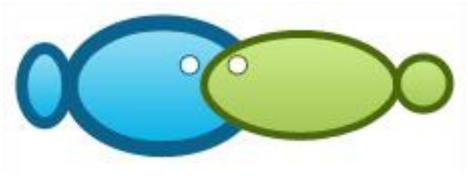

Effect of culture supernatant from *Haslea ostrearia* on the clearance rate and survival rate of adult Pacific oyster *Crassostrea gigas* infected with *Vibrio aestuarianus*

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Abstract. The microalga *Haslea ostrearia* is known as a diatom with the peculiarity of producing a water soluble blue-green pigment called marennine. This pigment has antibacterial activities against pathogenic bacteria of bivalves produced in aquaculture. The present study evaluates the effect of culture supernatant of the diatom *Haslea ostrearia* containing marennine on the clearance rate (CR) and survival rate (SR) of adult Pacific oyster *Crassostrea gigas* infected with the bacterial pathogen *Vibrio aestuarianus*. The greening of oysters was conducted by exposing bivalves to 0.5 mg L^{-1} of marennine for three days, and the effect of marennine on the CR and SR of oysters was evaluated by comparing four groups of treatment: control, greened, control+infected and greened+infected. The SR of oysters in each treatment was monitored for 11 days. Our results showed that marennine concentration decreased with time and dropped significantly on the last day of greening, and that marennine at an initial concentration of 0.5 mg L^{-1} did not significantly affect the CR of oysters in all treatments. Mortality of oysters was only observed in the control+infected group, but it was not significantly different from the control. Hence, the present study showed that the greening using 0.5 mg L^{-1} of marennine did not affect SR of adult oyster *C. gigas*, but further studies are needed to determine whether *H. ostrearia* supernatant could be used as an antibacterial and prophylactic agent in bivalve aquaculture.
Key Words: bivalve aquaculture, blue diatom, marennine, Pacific oyster, pathogenic bacteria.

Introduction. Mass mortalities in commercially important bivalves have been considered as a tremendous problem worldwide. In France, large scales of episodic massive mass mortalities occurred repeatedly along all coasts in 2008 and 2009, which caused detrimental economic loss due to a declined oyster production (Bédier et al 2011). Sampling juvenile oysters *Crassostrea gigas* during these outbreaks showed significant increase in the prevalence of the ostreid herpesvirus OsHV-1 compared to previous years (Segarra et al 2010) and also of several marine bacteria associated to mortality in *C. gigas* (Saulnier et al 2010). Moreover, the experimental studies of Dégremont (2011) and De Decker & Saulnier (2011) supported the hypothesis that bacterial isolates such as *V. splendidus* and *V. aestuarianus* induce mortality in healthy adult oysters *C. gigas*, which appeared to be more sensitive than the juveniles. In order to avoid vast economic losses due to disease outbreak, a solution could stem from natural antimicrobial agents, which are required to support sustainable aquaculture practices.

One of these natural antimicrobial candidates is marennine, the water-soluble blue-green pigment that is excreted by the non-toxic diatom *Haslea ostrearia* (Gaillon) Simonsen. Marennine is mostly known because it can adhere to the gills and labial palps of bivalves when they filter seawater, selecting and feeding on particles in suspension, during *H. ostrearia* blooming in oyster ponds, a phenomenon known as oyster greening (Gastineau et al 2018; Prasetiya 2015; Prasetiya et al 2017a). Until today, this anthropic greening phenomenon contributes significantly to the economic value of cultured oysters particularly in the French Atlantic coast due to organoleptic and essential fatty acids modification in green oysters (Robert 1975; Piveteau 1999; Prasetiya 2015; Prasetiya et al 2017b). Moreover, the greening phenomenon has also been historically observed in natural environments, in Great Britain (Sprat 1669), in Denmark (Petersen 1916), in the USA (Mitchell & Barney 1918) and in Australia (personal observation of Lean-Luc Mouget).

It has also been demonstrated that marennine has antibacterial properties and thus acts as an antibacterial and natural protective agent against pathogens in aquaculture, c.a. *V. aestuarianus* and *V. splendidus* (Gastineau et al 2012, 2014; Turcotte et al 2016). For instance, Turcotte et al (2016) demonstrated that the culture supernatant of *H. ostrearia* containing marennine (or the Blue Water, BW) could increase the survival rate of larvae of *Placopecten magellanicus* and *Mytilus edulis*, when exposed to bacterial pathogen *V. splendidus*. Therefore, marennine could be applied in shellfish aquaculture as a natural antibacterial agent and also possibly as an immunostimulant. However, to achieve optimum advantage exploiting marennine biological activities in aquaculture, the feasibility of using this pigment needs to be demonstrated.

In a previous study, Prasetiya et al (2017b) studied the impacts of purified marennine obtained according to the method of Pouvreau et al (2006) on the behavior (valve opening), physiology (clearance rate - CR, oxygen consumption, assimilation efficiency and scope for growth) and biochemistry (fatty acid composition of neutral and polar lipids) of blue mussel *M. edulis* juveniles and the American oyster *Crassostrea virginica*. Nevertheless, utilization of purified marennine is rather unpractical at an aquaculture scale, since it is a costly and time-consuming process. Therefore, utilization of marennine directly from the culture supernatant of *H. ostrearia* (hereafter named as blue water, BW) in bivalve aquaculture should be better considered. Recently, it has been demonstrated that BW at marennine concentration 3 mg L^{-1} has no effect on the bivalves, but it can alter the CR and the oxygen consumption of adult *M. edulis* (Prasetiya et al 2019). In contrast, BW at a concentration of 1 mg L^{-1} could be lethal to the larval stage in this species (Turcotte et al 2016). Furthermore, at a similar range of concentration, the BW could inhibit the growth of pathogenic bacteria such as bacterial pathogens relevant to aquaculture, *Vibrio harveyi* and *Staphylococcus aureus* (Permatasari et al 2019; Prasetiya et al 2020a). In line with current research on natural antimicrobials in aquaculture, the present study aimed to determine the effects of BW on the worldwide produced *C. gigas* species, at the adult stage, both on the CR and the possible protection against the bacterial pathogen *V. aestuarianus*.

Material and Method

Location and time of the experiment. This study was conducted at the Laboratory of Physiology, Genetic and Microbiology of Marine Molluscs Unit, L'Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Bouin Station, France. The experiment was conducted from September to December 2018.

Animals and diet preparation. Diploid adult oysters, *C. gigas* (81.6±1.2 mm mean shell length), were obtained from the Mollusc Experimental Platform located on the Ifremer station of Bouin, Atlantic coast of France. Unselected oysters (NSI) were produced according to a standardized protocol described by Petton et al (2015). All oysters (n=132) were cleaned from epibionts and other encrusting organisms. Animals were placed in 200 L maintenance tanks with 24 h per day aeration, salinity of 34 ppm,

temperature of 20°C and natural photoperiod. Animals were fed daily with *Tisochrysis galbana* at 1:1 equivalent volume of 4x104 cells mL^{-1}.

Algal culture and marennine preparation. The cultures of *H. ostrearia* (NCC-136) were obtained from the Nantes Culture Collection (NCC), Laboratory of Mer Molecule et Santé (MMS), Université de Nantes, France. The non-axenic stock cultures were maintained in sterilized Conway+Silicate medium at 100 µmol photons m^{-2} s^{-1}. Experimental cultures were produced in 500 mL Erlenmeyer flasks containing 250 mL of medium and maintained at 16°C with 14:10 h light/dark photoperiod (Arsad et al 2019). BW was harvested from the Erlenmeyer flasks and marennine concentration was estimated on cell-free culture medium (syringe-filtered on 0.20 µm, Sterile-R) using the Beer-Lambert law as described by Prasetiya et al (2016). The optical density was measured at 677 nm in a 10 cm cuvette using a spectrophotometer (Cary 100 Bio UV-Visible, Agilent Technologies), as well as the specific extinction coefficient for extracellular marennine (ε677 = 12.13 L g^{-1} cm^{-1}), as stated in Pouvreau et al (2007).

Greening of animals. For the present study, the oysters were greened in BW (equivalent to 0.5 mg L^{-1} of marennine) for 72 h and fed with *T. galbana* (3% of animal dry weight) as previously described. Similar microalga diet was used to feed the control. Optical density was observed daily for 72 h to monitor the decrease in marennine concentration due to filtration by the animals. Three animals were sampled on the last day of the greening experiment to verify the marennine fixation on the gills. Greened animals and control were then re-acclimatized in individual chambers with no marennine, and without any diet for 24 h prior to the physiological experiment.

Preliminary test on metabolic chamber. A preliminary test was performed to verify the stability of the static metabolic chamber in the physiological experiment. Briefly, two types of individual chambers filled with 400 mL of 0.2 µm-filtered, UV treated seawater, were used, the first ones containing an empty shell without the animal, and the second ones only containing seawater. Three replicates were provided for each treatment (total n=6). Afterwards, 200 cells μL^{-1} *T. galbana* were added into the chamber with aeration to generate the circulation in the chamber. Sampling was conducted at 0, 20, 40 and 60 min after the microalgae were added (T0, T20, T40 and T40, respectively). Cell density on each sampling time was measured using a flow cytometer (CyFlow-R Space, PARTEC). The validity of the stability of the metabolic chamber could be observed when the difference in cell density in each chamber was not significant.

Physiological rate measurement. 20 chambers filled with 1 µm-filtered and UV treated seawater were used simultaneously for 40 animals (10 in control, 10 in the greened group, 10 in control+infected group and 10 in greened+infected group). Two chambers of sedimentation contained only empty shells. Animals with valves that remained closed in the chamber during the measurement were excluded from the physiological analysis. Physiological rates were also standardized to mass-specific rates equivalent to individuals of 1 g dry mass using weight exponents as previously described in Bayne et al (1987).

CR is the volume of water containing suspended particles removed per unit time. It was measured using a static system as described in Prasetiya et al (2017b). Briefly, animals were placed in 0.5 L metabolic chambers 24 h prior to measurements, then fed with *T. galbana* at an initial concentration of 80 cells μL^{-1}. Circulation in the metabolic chamber was generated by aeration around the wall of the chamber to avoid the re-suspension of feces. Food particles were counted every 20 min during 60 min of measurement, using a flow cytometer machine as described in the previous section. The instrument was adjusted to measure *T. galbana* that fall in the size range of 4-8 µm, known to be efficiently retained by oysters (Ward & Shumway 2004; Prasetiya et al 2017b). The utmost difference between two consecutive measurements was used to calculate CR, as described in Gilek et al (1992), as follows:

$CR = [ln(T0)-ln(Tx)] \times Vol/t$

$CR_{final} = (CR_{max} - CR_{con})/(w)0.4$

Where: CR represents the clearance rate, T0 is the cell density at time zero, Tx is the cell density at time x, Vol and t represent the volume in the chamber and the interval sampling time, respectively. CR_{final} is the standardized CR, CR_{max} is the maximum value of CR obtained from each sampling time, CR_{con} and w are the CR obtained from the control chamber and the animal dry weight, respectively.

The survival rate (SR, %) was used to assess the protective effect of marennine. This parameter was monitored daily during 11 days post-cohabitation treatment. The SR was calculated as follows:

$SR (\%) = (Nf/Ni) \times 100$

Where SR is the survival rate, Nf and Ni are the final number of surviving oysters and the initial number of oysters, respectively.

Bacterial preparation. The virulent stains *V. aestuarianus* 07/011 were used together in this study and the protocol of bacterial culture was performed following Travers et al (2017). The strain was cultured in Marine Broth (Difco) prepared as follows: peptone 4%, yeast extract 1% diluted in Sterile Artificial Sea Water (SASW 2.3% NaCl, 20 mM KCl, 5 mM $MgSO_4$, 2 mM $CaCl_2$). Bacteria were grown at 20°C for 20 h under constant shaking. The bacterial culture concentrations were evaluated spectrophotometrically to obtain an optical density (OD) at 600 nm. Cells were washed with centrifugation at 3000 g for 10 min and the resulting pellet was re-suspended in 5 mL SASW to an OD of 2.0, corresponding to $2x10^8$ CFU mL^{-1}. A mix of both bacterial cells (v/v) was prepared for individual injection.

Animal bacterial infection by intramuscular injection followed by cohabitation. The protocol of bacterial infection of the adult *C. gigas* was performed according to Travers et al (2017). Briefly, oysters were anaesthetized for 5 h at 20°C in a solution of magnesium chloride (28 g L^{-1} $MgCl_2$), with added phytoplankton (*T. galbana*) and aeration to improve anesthetic efficiency (personal observations). Then, 100 µL of *V. aestuarianus* 07/011 bacterial suspension in equal doses of 10^8 CFU mL^{-1} were injected into the adductor muscle of oysters (n=60). Another group of oysters (n=12) were injected with SASW (as control). The rest of injected bacterial suspension was maintained to determine the presence of bacteria after 48 h of incubation time. After injection, oysters were transferred to tanks filled with 0.5 mm-filtered seawater disinfected with UV, maintained under static conditions at 20°C with aeration. Moreover, mortality of the bacteria-injected animals was monitored for 24 h after injection. When the mortality was observed, the contaminated seawater medium (CSM) was used for the cohabitation treatments of healthy oysters.

In the cohabitation treatment, the CSM was used and transferred into the individual chamber containing oysters (total n=20, 10 healthy oysters without marennine and 10 healthy oysters with BW, which hereafter are named as "control" and "greened", respectively). The contact between the animals and the CSM was performed for 24 h. After 24 h of cohabitation, the contaminated seawater in each individual chamber was replaced with 0.5 mm-filtered UV-treated seawater at 34 ppm salinity prior to the physiological experiment. 20 animals without pathogens were used as control (10 healthy oysters without BW and 10 healthy oysters with BW, which hereafter are named as "control+infected" and "greened+infected", respectively). All experiments were performed under static water conditions at 20°C with aeration, and supplementary food was added for the physiological experiment 24 h post cohabitation. The mortality was monitored for 11 days.

Statistical analysis. All statistical analyses were carried out using the GraphPad Prism Version 6 for the Macintosh Operating System. The significance value of all analysis was set at $p<0.05$. Normality and homoscedasticity were assessed by the Kolmogorov-Smirnov and Levene tests, respectively. The two-way ANOVA analysis was used to estimate the effect of shell's presence, sampling time and interaction in the control chamber in the preliminary test. The significance of reduction in marennine concentration in function of time was analyzed using one-way ANOVA, followed by Tukey's multiple comparison test when the significant result was detected. The significant differences in CR for each treatment were analyzed by one-way ANOVA to identify the effect of marennine and bacterial infection.

Results and Discussion

Stability of metabolic chamber. The preliminary test showed that the cell density of microalgae in both types of metabolic chambers remained steady from the beginning until the end of the experiment (ANOVA, $F_{3,5}=0.07$, $p=0.974$), indicating that no sedimentation occurred and microalgae circulated evenly in the chamber. The two-way ANOVA also showed no significant difference (ANOVA, $F_{1,5}=0.114$, $p=0.744$) in cell density observed between the chambers containing shell (+S) and without shell (-S) (Figure 1). In the literature, the indirect measurement of CR using a static chamber or a closed system is usually considered to have weakness, for instance, the accumulation of excretory products (pseudofeces) and declining particle concentration due to sedimentation (Gosling 2015). In the present work, the cell density of *T. galbana* in the control chamber (without animals) remained constant, indicating that the cells were evenly distributed in the experimental chamber and that sedimentation of the algae to the bottom of the chamber was absent or could be neglected. Moreover, in this study, the sample analysis using the flow cytometry avoided miss-identification between the living algae cells and the excretory products (feces and pseudofeces).

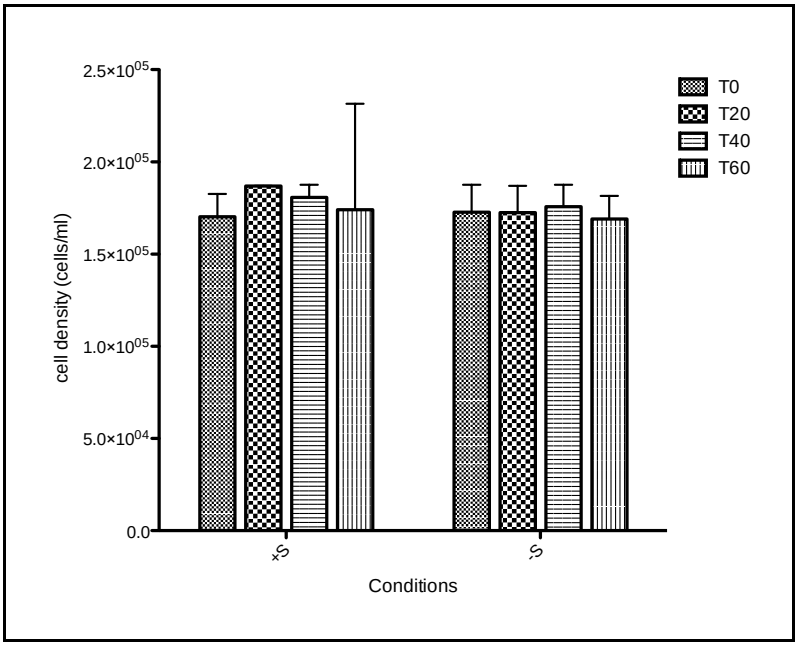

Figure 1. Microalgae cell density in individual chamber containing shells (+S) and without (-S) shells as control in the physiological experiment at different sampling time (T20-T60 - from 20 to 60 min, respectively); data are presented as means ± standard of error of mean, n=6.

Marennine fixation by oyster. In the present work, fixation of marennine in oyster gills was validated by the daily measurement of marennine concentration in the greening chamber from the beginning (day 0) until the end of greening experiment (day 3).

Marennine concentration decreased in function of time and dropped significantly on the last day of greening (Figure 2, ANOVA, $F_{3,8}$=8.414, p=0.007). Similar results were found by Prasetiya (2015) and Gastineau et al (2018), who observed a decrease in marennine concentration in the chamber containing *C. gigas*. The decrease in marennine concentration with time is likely due to the filtration activity of the oyster that results in the fixation of the pigment on the gills of the oyster. However, the greening intensity depends on both marennine concentration, exposure duration and animal physiology. For instance, by visual observation, it can be noted that the concentration of 0.5 mg L^{-1} marennine resulted in a weak greening coloration of the gills of oysters (Figure 3B), as compared to oysters exposed to 2 mg L^{-1} for the same duration (Prasetiya 2015).

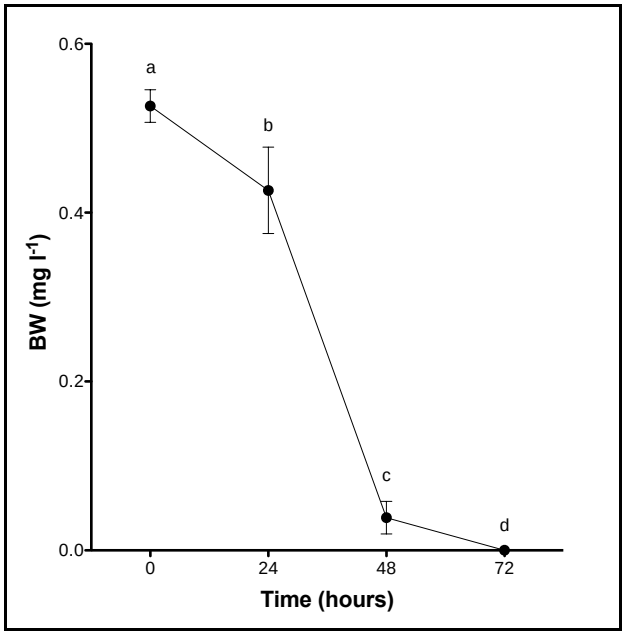

Figure 2. Marennine concentration (BW) in the chamber during the greening experiment; data are presented as means ± standard of error of mean (n=3); different letters indicate significant differences (p<0.05).

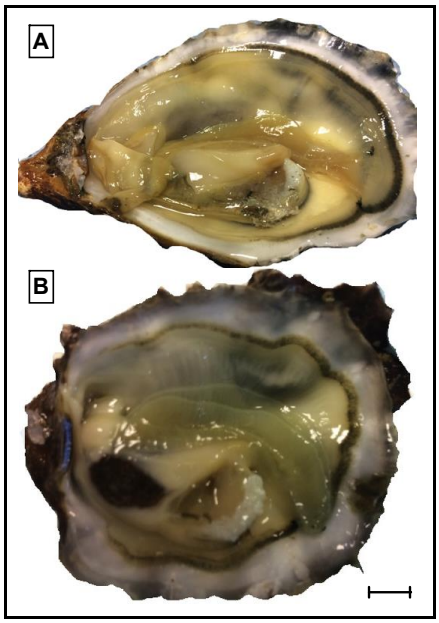

Figure 3. Greening intensity of oysters exposed to different concentration of marennine (0 and 0.5 mg L^{-1} that represent A and B, respectively) for 3 days; scale - 1 cm.

Marennine effect on CR and SR of oysters. Oysters filter the *T. galbana* suspension given in experimental chambers. Similar to the control chambers in the preliminary experiment, the cell density in the control chambers remained constant, suggesting that the experimental chambers appeared to be stable with no sedimentation and cells equally distributed. On the other hand, the cell density of *T. galbana* in the experimental chamber with oysters decreased due to the bivalve filtration and selection activity.

In the present work, BW containing marennine at 0.5 mg L^{-1} did not affect CR in adult oysters (Figure 4, One-way ANOVA, p=0.383). These results are comparable to previous studies (Prasetiya et al 2017b) that demonstrate that the effect of marennine was not only concentration dependent but also species and age-specific. In juveniles of *M. edulis* and *C. virginica*, significant effects of purified marennine on CR were only observed at 2 mg L^{-1}, while no effect was observed at 0.5 mg L^{-1} (Prasetiya et al 2017b). Recently, the ultrafiltered marennine at 3 mg L^{-1} appeared to decrease CR in adult *M. edulis* and *C. virginica*, but not in giant scallop *P. magellanicus* (Prasetiya et al 2019; Prasetiya et al 2020b).

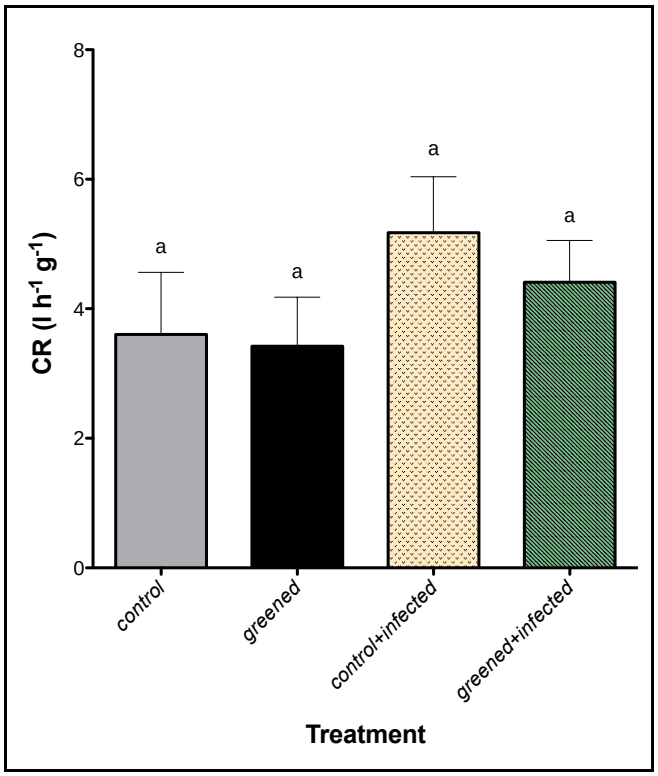

Figure 4. The clearance rate (CR) of adult oysters exposed to different concentration of blue water and bacterial infection; data are presented as means ± standard of error of mean (n=40); same letters indicate no significant differences (p>0.05).

In this study, we found that the bacterial infection did not significantly affect the CR in both control and greened oysters after 24 h of bacterial contamination. However, we observed the tendency of the CR of oysters in "control+infected" and "greened+infected" to be slightly higher than that of "control" and "greened" oysters. Yet, it seemed that the variation between treatments was not significant (p>0.05). Bacterial contamination in marine bivalves can influence the filtration activity that can eventually lead to bacterial cell removal from the water column such as *Salmonella enterica*, *Vibrio parahaemolyticus* and *Escherichia coli* (De Abreu Corrêa et al 2007; Love et al 2010; Phuvasate et al 2012). In the present study, the presence of *V. aestuarianus* in the experimental chambers probably accelerated the CR of oysters, but this has to be confirmed by further experiments.

In the presence of *V. aestuarianus*, no significant difference in SR in all treatments was observed, indicating that the bacterial infection using the cohabitation method did

not contribute significantly to oyster mortality (Figure 5). However, the presence of *V. aestuarianus* in the tissue of infected oysters should be tested, e.g., using RT-PCR, to validate this hypothesis. Travers et al (2017) recently demonstrated that the bacterial infection by *V. aestuarianus* could induce 90-100% mortality in *C. gigas*, this range of mortality occurring within 5 days post infection. Nevertheless, several experimental differences should be stressed. Firstly, in their experiment the bacterial infection was performed by intramuscular injection method instead of cohabitation method. Secondly, the oysters *C. gigas* used were juveniles. Thus, they are more sensitive to a pathogen like *V. aestuarianus* with a severe mortality rate. On the other hand, in our study, mortality of adult *C. gigas* was observed from day 8 to day 11 post-infection of CSM and only found in the "control+infected" treatment. However, no significant difference was found in SR between "control+infected" and "greened+infected" ($p>0.05$).

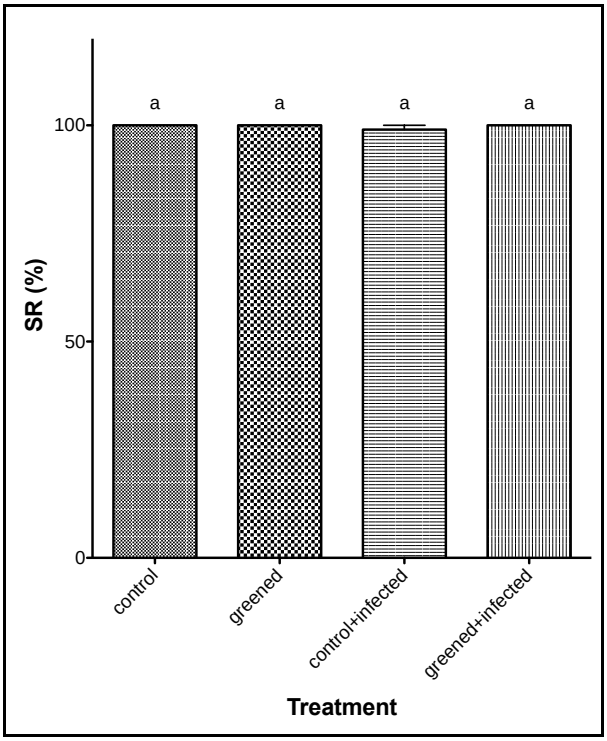

Figure 5. The survival rate (SR) of adult oysters exposed to different concentration of marennine and bacterial infection during the 11 days of observation; data are presented as means ± standard of error of mean (n=10).

Conclusions*.* The present study shows that the greening of oysters with BW containing 0.5 mg L^{-1} marennine did not affect filtration activity or survival rate of adult oyster *C. gigas* in all treatments. Given the low and not significant mortality observed, further studies need to be completed using different marennine concentrations within the range found in the natural oyster ponds to assess the feasibility of using this pigment as an antibacterial and prophylactic agent in shellfish aquaculture.

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

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