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# Does the freshwater mussel *Anodonta anatina* remove the fish pathogen *Flavobacterium columnare* from water?

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**Abstract** Global decline of freshwater mussels (Unionoida) is threatening biodiversity and the essential ecosystem services that mussels provide. As filter-feeding organisms, freshwater mussels remove phytoplankton and suspended particles from the water. By filtering bacteria, freshwater mussels also decrease pathogen loads in the water. The objective of this study was to evaluate whether the common freshwater bivalve *Anodonta anatina* (duck mussel) could remove the bacterial fish pathogen *Flavobacterium columnare* from the water. Mussels reduced bacteria in both of the two experiments performed, so that the bacterial concentration at the end of the 96-h monitoring in mussel treatments was only 0.3–0.5 times that of the controls. Surprisingly, mussels did not reduce algal cell concentration statistically significantly. Mussel behavior (shell openness, foot position, and movement) was not affected by the presence of bacteria or algae, except for biodeposition formation,

which was greatest in algal-fed treatments, followed by bacterial-fed treatments and controls, respectively. The intestines of bacteria-incubated *A. anatina* harbored *F. columnare*, suggesting that mussels ingested the bacteria. Present results suggest that freshwater mussels may also have a potential to mitigate aquaculture pathogen problems, as well as play a role in water quality management.

**Keywords** Biofiltration · Columnaris disease · Duck mussel · Unionidae · Bacteria · Ecosystem services

## Introduction

Freshwater mussels (Unionoida) are one of the most threatened animal group in the world (Lydeard et al., 2004; Lopes-Lima et al., 2017). In the recent decades, a number of freshwater mussel species have gone extinct (Haag & Williams, 2014). Mussel populations are declining due to pollution, habitat degradation and fragmentation, introduced species, and the loss of obligatory fish hosts (Bogan, 2008; Geist, 2010; Ferreira-Rodríguez et al., 2019), reducing the biological diversity in lakes and rivers. Freshwater mussels carry out important ecosystem services via their filtration and burrowing activity, including nutrient recycling and the deposition of organic matter from the water column to the bottom in the form of feces and pseudofeces (e.g., Vaughn, 2018). The

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biodeposition of feces and pseudofeces (the non-ingested particles loosely wrapped in mucus and ejected without undergoing the digestive process; Berg et al., 1996) by freshwater mussels provides a nutrient-rich and easily assimilated food source for benthic microbes and invertebrates, which supports fish populations and links multiple trophic levels in aquatic ecosystems (Howard & Cuffey, 2006; Vaughn et al., 2008; Vaughn, 2018). Therefore, in addition to biodiversity reduction per se, the decline of freshwater mussels can have a marked effect on the ecosystem.

Bivalves' potential role in water quality management, their "bivalve environmental services", has already been recognized in marine systems (e.g., nutrient trapping connected with mussel production) (Nielsen et al., 2016; Clements & Comeau, 2019; Taylor et al., 2019; Kotta et al., 2020). Mussels' effectiveness in nutrient and particle removal and in wastewater treatment has also been shown in freshwater systems (Strayer, 2014; Lummer et al., 2016; Mezzanotte et al., 2016; Hoellein et al., 2017; Kreeger et al., 2018). Thus, freshwater mussels can potentially be utilized in water quality management to reduce nutrient effluents and eutrophication (Kreeger et al., 2018). Depending on their ability to remove bacteria, freshwater mussels could also be used to reduce disease outbreaks. Several researchers have found that the dreissenid mussel *Dreissena polymorpha* (Pallas, 1771) can clear the pathogenic bacterium *Escherichia coli* (Migula, 1895) Castellani & Chalmers, 1919 from water in laboratory experiments (Silverman et al., 1995), in field conditions (Cotner et al., 1995), and from treated effluents of municipal waste water (Mezzanotte et al., 2016). Unionidae freshwater mussels are also capable of filtering bacteria (Jørgensen et al., 1984; Kryger & Riisgård, 1988; Nichols & Garling, 2002; Christian et al., 2004; Vanderploeg et al., 2011b), including *E. coli* (Silverman et al., 1995; Ismail et al., 2015). However, the ability of freshwater mussels to eliminate bacterial fish pathogens has not been investigated.

The aim of the present study was to investigate the capability of the duck mussel, *Anodonta anatina* (Linnaeus, 1758), to remove the bacterium *Flavobacterium columnare* Bernardet & Grimont 1989 from the water column in laboratory conditions. *Anodonta anatina* is a unionid mussel that commonly occurs in the lakes and rivers of Europe (Lopes-Lima et al., 2016). This mussel has been shown to filter

*Diplostomum pseudospathaceum* Niewiadomska, 1984 larvae from water, thereby reducing the transmission of this trematode parasite from aquatic snails to its fish host (Gopko et al., 2017). More importantly, *A. anatina* has the potential to filter and ingest colonial and filamentous cyanobacteria (Bontes et al., 2007). *Flavobacterium columnare* is a freshwater bacterium and an opportunistic fish pathogen that causes columnaris disease ("warm water disease") in fish. *Flavobacterium columnare* can infect a range of fish species throughout the world, such as salmon, trout, rainbow trout, channel catfish, carp, perch, pike, eel, and tilapia (Declercq et al., 2013). Columnaris disease is one of the most harmful diseases in channel catfish, *Ictalurus punctatus* (Rafinesque, 1818) farming in the USA, leading to approximately \$30 million yearly losses (Wagner et al., 2006). It is also a very difficult problem in young salmonid culture, causing high mortality and the use of antibiotics (Pulkkinen et al., 2010). This pathogen can also be found in natural lakes and rivers (Kunttu et al., 2012) and has been isolated from tissues of the unionid mussel *Villosa iris* (I. Lea, 1829) (Clinch River, USA; Starliper et al., 2008).

Our hypothesis was that *A. anatina* would be able to remove *F. columnare* from the water, manifested as a lower average bacterial concentration in the water with mussel treatment as compared to the control water without mussel. As a methodological control for the feeding of mussels, we established a treatment group fed with a commercial microalgae product commonly used for mussel feeding. As the bacterial suspension may affect the behavior of mussels, especially the filtration activity, monitoring of mussel behavior was included to the study. In addition, as the biodeposit formation can indicate filtration of matter from water, biodeposition formation was also monitored during the experiment. The present study is the first effort to evaluate the efficiency of freshwater mussels to remove the harmful bacterial fish pathogen from the water.

## Materials and methods

### Mussel collection and preparation

In total, 30 *A. anatina* individuals (Table 1) were collected from Lake Kojjärvi, Finland (60.97°N,

**Table 1** The number of mussels (*Anodonta anatina*), average length (mm, mean  $\pm$  SD) and age (y, mean  $\pm$  SD), sex (*F* = female, *M* = male), intensity of trematode infection*(Rhipidocotyle fennica)* (zero, light, moderate, heavy; see text for categorization), and occurrence of glochidia in mussels used in the 1st and 2nd experiment

	Number of mussels	Length Mean	Age Mean	Sex			Trematode				Glochidia Number
				F	M	Unknown	Zero	Light	Moderate	Heavy	
1st experiment	15	83 $\pm$ 4	5.6 $\pm$ 1	6	4	5	1	4	4	6	0
2nd experiment	9	87 $\pm$ 4	6.1 $\pm$ 1	1	4	4	2	2	2	3	0

23.73°E) during September 2016. The mussels were transported to Konnevesi Research Station, University of Jyväskylä and were kept in a 163 L flow-through tank with sand on the bottom until the experiments began. The mussels were not fed, but they received food from incoming Lake Konnevesi water during the maintenance period. In January 2017, the mussels were transported to the laboratory of the Department of Biological and Environmental Science, University of Jyväskylä. The mussels were placed in a large bucket of well water, aerated with an aquarium air pump, and kept on a 12 h light–dark cycle at  $\sim 17^\circ\text{C}$ . In the laboratory during the pre-experiment maintenance, the mussels were fed ad libitum with a commercial phytoplankton product (Shellfish Diet 1800®, SFD, Reed Mariculture, USA) containing six marine microalgae: *Isochrysis sp.*, *Pavlova sp.*, *Tetraselmis sp.*, *Chaetoceros calcitrans* (Paulsen) H.Takano, *Thalassiosira weissflogii* (Grunow) G.A.Fryxell & Hasle, and *Thalassiosira pseudonana* Hasle & Heimdal. The mussels were kept without food for 24 h before starting the experiments until they no longer released excrement. The water temperature at Konnevesi Research Station during the maintenance period was initially  $17^\circ\text{C}$  in September; it gradually declined to  $3^\circ\text{C}$  in December 2016, but it was artificially gradually increased to  $17^\circ\text{C}$  in the last two weeks before the experiments, which were conducted at  $17^\circ\text{C}$ .

#### Preparation of bacterial suspension, Shieh medium and agar plates

The *F. columnare* strain (B613) used in the experiments was isolated from a water sample taken from the shore of Lake Kynsivesi ( $62^\circ25''\text{N}$ ,  $26^\circ15''\text{E}$ ) in 2014; it was then cultivated in a modified Shieh medium

(Song et al. 1988) and stored at  $-80^\circ\text{C}$  with 10% glycerol and 10% fetal calf serum. In order to prepare the bacterial solution for the experiments, 20  $\mu\text{l}$  from the frozen stock was added to 5 ml of modified Shieh medium and incubated with continuous stirring at 120 rpm for 24 h at room temperature. One ml of the overnight-grown bacterial suspension was further added to 9 ml of modified Shieh medium, with another incubation at room temperature overnight. The optical density (OD) of the bacterial suspension was measured with a spectrophotometer at 595 nm and converted to a bacterial concentration using a pre-established relationship between OD and colony-forming units (CFUs); this was done to allow the researchers to adjust the initial bacteria concentration levels during the experiments.

Modified Shieh medium was prepared according to Song et al. (1988) to serve as a selective growth medium to isolate *F. columnare*. Agar plates were used to plate-count the viable bacterial concentrations; here, 10 g of agar powder were added per liter and supplemented with tobramycin in order to inhibit the growth of bacteria other than *F. columnare* (Decostere et al., 1997).

#### Experiment 1

The first experiment to evaluate *A. anatina*'s ability to remove *F. columnare* from water began in February 2017 and included 5 treatments with 5 replicates each (replicate = 1 sediment/sand-free aquarium filled with 5 L of well water with or without a mussel individual; see below). A schematic presentation of the experimental design is given in the Supplementary Fig. 1. The aquaria were aerated and kept on a light–dark cycle of 12 h:12 h at  $\sim 17^\circ\text{C}$ . The experimental treatments were as follows:

- (1) Bacteria and mussel: A known concentration ( $5 \times 10^5$  CFU ml<sup>-1</sup>) of *F. columnare* with an individual mussel to evaluate the potential of *A. anatina* to filter out *F. columnare*.
- (2) Bacteria control: A known concentration ( $5 \times 10^5$  CFU ml<sup>-1</sup>) of *F. columnare* without a mussel was used to study changes in the bacterial concentration of the water in the absence of the mussel.  
The null hypothesis was that there was no difference in bacterial concentration between treatments (1) and (2).
- (3) Algae and mussel: A known concentration of algae with a mussel; this served as a reference treatment to study *A. anatina*'s algal filtration.
- (4) Algae control: A known concentration of algae without a mussel was used to track changes in algae concentration in the water in the absence of the mussel.  
In treatments (3) and (4), 35 ml of Shellfish Diet 1800® was added to 1.1 L water; then, 100 ml of this suspension was added to each aquarium. The null hypothesis was that there was no difference in algae concentration between treatments (3) and (4).
- (5) Mussel control: A mussel without *F. columnare* and without algae was used to study mussel behavior without the presence of bacteria and algae.  
The null hypothesis was that there was no difference in behaviors between treatments (1), (3), and (5).

## Experiment 2

The second experiment began in March 2017 to verify the results of Experiment 1 in regard to bacterial filtration; it used a bacterial inoculation that was an order of magnitude lower than in Experiment 1 (see the Results section and Fig. 1). A schematic presentation of the experimental design is given in the Supplementary Fig. 2. The aquaria were aerated and kept on a light–dark cycle of 12 h:12 h at  $\sim 17$  °C. The experimental treatments were as follows:

- (1) Bacteria and mussel: A known concentration of *F. columnare* with an individual mussel to evaluate *A. anatina*'s potential to filter *F. columnare*; 9 replicate aquaria with a single mussel in each were used.

- (2) Bacteria control: A known concentration of *F. columnare* without a mussel was used to study changes in bacterial concentration in the absence of the mussel; 5 replicate aquaria were used.

The null hypothesis was that there was no difference in bacterial concentration between treatments (1) and (2).

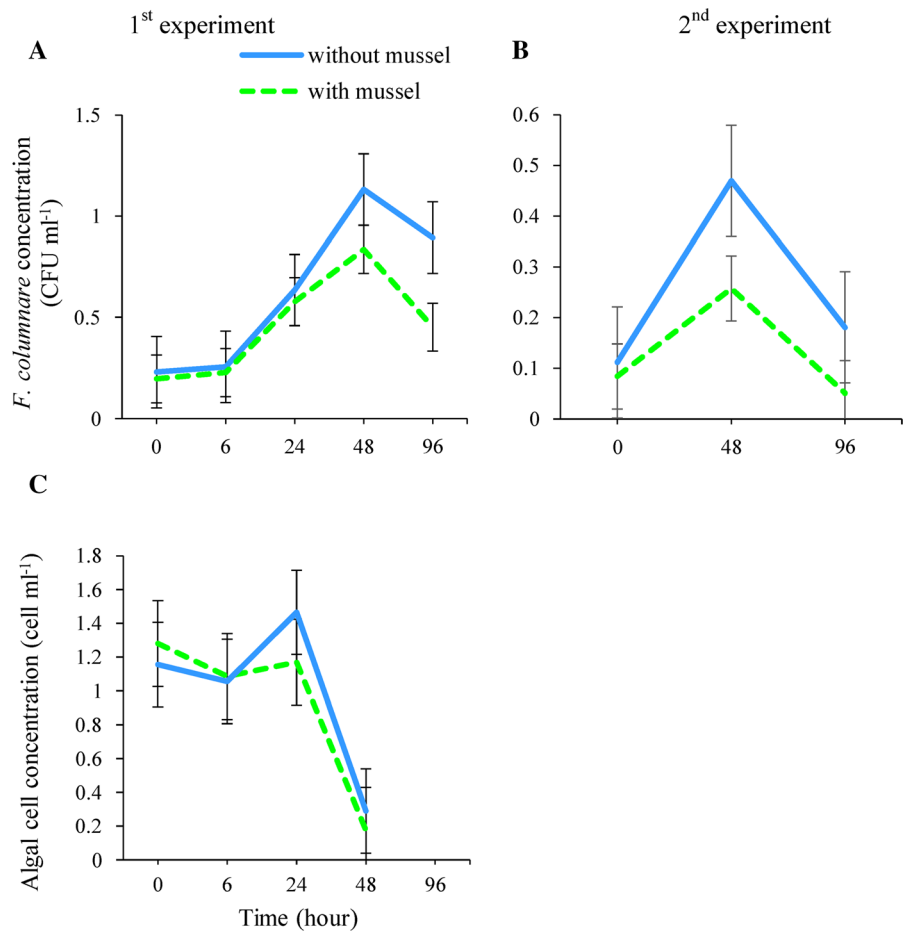
### Bacterial sampling from the water

Water samples were taken from all the aquaria prior to starting each experiment to exclude the possibility of prior contamination by *F. columnare*. The concentration of bacteria was evaluated from 100  $\mu$ l water samples from treatments with added bacteria (bacteria and mussel, bacteria control) at time points 0 h, 6 h, and 24 h; 1 sample per aquarium was evaluated. In order to decrease the observational error associated with reducing bacterial levels at time points 48 h and 96 h, the number of repeated water samples taken per aquarium was increased to three. In Experiment 2, 100  $\mu$ l of water was taken at time points 0 h, 48 h, and 96 h; 2 repeated samples were taken per aquaria. A dilution series ( $10^{-1}$ – $10^{-7}$ ) of each water sample was prepared and cultured on Shieh agar plates; colony formation was checked after 48 h. At the end of both experiments, treatments without bacteria were checked as described above ( $2 \times 100$   $\mu$ l water samples per aquarium) to exclude potential contamination from added bacteria during the experiment.

### Bacterial sampling from intestines and gonads

To examine if mussels can ingest bacteria and become infected with *F. columnare*, bacterial samples were taken from intestine and gonad of the mussels from all treatments at the end of both experiments. To avoid bacterial contamination from the aquarium water, mussel shell surfaces were sprayed with 70% ethanol before dissection. After opening the valves, the mussel body was cut in 2 so that a cross-section through the gonads—and the intestine winding through the gonads—could be accessed. Then, the bacterial samples were collected with a sterile loop and cultured on Shieh agar plates supplemented with tobramycin. In Experiment 1, bacterial sampling was done first from the intestine and then from the gonads. In Experiment 2, the sampling order was reversed to minimize

**Fig. 1** Bacterial concentration (CFU ml<sup>-1</sup>) (A) in aquaria with mussels and without mussels at different time points (N = 5) (mean ± SD) in the 1st experiment, in the 2nd experiment (B), and algal cell concentration (cell ml<sup>-1</sup>) (C) in aquaria with mussels and without mussels at different time points (N = 5) (mean ± SD)



possible contamination of the gonads from the intestine.

#### Algal cell sampling

To investigate *A. anatina*'s filtration capability, algal cell concentration was monitored from 3 replicate water samples per aquarium at different time points (0 h, 6 h, 24 h, 48 h) using a microscope counting chamber (Bürker, Marienfeld, Germany, depth = 0.1 mm, square width = 0.25 mm). After 24 h, algal cell concentration declined sharply in both treatments, and monitoring was discontinued.

#### Mussel behavior

In order to check the possible effect of the bacterial and algal diets on the mussels' behavior (for example, a complete inactivation of mussels when incubated

with bacteria), in both experiments, *A. anatina* behavior was recorded 3 times per day at 9:00, 12:00, and 15:00 h in a 2 min visual inspection for 99 h. The traits that were monitored included shell openness, foot position (extension or contraction), movement, and biodeposition (feces and pseudofeces) formation since the previous monitoring. Shell openness was scored as follows: fully closed (siphons completely invisible) = 0, slightly opened (siphons are still invisible) = 1, half open (siphons visible, partly protruding) = 2, fully open (siphons fully visible, fully protruding) = 3. Foot position was graded as follows: foot in = 0, foot partly out = 1, foot out = 2. Movement (position changes in the mussel) was monitored by comparing mussel position to a mark on the lid of each aquarium denoting the position at the previous inspection. It was scored as follows: movement = 1, no movement = 0. Biodeposition presence was classified into 2 categories:

without biodeposition = 0, and with biodeposition = 1.

#### Trematode parasitism, sex, and reproduction of mussels

Possible trematode parasitism (parasite species, intensity of infection) in the mussels was examined at the end of each experiment when the bacterial sampling from the intestine and gonads was performed; this was done because trematodes can have a marked influence on mussel physiology (Taskinen, 1998; Jokela et al., 2005). The gonads are the main site of trematode infection in *A. anatina* (Taskinen et al., 1997). Therefore, a 2–3 mm slice across the gonads was dissected after the bacterial sampling was conducted, pressed between large glass plates, and examined microscopically for trematode sporocysts using transmitted light. This was accompanied with the sex determination of the mussel and an estimation of the intensity of the infection (Taskinen et al., 1994; Taskinen & Valtonen, 1995). The intensity of the trematode infection was scored as follows: uninfected = 0, light infection (one sporocyst tubule or one group of sporocyst tubules) = 1, moderate infection from > 1 group of sporocyst tubules to almost complete replacement of mussel gonad tissue by sporocysts = 2, and heavy infection (gonad tissue completely replaced by trematode sporocysts) = 3 (Taskinen et al., 1994).

#### Statistical analyses

The statistical analyses of bacterial and algal concentrations were performed using IBM SPSS software (version 24.0.0). Cell concentrations (the CFUs of *F. columnare* and the counts of algal cells) were used as response variables; aquaria were used as the statistical units. General Linear Model (GLM) repeated measures analyses were performed to compare differences in bacterial and algal concentration in time (the within-subject factor) between treatments with and without a mussel (the between-subject factor). The differences between different time points were further examined using within-subject contrast (“simple”). Levene’s test was used to assess the homogeneity of variance, and the Greenhouse–Geisser correction was applied to correct the number of degrees of freedom when the sphericity assumption was not valid.

Differences in mussel behavior (shell openness, foot position, movement, biodeposition formation) at different time points between the treatments (mussel only, mussel + bacteria, mussel + algae) were analyzed using mixed effects logistic regression (GLMM) in R (version 4.0.2.) statistics software and Lme4 package (R Development Core Team, 2015). The interdependence of repeated observations from the same individual mussel during the experiment was accounted for by treating mussel identity as a random variable. To be able to fit the models using binomial (logit) distribution, all behavioral data were recoded as 0 or 1 before the analysis. Thus, shell openness was recategorized as 0 (valves fully closed) and 1 (valves slightly, half or fully open), and foot position as 0 (foot in) and 1 (foot partly or completely out).

The differences in the intensity of trematode infection between treatment groups in Experiment 1 scored from zero (uninfected) to 3 (heavily infected) could not be tested with a Chi-square test because expected values were less than 5. Instead, we used Fisher’s exact test extended to  $3 \times 3$  contingency table (McDonald, 2014). In addition, the difference in the mean intensity of the trematode infection was tested between the “bacteria and mussel” treatments in both experiments with a *t*-test. Differences between treatments were considered significant if  $p < 0.05$ .

## Results

### Effect of mussel filtration on bacterial concentration (Experiment 1)

At the beginning of Experiment 1, the mean concentration of bacteria was similar in both treatment groups with *F. columnare* (Fig. 1A). During the next 48 h, the bacterial concentration increased in both treatments, but the increase was more pronounced in the treatment without a mussel than in the treatment with a mussel. Between 48 and 96 h, the bacterial concentration decreased in both treatments (Fig. 1A). The bacterial concentration was lower in the treatment with a mussel than in the treatment without a mussel (Table 2, between-subject effects,  $F_{1,8} = 16.31$ ,  $p = 0.004$ ). However, this difference became significant only after 96 h of the experiment, as indicated by the significant interaction between time and treatment and the contrasts between the levels of the within-subject



**Table 2** Results of GLM repeated measures ANOVA for the mean concentration of *Flavobacterium columnare* (CFU ml<sup>-1</sup>) in the two treatments (with and without mussel) at different time points in the 1st and 2nd experiment

Source	First experiment				Second experiment			
	df	MS	F	<i>p</i>	df	MS	F	<i>p</i>
Between subjects								
Intercept	1	2.95E+12	654.47	< 0.001	1	4.75E+11	180.30	< 0.001
Treatment	1	7.36E+10	16.31	0.004	1	489E+10	18.55	0.001
Error	8	3.61E+10			12	2.63E+09		
Within subjects								
Time	2.335	1.78E+12	61.63	< 0.001	2	2.83E+11	61.17	< 0.001
Time × treatment	2.335	1.52E+11 <sup>a</sup>	5.26	0.012	2	2.74E+10	5.91	0.008
Error	18.676	2.88E+10 <sup>a</sup>			24	4.64E+09		
Within-subject contrasts <sup>b</sup>								
Time								
Time 6 h vs time 0 h	1	8.26E+09	3.79	0.087				
Time 24 h vs. time 0 h	1	1.55E+12	47.93	< 0.001				
Time 48 h vs. time 0 h	1	5.95E+12	139.97	< 0.001	1	9.09E+11	107.46	< 0.001
Time 96 h vs. time 0 h	1	2.11E+12	61.64	< 0.001	1	4.22E+09	0.46	0.511
Time × treatment								
Time 6 h vs time 0 h	1	6.76E+07	0.03	0.865				
Time 24 h vs. time 0 h	1	1.37E+09	0.04	0.842				
Time 48 h vs. time 0 h	1	1.72E+11	4.06	0.079	1	1.09E+11	12.92	0.004
Time 96 h vs. time 0 h	1	4.19E+11	12.21	0.008	1	3.36E+10	3.64	0.081
Error								
Time 6 h vs time 0 h	8	2.18 E+09						
Time 24 h vs. time 0 h	8	3.23 E+10						
Time 48 h vs. time 0 h	8	4.25 E+10			12	8.46 E+09		
Time 96 h vs. time 0 h	8	3.43 E+10			12	9.22 E+09		

Df = degrees of freedom, MS = mean squares

<sup>a</sup> Greenhouse–Geisser corrected values

<sup>b</sup> Within-subject contrast for the interaction between time (hour) and treatments. The contrast is “simple”, which compares each time point to the first time point (time 0 h). Note that for the 2<sup>nd</sup> experiment measurements were made at time points 0, 48 and 96 h

factor and treatment (time × treatment) (Table 2). Thus, it took 48 h from the beginning of the experiment before the removal of the bacteria by the mussels was apparent (Fig. 1A). Thereafter, the mussels reduced the bacteria so that the bacterial concentration in mussel treatment was 0.7–0.5 times that of the controls at 48 and 96 h, respectively (Fig. 1A). Thus, the mussels removed half the bacteria within 96 h as compared to the controls.

### Effect of Mussel Filtration on Bacterial Concentration (Experiment 2)

In Experiment 2, a similar pattern in bacterial concentrations to those in Experiment 1 was observed. The bacterial concentrations were similar in the beginning of the experiment in the treatments with and without a mussel, respectively (Fig. 1B). At 48 h, the mean concentration of *F. columnare* had increased in both treatments, but with a mussel the increase was approximately half of that in the treatment without a mussel. Between 48 and 96 h, the bacterial



concentrations decreased in both treatments (Fig. 1B). The bacterial concentration was lower in the treatment with a mussel than in the treatment without a mussel (Table 2, between-subject effects,  $F_{1,12} = 18.55$ ,  $p = 0.001$ ). Contrasts between the levels of the within-subject factor and treatment (time x treatment) indicated that this difference was significant after 48 h but not after 96 h of beginning the experiment (Table 2). Mussels reduced the bacterial concentration in mussel treatment to 0.5 and 0.3 times that of the controls at 48 and 96 h, respectively (Fig. 1B). Thus, the mussels removed two-thirds of the bacteria within 96 h as compared to the controls.

#### Gonad and Intestine Analysis (Experiments 1 and 2)

In Experiment 1, *F. columnare* was isolated both from the mussels' intestines and gonads in the treatment with added bacteria, but not in the other treatment groups (i.e., the mussels with algae and the mussels kept in clean water). At the individual (mussel) aquarium level, among the 5 replicates of mussel + bacterium treatment, 3 mussels had *F. columnare* in both the intestine and the gonads, 1 mussel had *F. columnare* in the intestine but not in the gonads, and 1 tested negative (no bacterium in either the intestine or in the gonads). In Experiment 2, among the 9 replicates of mussel and bacteria, *F. columnare* was only found in the intestines of 8 individuals; it was not found in the gonads.

#### Effect of Mussel Filtration on Algal Cell Concentration (Experiment 1)

The mean concentration of algal cells (Shellfish Diet) decreased between the start of the experiment and 48 h in both treatments with and without a mussel (Fig. 1C, Table 3, within-subject effect [time],  $F_{3,24} = 83.20$ ,  $p = 0.001$ ). However, the presence of *A. anatina* did not affect algal cell concentration (Table 3,  $F_{1,8} = 1.872$ ,  $p = 0.208$ ).

#### Mussel Behavior

##### *Shell openness (Experiments 1 and 2)*

In both experiments, the mussels in all treatments had open or partly open valves at the beginning of the

**Table 3** Results of GLM repeated measures ANOVA for the mean of algal cell concentration (cell  $\text{ml}^{-1}$ ) in the two treatments with and without mussel at different time points. Df = degrees of freedom, MS = mean squares

Source	df	MS	F	p
<i>Between subjects</i>				
Intercept	1	3.68E+13	1681.82	< 0.001
Treatment	1	4.10E+10	1.87	0.208
Error	8	2.19E+10		
<i>Within subjects</i>				
Time	3	2.46E+12	83.16	< 0.001
Time × treatment	3	8.33E+10	2.82	0.061
Error	24	2.96E+10		

experiment. The mussels gradually closed their valves during the first 24 h but started opening their shells again toward the end of the experiment (Fig. 2A and E). In Experiment 1, the presence of bacteria did not affect the mussels' shell openness as compared to the mussels with algal cells or those in clean water (GLMM,  $p > 0.05$ , Table 4).

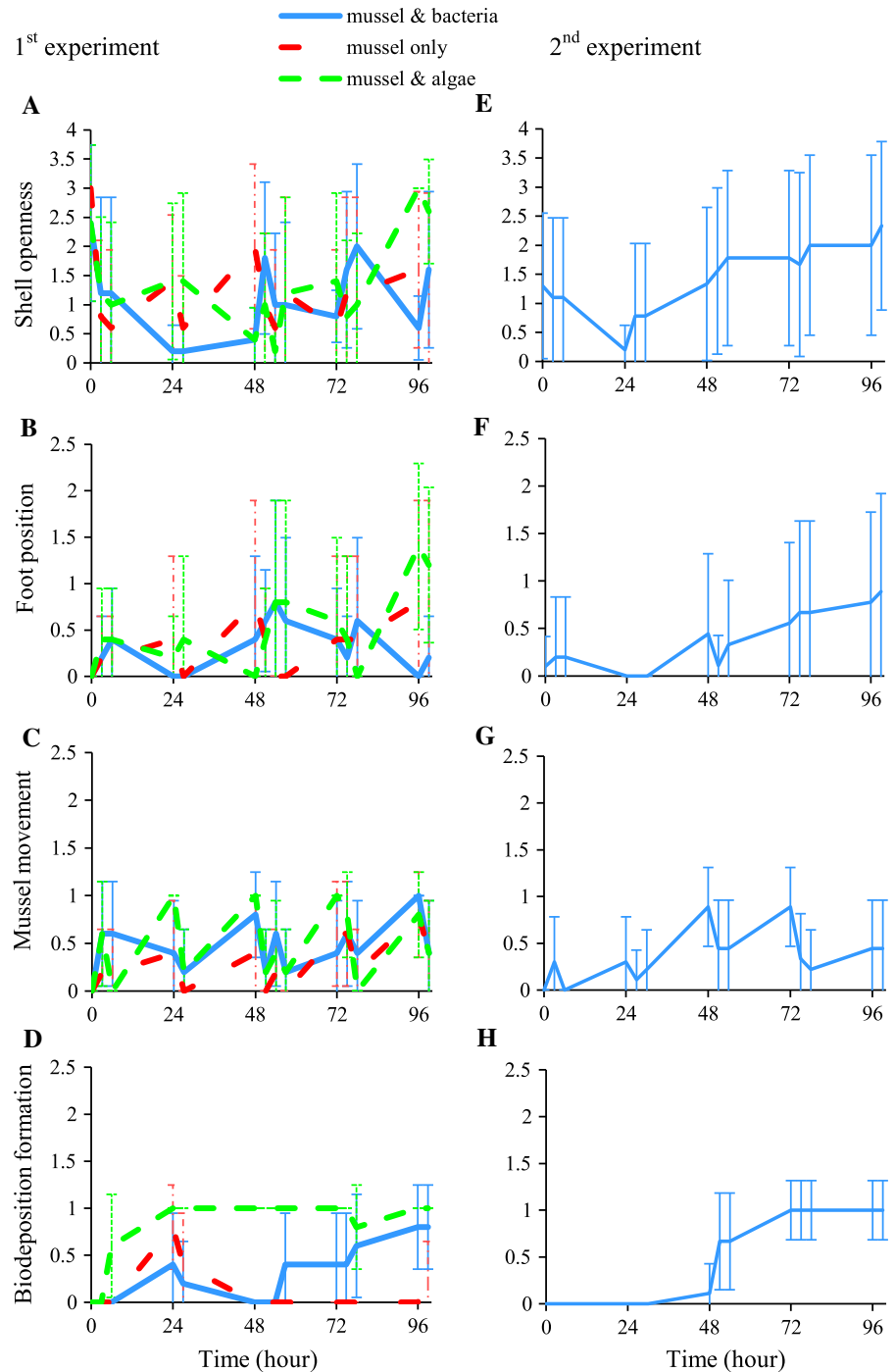
##### *Foot position (Experiments 1 and 2)*

Foot position scores revealed that the mussels kept their feet substantially contracted (Fig. 2B and F). In Experiment 1, the bacterial presence did not affect the foot position as compared to the mussels exposed to the algae or those kept in clean water (GLMM  $p > 0.05$ , Table 4).

##### *Movement (Experiments 1 and 2)*

*Anodonta anatina* in all treatment groups in both experiments exhibited a continuous and coherent pattern in movement behavior throughout the experiment (Fig. 2C and G). The presence of bacteria had a slight effect in decreasing mussels' movement activity (GLMM, the relative change in comparison to mussel only treatment  $0.69 \pm 0.36$ ,  $p = 0.053$ , Table 4), while the movement activity of mussels with algae was lower than mussels in clean water (GLMM, the relative change in comparison to mussel only treatment  $0.75 \pm 0.36$ ,  $p = 0.036$ , Table 4).

**Fig. 2** Shell openness (A), foot position (B), mussel movement (C) and biodeposition formation (D) in different treatments with mussels, added bacteria and algae at different time points (mean  $\pm$  SD) in the 1st experiment. Shell openness (E), foot position (F), mussel movement (G) and biodeposition formation (H) in treatment with mussels and bacteria at different time points (mean  $\pm$  SD) in the 2nd experiment



### Biodeposition (Experiments 1 and 2)

In Experiment 1, there were statistically significant differences between the bacterial, algal, and clean water treatments in terms of mean biodeposition

formation (GLMM, the relative change for mussel + bacteria treatment  $1.37 \pm 0.49$ ,  $p = 0.006$  and for mussel + algae treatment  $5.93 \pm 1.10$ ,  $p < 0.001$  in comparison to mussel only treatment, Table 4); thus, biodeposition formation was highest with the

**Table 4** Parameter estimates and standard errors (SE) of GLMMs for studying the effect of treatment (mussel + bacteria, mussel + algae) on mussel behavior (shell openness, foot position, movement and biodeposition) in the 1st experiment in comparison to the mussel only treatment (intercept)

Source	Estimate	SE	z-value	p
<i>Shell openness</i>				
Mussel (intercept)	0.08	0.40		
Mussel + bacteria	0.25	0.36	0.713	0.476
Mussel + algae	0.32	0.36	0.891	0.373
<i>Foot position</i>				
Mussel (intercept)	- 1.51	0.43		
Mussel + bacteria	0.27	0.43	0.642	0.521
Mussel + algae	0.74	0.41	1.800	0.072
<i>Movement</i>				
Mussel (intercept)	- 0.93	0.28		
Mussel + bacteria	0.69	0.36	1.929	0.053
Mussel + algae	0.75	0.36	2.093	0.036
<i>Biodeposition</i>				
Mussel (intercept)	- 1.93	0.41		
Mussel + bacteria	1.37	0.49	2.769	0.006
Mussel + algae	5.93	1.1	5.401	< 0.001

algal diet, second highest with the bacterial diet, and lowest with clean water (Fig. 2D). Biodeposits were found at time point 24 h in all treatment groups (Fig. 2D). After this time as well as toward the end of the experiment, biodeposition formation almost ceased in the group kept in clean water. In the treatment with added bacteria, biodeposition formation showed temporary cessation after 24 h, but it started again by 54 h and continued until the end of the experiment. In the treatment with algae, biodeposition formation remained high and constant until the end of the experiment (Fig. 2D). In Experiment 2, biodeposition formation largely resembled the pattern observed in the corresponding treatment (bacteria) of Experiment 1, with an increasing trend from 30 h onward (Fig. 2H).

#### Trematode Parasitism and Sex

Almost all the mussels in both experiments (14 out of 15 and 7 out of 9 in Experiments 1 and 2, respectively) were infected with the trematode parasite *Rhipidocotyle fennica* Gibson et al., 1992 (Table 1). One

individual in Experiment 2 was also infected with the trematode *Cercaria duplicata* von Baer, 1827 and with *R. fennica* (a double infection). There were no statistically significant differences in the intensity of trematode infection between the mussels in the bacterial, algal, and clean water treatments in Experiment 1 (Fisher's exact test  $p = 0.829$ ), nor were there differences between the mussels in the bacterial treatment in Experiments 1 and 2 ( $t = 0.350$ ,  $p = 0.732$ ). Because of the sterilizing trematode infection in the gonads (Taskinen & Valtonen, 1995), the sex of the mussels could not be determined for most of the individuals (Table 1).

#### DISCUSSION

As hypothesized, the presence of *A. anatina* reduced the mean concentration of the freshwater fish pathogen *F. columnare* in the water column so that at its lowest, only one-third of the concentration of the control level was observed in the mussel aquaria. Detecting *F. columnare* in the intestines of the mussels exposed to the bacteria, and formation of biodeposition in the presence of bacteria, suggests that the decrease in bacterial concentration was due to mussel filtration. In addition, other possible factors that could have caused or contributed to the reduction in concentration of *F. columnare* are, for example, competitive interactions between *F. columnare* and the microbiota present in and on the mussels (Rubiolo et al. 2019) or antimicrobial properties in mussel mucus (Cilia & Fratini 2018).

Filtration capacity in bivalves varies based on particle size and quality, among other things (Faust et al., 2009; Tuttle-Raycraft & Ackerman, 2018). Freshwater mussels can capture a wide range of particles sized 1–40  $\mu\text{m}$ , including bacteria—at least large bacteria (Strayer, 2008). *Flavobacterium columnare* is a rod-shaped bacterium measuring 4–10  $\mu\text{m}$  in length and 0.3–0.5  $\mu\text{m}$  in width (Declercq et al., 2013). Thus, by its size, *F. columnare* should be suitable for filtering by freshwater mussels, as suggested by the current results.

Isolation of *F. columnare* only from the aquaria with added bacterium indicates that the bacterium was not present in the experimental mussels originating from the lake, or in the borehole water source used in the study, and that there was no cross-contamination

between aquaria from adding the bacteria. In Experiment 1, a difference in bacterial concentration between the mussel treatment and control was not observed until 24–48 h. In addition, significant biodeposition production in the bacterial diet treatment also started late in Experiment 1 (not until 54 h). These results indicate that the removal of *F. columnare* from the water by *A. anatina* began after an initial delay. The reduction of bacterial concentration both in the mussel treatment and in the control group after 48 h in the present study could have been caused by, for example, an exhaustion of the nutrients required for steady bacterial growth, the bacteria's attachment to the aquaria and shell surfaces, sedimentation or changes in water quality. However, the difference between the mussel treatment and control remained through the end of the experiment.

*Flavobacterium columnare* has been isolated from mussel tissue in nature (Starliper et al., 2008), but the ability of *F. columnare* to infect *A. anatina* has not been demonstrated. The present study isolated *F. columnare* from mussel gonad tissue in Experiment 1, but not in Experiment 2. In Experiment 1, we performed the intestine sampling before the gonad sampling after the gonad/intestine complex was cut open, and therefore cannot rule out the possibility that presence of *F. columnare* in gonad was due to contamination from intestine. Therefore, the infection of the mussel gonads by *F. columnare* in Experiment 1 is, to some degree, uncertain. *Flavobacterium columnare*'s ability to infiltrate mussel tissue must be studied more closely in the future. *Flavobacterium columnare*'s ability to infect the mussels is an important question that can determine whether mussels can act as transient reservoirs for accumulated bacteria, potentially increasing the risk of the pathogen's spread (Ben-Horin et al., 2015; Bighiu et al., 2019).

There was no difference in the concentrations of algal cells between the experimental (with mussel) and control groups (without mussel), suggesting that the mussels did not significantly reduce the algal cell concentration. This was surprising and against the Hypothesis 2, especially when the biodeposition formation scores were high in the algal cell treatment. Algal cells tend to aggregate and accumulate at the bottom of the water (Berg et al., 1996). In addition, the algae used in the present study were preserved algae of marine origin, and it is possible that they started to

break up in the hypotonic freshwater environment, which could also have contributed to the observed decrease in algae concentration. The results from the bacterial treatment indicated that the mussels' filtration did not begin until after 24 h (Fig. 1A). This may have been too long a period of time for the preserved microalgae to maintain a good condition.

Mussels are highly sensitive to changes in their environment; therefore, mussel behavior can be used to monitor environmental conditions (Hartmann et al., 2015). In the present experiments, *A. anatina* behavior (shell openness, foot position, and movement) did not differ when the mussels were kept with bacteria, algal cells, or clean water, suggesting that the observed reduction of bacteria in the mussel treatment could not be explained by differences in the mussels' behavior. Freshwater mussels mainly hold their shells open for feeding and respiration and keep them closed for long periods under stressful conditions (Englund & Heino, 1994). Thus, shell openness, which also measured filtration activity in terms of siphon exposure, suggests that no marked differences in the activity of mussels took place between the treatment groups. This may imply that the other ecosystem services provided by mussels including nutrient excretion and bioturbation would not be hindered during the bacterial removal.

A very high proportion of mussels in both experiments were infected with trematodes, mostly the bucephalid *Rhipidocotyle fennica* (Taskinen et al., 1991; Gibson et al., 1992). *Rhipidocotyle fennica* infection has many adverse effects on *A. anatina* (Jokela et al., 1993, 2005; Taskinen et al., 1994; Taskinen & Valtonen, 1995; Taskinen, 1998). There is no information available about the influence of trematode parasitism on mussels' filtration rates; however, even if such an influence did exist, it would not skew the present results because all the treatment groups were equally trematode infected.

Bivalve mollusks consume a variety of particles like phytoplankton, organic detritus, and possibly dissolved organic matter (Ben-Horin et al., 2015). Phytoplankton is a primary food source for the majority of bivalves, but mussels can also utilize bacteria as a source of energy, surviving for long periods of time without any other food sources (Govorin, 2000; Gosling, 2008; Strayer, 2008; Ben-Horin et al., 2015; Burge et al., 2016). A high C:N ratio in the phytoplankton can meet mussels' carbon requirements (Muir et al., 1986), while bacteria with

a low C:N ratio as a food source provide them with the nitrogen they require (Seiderer et al., 1984). Therefore, it is not surprising that bivalve mollusks have been found to filter bacteria (e.g., Jørgensen et al., 1984; Muir et al., 1986; Kryger & Riisgård, 1988; Cotner et al., 1995; Silverman et al., 1995, 1997; Vanderploeg et al., 1995; Ben-Horin et al., 2015; Ismail et al., 2015; Burge et al., 2016; Mezzanotte et al., 2016). Indeed, finding *F. columnare* in the *A. anatina* intestines indicates that the mussels ingested the bacteria, suggesting that *F. columnare* could even be used as a food source by *A. anatina*, although this must be verified in further studies.

Filter-feeding bivalve mollusks often occur in dense beds; they provide many important ecological services (habitat modification, nutrient recycling and storage) and restorative functions through removing and concentrating suspended particles from the water column and improving water quality in aquatic ecosystems (Ben-Horin et al., 2015; Burge et al., 2016; Vaughn, 2018). Freshwater mussels filter large volumes of water and have shown their potential in water quality management as a tool for nutrient and particle removal (e.g., Strayer, 2014; Lummer et al., 2016; Mezzanotte et al., 2016; Hoellein et al., 2017). Therefore, they could be also used to decrease the environmental impact caused by organic wastes, to improve the hygienic condition of water, and to reduce pathogens and water-borne diseases (Jørgensen et al., 1984; Kryger & Riisgård, 1988; Cotner et al., 1995; Vanderploeg et al., 1995; Nichols & Garling, 2002; Christian et al., 2004; Mezzanotte et al., 2016), including *E. coli* (Silverman et al., 1995; Ismail et al., 2015).

The present study demonstrated the potential of *A. anatina* to remove the fish pathogen *F. columnare* from the water column in experimental conditions. Considering that *F. columnare* causes serious problems in fish farming (Wagner et al., 2002; Pulkkinen et al., 2010), it would be useful to investigate the efficacy of *A. anatina* in removing *F. columnare* at fish farms and their potential for reducing disease outbreaks in aquaculture. *Anodonta anatina* has been shown to markedly decrease the density of the cercarial larvae of the eye fluke *Diplostomum pseudospathaceum* (Trematoda), thereby decreasing the intensity of this harmful eye parasite in cultured rainbow trout (Gopko et al., 2017). *Anodonta anatina*'s potential to mitigate disease problems in

aquaculture can be justified, as it reduces the density of two key fish pathogens—*Flavobacterium* and *Diplostomum*—whose combined co-infection effect on fish mortality is even higher than in single infections (Louhi et al., 2015).

The present finding of *A. anatina*'s bacteria-removing capabilities may indicate a capacity to regulate bacterial communities. Based on the current results, it is not possible to determine whether *A. anatina* can significantly influence natural *F. columnare* populations or reduce pathogen transmission and disease outbreaks in field conditions. Overall, the ecosystem effect of freshwater mussels through bacteria removal is largely unknown.

The present experimental results showed a significant reduction of *F. columnare* concentration in the water column by *A. anatina*, which is a significant finding with regard to the potential of mussels in mitigation of disease outbreaks in aquaculture. Although our experimental setup does not allow indicating the exact mechanism for the bacterial reduction, the presence of the bacteria in the mussels' digestive systems suggests ingestion of bacteria by mussels. However, as there are challenges in extrapolating effects from laboratory or mesocosm experiments to field conditions, further studies are required to evaluate 1) the potential role of *A. anatina*, or freshwater mussels in general, in controlling natural bacterial populations; 2) the role of freshwater mussels in influencing the emergence and outbreaks of *F. columnare* disease in natural waters and in aquaculture; and 3) the possible role of bacteria as a food source of mussels. The present results indicate the potentially important, but poorly understood, effect of filter-feeding freshwater mussels on aquatic bacterial communities. Thus, freshwater mussels can provide potent ecosystem services including filtering and removing pathogens from water, which could be utilized in aquacultures and water quality management.

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**Author contribution** MH, SA, KP, and JT all conceived of and designed the experiments, performed the experiments, and collected the data. MH, SA, and KP analyzed the data. MH and SA wrote the first draft of the manuscript. Thereafter, MH, SA, KP, and JT contributed to writing the paper.

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**Data Availability** All the data and materials will be provided, if requested.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** Not applicable.

**Consent to participate** All authors have given their consent to participate.

**Consent for publication** All authors have given their consent to participate.

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