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Bioremediation of herbicide diquat dibromide from aqueous solution with white rot fungus *Phanerochaete chrysosporium*

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ABSTRACT

In this study, the fungal bioremediation of herbicide diquat dibromide (DD) by white rot fungus *Phanerochaete chrysosporium* was studied. After bioremediation, the reduction in toxicity of the remediated media was evaluated by *Daphnia magna* as a model organism. For bioremediation studies, flask-level batch experiments were performed at different DD concentrations (10, 20 and 50 ppm) under static and agitated conditions. The highest reduction rates of chemical oxygen demand and total organic carbon were determined as 85.92 (for 20 ppm DD) and 87.46% (for 10 ppm DD), respectively under agitated conditions. After bioremediation, *D. magna* bioassay was used for toxicity assessment during 24, 48 and 72-h periods. In current study, it was observed that *P. chrysosporium* has very effective bioremediation capacity for diquat dibromide. The *D. magna* bioassay revealed that *P. chrysosporium* provides effective reduction in toxicity of diquat dibromide.

Keywords: *Phanerochaete chrysosporium*; *Daphnia magna*; Chemical oxygen demand; Total organic carbon; Mortality

1. Introduction

The uncontrolled use of pesticides poses dangers to the living organisms in the receiving environments such soil, water, and air. Due to the long amount of time required for their decomposition and to their accumulation in the environment and food chain, pesticides are persistent environmental pollutants. Pesticides are an indispensable part of agricultural production that contributes to the reduction of food production losses due to pests and diseases [1]. As their effect is not specific to a species, pesticides kill or harm many organisms, including humans [2]. In the processes of precipitation and irrigation, pollutants enter nearby water bodies through farmland surface runoff, farmland drainage, and underground infiltration, causing water body pollution [3]. The overuse of pesticides has caused multiple adverse impacts [4]. This malpractice is one of the critical factors leading to agricultural non-point source pollution [1,5]. Among the pesticides that are used commonly in agriculture, herbicides represent the most significant group [6].

Diquat dibromide is one of the few herbicides registered for direct application to water systems in the United

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States to control aquatic poisonous weeds [7]. Diquat dibromide (DD) is a non-selective drying herbicide that affects quickly and only damages the parts of the plant applied. DD is ionic, odorless and is suitable for agricultural use with its high solubility in water, low evaporation during application and strong bonding ability to soil [8]. Its molecular formula is $C_{12}H_{12}Br_2N_2$. It is a potential pollutant of waters with a solubility of about 620 g/L at 25°C [9].

Among aquatic biota, micro crustaceans are model organisms widely used in ecotoxicology [10], as disturbances at this trophic level can affect the food chain, posing a threat to aquatic life [11]. *Daphnia* sp. is a key freshwater crustacean with important ecological roles. They are filter-feeders that regulate the population density of different microorganisms, as algae, cyanobacteria, and protozoa [12] and they are a food source for various species of predators.

White-rot fungi have been used as an effective microorganism for degradation of pesticides due to their low specific of the functional enzymes. Moreover, many species of white-rot fungi were applied to remediation applications [13]. Moreover, previous studies reported that whit-rot fungi can biotransformation and detoxification of some pesticides in pure cultivation medium [14]. *Phanerochaete chrysosporium*, which is in the group known as white rot fungi [14], is one of the most used fungal species in bioremediation studies [15]. *P. chrysosporium*, which can live in solid and liquid environments, can degrade xenobiotics even in a limited nutrient environment [16]. *P. chrysosporium* is the first fungus to be associated with the degradation of organo-pollutants and has been extensively studied as a model microorganism in research on the lignin degradation mechanism [17]. With its extracellular enzyme systems, this fungus has an important role in the bioremediation of pesticides, polycyclic aromatic hydrocarbons and polychlorinated biphenyls [18].

There are several physical, chemical, and biological treatment methods currently being implemented for remediation of persistent organic pollutants. All these approaches pose certain advantages and disadvantages, that is not only confined to the capital investment and operational cost but also is associated with efficiency, functionality, consistency, parameters dependency and generation of non-beneficial by-product during the treatment process [19]. Bioremediation, however, is one of the most efficient methods used to degrade a wide range of anthropogenic contaminants that depends largely on the indigenous microbes and their catalytic activities. The efficiency of environmental microbial communities and their involvement in diverse metabolic pathways has been one of the prominent reasons behind the biodegradation of pesticides like chemical recalcitrant. Due to simple cellular organization, small genome size, shorter replication time and rapid evolution and acclimatization to a contaminated environment, microbes especially bacteria are the most promising candidates for various in situ and ex situ bioremediation techniques [20].

In this study, it was aimed to reveal the bioremediation efficiency of *P. chrysosporium* on DD herbicide in static and agitated media with reduction rates of chemical oxygen demand (COD) and total organic carbon (TOC). In addition, it was aimed to evaluate the toxicity of the medias using *Daphnia magna* bioassay after bioremediation.

2. Material and methods

2.1. Fungus used in the study

The white rot fungus *P. chrysosporium* ME446 was used in experimental studies. The fungus was available in culture collection of Munzur University, Environmental Microbiology Laboratory. The main stock *P. chrysosporium* culture that was in 2% Sabouraud Dextrose Agar (SDA) solid medium in Petri dishes was passaged monthly for the purpose of renewal and kept in the refrigerator at +4°C until used in the experimental study.

2.2. Preparation of diquat dibromide

Diquat dibromide (200 g/L) that was used in the study was purchased from Ertar Chemicals (Tunceli, Turkey). 10, 20 and 50 ppm DD solutions used in experimental studies were prepared from this commercial product.

2.3. Fungus pellets for inoculation

Three plugs in 1 cm diameter from *P. chrysosporium* plates were inoculated to the 250 mL flask containing 200 mL of Sabouraud Dextrose Broth under sterile conditions in laminar flow cabinet. These flasks were placed in an orbital shaker incubator at 160 rpm at 27°C. At the end of 7 d of incubation, wet pellets were harvested. The pellets used for bioremediation studies.

2.4. Experimental design

Four different application groups were designed separately for both static and agitated conditions. These groups are illustrated at Table 1. Experimental studies were carried out in three replicates. Each application medias were sterilized using JSAT-80 model (Brand: JSR) autoclave (Ha Noi City, Vietnam). HF-safe 1200 model (Brand: Heal force) (Ha Noi City, Vietnam) was left to cool in laminar flow before the fungus inoculation until it reached room temperature.

2.5. Fungus inoculation

Each medium was studied in three repetitions. For static and agitated experimental applications, 6 g of *P. chrysosporium* wet pellets were inoculated into B, C and D media under sterile conditions. After inoculation, all media were placed in a static or agitated (160 rpm) incubator at 27°C.

2.6. Bioremediation of diquat dibromide

The samples (0.5 mL) from each experimental groups (A, B, C and D) in the static and agitated medium, on the 1, 3, 5 and 7th days were taken and transferred to falcon tubes under sterile conditions. The samples in the falcon tube were kept in the refrigerator available from the laboratory until the analysis process. All samples were analyzed for COD and TOC parameters.

2.7. COD and TOC analysis

For the COD analysis, the closed reflux method specified in the standard method 5220B was preferred. According

Table 1
Experimental groups

Conditions	Groups	Contents
Static	AS (negative control)	10 ppm DD solution
		20 ppm DD solution
		50 ppm DD solution
	XS	Without DD + 6 g wet *Phanerochaete chrysosporium* pellets
	BS	10 ppm DD + 6 g wet *Phanerochaete chrysosporium* pellets
	CS	20 ppm DD + 6 g wet *Phanerochaete chrysosporium* pellets
	DS	50 ppm DD + 6 g wet *Phanerochaete chrysosporium* pellets
Agitated	AA (negative control)	10 ppm DD solution
		20 ppm DD solution
		50 ppm DD solution
	XA	Without DD + 6 g wet *Phanerochaete chrysosporium* pellets
	BA	10 ppm DD + 6 g wet *Phanerochaete chrysosporium* pellets
	CA	20 ppm DD + 6 g wet *Phanerochaete chrysosporium* pellets
	DA	50 ppm DD + 6 g wet *Phanerochaete chrysosporium* pellets

to this method, with the HACH DRB200 model thermoreactor (Delfgauw, Netherlands), the Cat. 23459-52 model COD kits were used.

2.5 mL of the sample was added to the kits, and then kept in a thermoreactor at 150°C for 2 h, first left to come to room temperature, and then COD measurements were made in the Hach DR 890 Colorimeter device (Delfgauw, Netherlands).

COD % removal efficiencies were calculated according to Eq. (1):

$$\text{COD Removal}(\%) = 100 \times \left(1 - \left(\frac{\text{COD}_p}{\text{COD}_a}\right)\right) \qquad (1)$$

$$\text{COD}_p = \text{COD}_s - \text{COD}_x$$

COD_s: COD value of mediums after incubation,
COD_x: COD values of X (S/A) groups to eliminate COD from fungal pellets,
COD_a: COD value of the negative control A (S/A) groups

For TOC analysis, the high temperature combustion method specified in Standard Method 5310B was used. SHIMADZU TOC-V Total Organic Carbon Analyzer device (Tokyo, Japan) was used for these analyses. For TOC analysis, the samples were filtered through a 0.45 μm strainer before being placed in the vials of the device.

TOC % removal efficiencies were calculated according to Eq. (2):

$$\text{TOC Removal}(\%) = 100 \times \left(1 - \left(\frac{\text{TOC}_p}{\text{TOC}_a}\right)\right) \qquad (2)$$

$$\text{TOC}_p = \text{TOC}_s - \text{TOC}_x$$

TOC_s: TOC value of the mediums after incubation,
TOC_x: TOC values of X (S/A) groups to eliminate TOC from fungal pellets,
TOC_a: TOC value of the negative control A (S/A) groups.

2.8. Adaptation of D. magna

D. magna individuals used in mortality experiments were obtained from Munzur University Environmental Toxicology Laboratory. These individuals were adapted for 1 month in a 120 L aquarium at a temperature of 16°C–18°C (±1) and a 16:8 h light:dark photoperiod and were regularly fed with dry spirulina powder and *Saccharomyces cerevisiae* solution once a day, and the aquarium was regularly ventilated with an air pump. In addition, 25% of the water was renewed in 7-d periods.

2.9. D. magna mortality experiments

For mortality assessment, a mortality application design as following was created by using the treated or untreated media taken from the shake and static groups:

Untreated control mediums (C_1: 10 ppm DD, C_2: 20 ppm DD, C_3: 50 ppm DD)
Agitated treated mediums (A_1: 10 ppm DD, A_2: 20 ppm DD, A_3: 50 ppm DD)
Static treated mediums (S_1: 10 ppm DD, S_2: 20 ppm DD, S_3: 50 ppm DD) Natural living water.

For this purpose, 200 mL media were prepared from all application groups. These media were filled in polycarbonate containers and 20 first-stage juvenile *Daphnia* individuals were collected with a pipette and carefully transferred to these containers. The water temperature in each polycarbonate container was adjusted to 20°C (±1) with air conditioning and the changes in water temperature were checked regularly. *D. magna* individuals in the groups were not fed during the application. A 16:8 h light-dark photo period was applied for all environments. The number of dead individuals in each container was counted after 24, 48 and 72 h. At the end of each hour, mortality rates were calculated as a percentage in each experimental group [21].

2.10. Statistical analysis

Statistical analysis of all data was performed using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). ANOVA analysis of variance and Duncan's multiple range tests were used in the analysis of the data. All values are given as mean ± standard error. Different letters ($^{abc}p < 0.05$) on the bars in the graphs represent statistically significant differences.

3. Results and discussion

3.1. COD and TOC removal

The COD removal rates under static conditions after treatment in all media are illustrated in Fig. 1.

According to results in the static conditions, while the removal rate was 8.16% at BS group on the 1st day, it was achieved 79.93% at the end of the 7th day. At the end of the 7th day, the lowest percentage of COD removal was observed as 61.77% and 61.83% in CS and DS groups, respectively. After 7 d of treatment, the total COD reduction was achieved at the highest value at 10 ppm DD concentration, and no statistically significant difference was observed between the removal efficiencies obtained at CS and DS groups ($p > 0.05$) (Fig. 1).

The COD removal rates under agitated conditions after treatment in all media are illustrated in Fig. 2.

According to results in the agitated conditions, the removal rate was 21.89% in CA group on the 1st day, while it was 85.92% at the end of the 7th day. After 7 d of incubation at BA, CA and DA groups, the highest decrease in COD was detected at CA group. No statistical difference was observed in the removal efficiencies obtained at BA and DA groups. In agitated medium, the highest removal on the 7th day was 85.92% in CA group; In BA and DA groups, 81.43% and 81.75% were obtained, respectively. No statistically significant difference was observed when the removal efficiencies of all groups analyzed at the end of the 1st and 5th days were compared. At the end of the 3rd day, a statistically significant difference was obtained in all groups ($p < 0.05$) (Fig. 2).

The TOC removal rates under static conditions after treatment in all medium are illustrated in Fig. 3.

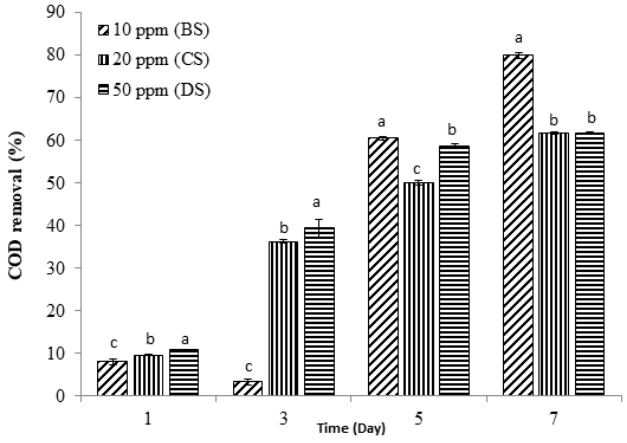

Fig. 1. Chemical oxygen demand removal rates in static conditions.

According to the results in the static conditions; at the end of the 1st day, the highest removal rates was determined as 8.39% at DS group, while it was observed as 5.15% and 5.41% for BS and CS groups. At the end of the 7th day, the highest TOC removal was obtained as 80.24% at BS group. No statistically significant difference was observed at BS and CS groups at the end of the 1st day, and at BS and DS groups at the end of the 3rd day ($p > 0.05$). A statistically significant difference was obtained at all concentrations on days 5 and 7 ($p < 0.05$) (Fig. 3).

The TOC removal rates under agitated conditions after treatment in all mediums are illustrated in Fig. 4.

According to the results in the agitated conditions; at the end of the 1st day, the highest removal rates were determined as 15.93% at DA group, while it was observed as 13.49% and 12.98% for BA and CA groups. At the end of the 7th day, the highest TOC removal was obtained as 87.46% at BA group. No statistically significant difference was observed at CA and DA groups for this period. At the end of the 3rd day, the removal rates were seen as 35.57%, 34.45% and 38.41% for all groups and at the end of the 7th day, the highest removal rates were 63.39% at BA group.

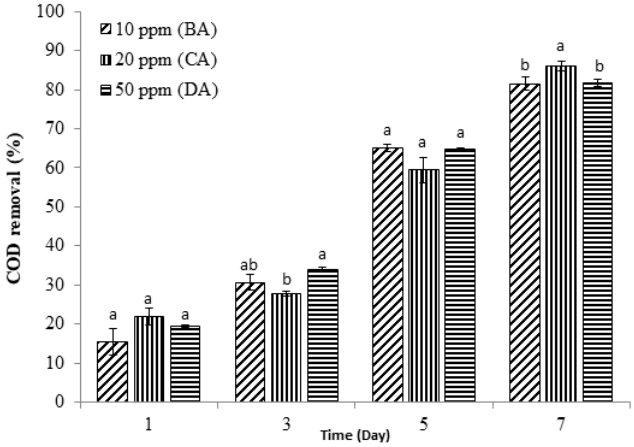

Fig. 2. Chemical oxygen demand removal rates in agitated conditions.

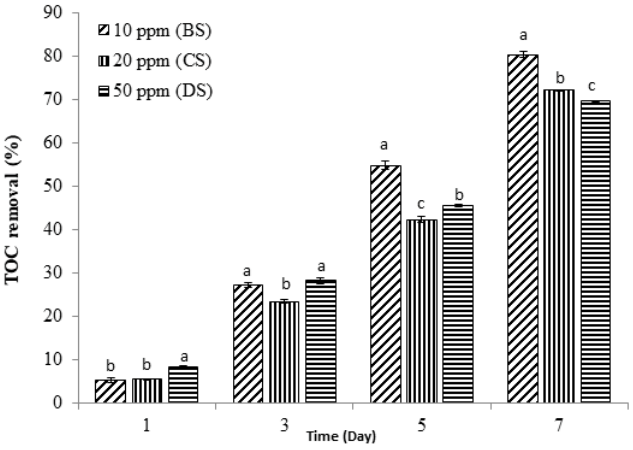

Fig. 3. Total organic carbon removal rates in static conditions.

For CA and DA groups, the removal rates were 63.39% and 59.48%, respectively. A statistically significant difference was obtained at all concentrations on days 5 and 7 ($p < 0.05$) (Fig. 4). It is thought that the increase in DD concentration in a static environment adversely affects the growth of *P. chrysosporium*. Thus, it was observed that the removal efficiency of the fungus decreased as the pesticide concentration increased, especially in the static application groups.

It was observed that the removal efficiency was higher in agitated environments than in static environments. The most important reason for this is thought to be since the *P. chrysosporium* fungus is an aerobic fungus and that the oxygenation of the environment with agitation has taken place at a sufficient level.

In a study carried out by Erguven et al. [13], COD removal rate of pesticide malathion by *P. chrysosporium* was seen at 99.6%, 98.8% and 98.7% at 50, 100 and 150 ppm concentrations, respectively after 15 d of incubation. In our study, bioremediation capacity of the same fungus species with different herbicide at 10, 20 and 50 ppm concentrations were studied. In our study, COD removal rate at 50 ppm DD concentration was 81.75% after 7 d of incubation. An effective COD reduction was detected in a shorter time compared to this previous study.

Valenzuela et al. [22] investigated the removal of the diquat pesticide by electrochemical cell method. In this study, diquat pesticide was used at concentrations of 25, 50 and 100 mg/L. It has been reported that the method provides 80% TOC and 70% COD removal, in the case of the undivided electrochemical cell, all the diquat is removed, and this value reaches approximately 92% in the case of the split H-type cell. In our study, in bioremediation experiments of diquat dibromide herbicide, higher efficiency was obtained by providing 87.46% TOC and 85.92% COD removal in agitated media after 7 d of incubation. Biological removal method we apply is more advantageous than the physicochemical methods in terms of energy consumption, chemical cost and environmental impact.

3.2. D. magna mortality

Mortality experiments were performed using the agitated and static treatment groups (B, C, and D) and the improved media taken at the end of the 7th day, using the control medium and natural living water (NLW). As a result of the experiments, *D. magna* deaths in all application environments were calculated as a percentage and shown in Fig. 5.

According to Fig. 5, at the end of the 24th hour, 91.66% death occurred in the control group (C_1) of 10 ppm DD concentration, while this rate was observed as 100% in the other control groups (C_2 and C_3). In the C_2 and C_3 groups, no living *D. magna* individuals could be detected after the 24th hour. Mortality was not detected in NLW and A_1 mediums at 24 h.

The highest mortality rate was 31.66% at static S3 medium at the 72nd hour, while this rate was 16.66% at the 72nd hour for agitated A_3 medium. In the studies carried out in agitated mediums, it was determined that the *D. magna* mortality rate observed at the end of the 24, 48 and 72nd hour was lower than in the static mediums. While the mortality rate was 100% in the untreated medium, it decreased significantly to 16.66% especially in the agitated medium. This indicates that the fungus species significantly reduces the toxicity of DD. It is known that metabolites formed because of the breakdown of pesticides may be more toxic substances [23]. However, such a situation was not observed in our study.

In a study by Sarigul and Bekcan [24] the 24 and 48th hour average lethal concentrations (LC_{50}) of the herbicide roundup containing 48% glyphosate were investigated for *D. magna*. The experiment was carried out in two series with the static bioassay method at five different concentrations (0.0115, 0.018, 0.021, 0.028, and 0.032) and a control group were used. As a result, the concentration of glyphosate that killed 50% of *D. magna* in 24 h was 0.019 mg/L concentrations and the concentration that killed 50% of them in 48 h was determined as 0.012 mg/L concentrations. This study supports that *D. magna* is a good bioindicator in terms of pesticide contamination, therefore, *D. magna* was preferred in our mortality study. Coelho-Moreira et al. [25] investigated the capacity of the white rot fungus *P. chrysosporium* to degrade the herbicide diuron in liquid cultures.

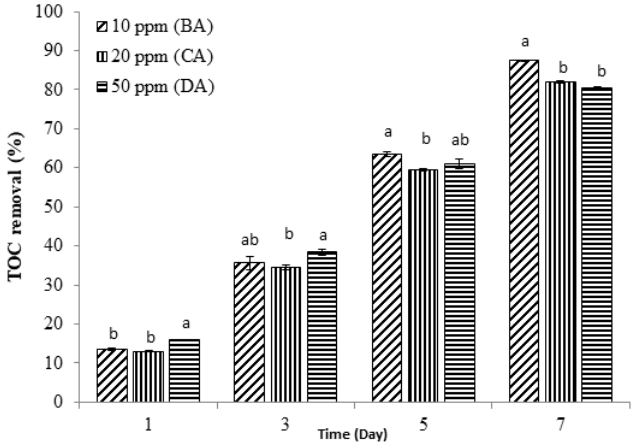

Fig. 4. Total organic carbon removal rates in agitated conditions.

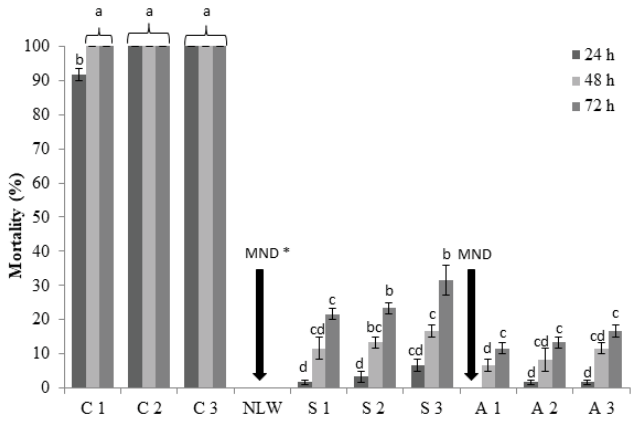

Fig. 5. *Daphnia magna* survival percentages after removal of diquat dibromide from application environments, *MND: Mortality not determined, NLW: Natural living water, C: Control, S: Static, A: Agitated) (concentrations; 1:10 ppm; 2:20 ppm, 3:50 ppm).

The herbicide at a concentration of 7 μg/mL did not cause any reduction in biomass production and removed 94% of the herbicide after 10 d of cultivation without significant toxic product accumulation. After 10 d of incubation, a significant reduction in toxicity as assessed by the *Lactuca sativa* L. bioassay was detected in cultures. This study provides evidence that *P. chrysosporium* can efficiently metabolize the herbicide diuron without accumulating toxic products. In this study, it was revealed that the toxicity values of the environment decreased similarly after the bioremediation with the same fungus species but different pesticide Villarroel et al. [26] observed the effects on survival, reproduction and growth of *D. magna* organisms exposed to 0.07, 0.10, 0.21 and 0.55 mg/L propanil. They found that when the propanil concentration increased in the medium, the growth was significantly reduced. Similarly, in this study we conducted, a 100% mortality rate in the control groups without bioremediation performed.

4. Conclusions

Since the removal of pesticides in the persistent organic pollutant class with conventional treatment methods is insufficient, new treatment technologies are needed. The cost and time consuming of the biological and physicochemical methods used for the removal of pesticides has made bioremediation applications with fungi even more important. Use of fungi in bioremediation applications; it provides many advantages such as ease of production, faster growth capabilities, economic viability of the media and environmental friendliness.

In this study, the bioremediation performance of *P. chrysosporium* on aqueous solution of DD investigated with mortality bioassays with *D. magna* before and after bioremediation steps. Higher removal efficiency was obtained from the studies carried out in agitated conditions compared to static conditions. In the toxicity tests performed after bioremediation, it was observed that the survival rate of *D. magna* indicator organism was high in proportion to the removal efficiency obtained in the agitated environment.

It was concluded that *P. chrysosporium*, which was used for the first time in the bioremediation of diquat dibromide herbicide, is effective in bioremoval of herbicides and in the elimination of toxicity in the environment after bioremediation, and it can be a suitable alternative tool for the improvement of aquatic environments contaminated with such herbicides. In addition, the data obtained in this study will set an example for new studies to reveal the effectiveness of other pesticides of this studied fungus species with similar methods.

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