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ORIGINAL PAPER

The investigation of bioremediation potential of *Bacillus subtilis* and *B. thuringiensis* isolates under controlled conditions in freshwater

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Abstract

Bioremediation is widely used to remove water pollution as environmentally friendly smart solutions. In this study, *Bacillus* isolates were investigated in terms of the effectiveness of single and multiple cultures in eliminating aquatic pollution related to aquaculture activities. In the established experimental setups, the environments where *Bacillus* isolates were inoculated with single and multiple cultures at 1×10^7 CFU/mL were evaluated comparatively with control groups without these isolates, and total aerobic mesophilic bacterial counts were performed in the petri dish by inoculation method. At the end of the 6 days of the experiment, in the environment in which single and multiple cultures of *Bacillus* isolates were presented with 17–20 ± 0.05 °C temperature and 5.1–8.1 pH 2–4.6 mg/l dissolved oxygen values (O_2), 2% increase in total phosphorus (TP) value was observed. On the other hand, 4% removal of Ammonia-nitrogen (NH_3–N), 80% removal of Nitrite-nitrogen (NO_2–N), and 100% removal of Nitrate-nitrogen (NO_3–N) were observed. In the changes in heavy metal concentrations, the removal of Ni, Cr, Se, Al, Cd, Mn, Fe, and B was observed from highest to lowest as 57%, 50%, 50%, 43%, 40%, 23%, 5%, and 2%, respectively. It also has been seen that *B. thuringiensis* isolate was observed to be more effective than *B. subtilis* in metal removal.

Keywords *Bacillus* · Bioremediation potential · Aquaculture · Heavy metal

Introduction

Organic substances from seafood waste can contaminate receptive water bodies and cause eutrophication (San Diego-McGlone et al. 2008; Burkholder and Shumway 2011). Organic materials from aquaculture can develop anoxic conditions in their environments and affect the ecology of benthic organisms adversely (Gowen and Bradbury 1987; Kristensen 2001). Advanced treatment methods have been developed which can be effective in eliminating deficiencies in existing treatment systems to restore the deteriorated natural balance and to clean the soil/water resources. Bioremediation is a globally applied environmental restoration method that promises to reduce environmental pollution. In this method, organic and inorganic pollution is destroyed by microorganisms (metallic and radionuclide pollution) (Adams et al. 2015; Vural et al. 2018; Nie et al. 2020). In many studies in this field, isolation of microorganisms, development conditions and resistance mechanisms against xenobiotics (substances that affect heavy metal/growth, antibiotics), as well as biotechnological and environmental compatibility, remediation potentials were also investigated in different environments or bacteria (Altuğ and Bayrak 2003; Altuğ et al. 2006; Altuğ and Okgerman 2008; Barlas Turan and Altuğ 2011; Çardak and Altuğ 2014; Chandrasekaran et al. 2015; Chen et al. 2018; Akay and Tezel 2019; Chang et al. 2019).

Bacillus emerges as the most promising bacterial group in this regard. Also, information on the use of these bacteria in aquatic ecosystem pollution and the remediation mechanism is very limited. The potential effects of water

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co-contaminations on the degradation process and path have not been evaluated at all or have been studied with a few limited parameters.

In this study, the biological healing potentials of domestic isolates of *Bacillus thuringiensis* and *B. subtilis* were investigated in the elimination of aquaculture wastes (feed waste, feces, etc.) under controlled conditions; total nitrogen, nitrite, nitrate, phosphorus, sulfate levels and some physic-chemical water parameters [dissolved oxygen, pH, temperature, conductivity, turbidity, color, free chlorine, total chlorine, alkalinity, total hardness] and heavy metal [aluminum (Al), iron (Fe), cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), boron (B) and selenium (Se)].

Materials and methods

Bacteria used in the study

Bacillus thuringiensis strain CP-1(Bt) and *Bacillus subtilis* strain FD-1(Bs) bacterial isolates used in the study were obtained from Atatürk University Faculty of Agriculture Plant Protection Department Culture Collection of Microbiology Laboratory. These bacterial isolates had been identified from *Ricania simulans* and *Malacosoma neustria* (Dadasoglu et al. 2013; Gokturk et al. 2018).

According to the differences in fatty acid methyl esters, it was identified in the microbial identification system (MIS) and nitrogen fixation properties were determined according to the development status in nitrogen-free media in the microbial identification system (MIS) according to differences in fatty acid methyl esters by isolating them from nitrogen fixation properties were determined according to the development status in nitrogen-free fattening medium. Bacteria were stored at – 86 °C in stock media containing Loria Broth (LB) and 18% glycerol.

Development of bacteria used in experimental setups

Frozen bacterial cultures [*B. thuringiensis* strain CP-1 (Bt) and *B. subtilis* strain FD-1 (Bs)] were planted in petri dishes containing NA medium, and they were incubated at 30° C to obtain fresh cultures for 24 h. Nutrient broth (NB) was taken from the fresh cultures, extracted from pure colonies, and transferred to the medium. It was developed for 24 h at 30 °C in a horizontal shaker incubator. Then, it was mixed in the vortex device and its concentrations were adjusted to 1×10^7 CFU/mL (colony forming unit/milliliter) according to the standard turbidity tube.

Determination of 16S-23S rDNA gene sequences of bacteria

DNA isolations were performed using the DNeasy Tissue Kit (QIAGEN) from the bacteria isolates. Then the 16S-23S rDNA region was amplified with Universal primers (27 Forward: AGAGTTTGATCCTGGCTCAG and 1492 Reverse: GGTTACCTTGTTACGACTT) (Tyler et al. 1995). The PCR reaction was done with minor changes of Moussa et al. (2013) method. The obtained PCR samples were visualized in 1% agarose gel after 50 min of execution at 90 V and purification was done with the PCR purification kit (Invitrogen). Performing a sequence analysis of the purified sample (Refg Biotechnology Firm, Turkey) was analyzed by Bioedit.

Designing the test setup and inoculation of bacteria into the test setup

At the Atatürk University Faculty of Fisheries Aquarium Fish Application and Research Center, the current (dirty) water of the aquariums that are in routine use has been transferred to the experimental setups created in an aseptic condition (using sterile materials) in an equal volume (2.7 L in plastic containers). For this purpose, a total of 15 aquarium arrangements are designed as control and application groups. As a qualitative bioindicator, a total of 30 goldfish (*Carassius auratus*, Linnaeus 1758) were distributed to these aquarium arrangements by chance, with 2 fish in each arrangement. Experiment groups are designed with three replications, each application group and its contents are given in Fig. 1

In the experiment, control groups; C(+): a commercial product containing *Bacillus* used in pollution control [(BioPure, Kaelsa Ltd. Sti., Ankara) (this product was applied as 500 mL to 1 scale (6gr) considering the user manual), and the other is C(−): does not contain any products/applications as a negative control (Fig. 1). In the experimental groups formed with pure bacteria was designed as singly and multiple (Bt group, Bs group and Bt + Bs group). These bacteria, which will be transferred to the experimental setup, are removed from the freezer and planted in petri dishes containing nutrient agar (NA) medium and incubated for 24 h at 30 °C and fresh cultures are obtained. After transported to each of these cultures to the medium containing 250-mL nutrient broth (NB), the isolate numbers of the bacteria were written on. The absorbances of these cultures, which were developed for 24 h in a horizontal shaker incubator, were adjusted to 1×10^7 CFU/mL with sterile distilled water. From the bacterial cultures in the concentration of 1×10^7 CFU/

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Fig. 1 Application contents in experimental setups

mL prepared into the experimental setup, application was made to each aquarium based on the ratio of 1/1000 vaccination culture considered in the production of biological products. In this application, taking into account the static test procedure, a solution containing bacteria was applied to every 100 mL as 1 mL, 1×10^7 CFU/mL.

Monitoring of devices by experiments

The samples of bacteria inoculations were taken from the aquariums at the Aquarium Fish Aquaculture Research and Production Unit Trial Unit and transferred to sterile bottles. The labeled samples were brought to the Faculty of Fisheries Laboratories, preserving the cold chain and stored until analysis at + 4 °C. Before the applications are carried out in the waters transferred to the test setups, all parameters were analyzed [total aerobic bacteria number (CFU/mL), water quality parameters and heavy metal content], and these data were recorded as trial start data (day 0 data).

Determination of total aerobic bacteria levels

For each water sample brought to the Faculty of Agriculture Plant Protection Department Laboratories; 10 mL of serial dilutions ($10^{-2-3-4-5-6-7-8-9}$) were prepared by transferring 1 mL of water to 9 mL of sterile peptone dilution tube. Dilution tubes were mixed separately and 100 µl of solution was transferred to NA and six petri dishes were inoculated. These petri dishes were left for incubation (48 h at 30 °C in aerobic conditions) and the formed colonies were counted. This was done three times at 2-day intervals (on the 2nd, 4th and 6th days).

Water parameters analyses

These analyses were done in the laboratories of Atatürk University Fisheries Faculty Laboratories and Erzurum Metropolitan Municipality Water and Sewerage Administration (ESKİ) General Directorate Treatment Plant. For this purpose, temperature (°C), dissolved oxygen (mg/L) and pH values were determined daily during the sampling process. Conductivity (µS/cm^{-1}), turbidity (NTU), color (Pt–Co), free chlorine (mg/L), total chlorine (mg/L), alkalinity (µg/L), ammonia (mg/L), sulfate (mg/L), nitrite (mg/L), nitrate (mg/L), total phosphorus (mg/L) analyzes were performed two times at begining (on the 0. day) and at the end of trial (6th days).

Some physico-chemical analyzes in water

During the trial period, water temperature, pH and electrical conductivity were measured daily with the Crison-MM40 multiparameter instrument, dissolved oxygen with WTW-340I oxygen meter and turbidity with Hach 2100AN turbidimeter device. Free chlorine, total chlorine, and alkalinity in water were determined in accordance with SM 4500 Cl-G chlorimetric method with Palintest Pooltest6 rapid test kits (0–5 ppm sensitivity) and color analysis was determined with Hachlange DR500 spectrophotometer device at 465 nm. For the total hardness analysis in water, titration was performed with N/100 EDTA in the titrimetric method. The results are calculated from the equation below (Anonymous 1995).

$$\text{Total hardness } \left(\text{mg/l } CaCO_3\right) = A \times 10,$$

A = Amount of N/100 EDTA spent (mL).

Total nitrogen and phosphorus analysis

Ammonia-nitrogen (NH_3–N) It was made with Nessler technique according to APHA (2010). In this method, the color intensity due to the concentration of the yellow color given by the Nessler reagent was read on the Backman brand spectrophotometer at a wavelength of 410 nm. *Nitrate-nitrogen (NO_3–N)* It was made according to APHA (2010) using brucine sulfate. The color change (formation of yellow

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color) formed in this reaction was detected at a wavelength of 410 nm. *Nitrite-nitrogen (NO_2–N)* It was made according to APHA (2010) using N-1-naphtylethylenediamine. In the method, diazonation of sulfanilic acid and color change with N-1-naphthylethylenediamine dihydrochloride were detected by reading at a wavelength of 523 nm in the spectrophotometer (Backman brand). *Total phosphorus (TP)* In the first stage (digestion process), persulfate disintegration technique was used, and it was determined according to APHA (2010) by the orthophosphate ascorbic acid method, which was released after disintegration. The results were read on the Backman brand spectrophotometer at a wavelength of 720 nm. *Sulfate (SO_4)* It was made according to APHA (2010) using barium chloride. The results were read on the Backman brand spectrophotometer at a wavelength of 410 nm.

Heavy metal analyses

Every heavy metal in the water (aluminum (Al), iron (Fe), cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), boron (B) and selenium (Se)] were performed at the end of the experiment with ICP-OES THERMO/ICAP 6300 DUO brand ICP-OES device in accordance with EPA 200.7 standard and the concentration values were calculated in µg/L.

Removal ratio was calculated as "%" in removing heavy metal and nitrite by accepting the control group as reference.

Statistical analyses

In this study, the appearence of difference between the data of some water quality parameters was analyzed in IBM SPSS 20 package program using two-factor variance analysis (ANOVA) in factorial order, DUNCAN test was applied in determining the significance level between the groups and it was important at $p < 0.05$. Levels were determined. Data were transferred to the tables as mean ± standard deviation. Bacterial count results were done three times and the results were reflected on average.

Results and discussion

Diagnostic results of bacteria

Sequence diagnosis results of bacteria used in the study according to 16S-23S rDNA gene sequences; FD-1 isolate was identified as *Bacillus subtilis* with a similarity of 98.8%, and CP-1 isolate was identified as *Bacillus thuringiensis* with a similarity rate of 81.1%. Both of these bacteria had been isolated from insects in previous studies, the *B. thuringiensis* strain CP-1 isolate has an entomopathogenic feature (Sarac et al. 2011; Tozlu et al. 2011; Dadasoglu et al. 2013; Tozlu et al. 2019a) and *B. subtilis* FD-1 strain has antifungal properties (Tozlu et al. 2019b; Tekiner et al. 2019).

It had been determined that *B. thuringiensis* strain CP-1 isolate causes 52% death in mosquito larvae (*Culex pipiens*) at 8×10^8 CFU/mL, it is well developed in nitrogen-free fattening medium, has a high tolerance to salt but not resistant to antibiotics (Dadasoglu et al. 2013).

Bacteriologic count results

The total number of aerobic mesophilic bacteria (CFU /mL) of water sampling with performed bacteriological analysis at the time intervals determined in the control and trial groups as a result of bacterial inoculations applied by the static method (day 0, 2, 4 and 6) are given in Fig. 2. At the beginning of the experiment (day 0), the number of bacteria

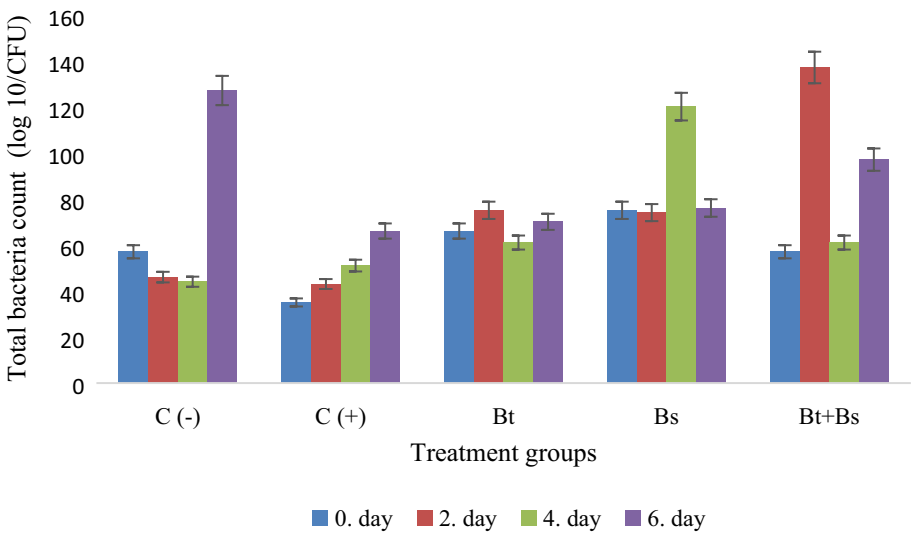

Fig. 2 Total bacteria count results for bacteria applied aquariums. C(−): Aquarium water without any application, C(+): Commercial product used aquarium water, *Bt Bacillus thuringiensis* CP-1 included aquarium water, *Bs Bacillus subtilis* FD-1, *Bt* + *Bs Bacillus thuringiensis* CP-1 + *Bacillus subtilis* FD-1 included aquarium water

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in all experimental groups was determined at the level of approximately 55–75 × 10^4 CFU/mL. The highest bacteria level was found in Bs, Bs + Bt groups. In the bacteriological counts of the control groups, the colony-forming bacteria were recorded as total aerobic mesophilic bacteria. The bacteria count results determined in the total counts during the trial are shown in Fig. 3.

Microbial enzymes have been an important research topic in recent years. One of the reasons for this trend is that most of the industrially used enzymes have microbial origin (Ortakaya and Fincan 2019). Species belonging to *Bacillus* genus play an important role in the synthesis of these enzymes. Gram-positive organisms with high ability to produce amylase, cellulose, and antibiotics include *Bacillus stearotermophilus, Zymomonas mobilis, Bacillus subtilis,*

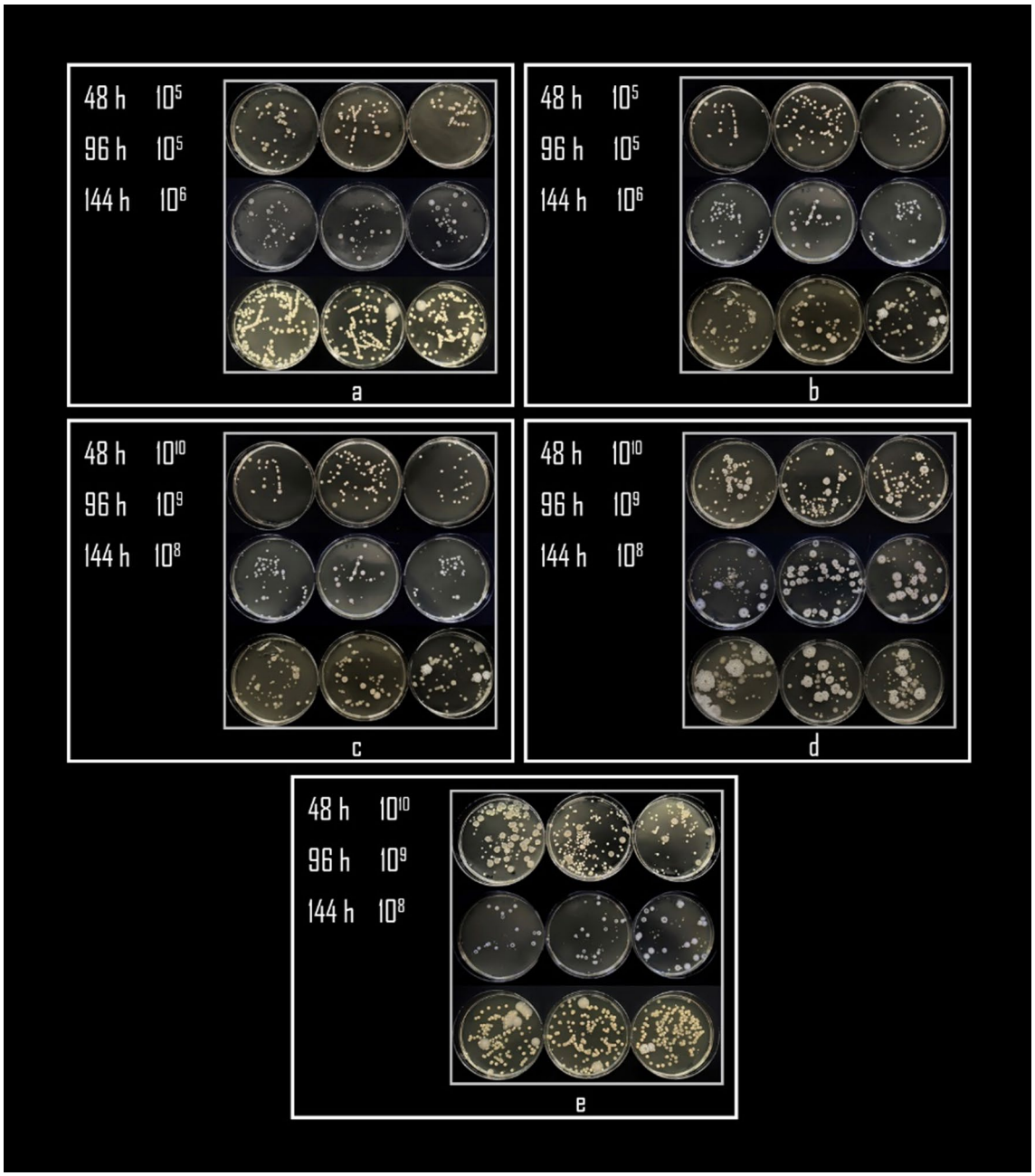

Fig. 3 The appearance of bacterial colonies grown in NA medium after incubation in water samples prepared from trial environments and prepared in different dilutions. **a** C(−); Aquarium water without any application, **b** C(+); commercial product used aquarium water, **c** *Bt Bacillus thuringiensis* CP-1 included aquarium water, **d** *Bs Bacillus subtilis* FD-1, **e** *Bt* + *Bs Bacillus thuringiensis* CP-1 + *Bacillus subtilis* FD-1 included aquarium water

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Bacillus licheniformis, and *Termoactinomyces* sp. microorganisms with this feature (Prem Anand et al. 2010; Ortakaya and Fincan 2019). In our study, decreases in bacteria applied groups were obtained at the end of the trial. This situation was explained by Dere and Sıvacı (2003) as losting their vitality by the effectes of experimental environment. *B. subtilis*, a Gram-positive, saprophyte bacterium, contributes to the food cycle in nature. Although these bacteria grow in the presence of oxygen, growth can also occur in airless environments in the presence of nitrate. In the hot and dry stages, *B. subtilis* can maintain its vitality by forming endospores. During the growth phase of metabolic activities, *B. subtilis* secretes various substances and enzymes, suppresses other microorganisms in the environment, benefits from nutrients and becomes dominant in the environment (Yonsel 2010). The findings of our research were coherent the previous data. The high number of bacteria in *B. subtilis* FD-1 and *B. thuringiensis* CP-1 applied aquariums is thought to be effective in the different metabolic properties of *Bacillus* as well as their wide physiological capabilities (psychrophilic to thermophilic; acidophilic to alkalinity; halotolerant to halophilic) (Uzunalioğlu, 2015). Besides, another thought is that the differences in the increases determined between bacteria may be due to NO_3^--N removal (Li et al. 2019).

It is known that *B. subtilis* is affected by temperature, humidity, UV rays very little and can maintain its vitality. In a study based on this situation, it was stated that *B. subtilis* suppresses many fungi and other bacteria with producing bacteriocytes, reduces bad odor, pH values and increases acidity and creates healthy conditions (Yonsel 2010). In our study, this situation explains that the total bacterial load in the application groups is less than the control group. We can attribute the increase of bacteria in 2 and 4 days in *Bs* and *Bs* + *Bt* applied groups can be attributed to the rapid growth of *Bs* bacteria in the treatment environment (Yonsel 2010).

At the end of the trial period, the determined decreases in all the groups (both bacteria are used singly and in combination) can be explained by the decrease of the protective effect of these bacteria and the inclusion of other bacteria in the environment due to the inclusion of the pollutant source.

When the obtained data evaluated, it can be thought that the bacteria can adapt to the changes in the presence of application bacteria in the environment. It is known that the adaptability of bacteria is an opportunity for bioremediation studies and can be used effectively in bioremediation applications (Türetken et al. 2019).

The previous studies indicate that bacteria do not have to develop resistance against xenobiotic substances (such as heavy metals, antibiotics, and pesticides) in their natural environment in the absence of these. Depending on environmental factors, as a result of the entry of pollution sources into aquatic environments, bacteria develop resistance to xenobiotics and produce certain resistance-related enzymes. In case of continued pollution, bacteria form resistance mechanisms such as accumulation or complexation of these substances, oxidation of metal ions, exopolysaccharide production and cellular mutations (Uzunalioğlu 2015; Ayangbenro and Babalola 2017; Marinescu et al. 2017; Akay and Tezel 2019).

Water quality parameters

Physico-chemical analyses, nitrogen fractions, total phosphorus, and heavy metal contents were determined at 2-day intervals in water samples taken from the devices. The difference between the application groups in the evaluated parameters was found statistically significant ($p < 0.05$). The obtained physico-chemical parameters, total nitrogen, phosphorus, sulfate, heavy metal nutrient salts and heavy metal contents are given as mean ± standard deviation ($\bar{X} \pm SS$) at the following tables (1–3).

Some physico-chemical analyzes in water

Changes in dissolved oxygen, pH, water temperature, electrical conductivity, turbidity, color, free chlorine (FC), total chlorine (TC), alkalinity, and total hardness (TH) values were determined and differences between groups were found significant at $p < 0.05$ (Table 1). The dissolved oxygen values showed significant differences among the groups and the amount of oxygen decreased due to the increasing temperature. In parallel with our study findings, it is known that there is less dissolved oxygen at high water temperatures. Low dissolved oxygen concentration is an indication of pollution and restricts the use of water for normal purposes. Dissolved oxygen changes in water due to physical, chemical and biochemical activities, but there is an inverse relationship with temperature and salinity (Saravanakumar et al. 2008). The reason for the low oxygen concentrations obtained in this research can be considered as the excessive consumption of oxygen as a result of the temperature and the activities of bacteria (such as their presence/multiplication and energy-producing requirements) in the environment. As a known situation, plants and living organisms (such as bacteria, fish) in the aquatic environment (photosynthesis during the day) consume significant oxygen (Jonnalagadda and Mhere 2001).

In our study, the lowest pH values among all application groups were determined in Bs, Bs + Bt and Bt groups, respectively (Table 1) in the water samples belonging to these groups which are more acidic than the control groups, the pH value may be affected by the metabolic activity of *Bacillus* (Yonsel 2010). Similar to our research findings, as a result of the inoculation of phosphate solubilizer *B. subtilis* SJ-101, it has been reported that there are significant decreases in pH values (7.5–4.8) in the current

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Table 1 Changes of physico-chemical properties of trial environments

Time (Day)	Treatment	DO (mg/L)	pH	Temperature (°C)	Conductivity (µS/cm)	Turbidity (NTU)	Color (Pt–Co Scale)	FC (mg/L)	TC (mg/L)	Alkanity (µg/L)	TH (mg/L $CaCO_3$)
0	C(−)	5.47 ± 0.20	7.89 ± 0.04	19.15 ± 0.40	790.0 ± 3	1.51 ± 0.03	7.00 ± 2.31	0.03 ± 0.00	0.36 ± 0.00	79.50 ± 2.887	16.02 ± 0.1
6	C(−)	2.89 ± 0.10^{a}	8.03 ± 0.08^{a}	17.85 ± 0.06^{ab}	893.00 ± 4.62^{c}	13.80 ± 4.16^{b}	18.00 ± 2.31^{a}	0.27 ± 0.09^{c}	0.63 ± 0.08^{b}	424.50 ± 46.76^{a}	35.65 ± 2.5^{a}
	C(+)	2.70 ± 0.46^{ab}	7.99 ± 0.03^{ab}	17.90 ± 0.12^{ab}	829.00 ± 6.93^{d}	27.90 ± 9.58^{a}	17.00 ± 1.16^{ab}	0.32 ± 0.04^{c}	0.49 ± 0.14^{b}	364.50 ± 25.9^{bc}	36.00 ± 5.8^{a}
	Bt	2.36 ± 0.10^{bc}	7.97 ± 0.06^{ab}	17.85 ± 0.06^{ab}	974.50 ± 16.74^{b}	33.75 ± 3.06^{a}	16.50 ± 1.73^{ab}	0.48 ± 0.07^{b}	0.75 ± 0.05^{a}	325.50 ± 6.35^{c}	28.00 ± 0.0^{bc}
	Bs	2.21 ± 0.18^{c}	7.94 ± 0.02^{b}	17.75 ± 0.29^{b}	989.00 ± 1.16^{ab}	28.00 ± 6.47^{a}	14.50 ± 1.73^{bc}	0.64 ± 0.02^{a}	0.85 ± 0.15^{a}	342.50 ± 13.28^{c}	26.50 ± 0.6^{c}
	Bt + Bs	2.14 ± 0.06^{c}	7.94 ± 0.01^{b}	18.05 ± 0.06^{a}	1028.50 ± 5.71^{a}	17.30 ± 4.97^{b}	13.50 ± 0.58^{c}	0.33 ± 0.03^{c}	0.76 ± 0.23^{a}	389.50 ± 32.9^{ab}	31.50 ± 1.7^{ab}

C(−): Aquarium water without any application, C(+): commercial product used aquarium water, *Bt Bacillus thuringiensis* CP-1 included aquarium water, *Bs Bacillus subtilis* FD-1, *Bt* + *Bs Bacillus thuringiensis* CP-1 + *Bacillus subtilis* FD-1 included aquarium water, *DO* dissolved oxygen, *FC* free chlorine, *TC* total chlorine, *TH* total hardness

There is a difference between groups in the parameter shown with different letters (a, b, c, d) between the same column and the same days

environment and the solubility of metals can also be effective (Uzunalioğlu 2015).

It has been reported that there is a directly proportional relationship between alkalinity and pH and a pH value of 8.3 should be 50 mg/L $CaCO_3$ alkalinity (Boyd 1998).

In our study, the average electrical conductivity value was measured as 872 ± 30 µS/cm.

In water, the electrical conductivity increase with high concentration of ion or total dissolved solids (Metcalf et al. 2000). In this study, the highest color value was determined on the 6th day (13.50–18.00) in all groups. These values are measured for C (−) and C(+)18.00 ± 2.31 Pt–Co and 17.00 ± 1.16 Pt–Co, respectively.

Hardness is an important parameter in the purpose of using water (drinking, urban or industry) and consists of calcium and magnesium salts. All examined aquarium waters in our study were determined in the range of 16–36 CaCO3 (mg/l) and were determined in soft waters (0–75 mg/l $CaCO_3$) class (Lawson 1995).

Total nitrogen, phosphorus, and sulfate values

Although the ammonia nitrogen level increased in all groups in parallel with the days in the experiment groups and deaths were observed in fish from the 5th day, the lowest values in terms of trial groups and days were recorded in *B. subtilis* FD-1 + *B. thuringiensis* CP-1 applications (Table 2). An improvement of 81% in total ammonia level and 4% in total nitrite level was determined. In trial environments using commercial products (C+), total ammonia level increased by 32% compared to control (C−), while nitrite level decreased by 2%. In low ammonia concentrations obtained in *Bacillus* applications compared to the treatment groups, it is thought that ammonium emission is prevented by the conversion into ammonia gas form since these bacterial spores develop rapidly in the environment after application and cause their pH to decrease due to their metabolic activities (Yonsel 2010). Konohana et al. (2000) reported that *Bacillus licheniformis* removed NO_3 and NH_4^+ from the environment under aerobic conditions.

Biological denitrification is observed in anoxic processes where there is no molecular oxygen and nitrate is used as the final electron acceptor. In conversions catalyzed by the separate enzyme system, $NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$ steps take place, respectively (McMillan et al. 2014). In the realization of these steps, *Achromobacter, Alcaligenes, Bacillus, Chrombacter, Corynebacterium, Halobacterium, Methanomonas, Moraxella, Paracoccus, Propionibacterium, Pseudomonas, Spirillum, Thiobacillus,* and *Xanthomonas* are the most known denitrification microorganisms (Koenig et al. 2005; Zhao et al. 2011; He et al. 2018). In our study, *B. subtilis* showed better development in the presence of nitrate. In a study conducted in parallel with our research findings,

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Table 2 Nitrogen fractions and total phosphorus alterations of trial environments

Time (Day)	Treatment	Ammonia nitrogen (NH_3–N, mg/L)	Nitrite (NO_2–N, mg/L)	Nitrate (NO_3–N, mg/L)	Total phosphorus (TP, mg/L)	Sulfate (SO_4, mg/L)
0	C(−)	0.76 ± 0.08	0.00 ± 0.00	1.70 ± 0.03	0.00 ± 0.00	0.47 ± 0.03
6	C(−)	1.35 ± 0.28^{b}	3.12 ± 0.00^{b}	1.17 ± 0.91^{c}	0.02 ± 0.01^{c}	0.52 ± 0.02^{c}
	C(+)	1.78 ± 0.00^{a}	3.06 ± 0.00^{a}	0.01 ± 0.01^{a}	0.93 ± 0.15^{a}	0.67 ± 0.04^{a}
	Bt	1.78 ± 0.00^{a}	1.65 ± 0.50^{b}	0.00 ± 0.00^{b}	0.81 ± 0.08^{b}	0.60 ± 0.03^{b}
	Bs	1.77 ± 0.00^{a}	1.68 ± 0.48^{b}	0.00 ± 0.00^{b}	0.93 ± 0.04^{a}	0.60 ± 0.05^{b}
	Bt + Bs	1.29 ± 0.52^{b}	0.60 ± 0.03^{c}	0.00 ± 0.00^{b}	0.43 ± 0.15^{c}	0.52 ± 0.03^{c}

C(−): Aquarium water without any application, C(+): commercial product used aquarium water, *Bt Bacillus thuringiensis* CP-1 included aquarium water, *Bs Bacillus subtilis* FD-1, *Bt* + *Bs Bacillus thuringiensis* CP-1 + *Bacillus subtilis* FD-1 included aquarium water

There is a difference between groups in the parameter shown with different letters (a, b, c, d) between the same column and the same days

B. subtilis, *B. licheniformis*, and *E. coli* bacteria observed anaerobic growth in nitrate-containing medium and determined 5%, 6%, and 16% growth in the density of the grown bacteria, respectively. They also reported that the maximum optical density achieved by *B. subtilis* during nitrate inhalation was approximately 5.7 times less than that achieved by *E. coli* (Clements et al. 2002). When we examine in terms of nitrate concentration, it had been determined that there is a 100% improvement in bacterial application groups compared to control (Table 2). In line with our results, Tang et al. (2018) reported the efficiency of *Bacillus* in the treatment of nitrate from wastewater in aquaculture. Again, in physicochemical parameters, the denitrification of nitrate is thought to be effective in the pH increases. In this case, pH increase occurs while nitrate is consumed (Rajta et al. 2019).

For living things, the main nutrient after nitrogen is phosphorus (P), an important chemical element for the DNA structures of all organisms. Different anion reagents of this element are associated with some cationic (Ca^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Mg^{2+} and Al^{3+}) in terms of movement possibilities. In these cases, there is a need for the presence of organic and phosphoric acids such as lactic acid, gluconic acid, acetic acid, formic acid, oxalic acid, tartaric acid, fumaric acid, and succinic acid in mineralization of phosphorus. *Bacillus polymyxa, B. megatarium, B. circulans, B. subtilis, B. firmus, Pseudomonas striata, P. rathonia, Rhizobium leguminosarum, and R. meliloti* are active in the production of these acids. It had been reported that *Bacillus* species can produce a mixture of lactic, isovaleric, isobutyric, and acetic acid groups (Nyangale et al. 2014.) In this study, it was found that total phosphorus value increased on the 6th day (Table 2). Since the total phosphorus level does not change during the day according to pH, dissolved oxygen or other water quality values, it is thought that the phosphorus value increases with the deaths of the bacteria on the last day. At the end of the trial period, the total sulfate level increased 28% in the C(+) group compared to the control (C−), while this ratio was determined as 15% in the bacteria-treated groups.

Concentration of heavy metals in water

Wastewater not only contains high nitrate and nitrite but also contains Cu^{2+}, Zn^{2+}, Cr^{6+}, Ni^{2+} and other heavy metal ions (Rajta et al. 2019). Under anaerobic conditions, nitrates, sulfates, Mn^{4+} or Fe^{3+} reducers can use oxygen as an electron acceptor (Liu et al. 2020). Due to their technological importance, heavy metals are widely used in various industries. However, these metals may cause permanent toxic effects in the waste waters of the areas where they are used. When the changes consider in heavy metal concentrations in the application groups at the end of the trial period (Table 3), can be seen that single and double usage of *B. thuringiensis* and *B. subtilis* removes Ni, Cr, Se, Al, Cd, Mn, Fe, B with the rate of % 57, 50, 50, 43, 40, 23, 5 and 2, respectively. It was observed that *B. thuringiensis* was more effective in Fe, Cr and B metals, especially in reducing Al, Cd, Ni and Se levels. It was observed. With this study, *Bacillus*-type bacteria were determined to be effective in heavy metal concentrations depending on the type of metal in trial environments. In a study, it was reported that *Bacillus* have a high resistance against heavy metals (such as copper, chromium and iron) in soil and wastewater (Uzunalioğlu 2015). Considering the low concentrations of Al, Cd and Ni metals determined in the Bt application groups obtained in our study findings, in accordance with the data we obtained; Uzunalioğlu (2015) stated that *Bacillus thuringiensis* MP7B strain (lead added to the feed medium) can use lead, *Bacillus subtilis*, *Pseudomonas putida* and *Enterobacter cloacae* are particularly preferred bacteria in the improvement of heavy metal contaminated environments. It is noted with different studies that *Bacillus subtilis* species can be used against to heavy metal pollution (Kanamarlapudi and Muddada 2019; Zhao et al. 2019).

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Table 3 Metal contents of aquarium environments during the trial

Time (Day)	Treatment	Al (µg/L)	Fe (µg/L)	Cd (µg/L)	Cr (µg/L)	Cu (µg/L)	Mn (µg/L)	Ni (µg/L)	B (µg/L)	Se (µg/L)
0	C(−)	8.46 ± 2.07	2.43 ± 0.14	0.65 ± 0.09	1.08 ± 0.05	8.57 ± 0.01	2.01 ± 0.04	0.66 ± 0.15	31.85 ± 0.63	2.90 ± 0.46
6	C(−)	2.42 ± 0.32^{a}	1.39 ± 0.06^{b}	0.30 ± 0.00^{a}	0.28 ± 0.01^{a}	1.16 ± 0.15^{b}	15.60 ± 0.46^{a}	0.53 ± 0.13^{a}	26.10 ± 0.693^{b}	1.68 ± 0.09^{ab}
	C(+)	1.84 ± 0.98^{b}	0.71 ± 0.00^{c}	0.35 ± 0.05^{a}	0.15 ± 0.00^{b}	1.58 ± 0.00^{a}	11.38 ± 0.14^{d}	0.51 ± 0.06^{a}	24.75 ± 0.751^{c}	1.52 ± 0.20^{b}
	Bt	1.38 ± 0.11^{c}	1.61 ± 0.31^{ab}	0.18 ± 0.02^{b}	0.30 ± 0.06^{a}	1.37 ± 0.14^{ab}	12.95 ± 0.28^{b}	0.23 ± 0.08^{b}	26.05 ± 0.635^{b}	0.84 ± 0.15^{c}
	Bs	1.63 ± 0.09^{bc}	1.32 ± 0.23^{b}	0.31 ± 0.10^{a}	0.25 ± 0.04^{a}	1.52 ± 0.09^{a}	12.05 ± 0.17^{c}	0.60 ± 0.11^{a}	25.65 ± 0.289^{b}	1.80 ± 0.05^{a}
	Bt + Bs	2.43 ± 0.18^{a}	1.89 ± 0.25^{a}	0.36 ± 0.05^{a}	0.14 ± 0.01^{b}	1.37 ± 0.19^{ab}	12.00 ± 0.46^{c}	0.64 ± 0.05^{a}	27.50 ± 0.346^{a}	1.63 ± 0.08^{ab}

C(−): Aquarium water without any application, C(+): commercial product used aquarium water, *Bt Bacillus thuringiensis* CP-1 included aquarium water, *Bs Bacillus subtilis* FD-1, *Bt* + *Bs Bacillus thuringiensis* CP-1 + *Bacillus subtilis* FD-1 included aquarium water

There is a difference between groups in the parameter shown with different letters (a, b, c, d) between the same column and the same days

The lowest values in terms of selenium and boron in the experimental groups were obtained in the Bt (*B. thuringiensis*) and Bs (*B. subtilis*) groups, respectively. It was stated by Uzunalioğlu (2015) that *B. subtilis* reduced Se, and this situation does not comply with our results. In our study, aluminum (A1) was observed in high amounts in the groups (Bt + Bs) where bacteria were used together on the last day of the experiment. When we consider the density of this metal in the experimental environments, we can attribute the ability of functional protein groups of the bacteria used to bind to the metal in question (Keloğlu, 2017). As a known case, the most important issues affecting the metal preference of biomaterials are especially their physic-chemical properties such as electronegativity and ionic radians. The higher these values, the higher the ability to connect (Bueno et al. 2008).

In the results obtained from the experimental environments, some metal groups generally decreased compared to the control (C−). It is known that proteins, bacterial species, and ambient pH are effective in the intake or binding of heavy metals in the cell surface by bacteria (detoxification). In aquatic environments, some factors (such as the specific features of microorganism, cell metabolism, and environmental influence) are effective in removing heavy metals by bacteria (Keloğlu, 2017).

In our experiment groups, the low pH was effective in some metal ion concentrations determined at higher rates than the control. In a study, it was stated that the best nickel (Ni) uptake by bacteria was at pH 6 and 7 (Gabr et al. 2008). The pH values in our study are far below the specified values and there is an acidic environment. The approach of the environment to neutral accelerates the metal uptake of bacteria. Otherwise, acidic pHs can affect the activities of carrier proteins and enzymes involved in the detoxification mechanism (removal of heavy metal from the cell, conversion of cations, especially sulfur-loving cations into complex compounds by means of thiol-containing molecules, and reduction of some metal ions to less toxic compounds) (Uzunalioğlu 2015; Keloğlu, 2017).

In another study, it was reported that *B. thuringiensis* developed in an environment containing high concentrations of heavy metals but could not remove the Cr (VI) metal from the environment. As an explanation of this situation, it is stated that B. thuringiensis bacteria protects itself by producing high concentration of surface layer proteins and binds heavy metal to this layer, under undesired conditions. Again, at the end of the experiment, it was determined that there were surface layer proteins in the bacteria in question, but no carrier proteins (binding heavy metals). This mechanism can be explained by the fact that bacteria increase protein synthesis in response to metal stressors (Kılıç 2008). Again, one of the most common strategies of bacteria is the production of siderophores, which are low molecular weight

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iron chelators. While Siderophore proteins can form stable complexes with Al, Cd, Cu, Ga, In, Pb, they are capable of removing high levels of iron complexes from minerals or organic compounds, especially in the absence of Fe. The production of these proteins is common at acidic pH and low pH value is preferred for hydroxamate siderophore synthesis by microorganisms (Uzunalioğlu 2015). It has been stated that *Bacillus* increases the use of ammonium and nitrate ions in the presence of 0.5 mmol/l Fe^{2+} (Takenaka et al. 2007). This situation supports our ammonium-nitrate results.

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