For the herbicide Linuron, the LC_{10} value at day 15 was between 0.0001 mg/l at the lowest and 0.044 mg/l at the highest (*Synechococcus sp.* number 41 and *Chroococcus sp.* number 4).

According to the LC_{10} values, the cyanobacteria with numbers 6 (from the Kızılırmak River), 41 (from the Uncalı Stream) and 4 (from the Mogan Lake) have the highest resistance to the herbicide concentrations in their environments.

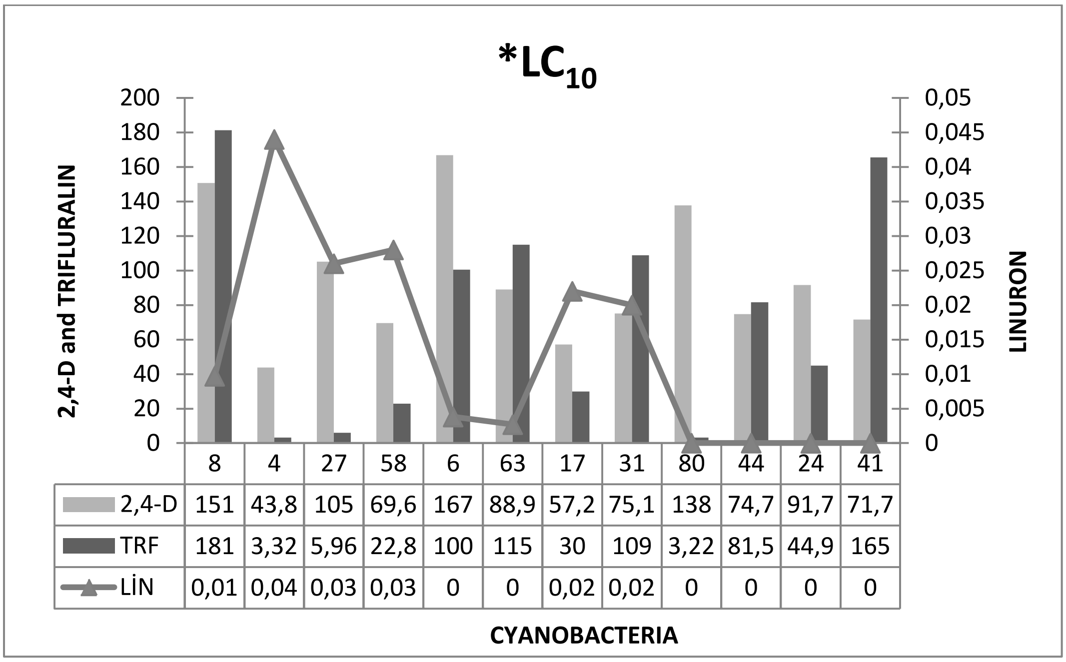
LC_{50} Assessment

For the herbicide 2,4-D, the LC_{50} value at day 15 was between 81.3 mg/l at the lowest and 747.8 mg/l at the highest (*Chroococcus sp.* with number 4 and *Synechocystis sp.* with number 6).

For the Trifluralin herbicide, the LC_{50} value at day 15 was between 139.5 mg/l at the lowest and 882 mg/l at the highest (*Chroococcus sp.* number 27 and *Synechocystis sp.* number 6).

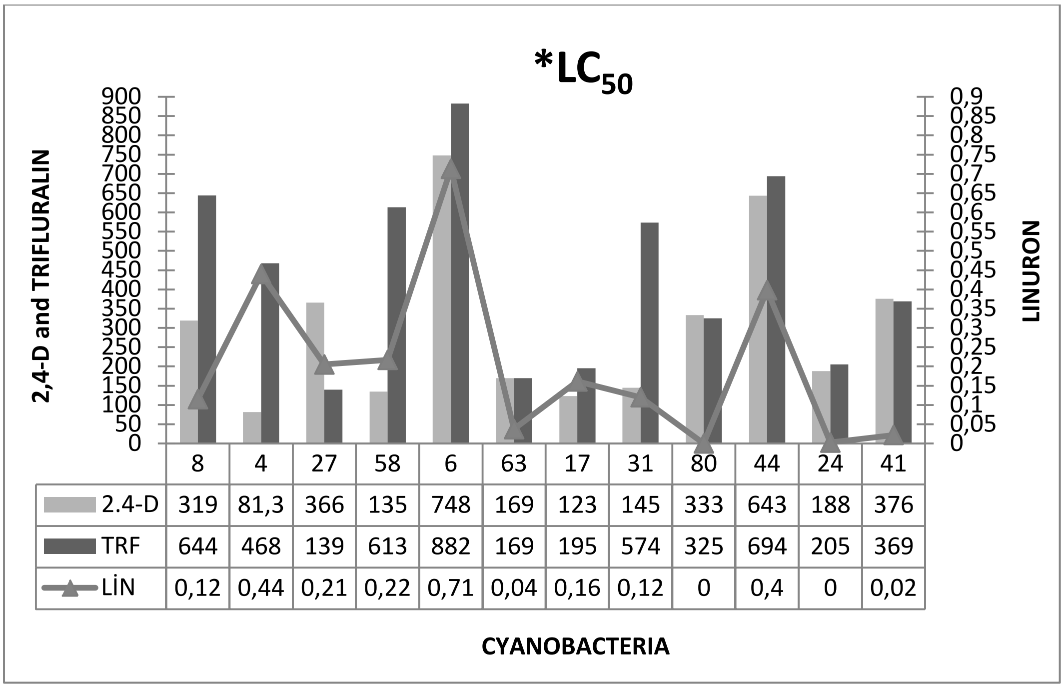
For the herbicide Linuron, the LC_{50} value at day 15 was between 0.0022 mg/l at the lowest and 0.71 mg/l at the highest (*Synechococcus sp.* number 24 and *Synechocystis sp.* number 6).

According to the LC_{50} values, the cyanobacterium number 6 (from the Kızılırmak River) had the highest resistance to the herbicide concentrations in their environments.



*Pearson's Chi Square Goodness-of-Fit in SPSS program with the calculated. (Significance level is 5%)

Figure 3. Probit Analysis Results for the LC_{10} Values of the Herbicides 2,4-D, Trifluralin and Linuron



	8	4	27	58	6	63	17	31	80	44	24	41
2.4-D	319	81,3	366	135	748	169	123	145	333	643	188	376
TRF	644	468	139	613	882	169	195	574	325	694	205	369
LİN	0,12	0,44	0,21	0,22	0,71	0,04	0,16	0,12	0	0,4	0	0,02

*Pearson's Chi Square Goodness-of-Fit in SPSS program with the calculated. (Significance level is 5%)

Figure 4. Probit Analysis Results for the LC_{50} Values of the Herbicides 2,4-D, Trifluralin and Linuron

In a ten day study conducted under light, the cyanobacteria *Microcystis aeruginosa* was able to survive at a 2,4-D dose of 1000 to 1500 ppm [18]. LC_{50} values were determined in a study that investigated the toxic effects of the herbicide 2,4-D on the two phytoplankton species known as *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*. According to this study, the LC_{50} of *Phaeodactylum tricornutum* against 2,4-D was determined as $362^{\pm}9$ ppm, and the LC_{50} of *Dunaliella tertiolecta* against 2,4-D was determined as $185^{\pm}11$ ppm [19]. In studies from the literature on herbicide degradation, it is seen that *Pseudomonas* bacteria breakdowns Trifluralin at doses of approximately 500 ppm [20]. In many studies conducted with pure and mixed cultures, it has been reported that 2,4-D could be used as a carbon and energy source by certain species belonging to the *Artrobacter*, *Pseudomonas*, *Xanthobacter* ve *Alcaligenes* genera [21, 22, 23]. In 1997, Fairchild et al. have comparatively measured resistance values against 16 herbicides with the algae *Selenastrum capricornutum* and the water plant *Lemma minor*. At the end of the 96 hour experiment, the results were evaluated in terms of LC_{50}. At the beginning of the experiment, 2.10^5 *Selenastrum capricornutum* cells and 12 *Lemna minor* (duckweed) plant had been placed in the study environment. According to the study results, the LC_{50} value for *Selenastrum capricornutum* at 96 hours was 41.77 µg/L for 2,4-D, and 673 µg/L for trifluralin. For *Lemna minor*, on the other hand, the LC_{50} value at 96 hours was > 100,000 µg/L for 2, 4-D and 170 µg/L for trifluralin [24]. In a study conducted in 2004 with the cyanobacterium *Chlorella vulgaris*, 20, 60 and 180 µg/L of the herbicide Linuron was applied to immobilized cells. While growth was observed in cyanobacteria at 60 µg/L concentration, no growth was observed at the highest dose [25].

In our study, the very high resistance values against herbicides in terms of LC_{50} for the *Synechocystis sp.* strain with code number 6 (isolated from the Kızılırmak River) were noteworthy. Yet, the tolerated values for all three herbicides vary between 0.1-1 ppm according to the EPA [26]. Based on the toxicity classification of the WHO, the herbicides are ranked, starting from the least toxic, as 2,4-D < Trifluralin < Linuron [27]. It can also be seen that cyanobacteria (strains with code numbers 6, 63, 80, 44, 24, 41), which were very sensitive to the LC_{10} values of the herbicide Linuron, have gained resistance to its LC_{50} concentrations (except for strains with code numbers 80 and 24) by means of the bleaching effect [28]. When the LC_{10} and LC_{50} values were evaluated together, it was seen that the *Microcystis sp.* strain number 44 had especially a high level of resistance to the 2,4-D and Trifluralin herbicides. This sample isolated from the Bafa lake can potentially be used for the biological treatment of the lake and its surroundings.

In the continuation of this study, the herbicide breakdown rate of the most resistant cyanobacteria will be determined with aid of chromatographic procedures (HPLC) The described section includes herbicide use and resistance studies These studies will focus particularly on strain number 6. Following the completion of the experiment, the propagation of this cyanobacteria in large scale fermenters and BG11 stock for use in herbicide-contaminated areas can be proposed. After taking the necessary precautions, strain number 6 can be used for cleaning the environment with the aid of a spray plane or pump. The economical aspect of the study, and the fact that it contributes to the protection of the environment, is promising in terms of biotechnology. Studies that can be performed at genetic level may allow for even better results to be obtained. We believe that this study will set a good precedent for other future studies.

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