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The bioremediation of glyphosate in soil media by some newly isolated bacteria: The COD, TOC removal efficiency and mortality assessment for *Daphnia magna*

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ABSTRACT

In this study, the bioremediation capacity of three isolates of *Bacillus aryabhattai*, *Pseudomonas azotoformans* and *Sphingomonas pseudosanguinis* and their consortia at glyphosate added soil medium investigated via the reduction of chemical oxygen demand (COD) and total organic carbon (TOC) parameters in filtrate waters obtained from a designed bioremediation setup. Additionally, mortality assays were performed on the filtrate waters with model organism *Daphnia magna*. According to the results of the experiments, at the end of the 11 days, the highest COD reduction rate seen in media with *S. pseudosanguinis* as 92.1% while TOC rate was 69.13% in consortia media. While the mortality rate was 10, 10 and 5% on B, P and S application groups at the end of the 72th respectively, these rates were 100% in control group at the end of the 48th. According to the results obtained from bioremediation and mortality assessment, these bacteria can reach the high bioremediation rate one by one and in consortia media.

1. Introduction

Glyphosate ($(HO)_2P(O)CH_2NHCH_2CO_2H$) is a phosphonate group herbicide generally used as active ingredient all over the world (Myers et al., 2016). This herbicide is used commonly in agriculture and forestry, for cleanup weeds in nonagricultural fields such as irrigation systems (Martínez et al., 2018). Glyphosate is known to be useful for non-target organisms, due to its active substance and presumed rapid degradation in agricultural areas. As such, this pesticide can reach receiving ecosystems, such as irrigated areas, through a combination of dry aerial and wet deposition, inadvertent overspray, and discharge from agricultural fields (Montiel-León et al., 2019). So many experimental studies have shown adverse effects on various non-target living organisms (Ruuskanen et al., 2019). Moreover, studies on recent years showed that glyphosate can stable and spread in habitat media, and that these residues of persistent organic pollutants can have a negative impact on non-target organisms and also plants (Muola et al., 2021).

Natural living water treatment mediums are an economic and sustainable alternative to intensive technologies in rural areas, although their efficiency needs to be improved (Pous et al., 2021). Bioremediation method is used as an environmentally friendly and cost-effective recovery method to treat contaminated environments such as soil, sludge

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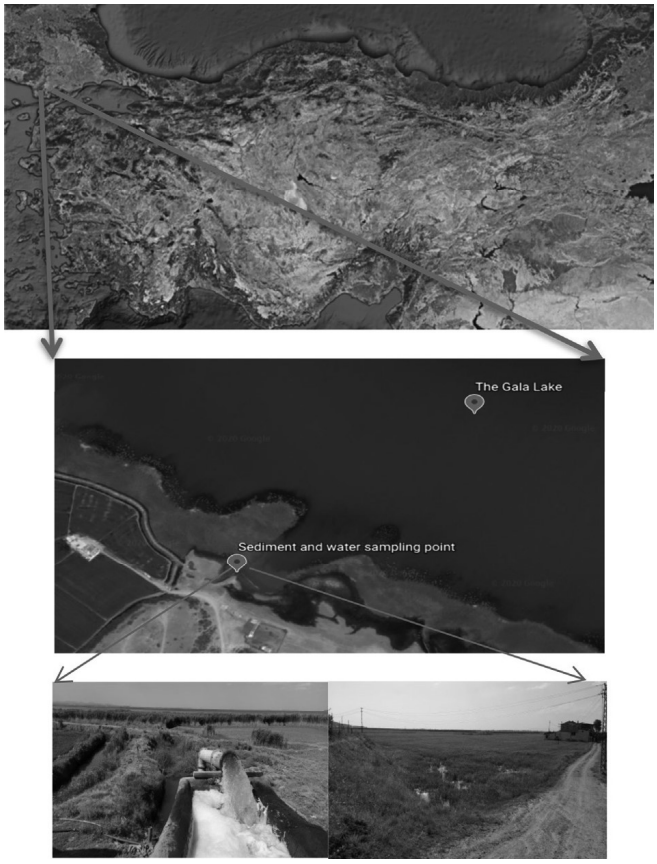

Fig. 1. Water and sediment sampling areas for isolation of novel bacteria.

and sediment (Lu et al., 2019). As one of the main type of the bioremediation method, bio-autogmentation can be a viable alternative to efficiently and quickly remove such contaminants. It is also known that the biodegradation of many persistent organic pollutants such as herbicides can be enhanced by bioaugmentation (Xu et al., 2018).

Daphnia is one of the most widely and oldest used test living in biological research. Their parthenogenetic reproduction, high fertility levels, short life cycles, and body size provide a great advantage in their use in scientific studies (Ebert, 2005). Daphnia is a food source for some fish and invertebrates. Due to these properties, Daphnia plays a substantial role in the worldwide by preparing energy transfer (Miner et al., 2012). The fact that Daphnia species are very suitable for laboratory studies has also allowed them to be used in different disciplines such as ecotoxicology and evaluation (Wu et al., 2019). Daphnia species, also called water flea, are the test organisms that are frequently used to investigate the toxic effects of numerous chemicals in the aquatic system (Edition, 2002). Examining the swimming behavior and mortality rates of water fleas, these parameters become effective biomarkers in toxicity studies (Bownik, 2017). *D. magna* have a significant role in aquatic ecosystems; there are many studies that have been used to evaluate the toxic effects of pollutants (Bao et al., 2020).

In this study we analyzed the toxicity of glyphosate herbicide in *D. magna* by waterborne exposure to environmentally relevant concentrations (1000 ppm) for two weeks. Decreasing of the herbicide from filtrated water gained from soil test units monitored via the most significant environmental parameters such as Chemical oxygen demand (COD) and total organic carbon (TOC) by 3 days period with some newly isolated bacteria one by one and consortia of them and the mortality tests were performed with the media that most reduction rate seen for these parameters.

Other control mechanism over resistance of plants includes increases in the concentration of glyphosate ingredients and gathering of highly resistant plant lines with special pesticide use. This has been the method for the use of this kind of herbicide resistant weed with glyphosate application

2. Material and method

2.1. Bacteria used in study

Water and soil sampling, irrigation water sample taken from drainage channel according to the method described in (Association et al., 1912) "Sampling standard for microbiological analysis" and sediment samples taken from rice cultivation area according to Zelles et al. (1991) in Edirne province at the Marmara region of Turkey in August 2020 (Fig. 1). These samples were taken to the laboratory at +4 °C. In the laboratory, the samples were diluted to 10^7 and

poured to the sterilized agar plate media and taken to the indicator at 25 °C for growing phase. At the end of the 5 day, the isolated cultures at the agar plates were signed and sent to REFGEN Company in Ankara province of Turkey for identification process.

DNA extraction and PCR amplification, the isolated cultures were chosen to identify the taxonomic patterns of bacterial communities. Microbial DNA was extracted using the Power Soil DNA Isolation Kit (REFGEN Laboratories, Ankara, TURKEY) according to manufacturer's protocols. The bacterial 16S rRNA gene was expanded by PCR using primers described in Figs. 2–4. PCR reactions were done in triplicate 20 μL mixtures containing 4 μL of 5x FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The PCR programs for bacterial amplification setup on a Codon Code Alligner® version 9.0 software for DNA sequencing. At the end of these procedures, *Bacillus aryabhattai*, *Pseudomonas azotoformans* and *Sphingomonas pseudosanguinis* were identified with 16SRNA method. The traces obtained from the system were described in Fig. 2. The similarity rate for these bacteria were 99, 98 and 99% respectively.

2.2. Preparation of the glyphosate solution

The herbicide glyphosate was supplied by an agricultural products store. pH of the prepared glyphosate was 7.0 and temperature was 20 °C. This herbicide contains 480 gr of glyphosate active material. Glyphosate was dissolved in distilled water.

2.3. Bioremediation setup

Holes with a diameter of 2 mm were drilled at the bottom of five sterilized plastic bottles with a diameter of 15 cm. These drilled parts were then cut out from bottom and placed at the bottom of the bottles for using as filters. 20 cm of sterilized agricultural soil (approximately 700 grams), that had not been exposed to herbicides or other PAHs previously (this has been confirmed with GC–ECD analyses) was filled in each bottles (Fig. 3).

The surface area of the five soil test units was 7.85×10^{-3} m^2. 700 ml of 1000 ppm glyphosate herbicide was added to each groups (Twice concentration of the recommended for use in agricultural fields). The bacteria used in the study were reproduced in sabouraud dextrose broth (SDB) enriched media, incubated for 7 days and had started to seek nutrients after consuming the existing substrate. Application groups were designed as control, B, P, S and consortia (approximately 10^9CFU/ml for each species of bacteria). All of the bacteria and consortia were transferred to these groups by a sterile pipette in sterile laminar flow cabinet (Fig. 3).

2.4. COD and TOC removal assays and pH determination

The COD experiments were done with HACH DRB 200 thermoreactor (heating phase to 148 °C and reaction time for 120 min) and HACH DR 890 colorimeter used for reading the concentration step. Hach COD kits that have measurement property in the range of 0–1500 mg O_2/dm3 (Cat number: 23459-52) used by the line of Standard Method 5220C closed reflux method. TOC measurements were performed with the method described in Standard Method 5310 A (burning at a high temperature) via SHIMADZU TOC-V Total Organic Carbon Analyzer device in Environmental Engineering Laboratory of Firat University. All of the experiments for COD and TOC were performed at room temperature three times (25 °C) according to the methods described in APHA (2005). The COD and TOC removal percentage were calculated by following equation.

$$\text{COD or TOC Reduction}\,(\%) = \frac{[\text{Initial COD or TOC}] - [\text{Final COD or TOC}]}{\text{Initial COD or TOC}} \text{x}100$$

The pH parameter was followed with the Standard Method 4500H+B, with the Orion 5 Star Multiparameter Device. In the experimental step, one drop of 1N H_2SO_4 was added to the filtered water to prevent the activity of cultures. This parameter was measured on filtrated water for each sample

2.5. Test organism and mortality assay

The test organisms *D. magna* used in the experiment were obtained from local markets and species determination made by Dr. B. Kutlu according to morphological properties. Water fleas were taken into the laboratory and were maintained at 16–18°C (± 1) temperature and 16:8 h light:dark photoperiod for a month to adapt to acclimatized laboratory conditions in 120 L aquarium. It was observed that the daily mortality rate during adaptation was less than 10%. The water fleas were regularly fed with mixture of a dry spirulina powder and bakers' yeast (*Saccharomyces cerevisiae*) once a day and the aquarium water was regularly aerated with an air pump. In addition, 25% of the water was renewed once every seven days with rested water.

Six experimental groups were designed for mortality assessment (Fig. 4). For this purpose 350 mL of filtrated water taken from all application groups (control, B, P, S, consortium) and natural living water (NLW) at 11th day were filled into polycarbonate containers and 10 first instar juveniles daphnia individuals were collected by pipette and gently

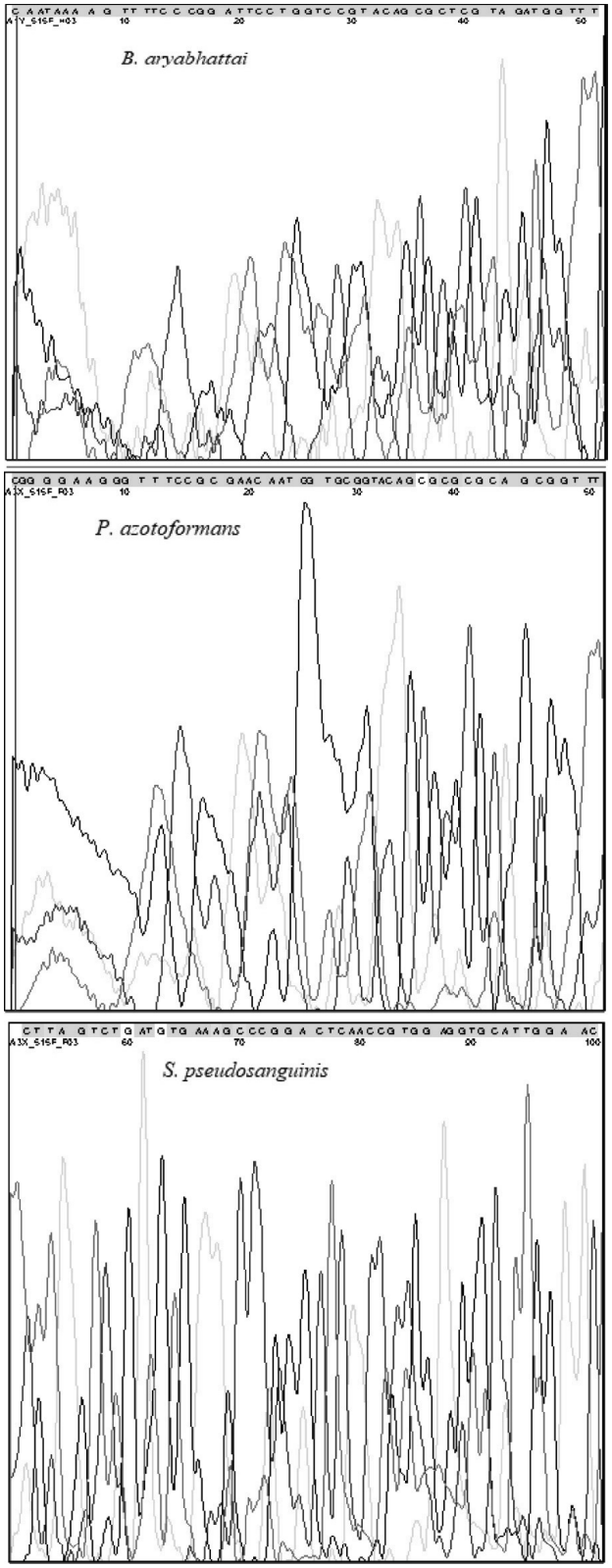

Fig. 2. Traces for *B. aryabhattai, P. azotoformans* and *S. pseudosanguinis*.

transferred to these containers. The water temperature in each polycarbonate container was adjusted to 20 °C (± 1) by an air conditioner and the changes in water temperature were regularly checked. The test organisms were not fed during the experiment and 16:8 h light:dark photoperiod was maintained. Three replicates were made for each experimental

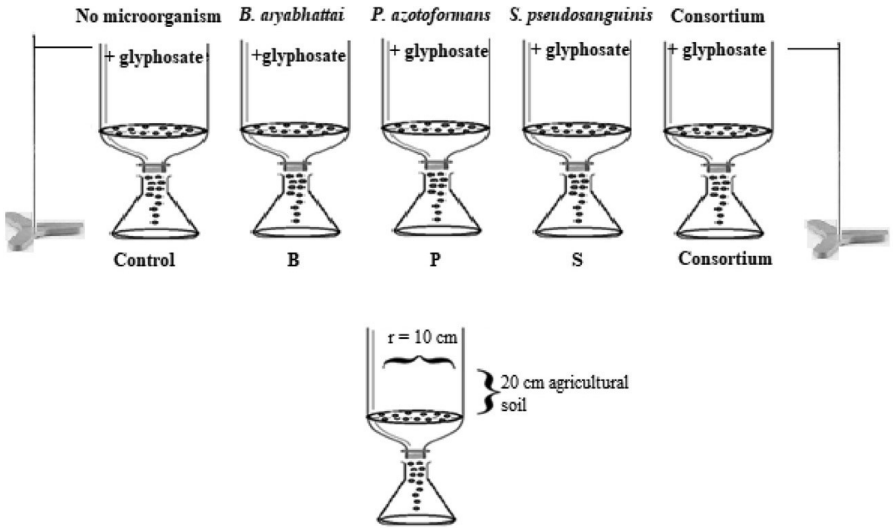

Fig. 3. The bioremediation setup, Control: soil + glyphosate without microorganism, B: soil + *B. aryabhattai* + glyphosate, P: soil + *P. azotoformans* + glyphosate, S: soil + *S. pseudosanguinis* + glyphosate, Consortium: soil + *B. aryabhattai* + *P. azotoformans* + *S. pseudosanguinis*.

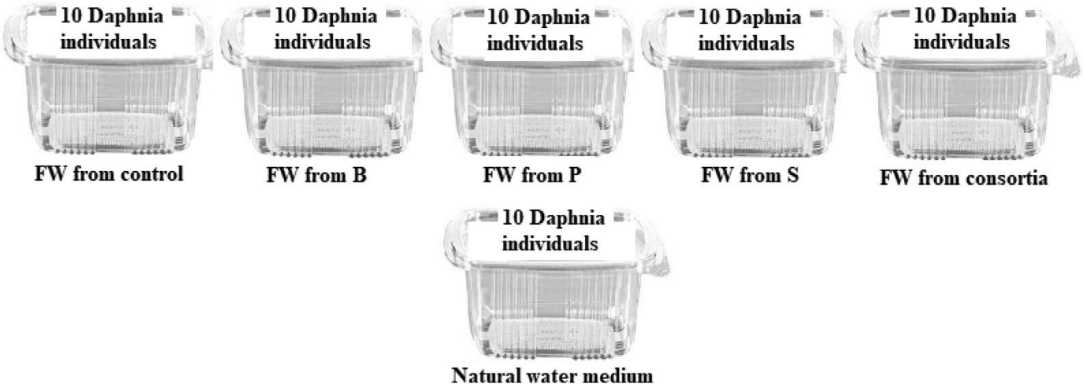

Fig. 4. Experimental setup for mortality assay, FW: filtered water.

groups. Number of dead water fleas in each containers were counted after 24, 48 and 72 h. At the end of the test period (72 h), mortality rates were calculated as percentages on each experimental groups (Babu et al., 2015).

2.6. Statistically analysis

SPSS version PASW Statistics 18 was performed for analysis of all data (SPSSInc., Chicago, IL, USA). The data were analyzed by analysis of variance (ANOVA) and the data presented are the averages of the results of three replicates with a standard error (SE).

3. Results

3.1. Reduction of COD and TOC and changing of pH in all application groups

The bioremediation study experiments lasted 23 days in total (7 days for isolation period, 5 days for enrichment period and last, 11 days for bioremediation studies). According to the results obtained, the COD and TOC removal rates of B, P, S, and consortia are shown in Fig. 5. The COD and TOC decreases in the all groups show that the system is functioning very efficiently. According to these results, the COD reduction rate of the glyphosate in B, P, S, and consortia were as 73.26, 87.1, 92.1 and 86.02% at the end of the 11th day. The TOC results were as 63.04; 67.80; 57.39 and 69.13% respectively at the end of the same time period.

pH changes in the control, B, P, S, and consortia groups were determined. These changes are shown in Fig. 6. When the pH results were discussed, it was demonstrated that the initial conditions of the study environment had a weak acidic and strong basic character within the range of 6–8. Although pH value was around 7, this situation is related with the

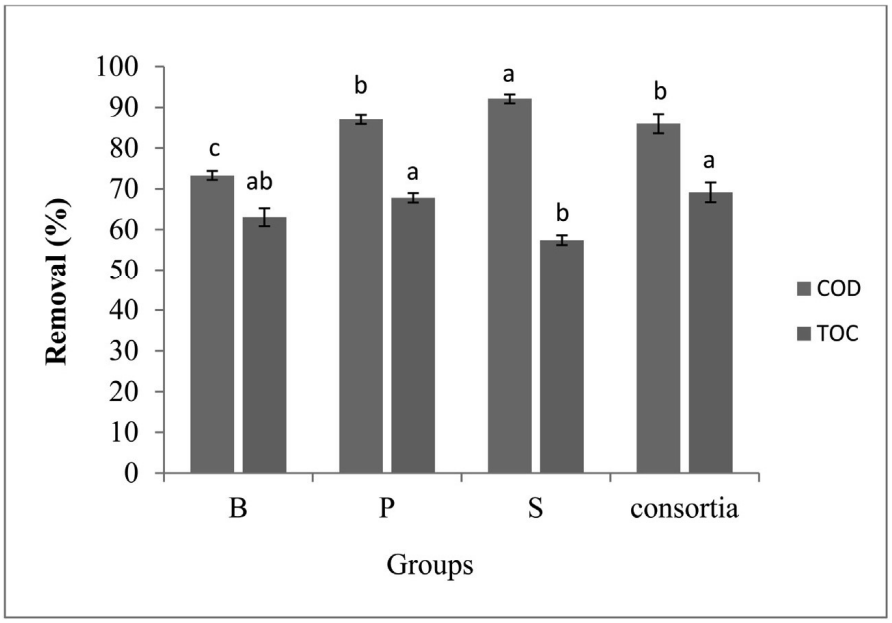

Fig. 5. Reduction of TOC and COD in application groups.

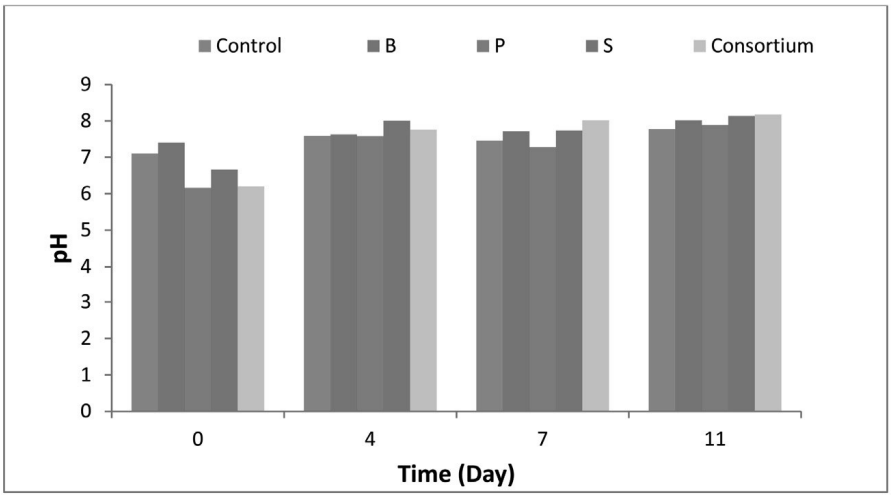

Fig. 6. Changing of pH in the soil media.

carbonic acid that is produced in the system. It is an expected result that the pH increases due to the microbial activity against glyphosate in system.

3.2. Mortality assessment

The mortality of the application groups was analyzed using *D. magna* as a model organism. Daphnia is one of the most useful model organisms for eco toxicological assays as it is sensitive for a wide range of pollutants. In the present study, the mortality rate of Daphnia in B or S groups were much lower, 5, 5 and 5% while, the control showed 85, 100 and 100% after 24, 48 and 72 h, respectively. All *D. magna* died at control group with an exposure time of 48 h. On the other hand 10, 10, 10% and 10, 10, 10% of the mortality rate was recorded for P and consortium, respectively. Additionally, the mortality rate in the natural water medium was the same rates with other application groups except for control (Fig. 7).

4. Discussion

The bioremediation process of herbicides related with the activity of microorganism (Field et al., 1995). Soil bacteria removed herbicide residues to gain their nutrient requirements (Erguven and Yildirim, 2016). It was determined that

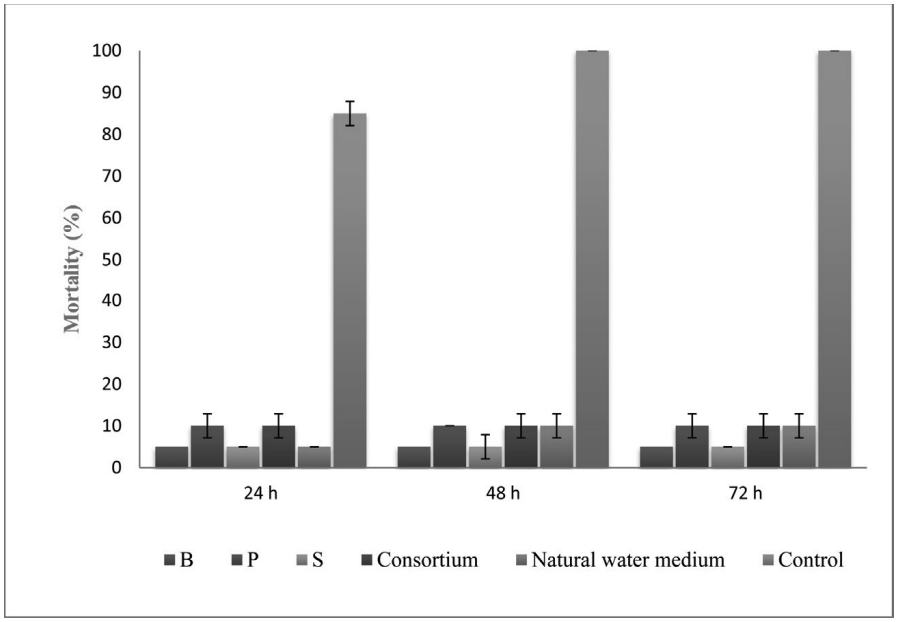

Fig. 7. *D. magna* mortality rates for all application groups.

Penicillium species enzymes could metabolize micro-pollutants such as herbicides and could remove the toxic effects of the chemicals that contain by 76% in 15 days, and 94% in 30 days by degrading the lignin (Siripong et al., 2009). In experiments conducted in aerobic and anaerobic facilities where mixed bacteria media were used, the literature demonstrated that endosulfan was degraded up to 96% biologically (Kumar and Philip, 2006).

Góngora-Echeverría et al. (2020) identified the degradation of glyphosate in pure strains and microbial consortium. They revealed out the bioremediation efficiency by inoculation, even in the remediation of agricultural soil contaminated by herbicides in agricultural fields higher than 90% for glyphosate with mixed consortia of Pseudomonas nitroreducens, *Ochrobactrum* sp. B18 and *Pseudomonas citronellolis strain ADA-23B*

Erguven and Yildirim (2016) investigated the bioremediation rate of chlorsulfuron herbicide via the reduction of COD parameter. They revealed that the removal rate of *B. simplex, B. muralis, M. luteus, M. yunnanensis,* and *C. tetani* were between 70 and 93% at the end of 4.5 days. In a study conducted on biodegrading chlorpyrifos by soil bacteria, Tatar et al. (2020) examined insecticide methomyl remediation toxicity. As a result of their study, at the end of 8 days, *Ochrobactrum thiophenivorans* and *Sphingomonas melonis* performed reduction as 94.7% and 96.8% on COD experiments.

There are examples where dinitroniline 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 to single cultures Erguven and Yildirim (2019) 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* (Zayed et al., 1983).

Belal and Elkhateeb (2014) conducted a study on bioremediation of pendimethalin, another diniroaniline 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 Elkhateeb (2014), 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 days, and 75%, 85%, and 95% removed at the end of 14, 21 and 28 days, respectively.

To check terrestrial and aquatic plants because it is a widely used herbicide glyphosate worldwide, evaluating the toxic potential against other non-target species is of great importance (Williams et al., 2000). It is also known that arthropods are more sensitive to toxic chemicals than other living organism like fish, birds and mammals. This is related with the increased sodium channel sensitivity, smaller size of their body and metabolism difference and increasing risk with decreasing of the metabolic rate (Maund et al., 2011). The level of a response of Daphnia is related with the contamination level and time of exposure to toxic substances (Ren et al., 2009). It has been reported that first instar juveniles of Daphnia are more susceptible to toxic pollutants than adults (Day and Kaushik, 1987; Hanazato, 1991). *Daphnia* cannot directly treat influent and primary wastewater. However, theoretically it is worth noticing that the inhibition of *Daphnia* in the presence of COD or BOD could be related to the toxicity of the organic matter, depletion of oxygen due to heterotrophic growth phase or to a combination of them (Pous et al., 2020). Because of in this case, we used 10–15 d-old *D. magna* in our experiments to examine mortality during short exposure periods (24, 48, and 72h). In our study, the r**e**duced mortality in application groups (B, P, S, and consortium) could be due to bacterial degradation of glyphosate. The increased mortality

rates in control group as the glyphosate is not degraded could be due to the effect of stressed and alteration of the various organs, enzymes or metabolites. Similarly Aghoghovwia and Izah (2018) reported that the environmental toxicants could be affecting the various organs or systems of *D. magna*. According to Colomer et al. (2019), *Daphnia magna* can survive longer when exposed to foreign materials than in calm conditions, provided food concentrations do not limit their capacity to biodegradation.

The results of mortality assays showed that our bacteria isolates (especially *S. pseudosanguinis*) are highly capable of detoxifying glyphosate. Similarly Zhao et al. (2015) reported that Pseudomonas spp. strains GA09, GA07 and GC04 established the best biodegradation proficiency against glyphosate and were used for the laboratory experiments of bioremediation of glyphosate.

5. Conclusion and future perspective

This study is the first report on the bioremediation of glyphosate-contaminated soil mediums with newly isolated bacterial strains and the evaluation of mortality with Daphnia. Decreased TOC and COD values in application groups (P, B, S, and consortia) showed that the inoculation of the newly isolated bacteria (*B. saryabhattai*, *P. azotoformans* and *S. pseudosanguinis*) enhanced the biodegradation of glyphosate in the designed soil bioremediation setup. This manner was also proven by the decrease in mortality values of *D. magna* compared to the control group. This bioremediation efficiency may be because the bacteria we used in our study were isolated from soil and water sources contaminated with glyphosate. Although, further investigation is needed, the bacterial isolates used in this study constitute a promising tool for bioremediation of receiving area contaminated with glyphosate.

CRediT authorship contribution statement

Volkan Korkmaz: Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Writing - review & editing. **Numan Yildirim:** Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Writing - review & editing. **Gokhan Onder Erguven:** Conception and design of study, Acquisition of data, Writing - review & editing. **Barbaros Durmus:** Writing - original draft. **Yasar Nuhoglu:** Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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All authors approved the version of the manuscript to be published.

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