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Research Article

Using The Thick-Shelled River Mussel (*Unio crassus*) Filtering Ability for Water Treatment Process in Aquaculture Systems: an In Vitro Study on Removal of the Bacteria from The Water

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ABSTRACT

The thick-shelled river mussel (*Unio crassus*) is listed as 'Endangered' on the IUCN Red List of Threatened Species and it is important to culture them for the conservation of natural stocks. Integrating mussels into the freshwater aquaculture system could be an efficient method, because of their filtering ability. In this study, it was aimed to determine the bacteria filtering ability of the thick-shelled river mussel on an aquaculture system to determine if the water quality got better in terms of bacteriology. Depuration, disinfection, and antibiotic treatments were applied to reduce the bacterial load in the mussels' bodies. Disinfection was made using $NaCl_2$, 2-Phenoxyethanol, Formalin, Virkon® S and Chloramine T. Antibiotic treatment was performed using Oxytetracycline and Florfenicol. The best result was obtained in the group to which 5 mg L^{-1} Oxytetracycline was added. However, since mussels can uptake the same bacteria into own bodies with their own pseudofeces, it was found that it is appropriate to use antibiotic treatment and depuration applications together. In the experiment of keeping them in the same environment with the pathogens (*Staphylococcus epidermidis, Aeromonas caviae*), intense growth of bacteria was inoculated into water. Thus, it has been determined that mussels clean the water by removing bacteria from the environment within 48 hours, so river mussels can be adapted to aquaculture systems to reduce aquatic bacteria.

Keywords: Unionidae, Filtering, Aquatic Bacteria, Aquaculture

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INTRODUCTION

Members of the Unionidae occur on all the continents except for Antarctica. *Unio crassus*, a thick shelled river mussel, has been classified as an endangered species in the IUCN Red list of threatened species since 1994. There was insufficient data found for taxonomic problems from these mussels in 2014 (Lopes-Lima et al., 2014). In Turkiye *U. crassus* has detected in Lake Sapanca basin, Sakarya (Ercan et al., 2013a), the Aras river, Erzurum (İşlıyen, 2017), Tersakan Stream, Muğla (Bahrioğlu, 2017), Karasu stream, Sinop (Coşkun et al., 2019), Çine Creek, and the Aydın (Serdar et al., 2019). In Particular, *U. crassus* was found to be more abundant than the other Unionids in the Maşukiye stream (Ercan et al. 2013a).

Unionids are highly endemic and sensitive to human impact (Strayer, 2008). Studies have shown that glochidia and juvenile mussels are sensitive to some chemicals (Gillis et al., 2008; Ingersoll et al., 2006). Also, since the breathing and exhalant mouths of freshwater mussels are positioned adjacent to each other, continuously flowing waters are suitable habitats for freshwater mussels. Since freshwater mussels can retain toxicants in their tissues and pseudo feces, they play an important role in maintaining water quality (Bauer & Wachtler, 2001).

Since the breathing and exhalant mouths of freshwater mussels are positioned adjacent to each other, continuously flowing waters are suitable habitats for freshwater mussels. Since mussels are fed by filtering the water in the environment, they assimilate suspended particles such as bacteria and plankton in their environment (Grizzle & Brunner, 2009; Starliper, 2001). This feature allows them to live symbiotically with bacteria as well as cause them to become infected (Antunes et al. 2010). Freshwater mussels may store toxicants in their tissues and pseudofeces (Bauer & Wachtler 2001) and the pseudofeces also contains bacteria (Ercan et al., 2013a), therefore it may play a key role in maintaining water quality (Bauer & Wachtler 2001). The pathogen bacteria species of the unionid mussels are also an undefined subject (Carella et al., 2016). Researchers recently found 47 bacterial genera from the hemolymph of freshwater mussels (Leis et al., 2019). The microbial biota is still unclear which of the species are endosymbionts and which are accidentally siphoned into the body. In addition, the microorganisms present in the body varied by collection locality, season, or density of contaminants (Grizzle & Brunner, 2009; Starliper et al., 2008).

The amount of particulate matter filtered from the water column by bivalves may be important, and freshwater mussels can be useful in the rehabilitation of organically contaminated waters, especially those associated with aquaculture (Ercan, 2009). There are studies in which mussels are used as filters for the outlet water, the purpose of re-use, instead of leaving the water to nature in the land aquaculture systems. Additionally, mussels are integrated into fish ponds and co-cultured with fish species (Zheng et al., 2017). Cultivation experiments were performed in order to recover thick-shelled mussels from endangerment (Serdar et al., 2018). Therefore, the depuration process is recommended before the replacement of mussels in hatcheries (Leis et al., 2019). This process is conducted to remove the bacteria that mussels take from the environment to their bodies. In addition, it is recommended to do quarantine and disinfection procedures to prevent mussels from carrying pathogens to new places during their relocation (Augspurger et al., 2003; Waller & Cope, 2019).

The depuration process of marine bivalves is applied to remove low and medium level contaminants from their intestines for a few hours to a few days by taking the bivalves into clean seawater tanks with normal water pumping movements (Lee et al., 2008). Few studies have been done on this process for freshwater mussels (Starliper, 2001). In addition, there is no research about disinfection with chemical or antibiotic treatment.

Although Unionid mussels are said to be endangered due to habitat destruction, pollution, and poor water quality, the large-scale death of mussels in streams with good quality water cannot be explained (Waller & Cope, 2019). Adult Unionids can tolerate low oxygen levels (Strayer, 2008). They can maintain their normal metabolism even at dissolved oxygen levels as low as 1mg L^{-1} and can even tolerate full anoxia for several weeks by simply covering their shells (McMahon & Bogan, 2001).

Many studies have been conducted on the health of mussels, and the scarcity of studies on the role of pathogens in the decrease in mussel populations draws attention (Waller & Cope, 2019). The most important step in determining whether these species are healthy or not requires knowledge about pathogens. Reported pathogens have the potential to disrupt the health conditions of Unionid mussels, but their role in disease is not well established (Carella et al., 2016).

Mussels are living communities that are involved in the removal of inorganic substances such as nutrient salts and/or heavy metals that cause accumulation in the aquatic ecosystem with their filtration functions and therefore in the improvement of water quality (Cummings & Graf, 2010; Lei et al., 1996; Strayer, 2008). During the nutritional activities of mussels, filtering and accumulating pollutants in their bodies is highly effective in improving water quality. The aim of this study was to determine the bacteria filtering ability of mussels before being adapted to fishponds in the aquaculture system.

MATERIALS AND METHODS

Collection and adaptation of mussels

Unio crassus were collected by hand and with a rake from the area of the Maşukiye stream with a depth of 10-80 cm and a length of 50 m, and 700 m away from the Sapanca Lake (40° 43′ 1.542″, 30° 8′ 29.0292″) in June 2019 in Sakarya city. The Maşukiye stream has high nutrient water and a sandy-muddy bottom. The water temperature was 16°C at the sampling time. Mussels were determined to be from the sampling area of the Maşukiye Stream, all experiments were done with a total of 28 specimens for three different treatments. They were transported to the "Aquatic Vertebrate Living Experiment Unit" of the Sapanca Inland Fisheries Production Research and Application Unit in a dry environment with net bags in 20 minutes.

They were quarantined and acclimated for 30 days at an enclosed holding facility. Accordingly, they were placed indoors, in a 200L circular polypropylene tank until used for experimental purposes. During the adaptation period, the tank was supplied with 16°C well water continuously which has between 7-8mg L^{-1} dissolved oxygen, 6pH and 5L min^{-1} flow. Mussels were fed daily at a rate of 1% body weight with dried algae (Algome, MarinBio). The algae suspension was mixed homogeneously in 1L water and added to the tanks (Bahrioğlu, 2017). The artificial lighting was adjusted to the summer photoperiod (12h light: 12h dark). After the adaptation period, 28 specimens were measured using digital calipers and electronic balance and grouped for the experiments. The mussels were measured as the mean length of 49.44 ± 2.29mm, a width of 27.72 ± 1.11mm, a height of 17.21 ± 0.82mm, and a weight of 15.30 ± 1.8g.

Firstly, three different methods were used; depuration, disinfection, and antibiotic treatment, to eliminate or reduce the natural bacterial biota of *Unio crassus*. After these steps, the pathogenicity of *A. caviae* and *S. epidermidis* in *U. crassus* and filtering ability for elimination of these bacteria from waters were investigated. During the experiment, the water parameters were adjusted as water was renewed. The constancy of the water temperature was provided by the air conditioner that controls the room temperature.

Detection of the bacterial density in mussels and water
At the beginning of the experiments, the bacterial density was determined in the body of the mussels and the water where the mussels were held in experimental tanks. One mussel was sampled and examined under aseptic conditions to determine the bacterial biota. After that the shells were disinfected with 70% alcohol, the internal organs of the mussel were separated from the shell with the scalpel and taken into sterile sampling bags. The mussel organs were homogenized in an equal amount (w/v; 1:2 dilution) of sterile peptone water for 10 minutes by Stomacher. Homogenate was diluted 10-fold serially with peptone water. 0.1mL of each dilution was streaked onto TSA plates with the drigalski. The plates were incubated for 24-48 hours at 22°C and the total bacteria were enumerated and recorded as CFU g^{-1} (Whitman, 2004).

For determination of the bacterial density in water, the water samples (0.1mL) were streaked onto tryptic soy agar (TSA) plates. Plates were incubated for 24-48 hours at 22°C and the total bacteria were enumerated and recorded as CFU mL^{-1} (Whitman, 2004). Bacterial observation was done only by macroscopically examining the colonial morphology. Additionally, water samples were streaked onto Baird Parker Agar (w/RPF Supplement) and Blood Agar to determine presence of *Staphylococcus* spp. and *Aeromonas* spp.

Elimination of the bacterial biota of mussels
After the detection of bacterial biota in the mussels, depuration, disinfection, and antibiotic treatment experiments were made for the elimination of bacterial density. Measurement of bacterial density was the same in all the applications. Water samples were taken by micropipette from each group. To measure the bacterial density, the water samples (0.1mL) were streaked onto tryptic soy agar (TSA) plates at 0-2-24 hours and 24-hour intervals for all experiments. Plates were incubated for 24-48 hours at 22°C and the total bacteria were enumerated and recorded as CFU mL^{-1} (Whitman, 2004). The removal degree of total bacteria was calculated as a percentage according to Lekang (Verdegem, 2007).

Depuration experiment
The mussels were subjected to the depuration process using ultra-pure water (Bighiu et al., 2019). Each mussel was held in different beakers. A total of 9 mussels were used for the depuration experiment. To sustain the filtration process, aeration was continued during depuration. To prevent contamination from an external source, the beakers in which the mussels were kept were covered with aluminum foil. The water in each beaker was renewed by using ultra-pure water at 24-hour intervals for 168 hours (7 days), and 0.1mL of water samples was taken from the containers before the water change. Water samples were spread onto TSA plates in duplicate. The survival rate during the experiment was 100%.

Disinfection experiments
Three mussels in each beaker were treated with three different disinfectants. In the first application, three different groups of mussels were held in ultra-pure water with $NaCl_2$ (20g L^{-1}), 2-Phenoxyethanol (1.5mL L^{-1}) and Chloramine T (0.5g L^{-1}) for one hour then taken into ultra-pure water. In the second application, one mussel was held in ultra-pure water with NaCl2 (20g L^{-1}) for 24 hours then taken into ultra-pure water (Stockton & Moffitt, 2013; Garcia et al., 2014). In the third application, the shells were brushed with toothbrushes soaked in solutions of Formalin (10%), Virkon® S (1:100) and Chloramine T (5 g L^{-1}) separately. After the mussels were brushed for 30 minutes then they were washed and taken into ultra-pure water. They were observed for 120 hours (5 days). In the experiments, the used water was renewed at 24-hour intervals with ultra-pure water. Water samples were spread onto TSA plates in duplicate.

Antibiotic treatment experiment
An antibiogram test was performed on bacteria taken from water samples with fish antibiotic discs on Mueller Hinton Agar. As a result of this experiment, the use of oxytetracycline and florfenicol was found appropriate in the treatment of mussels. Three different doses of Oxytetracycline (0.5, 1, and 5mg L^{-1}) and Florfenicol (0.05, 0.1, and 0.5mg L^{-1}) were added to the ultra-pure water every day for 5 days. During this experiment, the water was not changed. After the experiment ended, the mussels were held in the same beakers for a week without any manipulation. Afterward, the water samples were taken from each beaker for control purposes. Water samples were spread onto TSA plates in duplicate.

Preparation of bacterial inoculum
The strain of *Aeromonas caviae* reference strain ATCC 15468 was taken from ATCC and *Staphylococcus epidermidis* was originally isolated from fish in 2017 (Çanak & Timur, 2020).

The bacteria were cultured in nutrient broth (NB) at 23°C on a 150-rpm shaker for 48 hours. The culture was centrifuged for 10 minutes at 3000g, the pellet was washed with phosphate buffered saline (PBS, Oxoid) and adjusted to an optical density of 1.0 at 600nm. Tenfold dilutions of the cells were prepared in PBS for determination of viable colony-forming units (CFU). 0.1mL of each dilution were placed on the surface of Tryptic Soy Agar (TSA) (Paniagua et al., 1990; Zepeda-Velázquez et al., 2017).

Elimination of *A. caviae* and *S. epidermidis* by mussels
According to results of earlier experiments, oxytetracycline was used at a dose 0.5mg L^{-1} for 5 days before beginning the exposure experiment. Then, the water was changed with autoclaved distilled water before the inoculation of bacteria.

The growth bacterial colonies were enumerated, and prepared dilutions inoculated into 1L water. Mussels in experimental groups formed in 5L beakers were exposed to two bacterial species at a final concentration 10^8 CFU mL^{-1} or 10^7 CFU mL^{-1} *S. epidermidis*, 10^7 CFU mL^{-1} or 10^6 CFU mL^{-1} *A. caviae*. An aliquot of PBS was added to the control group. Water samples were taken by micropipette from beakers to measure the bacterial density. The water samples (0.1ml) were spread onto TSA at 0, 2, 4, 6, 24, 48, 336 hours after bacterial inoculation. Growth colonies were enumerated and recorded.

Statistical analysis
An assessment of the correlation between the experiment groups for elimination of bacteria was determined using SPSS version 28, and an analysis by bivariate Pearson correlation coefficient ($p<0.01$).

RESULTS AND DISCUSSION

At the beginning of the experiment, all mussels were kept in the same tank. Total bacteria were determined 1.1x10^5 CFU mL^{-1} in the water where the mussels were kept. This number was used as the initial (I) amount of bacterial density for all experiments. These colonies had very high concentrations and some of them were swarm type colonies.

It was observed that the bacterial endobiota contained different types of bacteria and very intense growth on TSA. Total bacterial recovery from the tissues was found as 4.1 x10^7 CFU g^{-1}. Considering the morphological features, five different types of bacterial colonies

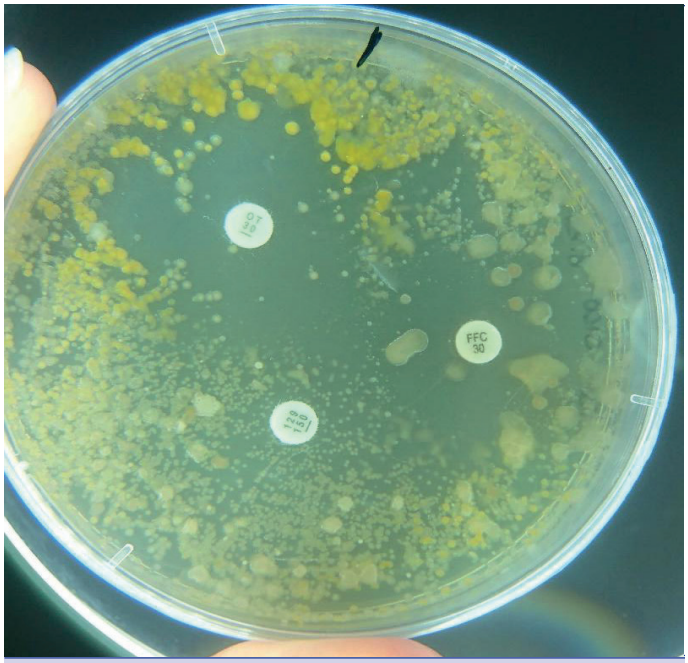

Figure 1. Antibiogram test on TSA plate (OT, FFC) and microbial variation in the water at the beginning of the study

were observed on TSA, but the species were not identified (Fig. 1).

As a result of the 7-days depuration process at 22 °C performed to reduce the natural bacterial biota, it was determined that 68.2 % of the total bacteria were removed and decreased to 3.5 x10^4 CFU mL^{-1} (Fig. 2).

In the groups kept for 1 hour in $NaCl_2$ (20g L^{-1}, NCL), phenoxyethanol (1.5mL L^{-1}, PHN) and Chloramine T (0.5g L^{-1}, CHL1), a decrease in bacterial load was observed after 120 hours but could not be eliminated. The total amount of bacteria was found to be 5.5 x10^1 CFU mL^{-1}, 4.3 x10^2 CFU mL^{-1} and 8.2 x10^2 CFU mL^{-1} at 2 hours and 1.15 x10^3 CFU mL^{-1}, 3.59 x10^3 CFU mL^{-1} and 2.83 x10^3 CFU mL^{-1} at 120 hours, respectively (Fig. 2). Removal of bacteria was determined as 91.36%, 67.72% and 73.72% respectively. However, the total bacteria amount was found 10-fold higher in all groups after 144 hours when the experiment was terminated.

Although the bacterial density decreased to almost 3 x10^4 CFU mL^{-1} after 24 hours in all groups where the mussel shells were brushed with solutions of formalin (7%, FRM), Virkon® S (1:100, VRN) and Chloramine T (5 g L^{-1}, CHL2) (Fig. 2), all mussels were dead at the 48th hour.

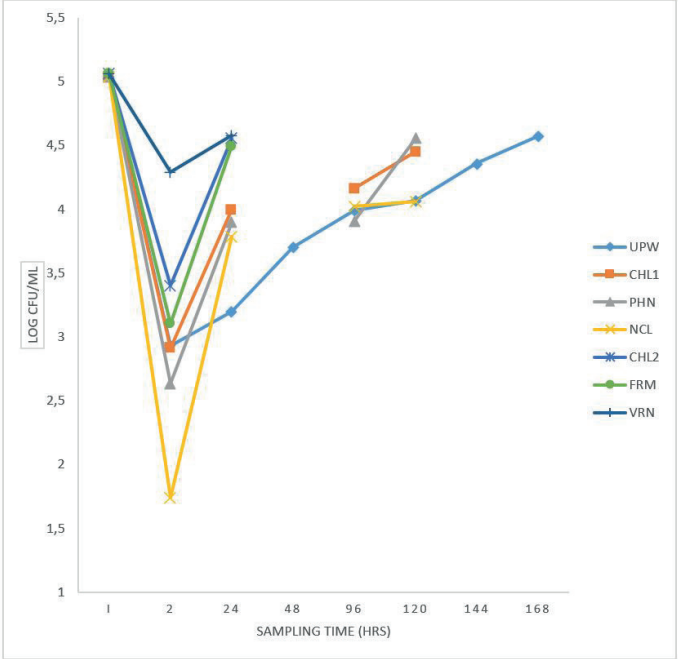

Figure 2. Time-dependent variation of total bacterial load (log CFU mL^{-1}) in the water in which the mussels were kept during different treatments. UPW: depuration with ultra-pure water change daily; CHL1: Chloramine T (0.5g L^{-1}); PHN: Phenoxyethanol (1.5mL L^{-1}); NCL: NaCl2 (20g L^{-1}); CHL2: Chloramine T (5g/L); FRM: Formalin (%7); VRN: Virkon (1:100).

In the antibiotic treatment groups (Oxytetracycline and Florfenicol), after 5 days, the best results were obtained as 5 x10^2 CFU mL^{-1} from the group in which 5mg L^{-1} oxytetracycline was added (Fig. 3).

Oxytetracycline reduced both the number and types of bacteria. While five types of colonies were grown at the beginning of the study (Fig. 1), two types of bacteria were grown at the end in the oxytetracycline treatment group. However, after a week with no treatment, bacterial concentration increased again.

According to results of the bacterial elimination experiment; Bacterial density decreased 10 times in the group that added 10^8 and 10^7 CFU mL^{-1} of *Staphylococcus epidermidis* bacteria to the water. 10^6 CFU mL^{-1} *Aeromonas caviae* (ATCC 15468) bacteria in the water was filtered from the water after 24 hours and 10^7 CFU mL^{-1} *Aeromonas caviae* (ATCC 15468) bacteria was reduced to 10^1 CFU mL^{-1}. No death was observed during the experiments with both bacteria.

It has been found that the natural biota of mussels has very dense bacteria. Although sterile water was used, it destroyed patho-

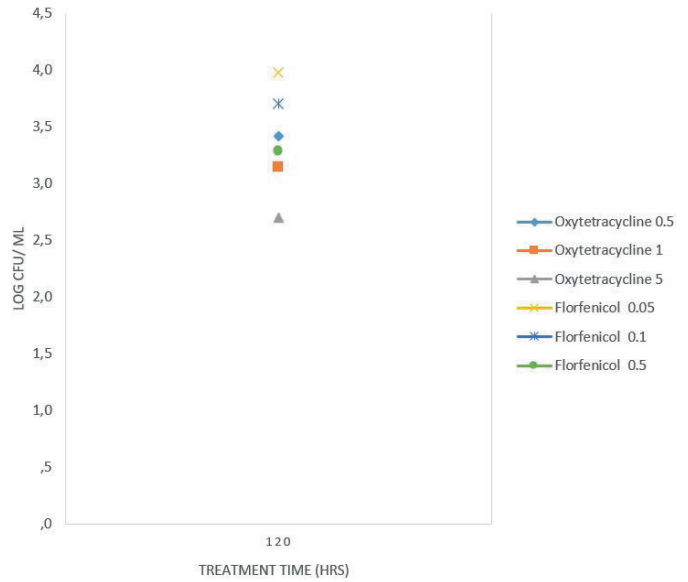

Figure 3. The total bacteria amount in water after 120 hours of the antibiotic treatments; Oxytetracycline and Florfenicol with different doses (mg L^{-1}).

genic bacteria but reproduced other bacteria within itself. Different from *A. caviae*, yellow to orange colonies grew on the TSA of the control group 2 hours later and yellow colonies were seen on the TSA of 10^6 CFU mL^{-1} *A. caviae* inoculated group after 24 hours. After 48 hours, *A. caviae* was eliminated in this group while yellow colonies increased on the TSA. After 14 days, all groups of the *A. caviae* experiment and control were checked again and it was found that all contained the same number of bacteria (Fig. 4). The control with 10^7 CFU mL^{-1} *A. caviae* and with 10^6 CFU mL^{-1} *A. caviae* added groups were found with a negative correlation (r= -.439, p=0.01 and r= -.444, p=0.01, respectively). But between the two groups, 10^7 CFU mL^{-1} *A. caviae* and 10^6 CFU mL^{-1} *A. caviae*, was found to be a strong positive correlation (r=1, p=0.01) by SPSS version 28, and an analysis by bivariate Pearson correlation coefficient.

Also, in this study we observed that, mussels could have cleaned the water of 10^6 CFU mL^{-1} *A. caviae* inoculated group and 10^7 CFU mL^{-1} of *Staphylococcus epidermidis* inoculated group (Fig. 5) and made pseudo feces.

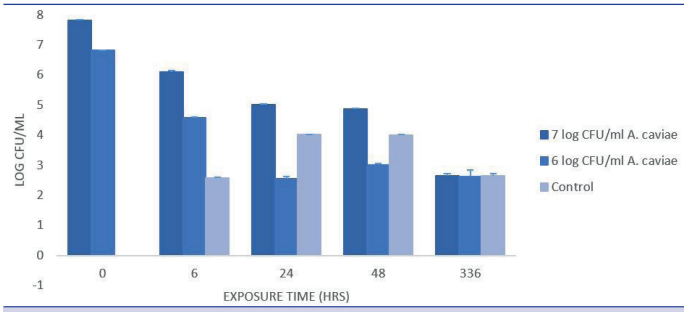

Figure 4. Time-dependent variation of total bacterial load (log CFU mL-1) in water, during the exposure of different concentration for *A. caviae* (The bars showed SD).

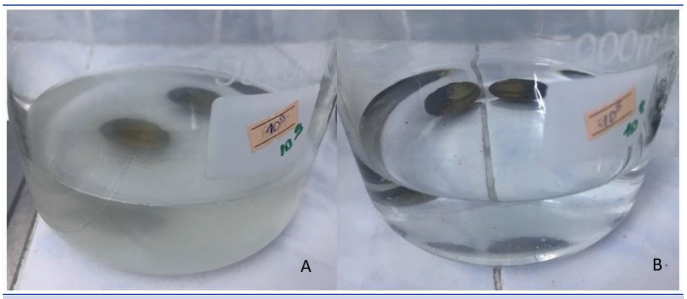

Figure 5. Mussels filter bacteria from water; a view of water at the beginning of experiment (A) and view of cleared water after 48 hours (B).

In this study, it was determined that there were a wide variety of bacteria in the biota of mussels sampled from the Maşukiye stream. The sample taken from the water of mussels placed in sterile water was streaked on selective media and it was found to contain *Staphylococcus* spp. and *Aeromonas* spp. in that water sample. Therefore, experiments were made to reduce the natural bacterial load from the environment so that bacteria, which are present in the mussel, do not mix with *Staphylococcus epidermidis* and *Aeromonas caviae*, which were required to be filtered in this study. For this reason, depuration was the first step, and after this process, different disinfectants and antibiotics were applied because the bacterial density was still high. Despite all the interventions to destroy bacteria found in the natural biota it was achieved only to a limited extent. But a significant reduction was achieved for a short time at 48 hours.

Two bacterial species that can be pathogenic for fish, *A. caviae* and *S. epidermidis*, were selected and mussels were expected to be able to filter and eliminate them from water. As a result of the study, a significant difference was found between the control group and 10^6 CFU mL^{-1} *A. caviae* and with 10^7 CFU mL^{-1} *A. caviae* groups. However, no difference was found between 10^6 CFU mL^{-1} *A. caviae* and 10^7 CFU mL^{-1} *A. caviae* groups in terms of the bacterial filtration. The previous studies showed that mussels assimilated the various bacterial genera by siphoning contaminated water in the tank or natural habitats (Ercan et al., 2013b; Leis et al., 2019; Starliper, 2001; Starliper et al., 2008). According to our results, mussels can be used for elimination of *A. caviae,* but it is not appropriate for *S. epidermidis*. In other words, the two different bacterial species were assimilated at different rates. In previous studies mussels assimilated bacteria at different rates, even if they belonged to the same genus (Bighiu et al., 2019).

According to the results, there was a high concentration of bacteria in the whole-body of the homogenate sample, and the biota on the shell was thought to be strong. It is known that bacteria make a biofilm layer on hard substances. The possibility of formation of a biofilm layer on the shell was considered, and the shell was brushed to remove bacteria. The shell brushing method was not successful because even if the shell is resistant to

chemicals, the mussel may have absorbed these chemicals during the purification from the chemical process. Or, the destruction of bacteria on the shell and the normal life cycle of the mussel, which can feed on bacteria, may have been adversely affected.

Some studies showed that glochidia and juvenile mussels were sensitive to certain chemicals compared to cladoceran, amphipod and different fish species (Waller and Fisher 1998; Ingersoll et al., 2006; Gillis et al., 2008). Furthermore, the sensitivity of different mussel species to chemicals was also found to be different in a report (Waller & Fisher, 1998). In their study, high mortality was observed in some mussel species in the groups that were kept in water with 2% $NaCl_2$ added for 6 hours, while no mortality was observed in others. In our study, we kept *U. crassus* in water with 2% $NaCl_2$ for 1 hour, and no deaths were observed during the experiment (approximately 1 week). While no death was observed in the group that held 1 hour with 0.5g L^{-1} Chloramine T in the study, it is thought that the reason for death by brushed shell with 5g L^{-1} Chloramine T may be due to the increased chemical concentration or the deterioration of bacterial biota on the shell.

Starliper et al., (2001) found 1.88 x10^5 CFU g^{-1} bacteria in soft tissues of *Amblema plicata*, which was less than our findings. But a mussel's body fluid was separated from organs and tissues and were rinsed with sodium hypochlorite in that study. In another study, researchers found a maximum of 1.52 x10^7 CFU g^{-1} in soft tissues of *Villosa iris* (Starliper et al., 2008). The fact that we sampled all tissues and body fluids together and the tissues were not washed with sodium hypochlorite caused the bacterial load to be a bit higher as 4.1 x10^7 CFU g^{-1} in all soft tissues. Different mussel species may contain different concentrations of bacteria in their body depending on the living habitat. Although the bacterial density was high in the soft tissues, the number of mussels was not low in the sampling area of the stream. This situation showed that thick shelled river mussels could tolerate this high density of bacteria.

The antibiotic dose selection was chosen as similar as the doses applied in fish but was concentrated in the water and accumulated in the mussel tissue as there was no water change, therefore the dose was not chosen too high. According to our results, oxytetracycline treatment was found as the best method to reduce bacterial biota of mussels. However, it is thought that this method alone is not sufficient, and it would be more effective when used together with the depuration method. Because mussels can retake the same bacteria with the feces that they leave in the water. The daily cleaning would change the quality of the water and the biota of the mussel. All species of bacteria need different antibiotic treatment, so the antibiotic treatments would be decided according to the result of the antibiotic susceptibility test following the determination of the mussel bacterial biota.

While fish pathogen bacteria, *Flavobacterium columnare*, could be destroyed in one day by the depuration of mussels (Starliper et al., 1998), *Aeromonas salmonicida* was only reduced by 70% in another study by the same researcher (Starliper, 2001). Starliper (2001) also mentioned that the results of the 1 day and 5 days' depuration process were not different. Based on this, different bacterial species can give different responses to the depuration process in mussels. According to this study, a limited decrease in total bacterial load with depuration was due to the presence of different species in the bacterial biota of mussels. Some species still existed, and some were eliminated after the depuration process. Moreover, a rapid decrease was observed within 24 hours, and an increase in the total number of bacteria was observed in the following days. For this reason, it suggested that 24 and 48 hours were sufficient for the depuration process, and it should not be forgotten that different reductions can be obtained for different bacterial species. Furthermore, unculturable bacteria cannot be cultured in artificial media and some bacteria prevent the growth of other bacteria in the same media, the mussel microbiota, which can be found in many more bacterial species, can be revealed in more detail by metagenomics studies.

The symbiotic life of mussels with bacteria suggests that there may be an important relationship to protecting the ecosystem. Because the number of bacteria decreased in the first days of the applications and even though the contaminated water was replaced with ultrapure water every day, the bacterial load continued to increase day by day in the following days. This might be an effort to create a food for the mussel since there is no food in its environment. Although it has been reported in previous studies that bacteria were eliminated by depuration (Lee et al., 2008), in this study, it was observed that depuration was not valid for many bacteria in *U. crassus,* and individual experiments should be made for each bacteria.

Leis et al., (2019) suggested that future studies should investigate associations between *Aeromonas* spp. and unionid health and disease. Sicuro et al., (2020) worked on the use of freshwater bivalves in rainbow trout (*Oncorhynchus mykiss*) farm wastewater filtering. In this research Unionid species (*Sinanodonta woodiana*) were used. They found that the efficiency of freshwater bivalves in reducing the bacterial load, against *A. hydrophila* was successfully done. In this study, we investigated the association between *Aeromonas caviae* and *Unio crassus*. According to our results, a high concentration of (10^8 CFU ml^{-1}) *Aeromonas caviae* was added to the water of mussels, and after 21 days, these bacteria were found not to be associated with the disease of *Unio crassus*. During the 21-day observation period, there was no mortality in all mussel groups and no signs of disease of the mussel organs. It was determined that this bacterium, which is a fish pathogen, did not show a pathogenic effect for mussels. Additionally, mussels survived six months more at around 20°C without any addition of food or air in the beakers covered with aluminum foil after the study. These findings are similar to those reported in previous reports (McMahon & Bogan, 2001). This event suggests that the balance between oxygen production and consumption can be established with the help of endosymbiont bacteria. Besides, it was known that they could use bacteria as food sources (Silverman et al., 1997; Leis et al., 2019). More detailed work needs to be done to understand the complex relationship between bacteria and with these mussels.

Unionids and other freshwater bivalves are important components of the freshwater ecosystem. Several species of potentially pathogenic bacteria have been isolated from freshwater bi-

valves, but their role in diseases of bivalves has not been established (Grizzle & Brunner, 2009). According to some reports, the bacterial biota in mussels changes very quickly (Starliper et al., 1998; Nichols et al., 2001). Starliper et al., (1998) demonstrated that the bacterial biota in mussels changed significantly within 24 hrs of a change in water supply. The finding that the bacterial biota rapidly responds to changing water supplies could be used favorably to minimize the risk for introduction of pathogens. These findings should be supported by further studies.

As Waller and Cope (2019) mentioned earlier, there are many questions that need to be answered in order to protect mussel health. Comparisons can be made with the data in healthy populations by examining the water and mussel tissues, especially in regions with intense mussel death. In this way, it can give us information about the main factor that causes death for mussels. In addition, by detecting the presence of pollutants in regions with concentrated deaths, it can be determined whether the cause of death is due to organic pollutants or chemical pollutants.

One of the aims of the study was to remove any potentially harmful bacterial biota before placing mussels at the bottom of a fish pond within aquaculture conditions. It has not been found appropriate to be kept in the same environment with fish before antibiotic treatment and depuration, due to its symbiotic life with motile *Aeromonas* spp. For this reason, it is thought that mussels collected from a stream can be adapted to fish ponds after investigating the natural bacterial biota and treating them with appropriate antibiotic.

This is the first study carry out in the application of disinfection and antibiotic therapy for reducing the bacterial load of river mussels. According to our experience, it was concluded that mussels increase the bacterial load consciously and can keep the normal biota in balance. There is a symbiotic lifestyle with some bacteria. Moreover, mussels have a complex bacterial biota. Despite all the used interventions, it was impossible to eliminate the bacteria in the mussels' microbiota. It is an original finding that mussels regenerate their bacterial biota continually. It was concluded that mussels can filter and digest some bacterium types (Fig. 5), and some cannot or willingly do not, so it was suggested that if this feature is to be used in rearing conditions, it is necessary to conduct separate trials with each type of bacteria.

CONCLUSION

Mussels can be adapted to mussel-fish integrated culture systems, as it minimizes the amount of *A. caviae* polluting the water in the culture tanks after 48 hours, although it is not highly effective for *S. epidermidis* contamination in culture tanks. To successfully use freshwater mussels to filter bacteria in the water, further studies are needed with mechanical and UV filtration systems.

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