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Aquat. Living Resour. 29, 401 (2016)

DOI: 10.1051/alr/2016032
www.alr-journal.org

Aquatic Living Resources

Prophylactic effect of *Haslea ostrearia* culture supernatant containing the pigment marennine to stabilize bivalve hatchery production

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Received 14 July 2016; Accepted 21 October 2016

Abstract – This paper explores the possibility of using the supernatant of *Haslea ostrearia* culture containing marennine, a natural microalgal pigment, as an antimicrobial in bivalve hatcheries. The blue mussel *Mytilus edulis* and the scallop *Placopecten magellanicus* were used as model animals, and the pathogenic marine bacteria *Vibrio splendidus* was used to induce larval mortality. The hypothesis tested was that *V. splendidus* pathogenicity in larval rearings can be controlled by using marennine-containing culture supernatants. The effect of three marennine concentrations was tested on a larval rearing over 20 days for *M. edulis* and 9 days for *P. magellanicus*. At a low dose (0.1 mg L^{-1}), survival and physiological condition were both higher than in the control. In bacterial challenges, larvae were exposed to *V. splendidus* for 72 h, with or without marennine. The bacterial challenge caused significant mortality when compared to controls, while the marennine-treated larvae showed significantly higher survival. Results show that marennine is an interesting molecule for pathogen control in hatcheries as it is active at low concentrations and significantly enhanced larval survival and physiological condition.

Keywords: Bivalve hatcheries / microalgal by-products / *Haslea ostrearia* / *Mytilus edulis* / *Placopecten magellanicus* / *Vibrio splendidus*

1 Introduction

A primary requisite in any shellfish culture or farming operation is an abundant, reliable and inexpensive supply of juveniles, commonly called seed (Helm et al. 2004). Hatchery-produced seed is increasingly becoming the standard raw material for aquaculture, a trend that is likely to develop in the future (FAO 2011). One of the major difficulties in bivalve hatchery production is repeated bacterial infections resulting in significant mortality, causing major losses and great expense for shellfish growers. These mortalities are generally related to bacteria from the genera *Vibrio*, *Pseudomonas* and *Aeromonas*, with members of the genus *Vibrio* being the most frequently observed (Tubiash et al. 1965; Estes et al. 2004; Paillard 2004). The pathogenic bacteria *V. splendidus* is considered as a widespread bivalve pathogen, and its pathogenicity has been thoroughly studied (Duperthuy et al. 2010, 2011; Decker and Saulnier 2011; Tanguy et al. 2013).

The two bivalve species studied here, *Placopecten magellanicus* and *Mytilus edulis*, are good representatives of the most commonly cultured species, the first being reputed to be difficult to produce compared to mussels or oysters (Robert et al. 1996; Helm et al. 2004). In pectinid hatcheries, bacterial infections are known to be the major cause in massive mortality events around the globe, whatever the production system used (Devauchelle and Mingant 1991; Riquelme et al. 1995; Nicolas et al. 1996; Jorquera et al. 2001; Torkildsen et al. 2004; Torkildsen et al. 2005; Andersen et al. 2011). The use of known antibiotics to control diseases is generally avoided in hatcheries, as they are expensive, may leave harmful compounds, and may cause the development of antibiotic-resistant bacterial strains (HHS 1999; Helm et al. 2004). Their utilization in hatcheries is now controlled or prohibited in many countries (Arkinbowale et al. 2006). Hence, there is a need to identify new molecules with antimicrobial activities. Natural compounds are unsurpassed in their ability to provide novelty, complexity and diversity (Spížek et al. 2010), which should help prevent the development of antibiotic resistance.

Marennine (Lankester 1886) could be such a molecule. It is a blue-green water-soluble pigment synthesized by the marine pennate diatom *Haslea ostrearia* (Gaillon) (Simonsen 1974).

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This microalga has long been considered the only one within the *Bacillariophyceae* able to produce such a pigment (Neuville and Daste 1978). Recently, two new species of blue *Haslea* have been described, one producing a pigment similar to marennine, *H. provincialis* (Gastineau et al. 2016), the other, *H. karadagensis*, a different pigment but belonging to the same chemical family (Gastineau et al. 2012b). Marennine accumulates in the apical regions of the cell before being released into the growth medium (Nassiri et al. 1998), allowing the predominance of *H. ostrearia* due to marennine's allelopathic activity (Pouvreau et al. 2007a; Prasetiya et al. 2016). Purified marennine has been shown to display antioxidant (Pouvreau et al. 2008), antiproliferative, antiviral, antifungal, and antibacterial activities (Gastineau et al. 2012a, 2014). In vitro, marennine inhibited the development of pathogenic marine bacteria *Polaribacter irgensii*, *Pseudoalteromonas elyakowii*, and *Vibrio aestuarianus* at concentrations as low as 1 μg m L^{-1} (Gastineau et al. 2012a). The molecular structure of marennine is unknown and many hypotheses have been formulated regarding its chemical nature. The most recent hypothesis proposed marennine as a polyphenol (Pouvreau et al. 2006a), possibly with a glycosidic component (Gastineau et al. 2014). The molecule's interaction with bacteria has been investigated briefly. As evidenced by Tardy-Laporte et al. (2012) in *Escherichia coli*, a Gram-negative bacterium, marennine appears to exert its antimicrobial activity by interacting with lipopolysaccharides (LPS) in the outer membrane. In recent years, molecules disturbing biological membranes via non-specific interactions with the membrane lipid components have drawn attention as they may be an interesting solution for the development of new efficient antibiotics against resistant bacteria (Arouri et al. 2009). With the development of high-volume production systems (Gastineau et al. 2014), marennine could be used as a natural antimicrobial to control bacterial pathogenicity in larval cultures.

The main hypothesis of this work was that the supernatant of *Haslea ostrearia* culture containing marennine (blue water, BW) could exert a prophylactic effect in larval rearing of *P. magellanicus* and *M. edulis*, by controlling *V. splendidus* pathogenicity in bacterial challenge tests with these two bivalves. More specifically, the main objectives were to (1) improve larval survival and physiological condition; (2) reduce the total bacterial load; and (3) inhibit the pathogenicity of *V. splendidus* in challenge conditions by using BW with marennine in *P. magellanicus* and *M. edulis* larval rearing. Additionally, the effect of marennine at the concentrations used in the larval tests was tested on the growth of microalgae species used as feed for the larvae.

2 Materials and methods

All experiments were conducted at the *Station aquicole de Pointe-au-Père* (UQAR, 48°31′ N; 68°28′ W, Quebec, Canada). Experiments were run with axenic cultures of *Haslea ostrearia* (NCC-136) isolated from Bourgneuf Bay, France and provided by NCC (Nantes Culture Collection). Axenic stock cultures were grown in autoclaved F/2 medium at low irradiance (<20 μmol photons m^{-2} s^{-1}) and maintained in the laboratory by regular subculturing with axenic fresh media every 14 days. Pilot-scale cultures were grown in semi-continuous mode in 50 kDa (Romicon, KOCH Membrane) ultrafiltered seawater enriched with F/2 medium (Guillard 1975) and 30 mg L^{-1} silicates. Cultures were conducted in two 100 L flat bottom circular photobioreactors (PBRs) at a light level of 180 μmol photons m^{-2} s^{-1}, 14/10 h light/dark cycle, temperature of 20 °C and salinity of 28. Marennine concentration was determined on cell-free culture water (syringe-filtered on 0.22 μm) using the Beer-Lambert law. Optical density was measured at 677 nm in a 10 cm cell using a spectrophotometer (Cary 100 Bio UV-Visible, Agilent Technologies), as well as the specific extinction coefficient for extracellular marennine (ε_{677} = 12.13 L g^{-1} cm^{-1}), as stated in Pouvreau et al. (2007b). When maximum marennine concentrations were reached (around 6.2 mg L^{-1}), the supernatant containing the marennine (BW) from each of the PBRs was concentrated 100 times (from 100 L to 1 L) using two ultrafiltration units (30 and 3 kDa), as described in Pouvreau et al. (2006b) and stored for less than one week in high-density polyethylene bottles at 5 °C in the dark. For the present work, concentrated BW, a solution containing high concentrations of marennine, was not purified according to Pouvreau et al. (2006b), as it is a costly and time-consuming process, based on anion-exchange chromatography. In fact, for possible applications in aquaculture, using concentrated BW seems more realistic and appropriate for commercial hatchery.

The effect of BW containing marennine on microalgal cultures of *Pavlova lutheri*, *Isochrysis galbana* and *Chaetoceros gracilis* was assessed at concentrations used for in vivo tests with the larvae (final marennine concentrations of 0.1 and 1.0 mg L^{-1}) and compared to control cultures without marennine. Each strain was cultivated in triplicate for 12 days after inoculation in 1 L borosilicate Erlenmeyer flasks. Microalgal stocks were obtained from the Center for Culture of Marine Phytoplankton (CCMP) Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, ME, USA), and were cultured in batch-mode in F/2 medium and silicates (Guillard 1975) in 6 L borosilicate Erlenmeyer flasks with 0.1 μm filtered and UV-treated seawater at 20 °C under continuous illumination. Three treatments were applied to the cultures by adding BW to the seawater. Cell counts were made in triplicate generally each day (for 10 days out of 12) with a Beckman Coulter-counter Z2, fitted with a 70 μm orifice tube (Beckman Coulter Canada, Mississauga, ON, Canada).

2.1 Mytilus edulis rearing procedures

Spawning adults were obtained in June 2013 from the Havre-aux-Maisons lagoon (Iles-de-la-Madeleine, QC, Canada, 47°25′ N, 61°50′ W), where *M. edulis* accounts for over 98% of the mussel population (Moreau et al. 2005). Thirty mussels were used to characterize their histopathological status according to McGladdery et al. (1993) and no diseases were observed. Only very low intensity and prevalence of some ciliate in gills (*Ancistrum mytili*) and digestive glands (mussel protozoan X) were noted. Generated gametes, derived from 60 different individual parents, were used in a pool-cross designed to produce one random larval family carried

out at the *Station aquicole de Pointe-au-Père* from UQAR. Spawning was induced by thermal shock from 12 to 25 °C, fertilized eggs were transferred to 180 L tanks at densities of 30×10^6 eggs tank^{-1} and left undisturbed at 18 °C for 48 h in filtered (1 μm) and UV-treated seawater.

D-larvae were collected by filtering the water on a 20 μm mesh screen and transferred at densities of ten larvae mL^{-1} into specific rearing tanks, depending on the experiment. Larvae were fed every 2–3 days with a mixture of *Pavlova lutheri*, *Isochrysis galbana*, and *Chaetoceros gracilis* at 30 cells μL^{-1} of each component of the diet, cultured with treated seawater, illumination, and temperature as described earlier. At each feeding period, water in each tank was entirely renewed. To feed the larvae, algae were harvested in the late exponential growth phase, 6 to 10 days after flask inoculation. The BW marennine effect was tested on larval rearing with 10 larvae mL^{-1}, using 2.8 L Nalgene Erlenmeyer flasks at 20 °C with gentle aeration. Filtered (1 μm) and UV-treated seawater was used with addition of BW to obtain marennine concentrations of 0.1 and 1.0 mg L^{-1}. The control consisted of only seawater and each treatment had four replicates. No significant initial effect on the larvae (survival, behavior) was observed when marennine was added to the seawater. After 20 days of development, three sub-samples per flask were taken for survival estimation and bacterial analysis. The flask content was then filtered on a 100 μm mesh to remove bacteria and microalgae. Remaining larvae were filtered onto pre-combusted (450 °C) Whatman GF/F filters. Samples were stored in pre-cleaned amber glass vials with Teflon-lined caps under nitrogen with 2 mL of 2:1 (v:v) mixture of dichloromethane:methanol and stored at –80 °C until lipid extraction.

2.2 Placopecten magellanicus rearing procedures

As for mussels, 30 scallops from this site were used to characterize their histopathological status by the methods of McGladdery et al. (1993). No diseases were observed and only very low intensity and prevalence of ciliates in gills (*Ancistrum mytili*) were observed. In 2014, two spawns were produced, the first for larval rearing and the second for bacterial challenge. For the first spawn, 70 mm scallops cultured in Baie de Gaspé, Quebec were provided by Fermes Marines Québec Inc. (FMQC) in late August. For the second spawn, scallops of the same size were harvested by scuba diving in Pointe Saint-Pierre, near Baie de Gaspé in September. The scallops were kept in a flow-through seawater system (for a maximum of 12 h) until spawning was induced by thermal shock from 5 °C to 15 °C. Gametes from five to ten individuals were mixed at a ratio of about ten sperms per oocyte. Fertilized eggs were left undisturbed for 24–48 h in a 500 L Xactic® tank filled with filtered (10 and 1 μm) and UV-treated seawater. Trocophore larvae were then siphoned into another tank with little aeration. At 4 days post-fertilization (dpf), D-larvae were collected on a 20 μm square mesh screen and transferred into another 500 L Xactic® tank. Larvae were fed the same microalgal mixture as *M. edulis*. Larvae were filtered every 2–3 days, concentrated in a volume of 2 L, where survival and shell length were measured on at least 50 larvae in a 1 mL subsample.

For the first hatching, the number of larvae necessary for the experiment was harvested at 6 dpf and put in a flow-through system, and the remaining larvae were reared in the 500 L Xactic® tank to have an external rearing control, as this method was demonstrated to be successful for this species (Pernet et al. 2003). Survival and growth were measured as described earlier. Larvae were reared in the flow-through system at an initial density of 1 larva mL^{-1}, with two water renewals per day and fed continuously with the same microalgal mix as described earlier. Filtered seawater (10 and 1 μm) entered the tanks by the bottom and the overflow was evacuated on top by a mesh screen, for a constant tank volume of 180 L. The flow was adjusted to keep water temperature under 15 °C. Two treatments were used: marennine treatment (0.1 mg L^{-1}) and a control without BW marennine, with two tanks per treatment (n = 2). No significant initial effect on the larvae (survival, behavior) was observed when marennine was added to the seawater. The experiment was stopped at 15 dpf, after 9 days of marennine treatment. Larvae were filtered and concentrated in 5 L, where survival and shell length were evaluated. The remaining larvae were filtered on a GF/F filter and stored in a dichloromethane:methanol (2:1, v:v) solution at –80 °C for lipid analysis. Water from each tank was sampled for bacterial analysis.

2.3 Marennine effect on larvae exposed to pathogen

Larvae were exposed to *Vibrio splendidus* 7SHRW, a wild strain isolated by Mateo et al. (2009) from sediments from Hillsborough River, Prince Edward Island (Gulf of Saint Lawrence, Canada) and identified on conventional biochemical tests and BIOLOG automated identification. These authors have demonstrated the pathogenicity of *V. splendidus* on soft shell clam hemocytes. For *M. edulis*, 10-day-old larvae reared in 60 L cylindrical tanks at 19 °C with gentle aeration in filtered (1 μm) and UV-treated seawater were challenged with *V. splendidus*. Larvae were collected on a 53 μm square mesh filter, visually counted, pooled, and distributed equally into 1 L beakers (10 larvae mL^{-1}). Larvae were cultured at 20 °C in duplicate with four treatments: *Control*, *Marennine*, *Vibrio*, and *Vibrio* + *Marennine*. An initial concentration of 6.6×10^5 bacteria mL^{-1} for *V. splendidus* was used, and 0.5 mg L^{-1} for marennine. Samples were collected for larvae survival assessments and bacterial analyses after 72 h. Prior to using *V. splendidus*, the strain kept in glycerol at –80 °C was streaked onto Marine Agar plates to ensure purity and was further cultured in 100 mL Marine Broth medium (Difco, Lawrence, KS, USA) before incubation at 25 °C for 24 h prior to use. Bacterial culture was centrifuged (6000 g, 15 min), the supernatant discarded, and the pellet re-suspended in 10 mL of sterile seawater. Optical density of the bacterial suspension was measured at 600 nm in five replicates and compared to a standard of sterile seawater to determine the acterial concentration.

For *P. magellanicus*, larvae from the second hatching were harvested after 5 dpf for the bacterial challenge with *V. splendidus*. The same protocol and treatments (n = 3) were applied but 2.8 L Erlenmeyer flasks filled with 2 L autoclaved seawater were used, with an initial density of 1 larva m L^{-1}, 0.1 mg L^{-1} marennine, and an initial bacterial concentration

of 5.75×10^5 cell mL^{-1}. In this case, *I. galbana* was provided at 60 cells μL^{-1} and the experiment was conducted in a 15 °C room. The challenge was stopped after 72 h (8 dpf) and samples were taken as described for *M. edulis*.

2.4 Bacterial analysis

Triplicate 4 mL aliquots of seawater samples for challenge test experiments were fixed in the dark for 15 min using 2% glutaraldehyde (final concentration; pH 7), and kept frozen at –80 °C until analysis. The total free bacteria were enumerated using an EPICS ALTRA cell sorting flow cytometer (Beckman-Coulter Inc., Mississauga, ON, Canada) equipped with a 488 nm blue laser operating at 1 mW and running at a flow rate of 60 mL min^{-1} as described in Seychelles et al. (2013). Heterotrophic bacteria were stained (SYBR Green I, Molecular Probes Inc., Eugene, OR, USA) and subpopulations with low nucleic acid (LNA) or high nucleic acid (HNA) content were discriminated according to Belzile et al. (2008). Data analysis was performed with Expo321 software (Beckman Coulter Inc., Fullerton, BC, CA). For the purpose of this study, the percentage of HNA (%HNA being the ratio of HNA cells to total bacterial count) was used as a proxy for the physiological state of the bacterial population (Seychelles et al. 2013).

2.5 Lipid analysis

To examine the physiological condition, lipid class composition was compared between larvae under the different treatments. Bivalve larvae generally store large amounts of triacylglycerol (TAG) as the primary endogenous energy reserve to fuel basal metabolism and growth (Holland 1978; Gallager et al. 1986). The TAG level is also a predictor of survival in bivalve larvae (Pernet et al. 2004), as successful growth and development depend on sufficient energy acquisition to sustain demands during embryogenesis and metamorphosis. Thus, the combination of a rapid response to environmental change and the integrative nature of lipid energy reserves suggest that they are an ideal tool for evaluating physiological condition in larvae populations.

Lipid extractions were carried out on ice according to the modified Folch's method (Folch et al. 1957) as described by Parrish (1987). Extracted lipids were spotted onto S-III Chromarods (Iatron Laboratories, Tokyo, Japan) for thin layer chromatography and lipid classes were separated using a four-solvent system as described in Parrish (1987). Chromarods were scanned by a flame ionization detection system (FID; Iatroscan Mark-VI, Iatron Laboratories, Tokyo, Japan). Integration software (Peak Simple version 3.2, SRI) was used to analyze chromatograms of each lipid class, as described by Parrish (1999).

2.6 Data analysis

Univariate variables (microalgae cell concentration, larval survival, total bacterial concentration, % HNA) were compared using one-way ANOVA as a function of marennine concentration or *V. splendidus* treatment for flask experiments. A

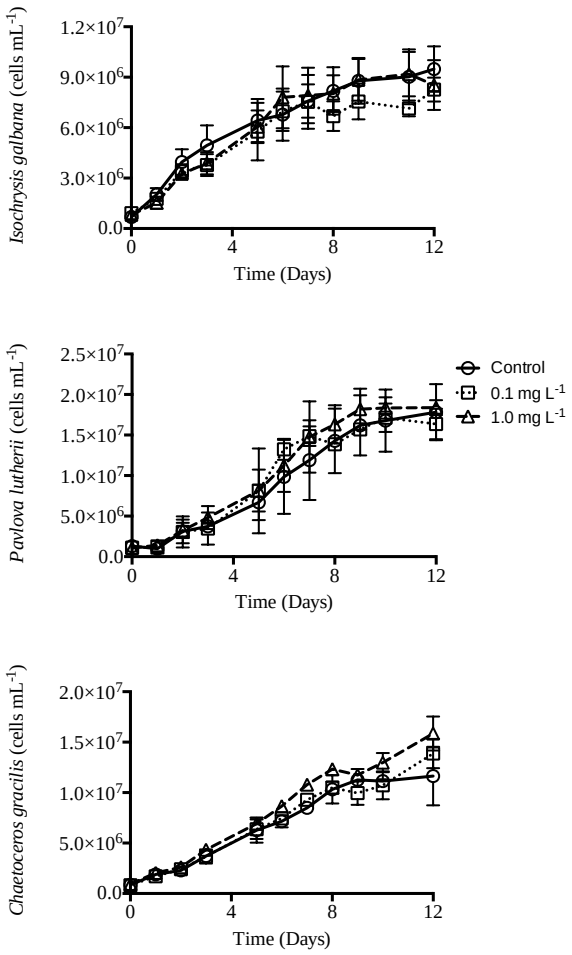

Fig. 1. Growth kinetics of different microalgae species (*Isochrysis galbana* (up), *Pavlova lutheri* (center) and *Chaetoceros gracilis* (down)) in the presence of blue water (BW) containing marennine (0.1 and 1 mg L^{-1}) and without BW marennine (control). Data are means ± SD (n = 3).

T-test was used to compare the effect of marennine (control and 0.1 mg L^{-1}) on dependent lipid variables (total lipid concentration and TAG/Sterol ratio) in rearing experiments. The assumption of homoscedasticity was verified visually by the spread of residuals, as suggested by Quinn and Keough (2002); normality was tested by a Shapiro-Wilk test and data were transformed (arcsine square-root for percentage and square-root for concentrations), when necessary. Post hoc Tukey's pairwise multiple comparison tests were used to determine which means were significantly different. All statistical analyses were carried out at a significance level at $x = 0.05$ and performed using JMP 9.0.1 (SAS Institute) software.

3 Results

3.1 BW marennine effect on microalgae

The presence of marennine below a concentration of 1.0 mg L^{-1} had no effect on microalgae growth for each of the species under study (Fig. 1). No significant differences

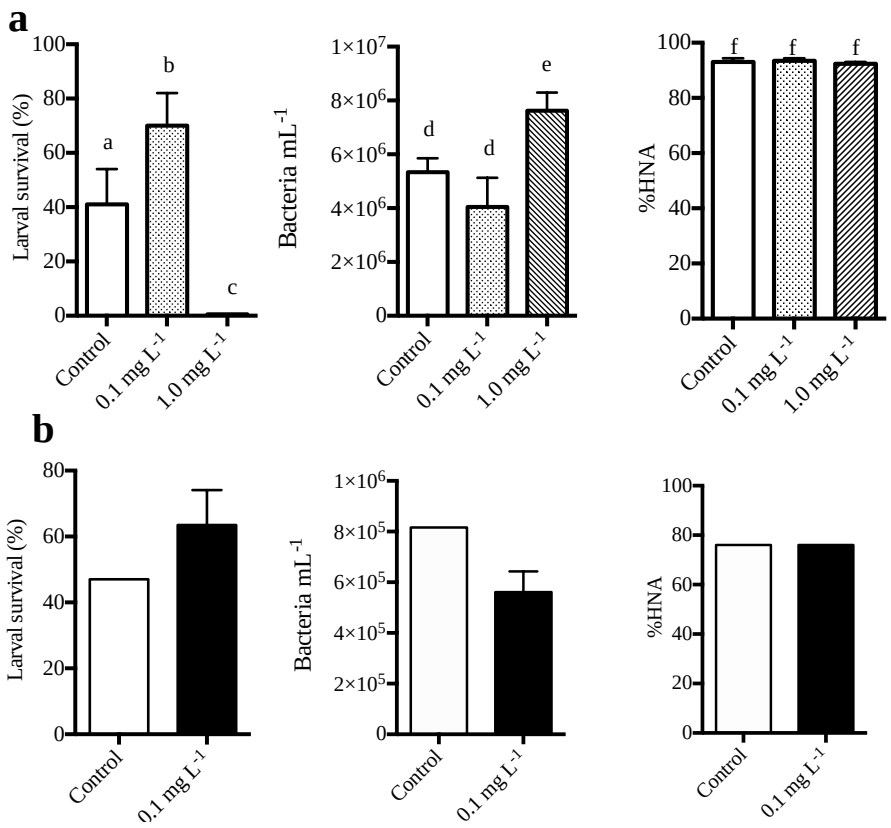

Fig. 2. Larval rearing success of *Mytilus edulis* (a) and *Placopecten magellanicus* (b) and bacterial concentration (cells mL^{-1}) and high nucleic acid bacteria proportion (% HNA) of larvae culture water for blue water marennine treatments and the control without BW marennine. Data are means ± SD (*M. edulis* $n = 4$; *P. magellanicus* $n = 2$) and different letters indicate significant differences at $p < 0.05$.

were found among the three treatments (0, 0.1 and 1.0 mg L^{-1} of marennine) in final cell concentration (day 12) for the culture of *I. galbana* ($F_{2,7} = 0.82$; $P = 0.48$), *P. lutherii* ($F_{2,7} = 0.43$; $P = 0.67$) and *C. gracilis* ($F_{2,7} = 3$; $P = 0.12$).

3.2 BW marennine effects on Mytilus edulis rearing

In 2.8 L flask cultures, a significant effect of marennine concentration on larval survival ($F_{1,7} = 9.97$; $P = 0.02$) and on bacterial load ($F_{2,10} = 20.82$; $P = 0.001$) was observed after 20 days of development (Fig. 2a). Larval survival was higher at 0.1 mg L^{-1} than in the control, while almost no larvae survived at 1.0 mg L^{-1}. The total bacterial load was lower in the 0.1 mg L^{-1} treatment and control than in the 1.0 mg L^{-1} treatment, while no significant differences were found between treatments in % HNA ($F_{2,10} = 1.12$; $P = 0.37$). Table 1 shows results from the lipid analysis. The 1.0 mg L^{-1} treatment was not included due to the absence of sufficient larvae to perform the analyses, following the very high mortalities observed with this treatment. No significant changes were observed in total lipid content (t-test$_{1,7} = 0.19$; $P = 0.67$) or TAG/Sterol ratio (t-test$_{1,7} = 1.44$; $P = 0.28$). However, the BW marennine-treated larvae at low concentration had a mean TAG/sterol ratio more than twice that of the control; variability was high between replicates and the difference was not significant. Final shell length was significantly higher in the marennine 0.1 mg L^{-1} treatment (t-test$_{1,7} = 11.15$; $P = 0.02$).

3.3 BW marennine effect on Placopecten magellanicus rearing

Survival and growth in larvae maintained without treatment in the 500 L Xactic® showed results similar to the literature with a survival of 45% at 15 dpf and an increase in shell length of 4 μm day^{-1} (Pernet et al. 2003, 2006).

In 150 L flow-through systems, larval survival was higher in BW marennine-treated tanks after nine days of rearing (Fig. 2b), like the 500 L static tank. Due to problems in maintaining flow-through rearing conditions in one of the control tanks, high mortality occurred with a final survival of 6%, compared to 45% in the other control tank. Thus, it was not possible to sample the control tank with high mortality and the results of the control were not replicated. In the treated tanks, the TAG/ST ratio was 60% to 95% higher than in the control (Table 1). Bacterial load was also lower in the treated tanks (37% and 25% reduction), whereas shell length and % HNA were the same (Table 1, Fig. 2b).

3.4 BW marennine effect on challenged larvae

In *M. edulis*, treatment had a significant effect on survival ($F_{3,5} = 15.49$; $P = 0.01$), bacterial load ($F_{3,5} = 168.84$; $P = 0.001$) and % HNA ($F_{3,5} = 221.58$; $P = 0.001$). After 72 h, larval survival was 88% in the control and was similar to Marennine and *Vibrio* + Marennine treatments. Survival

Table 1. Total lipids (*Mytilus edulis*: ng/larvae *Placopecten magellanicus*: ng/μg larvae), triacyglycerol over sterol ratio (TAG/ST) and final shell length (μm) for different marennine treatments and controls in *Mytilus edulis* and *Placopecten magellanicus*. Data are means ± SE (*M. edulis*, $n = 4$; *P. magellanicus* $n = 2$).

	Treatment	Total lipids	TAG/ST	Shell length (μm)
Mytilus	Control	38.4 ± 12.3	12.3 ± 6.4	215.3 ± 10.9
edulis	Marennine 0.1 mg/L	32.6 ± 4.3	28.5 ± 11.9	237.4 ± 8.2
Placopecten	Control	5.7	2.2	117.4 ± 11.8
magellanicus	Marennine 0.1 mg/L	8.13 ± 0.83	3.9 ± 0.6	120.5 ± 5.5

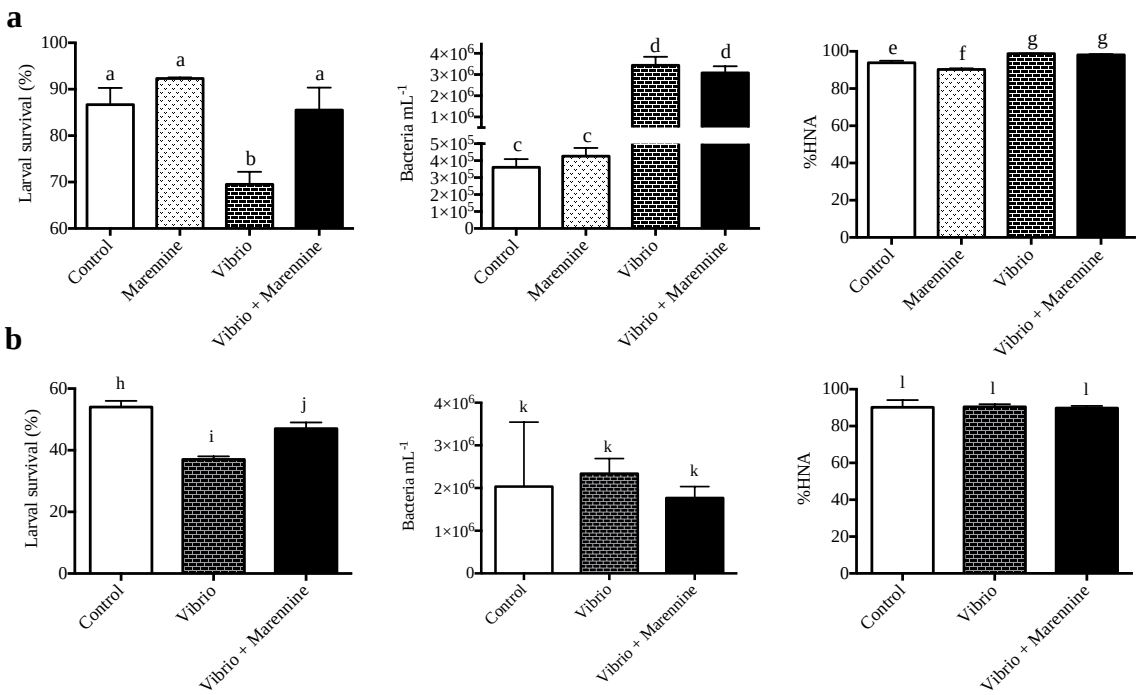

Fig. 3. Larval rearing success of *P. magellanicus* (**a**) and *Mytilus edulis* (**b**) and bacterial cell concentration (cells mL^{-1}) and high nucleic acid bacteria proportion (% HNA) of larvae culture water for the control without addition, blue water containing marennine treatment (0.1 mg L^{-1}), *Vibrio* treatment (10^5 bacteria mL^{-1} of *V. splendidus*) and the *Vibrio* + BW marennine treatment (0.1 mg L^{-1} of marennine and 10^5 bacteria mL^{-1} of *V. splendidus*) after 72 h of exposure. Data are means ± SD ($n = 2$ for *M. edulis* and $n = 3$ for *P. magellanicus*) and different letters indicate significant differences.

for larvae challenged with *V. splendidus* without marennine (*Vibrio* treatment) was significantly lower (Fig. 3a). Both treatments with *V. splendidus* had higher total bacterial counts and % HNA. The *Vibrio* + Marennine treatment did not show different bacterial counts or % HNA from the *Vibrio* treatment (Fig. 3).

In *P. magellanicus*, treatment had a significant effect on larval survival ($F_{2,7} = 60.61$; $P = 0.001$) but not on total bacterial load ($F_{2,7} = 0.29$; $P = 0.75$) or % HNA ($F_{2,7} = 0.05$; $P = 0.94$). *Vibrio* treatment greatly reduced larval survival, and the marennine treatment improved survival, although it did not completely inhibit *V. splendidus* pathogenicity (Fig. 3b).

4 Discussion

BW containing marennine had a positive effect on larval survival in rearing experiments, for both bivalve species tested. Typical survival from D-larvae to pediveliger stage is variable upon cultured species, but it can be as high as 50 to 70% for oysters and clams and as low as 15 to 30% for some pectinids (Helm et al. 2004). The 70% survival for BW marennine-treated *M. edulis* larvae, with 75% survival improvement compared to non-treated larvae (control), is thus on the upper end of the expected survival for bivalves. In pectinids, elevated variability, massive mortalities or survival between 0 and 10% are frequent in *Pecten maximus* larval rearings (Torkildsen and Magnesen 2004). Here in *P. magellanicus*, larval survival decreased to 6% after 30 dpf in the Xactic® rearing, which is a common result for a pectinid rearing without treatment. In the BW marennine treated flow-through rearing, final survival at 15 dpf between 55 and 70% is very high, and mostly higher than in the flow-through control (6 and 45%) and the static Xactic® rearing after the same rearing time (45% survival at 15 dpf).

Results showed that the physiological condition of larvae was also improved by the BW marennine treatment in both species. In *M. edulis*, a marennine concentration of 0.1 mg L^{-1} had no significant effect on larvae capacity to accumulate lipids. Their total lipid content and lipid class composition

after 20 days of exposure to BW marennine were similar to the control. However, even though the differences were not significant, mussel larvae exposed to BW containing marennine accumulated 40% more TAG (energetic lipid reserve) than the control larvae. This trend was also reflected on the TAG/ST ratio generally used in larvae as a physiological indicator (Fraser et al. 1989; Pernet et al. 2004). BW marennine treated larvae had a TAG/ST ratio more than two times higher than the control, indicating a better physiological state, but as the variability between rearing tanks was very high, this difference was not significant. This trend was validated with *P. magellanicus* reared in higher volume tanks, where rearing conditions were more stable. A high culture volume stabilizes the environment by slowing down the variation in culture factors, and lowering the surface-to-volume ratio, explaining the better survival results generally obtained in larger rearing volumes (Gruffydd and Beaumont 1972; Harboe et al. 1994; Andersen et al. 2000). A higher TAG content associated with higher survival in BW marennine treated larvae would be in accordance with the results of Pernet et al. (2004), who found a positive relationship between *M. edulis* larval survival and higher energy reserve content estimated by TAG level. In our experiments, BW marennine treatments did not affect food availability, as the growth of each microalga used for larval feeding was similar with or without marennine until a concentration of 1.0 mg L^{-1}. Furthermore, as larvae treated or not with BW marennine accumulated similar lipid contents with same lipid class composition, BW marennine treatment does not seem to affect microalgae quality, feeding behavior of the larvae, and accumulation of lipid reserves by the larvae.

Information about marennine action on the rearing can be deduced from the bacterial challenge experiments, where larval survival was improved by the treatment for both species. Considering that the pathogen caused significant larval mortalities, that the BW marennine treatment reduced or inhibited the mortalities, and that the bacterial load and % of cells with high nucleic acid content (% HNA used as a proxy for the physiological state of the bacterial population) did not change under the treatment (or only slightly for % HNA in one case), the positive effect of BW marennine could result from a reduction in bacterial pathogenicity. In the work of Tardy-Laporte et al. (2012), marennine was found to interact with the LPS in bacterial membranes, lipids related to Gram-negative bacteria toxicity (Beutler 2004). Also, the outer membrane protein (OMP) OmpU was shown to be a major determinant of *V. splendidus* strain LGP32 virulence, contributing to host antimicrobial peptide/protein (AMPs) resistance, to host cell adherence, and to pathogen recognition (Duperthuy et al. 2010). Thus, we suggest that marennine could interact with the bacterial cell outer structures responsible for *V. splendidus* pathogenicity, but the mechanisms of action need to be determined. As marennine was shown to exhibit antioxidant activity (Pouvreau et al. 2008), it could also provide a protective action to the host against the oxidative stress burst in larvae exposed to pathogenic bacteria (Genard et al. 2013). The marennine mechanism of action could thus be explained by both activities (antimicrobial and anti-oxidative).

To demonstrate the overall benefit of marennine antimicrobial action, study of the dose that should be used in a rearing of any species should be conducted. As shown here, a dose of 0.1 mg L^{-1} was beneficial for both species, but a 10-fold higher dose was lethal to *M. edulis* larvae. The marennine effect was also unequal in reducing *V. splendidus* pathogenicity toward the species. Larvae of different species may not be as easy to rear (scallops are known to be difficult), and pathogenic effects of the same bacteria will not always be the same for different hosts (Anguiano-Beltrán et al. 2004).

5 Conclusion

This study is the first experimental demonstration that blue water (BW) containing marennine produced by the diatom *Haslea ostrearia* used as a prophylactic agent in bivalve hatcheries has promising results on animal survival. Indeed, BW marennine was shown to enhance larval survival in challenged and normal rearing conditions in both commonly used species for aquaculture, *Mytilus edulis* and *Placopecten magellanicus*. Larval condition was also increased in one BW marennine treatment. This protective effect could be the result of an interaction with the bacterial membrane, reducing microbial pathogenicity. There is a real need for natural molecules that can enhance larval survival and quality in hatcheries, and the present work demonstrates that BW marennine could be a good candidate. Further research should be conducted to precisely determine the optimal marennine concentration for larval performance, to determine the effect of marennine on a wider range of species relevant for aquaculture (including fish), and to estimate the efficiency of this bioactive compound against different pathogenic bacteria in challenged larvae conditions or adults.

Acknowledgements. The authors would like to thank Nathalie Gautier, Nathalie Morin, Mathieu Babin, Claude Belzile and Alexandre Boudreau for their technical assistance. This publication benefited from funding from the National Scientific and Engineering Research Council (NSERC-Discovery Grant 299100-2010 to R. Tremblay and Engage Grant EGP 468788 – 14 to JS Deschênes), from the Fonds de Recherche du Québec – Nature et Technologies (FRQNT) under Ressources Aquatiques Québec (RAQ), and from the European Commission under the Community's Seventh Framework Programme BIOVADIA (Contract No. FP7-PEOPLE-2010-IRSES-269294).

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