Harmful or harmless: biological effects of marennine on marine organisms

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Highlights:

- The biological effects of marennine were studied on different marine organisms.
- Marennine is the blue water-soluble pigment produced by the diatom *Haslea ostrearia*.
- Blooms of blue *Haslea* are recorded worldwide.
- Marine organisms can be exposed to significant amount of marennine during blooms.
- Marennine significantly affects early developmental stages of the tested organisms.

Abstract

Marennine is a water-soluble blue-green pigment produced by the marine diatom *Haslea ostrearia*. The diatom and its pigment are well known from oyster farming areas as the source of the greening of oyster gills, a natural process increasing their market value in Western France. Blooms of blue *Haslea* are also present outside oyster ponds and hence marine organisms can be exposed, periodically and locally, to significant amounts of marennine in natural environments. Due to its demonstrated antibacterial activities against marine pathogenic bacteria (*e.g. Vibrio*) and possible prophylactic effects toward bivalve larvae, marennine is of special interest for the aquaculture industry, especially bivalve hatcheries. The present study aimed to provide new insights into the effects of marennine on a large spectrum of marine organisms belonging to different phyla, including species of aquaculture interest and organisms frequently employed in standardised ecotoxicological assays. Different active solutions containing marennine were tested: partially purified Extracellular Marennine (EMn), and concentrated solutions of marennine present in *H. ostrearia* culture supernatant; the Blue Water (BW) and a new process called Concentrated Supernatant (CS). Biological effects were meanwhile demonstrated in invertebrate species for the three marennine-based solutions at the highest concentrations tested (*e.g.*, decrease of fertilization success, delay of embryonic developmental stages or larval mortality). Exposure to low concentrations did not impact larval survival or development and even tended to enhance larval physiological state. Furthermore, no effects of marennine were observed on the fish gill cell line tested. Marennine could be viewed as a Jekyll and Hyde molecule, which possibly affects the earliest stages of development of some organisms but with

no direct impacts on adults. Our results emphasize the need to determine dosages that optimize beneficial effects and critical concentrations not to be exceeded before considering the use of marennine in bivalve or fish hatcheries.

Key words: diatom; *Haslea ostrearia*; marennine; marine organisms; natural bioactive compound

1. Introduction

Haslea ostrearia is a cosmopolitan marine pennate diatom that synthesizes and releases a water-soluble blue-green pigment called marennine. This species can bloom erratically in oyster ponds in Western France (Baie de Marennes-Oléron, Baie de Bourgneuf), a phenomenon that has long been known to be responsible for the greening of oyster gills. This phenomenon is of economic interest for the French oyster farming industry as the market value of green oyster is significantly increased (by 20 to 30 %) due to changes in the organoleptic properties of the oysters and the scarcity of the product, blooms in ponds being an erratic phenomenon. In the last decade, new species of blue *Haslea* have been discovered, some of them producing marennine-like pigments chemically distinct from the originally described marennine molecule (Gastineau et al., 2012a, 2016). Blooms of blue *Haslea* are also observed in natural environments, such as in the Mediterranean Sea and East coast of the USA (Figure 1). Moreover, oysters with green gills have long been observed worldwide, *e.g.*, in Great Britain (Sprat, 1667), Denmark (Petersen, 1916), the USA (Mitchell and Barney, 1917) and Australia (Hallegraeff and Mouget, personal communication). Furthermore, the greening of gills has been reported in other organisms, such as polychaetes, crabs, littorina, mussels (Ranson, 1927), sea-anemones (Gaillon, 1820), scallops and cockles (Gastineau et al., 2018), illustrating that many marine organisms can be exposed to blue *Haslea* populations and marennine-like pigments not only in artificial (oyster) ponds, but also in natural environments.

Although our knowledge of the blue *Haslea* biodiversity has recently increased, little is known about the chemical properties of marennine and marennine-like pigments or their

functions for the microalgae. Marennine is thought to be produced *via* a cytoplasmic synthesis pathway (Nassiri et al., 1998) and transiently accumulates at the cell apices (intracellular form of the pigment, IMn). Marennine is excreted from the cells, possibly by exocytosis via small vesicles, which collapse and release an extracellular form of marennine (EMn). The two forms of the pigment differ in their UV-visible spectral characteristics and molecular mass (Pouvreau et al., 2006b). Marennine is a complex molecule composed of glycosidic units (Gastineau et al., 2014) attached to one or various aromatic rings (Pouvreau et al., 2006b). A protocol to obtain a purified form of EMn or IMn has been developed (Pouvreau et al., 2006c) and several authors have proposed different methods to estimate marennine concentration in solution, despite incomplete knowledge of its chemical structure (Pouvreau et al., 2006a; Robert et al., 2002).

Various studies have demonstrated that marennine (as purified molecule or raw extract) has multiple biological activities, such as antioxidant (Pouvreau et al., 2008), antiproliferative (Carbonnelle et al., 1998; Gastineau et al., 2012), antiviral (Bergé et al., 1999; Gastineau et al., 2012) and antibacterial (Falaise et al., 2016; Gastineau et al., 2014, 2012). It has also been shown that marennine possesses allelopathic properties, limiting the growth of various microalgae (Pouvreau et al., 2007; Prasetiya et al., 2016). The biological activities of marennine are species- and even strain-dependent in the case of bacteria (Falaise et al., 2016), suggesting that marennine could act on specific molecular targets. Tests conducted with Gram-negative bacteria have demonstrated activity of the marennine-like pigment produced by *Haslea provincialis* (Gastineau et al., 2016) and marennine produced by *H. ostrearia*, on the lipopolysaccharidic cell membrane of *Escherichia coli* (Tardy-Laporte et al., 2013) and of *Vibrio splendidus* (Bouhlel et al., 2018), rendering it more rigid.

In line with these results, particularly those demonstrating the capacity of marennine to limit the proliferation of certain pathogenic marine bacteria (Falaise et al., 2016; Gastineau et al., 2012, 2014), further research has confirmed the protective effect of marennine on the giant scallop (*Placopecten magellanicus*) and blue mussel (*Mytilus edulis*) larvae when challenged with *V. splendidus* (Turcotte et al., 2016). The same study also revealed that a 20 d (day) exposure of mussel larvae to low concentrations of marennine (0.1 µg mL^{-1}) provided a significantly higher survival rate than the control, although exposure to higher concentrations of marennine (1 µg mL^{-1}) resulted in 100 % larval mortality. Even brief exposure to marennine has

been demonstrated to result in behavioural, physiological and biochemical changes that were still present eight weeks after exposure (Prasetiya et al., 2017).

These results underpin the interest to further study the effects of marennine on early developmental stages of marine organisms, to identify which are possibly sensitive to marennine, as well as defining exposure concentrations and durations that would be beneficial to species of aquaculture interest. The present work presents a broad panel of experiments conducted to provide an overview of the biological effects of marennine solutions on various marine organisms belonging to different phyla. The organisms were selected based on aquaculture interest or their established role in ecotoxicological model assays. Different solutions containing marennine, prepared from *H. ostrearia* culture supernatants, were used in the exposures: the purified EMn (Pouvreau et al., 2006c), the Blue Water (BW; (Turcotte et al., 2016) and a newly patented Concentrated Supernatant (CS; see Materials). Although EMn represents the most purified form of marennine currently available, BW and CS are of particular interest if the use of marennine-based solutions is considered at larger scales than laboratory experiments due to their easier and cheaper method of production. Marennine exposures were conducted on embryos and/or larvae of the mollusc *M. edulis*, the crustacean *Chthamalus bisinuatus*, the chordate *Pseudopleuronectes americanus* and the echinoderms *Sphaerechinus granularis* and *Paracentrotus lividus*. The effects of the blue pigment solutions were also investigated *in vitro* on the fish gill cell line RTgill-W1, on the oyster *Crassostrea gigas* haemocytes, and on prokaryotic models with bacteria of the genus *Vibrio*, providing a broad view of the diversity of marennine effects on marine organisms.

2. Materials and Methods

Purified form of extracellular marennine (EMn), Blue Water (BW) and Concentrated Supernatant (CS) were all obtained from *H. ostrearia* culture supernatant (Table 1), but the process to obtain BW and CS is faster and has a better yield in comparison with purified EMn. BW and CS are not purified marennine *per se*, but they allow preparing concentrated solutions of marennine needed to run dose-response experiments between *Haslea* pigment and target organisms. Experiments are presented in the Results section in the following order: 1) Experiments using the BW solution, 2) CS solution, and 3) purified EMn solution.

2.1. Preparation of the purified extracellular marennine (EMn), Blue Water (BW) and Concentrated Supernatant (CS)

The Blue Water (BW) and purified extracellular marennine (EMn) were produced at the *Station aquicole de Pointe-au-Père* (Québec, Canada) and provided by the *Institut des sciences de la mer de Rimouski-Université du Québec à Rimouski* (ISMER-UQAR; Québec, Canada). The production process of BW and purified EMn were previously described (Turcotte et al., 2016; Pouvreau et al., 2006c). Briefly, *H. ostrearia* strains (NCC 136), isolated from Bourgneuf Bay (France) and provided by NCC (Nantes Culture Collection), were cultured in 100 L photobioreactors until the extracellular marennine concentration reached a maximum of 6 to 8 µg mL^{-1}. Marennine concentration was determined on cell-free culture supernatant (filtered through Sarstedt 0.2 µm syringe filters) using a spectrophotometer (Cary 100 Bio UV-Visible, Agilent Technologies) and the Beer-Lambert's equation (ε_{677}=12.13 L $g^{-1}cm^{-1}$) as proposed by Pouvreau et al. (2006c). The BW was then obtained by concentration of the culture supernatant containing EMn by ultrafiltration (double cut off 3-30 kDa; (Turcotte et al., 2016). To obtain the purified EMn, the BW was further treated by an anion-exchange chromatography process and the fraction collected was dialyzed and freeze-dried (Pouvreau et al., 2006c). BW and purified EMn were stored in the dark at 4 °C and -20 °C respectively. Concentrated Supernatant (CS) was produced in the *Mer Molécule Santé* (MMS) and *Institut des Molécules et Matériaux du Mans* (IMMM) laboratories (Le Mans, France). A strain of *H. ostrearia* (NCC 495) was batch cultured in 500 mL Erlenmeyers flask containing 250 mL of autoclaved sea water prepared from a commercial sea salt mix (Instant Ocean, Aquarium Systems®; pH 7.6 ± 0.2; salinity 32) with an enrichment solution as described in Mouget et al. (2009). Microalgal cultures were maintained in a 16 °C temperature-controlled room at an irradiance of 200 µmol photon m^{-2} s^{-1}, with illumination provided by cool-white fluorescent tubes in a 14/10 h light/dark cycle. At the beginning of the stationary growth phase the culture supernatant containing EMn was collected by decantation of the microalgal cells and subsequent vacuum filtrations through 15 µm (150 mm Filter paper, Fisher Scientific®) and 1.2 µm (37 mm glass microfiber filters, Whatman®). Filtered culture supernatant was collected in 1 L glass bottles and the EMn was then concentrated using an innovative technique recently patented (patent n°: 1872316). The resulting solution was then dialyzed using a 2 kDa dialysis membrane (Spectra/Por®6, Spectrum®). Dialysis tubes were placed in ultra-pure water tanks for 3 d under agitation with the water changed every 24 h. The

dialyzed solution was then ultra-filtered (double cut-off 1 kD-30 kDa; Kros Flo® Research IIi TFF System and Kros Flo® Automatic Backpressure valve, Spectrum®) and further concentrated by evaporation with a Rotavapor (Vacuum controller CVC2, Vacuubrand®; Rotary Elevator, Heating Bath Hei-Vap, Base Hei-Vap ML Adv/Pre, Heidolph®). The pH of the resulting CS was neutralized if required to 7.5 ± 0.2 by addition of NaOH 0.1 M. The CS was stored one week in the dark at 4 °C until use. The concentration of marennine in the CS was determined using a spectrophotometer and the Beer-Lambert equation (ε_{669}=17.2 L $g^{-1}cm^{-1}$) as proposed by (Robert et al., 2002). UV-Vis spectra of the different marennine based solutions were conducted using quartz cuvettes with 1 cm path length (UV/Vis Lambda 25 Perkin Elmer spectrophotometer, UV Winlab software).

2.2. Exposure of the mussel *Mytilus edulis* larvae to BW

Adult mussels were obtained in summer 2017 from a farm in the Magdalen Islands (47° 25'N, 61° 50'W, Quebec, Canada), characterized by pure *M. edulis* populations (Myrand et al., 2009) and conditioned for spawning in the *Station aquicole de Pointe-au-Père* (ISMER-UQAR; Québec, Canada) facilities for one month. Mussels were maintained in 180 L tanks in 1 µm filtered seawater at 20 °C flowing at 1 L min^{-1} and were continuously fed with a mixture of *Pavlova lutherii*, *Isochrysis galbana*, and *Nannochloropsis oculata* (ratio 1:1:1) supplied with a peristaltic pump at constant flow to maintain food load at 0.5 mg L^{-1} as described in Hennebicq et al. (Hennebicq et al., 2013). Before spawning, a dozen individuals were removed from the tank and washed by gently rubbing the shell with a brush and diluted bleach and finally rinsed with filtered, UV sterilized sea water. Spawning was induced by successive thermal shocks from 10 to 25 °C. Fertilized eggs were transferred to 60 L tanks at densities of 10 eggs μL^{-1} and embryos were maintained at 18 °C for 48 h. D-larvae were collected by filtration of the water through a 20 µm mesh screen and transferred to 60 L rearing tanks at densities of 10 D-larvae mL^{-1}. Every 2-3 d, tanks were washed and sterilized and the larvae fed with a mixture of *P. lutherii*, *I. galbana* and *Chaetoceros gracilis* at a final concentration of 90 cells μL^{-1} (30 cell μL^{-1} for each component of the diet; Turcotte et al., 2016).

M. edulis embryos were collected prior to the transfer to the embryogenesis tank, and D-larvae prior to the transfer to the rearing tank (48h post fertilization). Veliger larvae were collected in the rearing tanks (14 d post fertilization) by filtration of the water through a mesh

screen. Embryos and larvae were exposed to BW in 12-well flat bottom sterile microplates with each well containing 3 mL of diluted BW and 10-15 embryos or 10-15 larvae per well. Five concentrations of marennine from BW were tested: 0, 0.1, 0.5, 1 and 5 µg mL^{-1}. Marennine concentration from BW was estimated as described in section 2.1 and Table 1. BW was syringe-filtered through 0.2 µm and diluted in sterile sea water. Microplates were kept in a controlled temperature room at 18 °C without addition of food during the time of the experiment. The mortality of embryos and veliger larvae was assessed using a light microscope (Olympus BX41; W. Carsen Co., Ltd., Don Mills, ON, Canada) coupled to a digital camera (Evolution VF Color, MediaCybernetics, Silver Spring, MD, USA). The percentages of swimming and motionless D-larvae were assessed by observation of the microplates using an inverted microscope (Axiovert 100, Zeiss). Larval size was measured with Image Pro-Express (Media Cybernetics) after addition of formaldehyde to the wells. A minimum of 10 larvae were counted per well with at least 3 wells per concentration tested.

2.3. Exposure of the barnacle *Chthamalus bisinuatus* larvae to BW

Experiments were conducted in June 2015 in the *Center for Marine Biology of Sao Paulo University* (CEBIMar/USP; Sao Paulo, Brazil). Spawners were harvested by collecting rock fragments in the intertidal zone of Calhetas Beach (23°49’ 28”S, 45°25’11”W). The fragments were transferred to the laboratory and placed in seawater pumped from the adjacent bay, as described in Kasten and Flores (Kasten and Flores, 2013). A light source was placed above the tanks containing the breeders to recreate the natural photoperiod. Broodstock was submerged for 30 min every 12.4 h to simulate the natural effect of the tide and stimulate spawning. After a first submersion, the water used to submerge the broodstock was siphoned and filtered to harvest the larvae. The larvae were placed in filtered seawater and isolated using a dissecting microscope to obtain a number of larvae sufficient to run the experiment. Larvae were placed individually in the wells of a 96-well microplate in 2 mL of seawater containing 0, 0.05 or 0.1 µg mL^{-1} of marennine from BW without food to test only the potential toxicity of BW. The BW was prepared and the concentration estimated as described in section 2.1 (Table 1). Four larvae were tested at each concentration. The microplate was placed on a stirring plate and no food was provided during the experiment. Larvae were observed once a day and survival recorded over 9 d. The experiment was repeated once with spat from a different production.

2.4. Exposure of the winter flounder *Pseudopleuronectes americanus* larvae to BW

Larvae were reared as described by (Khemis et al., 2003) in 57 L cylindro-conical polyethylene tanks and fed rotifers from d 4 to d 26 (Fraboulet et al., 2010). Rotifers were fed a cocktail of microalgal concentrates (1:1:1 *N. oculata*: *I. galbana*: *P. lutherii*, Instant algae, REED Mariculture) and a SELCO food supplement (1 g M^{-1} rotifers, INVE Aquaculture Nutrition, Gransville, UT, USA). The larvae were kept in green water (addition of *N. oculata* culture directly to basins). From d 2 to d 14, two concentrations of marennine from BW were tested on the larvae, 0.05 and 0.1 µg mL^{-1}, with three tanks per treatment (n = 3). BW preparation, as described in section 2.1 (Table 1), was added to the tanks in the morning, when water flow was cut to feed the larvae. Water flow was restarted at the end of the day (for a total of about 8 h of exposure) and BW was gradually evacuated from the tanks with the flow of water outlet. A dose of BW was given every two days, for a total of seven treatments per tank for the duration of the experiment. Procedures were the same for the control treatments, without the addition of BW in the control tanks. The effect of BW treatment on bacterial load and larval size was evaluated on d 2, d 6, d 10, and d 14, and the size and physiological condition (assessment of energy reserves) of the larvae were determined at the end of the experiment, *i.e.* at d 14 (12 d of treatment). Standard length was measured on formaldehyde-preserved larval pictures as described in (Hjörleifsson and Klein-MacPhee, 1992) using a dissecting microscope (Olympus SZ61) coupled to a digital camera (Evolution VF; Media Cybernetics) and Image Pro-Plus measurement software 5.0 (Media Cybernetics). Lipids were extracted in a 2:1 mixture of dichloromethane: methanol according to (Folch et al., 1957), the lipid classes (triglyceride [TAG], sterol [ST], acetone mobile polar lipids [AMPL], and phospholipids [PL]) concentrations were quantified by TLC-FID as described by (Parrish, 1987), and chromatograms analyzed using PeakSimple v3.21 software (SRI Inc.). To estimate the potential effect of BW on bacterial load in rearing water, bacterial analyzes were carried out using a flow cytometer on water samples taken from the tanks and frozen with glutaraldehyde according to (Seychelles et al., 2011).

2.5. Exposure of the sea urchins *Sphaerechinus granularis* and *Paracentrotus lividus* to EMn and CS

A first series of assays were conducted in May 2016 with purified EMn on *S. granularis* and *P. lividus*, model animals frequently used for *in vivo* ecotoxicological bioassays (Buttino et al., 2016; Pinsino et al., 2010). Sea urchins were collected in the Brest area (France) and obtained from the *Centre de Ressources Biologiques Marines* (CRBM) at the *Roscoff Biological Station* (Roscoff, France). Other experiments on sea urchins were run in March 2017 using CS rather than purified EMn due to its limited production, and the species *S. granularis* because it is available throughout the year.

Marennine-based solutions were prepared by weighing purified EMn freeze-dried powder or after the estimation of CS concentration as described in section 2.1 (Table 1). Solutions were diluted in 0.22 µm Millipore-filtered seawater (FSW) and the final solutions were syringe filtered through 0.2 µm. Sea urchin spawning was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in FSW, rinsed twice by centrifugation (2.000 *g*, 2 min) and re-suspended in FSW for a final 2 % (v/v) egg solution containing 0.1 % (v/v) glycine. Sperm was kept dry at 4 °C until use. Experiments took place in a temperature-controlled room at 16 °C.

Effects of purified EMn and CS were first assessed on fertilization. Eggs were transferred to a 24-well culture plate and incubated during 10 min in marennine-based solutions prior to addition of sperm. Solutions at different concentrations were added in wells containing 1 mL of the egg suspension. Only FSW was added for the control condition. pH strips were used to ensure that the pH did not vary between wells of the different concentrations tested. For fertilization, 50 µL of dry sperm was diluted in 1 mL of FSW shortly before use and 4 µL of diluted sperm added per mL of egg suspension. Observation of the culture plate under phase contrast inverted microscopy allowed the determination of the fertilization rate by counting at least 60 eggs per well (n = 3 wells per concentration tested). For experiments conducted with CS, unfertilized eggs exposed to the highest CS concentrations were rinsed 3 times in FSW using a benchtop centrifuge for 5 to 10 seconds and exposed to sperm for fertilization as described above. Two hours post fertilization, eggs exposed to CS were fixed on a DNA fluorescent stain (Hoechst fixative: Bisbenzymide 0.1 µg mL^{-1}; methanol 75 %, glycerol 25 %) and observed under a fluorescence microscope (ApoTome, Zeiss).

The effects of CS were also assessed post fertilization (kinetics of first cleavage and early embryonic development). For fertilization, diluted sperm was added in a 50 mL tube containing 25 mL of eggs suspended in FSW (2 % (v/v) egg suspension). When egg batches exhibited

greater than 90 % fertilization, sperm was removed by centrifugation in Heraeus Labofuge centrifuge (2.000 *g*, swinging bucket rotor 2 min). Fertilized eggs were transferred to a 24-well plate and CS solutions at 1; 10; 50 and 100 µg mL^{-1} added 10 min post fertilization. The percentage of dividing eggs was determined under phase contrast inverted light microscope; at least 60 eggs per well were counted. Three different sea urchin couples were used to replicate the experiment. For the "washed" condition, embryos from 3 wells per concentration tested were rinsed 6 h post fertilization as described earlier. Culture plates were maintained under constant agitation at 16 °C and embryos were observed 8, 48 and 72 h post fertilization with a Leica DMi8 inverted microscope and pictures acquisition done with LASX software. For each observation time, a 75 µL sample of each well was transferred to a glass slide and pictures of the developmental stages predominantly observed were taken. For post hatching developmental stages, a 1 µL drop of Janus green was added to the samples. Bright Field microscopy technique (BF) was applied for embryos 8 h post fertilization and a Differential Interference Contrast technique (DIC) for embryos 48 h and 72 h post fertilization.

2.6. Exposure of *Vibrio* species to BW and CS

Experiments were conducted during spring 2018 in *MMS* laboratory (Le Mans, France). BW and CS were prepared as described in section 2.1 (Table 1), and the concentration of marennine was determined with spectrophotometric measurements using the Beer-Lambert law with the specific extinction coefficient (ε_{677}=12.13 L $g^{-1}cm^{-1}$) proposed by (Pouvreau et al., 2006b). CS and BW were diluted with sterile ultra-pure water. Salinity and pH were adjusted by addition of NaCl and 0.1 M of NaOH or HCl (pH 7.5 ± 0.2; salinity 32) and the solutions were then syringe-filtered through 0.22 µm (Sarstedt). The three *Vibrio* strains tested, *Vibrio chagasii* (strain 8T3_5), *Vibrio crassostreae* (strain 8T2_1) and *Vibrio sp.* (strain 7G1_11) were previously identified (Bruto et al., 2017) and kindly provided by the *Laboratory of Integrative Biology of Marine Models* (CRBM/Roscoff, France). Bacterial strains were kept at -80 °C in 25 % glycerol, inoculated in Mueller Hinton Broth (MHB) + 1 % NaCl (pH 7.5 ± 0.2; salinity 32) and incubated overnight at 25 °C. Isolations were done on Petri dishes containing agar prepared with Nutrient Agar (Biokar) + 2.3 % NaCl (pH 7.5 ± 0.2; salinity 32). The plates were incubated at 25 °C for one day. The antibacterial activity of BW and CS was assessed according to a method described in the Clinical and Laboratory Standards Institute (CLSI) antimicrobial microdilution guidelines (Clinical and

Laboratory Standards Institute (CLSI), 2012). The day prior to the experiment, three different colonies per Petri dish were inoculated in broth media and grown overnight at ambient temperature. Wells of a 96-well sterile microplate with cover and flat bottom were filled with 50 µL of either CS or BW at a final concentration of 10 µg mL^{-1} (or sterile saline water for the controls) and 50 µL of a bacterial inoculate. A negative control was also run with sterile saline water and broth media without bacteria to ensure that no contamination occurred during the experiment. To prepare the bacterial inoculate the optical density (OD) of the broth culture was measured at 630 nm (V-10 Plus Onda Spectrophotometer), the OD was adjusted to 0.1 by dilution in MHB + 1 % NaCl and the solution was then further diluted by 1/100 as recommended by the CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2012). Microplates were inserted in a microplate spectrophotometer (xMark Bio-Rad) for a 20 h run at ambient temperature. Growth was managed with microplate Manager 6 Software by taking the OD in each well every 30 min at 600 nm.

2.7. Exposure of fish gill cell lines to EMn

Experiments took place in August 2017 at the Institute for Marine and Antarctic Studies (IMAS; Hobart, Australia). The gill epithelium cell line RTgill-W1 was obtained from the American Type Culture Collection (ATTC; and originally isolated from the Rainbow trout *Oncorhyncus mykiss*). The cell line was maintained and exposures to purified EMn conducted as described in Dorantes-Aranda et al. (2011) in conventional 96-well plates. Gill cells were seeded into a flat-bottom 96-well plate (655180, Greiner) at 2.5 x 10^5 cells mL^{-1} in L-15 medium (L-1518, Sigma) and allowed to attach for 48 h in the dark. Confluence of cell cultures was verified 12 h before experimental exposure and the L-15 medium replaced by L-15/ex (Schirmer et al., 1997). The concentration of the original purified EMn solution was estimated (540 µg mL^{-1} in 50 % methanol) as described in section 2.1 (Table 1), and the solution was diluted in L-15/ex by factors of 1 x 10^{-1}, 5 x 10^{-2}, 1 x 10^{-2}, 5 x 10^{-3}, 1x 10^{-3}, 5 x 10^{-4} and 1 x 10^{-4}, yielding final exposure concentrations of 0-54 µg mL^{-1}. Methanol was added to all dilutions to achieve a constant final MeOH concentration of 5 % across all treatments, including the non-toxic control (L-15/ex). Gill cells were exposed to these solutions for 2 h at 20 ± 1 ˚C in the dark (quadruplicate wells per concentration). After the exposure, wells were rinsed twice with saline phosphate buffer (100 µL per well) and incubated for a further 2 h in the dark with 100 µL of 5 % resazurin viability stain in L-15/ex medium.

Metabolic reduction of resazurin to resorufin by the gill cells was measured in a microplate reader (Fluostar Omega, BMG Labtech) at excitation and emission wavelengths of 540 and 590 nm, respectively. Results are expressed as percentage viability of the nontoxic control (5 % MeOH in L-15/ex).

2.8. Exposure of the oyster *Crassostrea gigas* haemocytes to EMn

Pacific oysters *C. gigas* were harvested in 2015-2016 in the hatchery of *Laboratoire de Génétique et de Pathologie des Mollusques Marins* (LGPMM; La Tremblade, France) in raceways supplied with a constant flow of seawater enriched with phytoplankton (*Skeletonema costatum, Tetraselmis suecica, I. galbana* and *C. gracilis*). They were maintained in safe conditions, free of known infectious pathogens. The shell of 20 adult oysters was broken with metal clamps and haemolymph withdrawn from the adductor muscle sinus (1 to 1.5 mL of haemolymph per oyster) using 1 mL sterile plastic syringes equipped with a needle (0.90 mm x 25 mm). The haemolymph of the different individuals was pooled, filtered through 60 µm nylon mesh and held on ice to prevent haemocyte aggregation (Auffret and Oubella, 1997). Haemocytes were observed under light microscopy using a Malassez-cell. Haemocytes were then exposed to different concentrations of purified EMn: 0, 1, 50 and 100 µg mL^{-1}. The purified EMn was obtained as described in section 2.1 (Table 1) and the solutions were prepared by weighing of the EMn dried powder on an analytical balance (Sartorius Entris®) and by dilution on sterile sea water. Purified EMn solutions were added to the haemolymph (1:1 ratio) for a final haemocyte concentration of 2.10^4 cells mL^{-1}. Haemocytes were exposed to EMn during 1, 3 or 6 h and mortality was quantified using 200 µL of cell suspension. Cells were incubated in the dark for 30 min on ice with 50 µL of Propidium Iodide (PI, 1.0 g L^{-1}, Interchim), a fluorescent DNA/RNA-specific dye that only permeates through the membranes of dead cells and stains the nucleic acids. Haemocyte samples were analyzed with flow cytometry using an EPICS XL 4 (Beckman Coulter) and red fluorescence following the protocol of (Morga et al., 2009). Based on size discrimination, only haemocytes were taken into account with 5.000 events counted per sample. Results were depicted as cell cytograms and reported as log scale fluorescence levels. Data were analyzed with *Flowing Software 2*.

2.9. Statistics

Statistical analyses were run using *SigmaPlot 12.3* software for Windows. Differences between treatments were assessed with One-Way or Two-Ways ANOVAs. Normality was tested by the Shapiro-Wilk test and the assumption of homoscedasticity of variance with Fisher's test (F-test) and/or verified visually by the spread of residuals, as suggested by (Quinn and Keough, 2002). Post hoc Tukey's pairwise multiple comparison tests were used to determine differences between means. Unless specified, alpha value used was 0.05.

3. Results

3.1. Differences in the solutions containing the extracellular marennine

BW and CS presented the same UV-Vis spectral characteristics in comparison with purified EMn (Pouvreau et al., 2006b), with one peak around 670 nm in the visible region and two others in the UV region around 250 and 320 nm. Different coefficients of extinction were used to assess the extracellular marennine concentration depending on the preparation and concentration process and on the series of experiments (summarized in Table 1). Despite a difference in calculated concentrations of about 30-40 % resulting from the use of either the apparent extinction coefficient proposed by (Robert et al., 2002) or the one proposed by (Pouvreau et al., 2006a), the solutions tested had comparable concentration ranges and activities as described below.

3.2. Effects of BW on *Mytilus edulis* larvae

After a 48 h exposure of embryos to BW, the percentage of larvae that reached the D-larval stage and the mortality rate were assessed (Figure 2A). The three lowest concentrations tested did not delay larval development; however, when embryos were exposed to 1 µg mL^{-1} of BW, the percentage of D-larvae was significantly lower (22 ± 1 % of D-larvae compared to 52 ± 2 % for the control; *p*-value = 0.026). Exposure to 1 µg mL^{-1} significantly increased the mortality rate, with 47 ± 1 % of mortality while no mortality was recorded in control condition. Exposure to 1 µg mL^{-1} of BW significantly delayed the development with a D-larvae mean length of 88 ± 7 µm compared to 122 ± 1 µm for the control (*p*-value <0.001; Figure 2B). At 5 µg mL^{-1}, none of the embryos survived after a 48 h exposure to BW (Figure 2A).

In a second series of experiments, D-larvae were exposed to BW in order to assess the effects on larval swimming (Figure 3A). After 24 h in BW, the percentage of swimming larvae

exposed to 1 µg mL^{-1} was significantly lowered (80 ± 9 % compared to 100 ± 0 % for the control; *p*-value <0.001). D-larvae exposed to 5 µg mL^{-1} of BW were totally motionless, with the velum highly coloured with marennine (Figure 3B). After 72 h in BW, the percentage of swimming D-larvae exposed to 1 µg mL^{-1} of BW decreased significantly in comparison with the day one with only 45 ± 2% of swimming larvae (*p*-value 0.012).

When veliger larvae were exposed over a 5 d period to BW (Figure 4A), a significant increase in mortality was only observed at a concentration of 5 µg mL^{-1}, with 64 ± 7 % of dead veliger larvae compared to 7 ± 6 % of mortality in control (*p*-value <0.001). Larval length only differed for treatments of 0.5 and 1 µg mL^{-1} compared to 5 µg mL^{-1} of BW (Figure 4B; *p*-values 0.036 and 0.030 respectively).

3.3. Effect of BW on the barnacle *Chthamalus bisinuatus* larvae

The survival rate of *C. bisinuatus* larvae was measured at two concentrations of BW, 0.05 and 0.1 µg mL^{-1} over a 9 d period, the maximum period to maintain barnacle larvae without food. At both concentrations, exposure to BW had no significant effect on *C. bisinuatus* larval survival rate in comparison with the control (Figure 5; *p*-value 0.1427).

3.4. Effect of BW on the winter flounder *Pseudopleuronectes americanus* larvae

The addition of BW at 0.05 and 0.1 µg mL^{-1} had no effect on larval growth (*p*-value 0.287). Indeed, the three groups experienced a growth of 0.1 mm d^{-1} from d 2 to d 14 (Figure 6A). The bacterial load remained comparable in all treatments (*p*-value 0.868), demonstrating that, at such concentration, marennine had no impact on bacterial development in fish rearing tanks (Figure 6B). Energy reserves in larvae treated with marennine seemed to increase with 4.6 ± 0.7 µg $larva^{-1}$ under 0.1 µg mL^{-1} of BW exposure *vs* 3.9 ± 0.7 µg $larva^{-1}$ for the control, but the difference from the control was not significant due to the high variability among tanks (total lipids, *p*-value 0.1092; triacylglycerol/sterol (TAG/ST) ratio, *p*-value 0.0767). However, the larvae showing the highest lipid content, and the only ones containing TAG, were sampled from the marennine treated tanks (Figure 6C).

3.5. Effects of CS and purified EMn on *Sphaerechinus granularis* and *Paracentrotus lividus*

A first series of experiments was conducted on the sea urchins *S. granularis* and *P. lividus* to assess if purified EMn had an effect on fertilization and first cleavage of the egg. In both species, fertilization was totally blocked at 50 and 100 µg mL^{-1} and the phenotypes of embryos exposed to 10 µg mL^{-1} were either delayed in comparison with the control or abnormal (*e.g.*, flattened). Four different incubation periods were tested, from 0 to 20 min prior to the addition of purified EMn, but the effects on fertilization were similar. A 10 min incubation of sperm prior to fertilization was also conducted, but the fertilization rate remained unaffected with 100 % fertilization observed even when sperm was incubated in EMn at 100 µg mL^{-1} (data not shown). It was checked if purified EMn prevented fertilization or if the absence of fertilization membrane around the eggs was caused by an effect of EMn on the elevation of fertilization membrane steps. To do so, a calcium ionophore (*i.e.*, a chemical that can activate echinoderm eggs by a release of intracellular calcium ions and inducing the membrane elevation (Steinhardt and Epel, 1974)) was added in EMn incubated eggs. For all concentrations of EMn tested, from 1 to 100 µg mL^{-1}, the calcium ionophore induced the egg activation demonstrating that purified EMn did not interfere with the elevation of the fertilization membrane steps (data not shown).

Incubation of *S. granularis* eggs with CS prior to the addition of sperm also exhibited a dose-dependent effect on fertilization (Figure 7A). Exposure to 10 µg mL^{-1} significantly lowered the fertilization success with 85 ± 8 % of fertilization compared to 99 ± 2 % for the control condition (*p*-value 0.032). When eggs were incubated in 50 µg mL^{-1}, no fertilization occurred although the sperm was highly active around the eggs (Figure 7B). When eggs previously exposed to 50 µg mL^{-1} of CS were rinsed and removed in FSW without CS, the fertilization rate was similar to the control condition (*p*-value 0.58) demonstrating that the effect of CS on *S. granularis* eggs and fertilization was reversible (Figure 7A).

Incubation of newly fertilized *S. granularis* eggs with CS demonstrated a dose dependent effect on the kinetics of first cleavage (Figure 8A) and on the early embryonic development (Figures 8B-C). The two lower concentrations tested, 1 and 10 µg mL^{-1}, had no effect on the kinetics of first cleavage: as for the control, divisions started 95 min post fertilization with embryos dividing symmetrically and synchronously, and at 180 min post fertilization all embryos reached at least the 2-cell stage. At higher incubation concentrations, a dose-dependent effect of CS was observed with fewer dividing eggs, asymmetrical and asynchronous divisions. At 180 min post fertilization, less than 85 % and 50 % of embryos incubated in 50 and 100 µg mL^{-1} of

CS, respectively, started to divide and the others quickly degenerated. Similar observations were made for the 3 series of experiments.

Eight hours post fertilization, embryos of the control and those incubated in the presence of CS (1 and 10 µg mL^{-1}) reached the blastula stage (Figure 8B) while cell division for embryos incubated in 50 and 100 µg mL^{-1} was quickly halted after a few and incoherent divisions or no division at all. Twenty-four hours post fertilization, the gastrula stage was observed for the control embryos and those exposed to 1 µg mL^{-1} while embryos incubated in 10 µg mL^{-1} were still at the blastula stage and did not hatch (data not shown). Forty-eight hours post fertilization, embryos incubated in 1 and 10 µg mL^{-1} exhibited a dose dependent developmental delay with embryos still at the blastula stage and at the early prism stage, respectively, while control embryos reached the late prism stage (Figure 8C). Three days post fertilization, the early pluteus stage was observed in controls while the early prism stage and hatched blastula stage were observed in those incubated in 1 and 10 µg mL^{-1} of CS, respectively. Washed embryos were able to recover; embryos initially exposed to 1 µg mL^{-1} presented similar developmental stages to the control, 48 and 72 h post fertilization and the development of embryos initially exposed to 10 µg mL^{-1} and rinsed 6 h post fertilization was still slightly delayed in comparison with the control but more advanced than in embryos incubated in the CS.

3.6. Antibacterial effects of BW and CS on *Vibrio* species

This series of experiments aimed to compare the antibacterial activity of two different solutions of marennine, BW and CS. The bacteria *V. chagasii*, *V. crassostreae* and *V. sp.* were exposed to 10 µg mL^{-1} of BW and CS for 20 h and relative growth inhibition in comparison with the control was estimated (Figure 9). *Vibrio sp* was the most sensitive strain with 37 ± 2 % and 40 ± 1 % of growth inhibition when exposed to 10 µg mL^{-1} of BW and CS, respectively. The growth of *V. chagasii* exposed to BW and CS also decreased significantly, with percent inhibition as a fraction of the control of 9 ± 1 % (*p*-value <0.001) and 6 ± 2 % (*p*-value 0.003), respectively. *V. crassostreae* was the least sensitive strain, although growth was also significantly lower than in controls when exposed to BW (5 ± 2 % of inhibition, *p*-value 0.034) and to CS (6 ± 2 % of inhibition, *p*-value 0.020). For each strain tested, growth inhibition induced by BW was statistically similar to the growth inhibition induced by CS (*p*-values >0.05) indicating that the two different solutions had similar antibacterial effects at 10 µg mL^{-1}.

3.7. Effects of EMn on fish gill cell line RTgill-W1

After 2 h exposure to purified EMn, marennine showed no significant effect on fish gill cell viability in the concentration range tested (0-54 µg mL^{-1}).

3.8. Effects of EMn on *Crassostrea gigas* haemocytes

After an exposure of *C. gigas* haemocytes to 1 µg mL^{-1} of purified EMn, the percentage of lysed cells was not significantly different from the control, 6 ± 1 % compared to 5 ± 1 %, respectively (Figure 10). The percentage of lysed haemocytes increased in a dose dependent way at 50 (8 ± 1 %, *p*-value 0.009) and 100 µg mL^{-1} (10 ± 1 %; *p*-value < 0.001). Different durations of exposure were tested (1, 3 and 6 h) without any difference observed in number of lysed cell suggesting that the effects on haemocytes result from the immediate contact with the BW (data not shown).

4. Discussion

This work represents a broad panel of experiments conducted in different laboratories to study the biological effects of marennine on various marine model organisms of aquaculture interest. The results confirm that independently of the mode of preparation, solutions containing marennine can exert biological activities, ranging from growth inhibition of pathogens to the death of bivalve larvae. The effects observed depend on the species, the life cycle stage and concentrations of marennine used. The results are summarized in Table 2 and raise two questions regarding: 1) limitations of the study due to diversity of marennine solutions tested, and 2) interpretation of data in view of importance of these biological activities for the marine environment and potential applications in aquaculture.

4.1. Limitations of the study

This work brings together the results of different experiments, conducted over a three-year period, in different laboratories and using three different marennine-based solutions (purified EMn, BW and CS), as well as different methods to quantify marennine concentrations. Moreover, cultures of *H. ostrearia* were grown on different media, which also may have impacted

marennine production and quality. The first series of experiments using purified EMn and CS exposed against *S. granularis* and *P. lividus*, showed similar effects on sea urchin egg fertilization. Furthermore, growth inhibition induced by BW and CS was comparable within the same *Vibrio* species at 10 µg mL^{-1}. We conclude that biological activity was not impaired related to marennine production conditions.

The concentration ranges tested could vary by about 30-40 %, depending on the extinction coefficient used. Various marennine quantification methods have been published so far, using an extinction coefficient determined from raw extract (Robert et al., 2002) or purified marennine (Pouvreau et al., 2006a), or weighing of purified marennine dried powder (Prasetiya et al., 2017; Turcotte et al., 2016 and Gastineau et al., 2012, respectively). As the absolute quality and quantity of the pigment in different solutions could not be clearly assessed without a complete characterization of this pigment, our results provided a range of concentrations showing biological activities against various marine organisms. While the higher concentrations (50 and 100 µg mL^{-1}) provide clues about doses with deleterious effects, the lower concentrations (from 0.01 to 10 µg mL^{-1}) are more ecologically relevant. In natural environments, marennine measurements indicate a range of 1-10 µg mL^{-1} (Turpin et al., 2001), and natural blooms of blue *Haslea* have been recorded worldwide (Figure 1). *Haslea* blooms are regularly observed in Calvi Bay (Corsica, France) as part of seaweed surveys. These blooms, which mostly occur in spring, result in the development of a blue-green biofilm at the surface of the thallus of seaweeds (*i.e., Padina, Halopteris, Acetabularia*), on short turfs, and to a lower extent on sediments and rocks (STAtion de REcherche Sous-marines et Océanographique - STARESO and Liège University; V. Demoulin, D. Sirjacobs, S. Gobert and P. Lejeune, personal communications). Blue *Haslea* can display very large patches reaching several square meters and be observed at depths ranging from 2 to 8 meters. Such extensive blooms are also annually recorded from November through March in central and southern coastal estuaries of North Carolina (USA), in particular along the central coast from lower Core Sound westward across the lower North River estuary and Back Sound to the western portions of Bogue Sound. These blooms of a blue *Haslea* possibly different from *H. ostrearia* do not seem to be associated with the development of biofilms on *Padina* sp. thalli, however they cause a wide-spread greening of oysters, which could underpin emerging *green oyster* industries in the USA (N. Lindquist, personal communications).

Thus it can be inferred that, at the microenvironment scale, high concentrations of marennine could be encountered, and that many marine organisms can periodically and locally face acute marennine exposure. All laboratory experiments to study biological activity of marennine lasted hours or days, and the cumulated effect of long-lasting exposure to low concentrations of marennine has not yet been tested. Greening of oyster gills can be viewed as a cumulative, longer term effect. According to green oyster producers, the greening in oyster ponds can occur in a matter of days, depending on *Haslea* growth and density. In the laboratory, EMn rather than IMn was preferentially responsible for greening, which proved to be time dependent and long-lasting (Gastineau et al., 2018, 2014). However, green oysters have also been observed in natural open environments, in absence of any record of *Haslea* blooms, for instance in oyster leases in Australia (New South Wales and Tasmania). This means that marennine could have biological activities in the long-term, from subacute and chronic exposure, and further work is required to better assess the impact of this pigment in the natural environment.

4.2. The unpredictable "Jekyll and Hyde", good and bad nature of marennine

On the one hand, our results showed that exposure to the tested marennine solutions could lead to adverse effects against many marine animals, depending on the exposure dose. Concentrations from 1 µg mL^{-1} significantly lowered the survival rate of *M. edulis* embryos and locomotion of the D-larvae. Significantly higher mortality rates were observed for veliger larvae exposed to 5 µg mL^{-1} of BW while no mortality was recorded at 1 µg mL^{-1}, which contrasts with results of a previous study where exposure of veliger larvae to 1 µg mL^{-1} of BW led to 100 % mortality, but the duration of that experiment was 4 times longer, 20 d *vs* 5 d (Turcotte et al., 2016). Significant dose-response effects of marennine were also observed with the sea urchin model. While high concentrations (50 and 100 µg mL^{-1}) resulted in a total blockage of fertilization and embryonic development, lower concentrations (1 and 10 µg mL^{-1}) of CS induced a developmental delay of *S. granularis* embryos. Previous works also have demonstrated the effects of a water-soluble extract of the diatom *Thalassiosira rotula* on cell division of the sea urchin *P. lividus*, with a blockage of cell division at the higher doses tested and a delay and abnormal development of embryos at lower doses (Buttino et al., 1999). Also, intact diatom cells of six different species could inhibit the egg first cleavage of the sea urchins *Strongylocentrotus droebachiensis* and *Echinus acutus* (Gudimova et al., 2016). Most interestingly, our results indicated that even at

high concentrations, the effects of CS on fertilization and embryonic development were reversible after rinsing the eggs or embryos. This confirms previous observations of the effects of water-soluble diatom extracts against various marine invertebrates, including the sea urchin *Psammechinus miliaris* (Caldwell et al., 2002).

The present results could suggest an inability of marennine to bind tightly at the cell surface if considering the reversible effects observed in sea urchins, and to penetrate the cell and exert irreversible damages given the high molecular weight of the molecule (> 10 kDa; Pouvreau et al. 2006b). Marennine could however link to cell surface or membrane receptors and trigger stress responses or even apoptotic signals by inducing changes in cell metabolism. Furthermore, the variability of BW and CS effects observed at both the interspecific and intraspecific levels on the three *Vibrio* species tested, but also those previously shown with *V. aestuarianus, V. coralliilyticus* and *V. tubiashii* strains (Falaise et al., 2016) could reflect differences in membrane receptor affinities and in mechanisms of action. Such variability should be considered before application of marennine in aquaculture as an antibacterial agent. However, more data is necessary to confirm these hypotheses.

On the other hand, despite observed deleterious effect at the higher concentrations tested, very low concentrations (0.05 and 0.1 µg mL^{-1}) of BW did not exhibit a negative effect on the barnacle *C. bisinuatus* survival nor on the winter flounder *P. americanus.* Both flounder growth rate and bacterial load in the water remained unaffected throughout the marennine exposure (0.05 and 0.1 µg mL^{-1}), and physiological condition (energetic reserves) improved in response to marennine treatment. These results compare with no observable effect of marennine (0-54 µg L^{-1}) on the viability (metabolic activity) of the rainbow trout gill cell line RTgill-W1. Although the gill cell line exposure was conducted for only 2 h, results provide a strong indication that marennine does not impair fish gill function at the concentration ranges tested here. These results are further supported by (Turcotte et al., 2016) who demonstrated that low exposure to BW (0.1 µg mL^{-1}) enhanced survival and physiological condition of *M. edulis* and *P. magellanicus* larvae. In addition, our results showed that low concentrations of EMn (1 µg mL^{-1}) did not present a cytotoxic effect against haemocytes of adult oysters. Even though cytotoxic effects were evidenced at the highest concentrations (50 and 100 µg mL^{-1}), the point of inflection could not be determined with the concentration range tested. However, it should be emphasized that even after

centuries of production of green oysters, no negative impacts on adult oysters, nor green oyster consumers have been reported.

Despite the fact that diatoms are traditionally considered a high quality food source enhancing growth and survival of many marine organisms, they can produce secondary metabolites that function as grazing deterrents (Ianora and Miralto, 2010). Most of these secondary metabolites are polyunsaturated fatty acids (PUFAs) and polyunsaturated aldehydes (PUAs) with a special focus on oxylipins (Caldwell, 2009). Effects of diatom aldehydes on reproductive and fertilization success or embryonic development of various organisms such as crustaceans, echinoderms or arthropods have been widely studied (Caldwell, 2009; Ianora and Miralto, 2010). Aldehyde production is a diatom chemical defense strategy to limit growth of their grazers (Ban et al., 1997; Pohnert, 2005), but other studies reported the absence of a correlation between diatom aldehyde and reproductive biology and early development of marine invertebrates, suggesting the involvement of other unidentified diatom metabolites (Poulet et al., 2006; Wichard et al., 2008). Unlike aldehydes, that are likely to exert their activity following diatom cell destruction and ingestion, the water-soluble marennine may target a larger spectrum of marine organisms and not only diatom grazers.

In conclusion, the present work demonstrates that *H. ostrearia* supernatant containing marennine represents a biologically active water-soluble solution with potential effects on various marine organisms. More studies are required to better estimate the long term impacts of blue *Haslea* blooms in natural environments, facilitated by laboratory studies, investigating chronic exposure to low concentrations of marennine. If the use of *H. ostrearia* supernatant is considered in hatcheries as a preventive or curative anti-infectious agent, concentrated solutions such as BW or CS could be of interest in order to control the delivered marennine doses. It is likely that the adverse effects of marennine only target the early and more succeptable fragile developmental stages such as embryos and larvae, depending on the exposure concentration, whereas adults remain unaffected.

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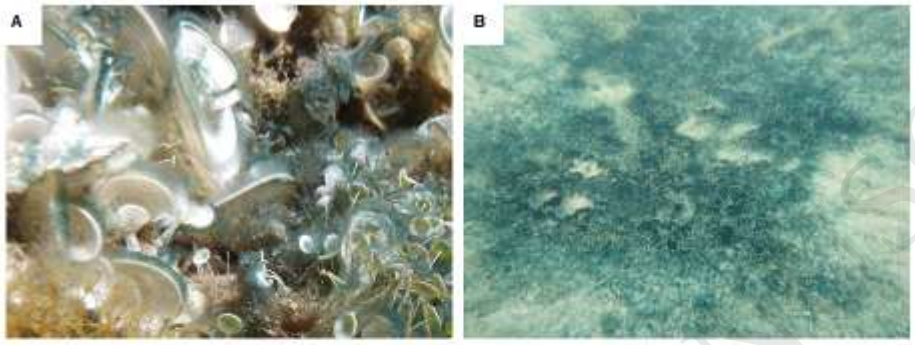
List of Tables and Figures (in order of apparition in the text):

Figure 1. Underwater pictures of natural blue *Haslea* blooms forming biofilms observed **A)** in macro-algae *Padina sp*. in the Mediterranean sea, Corsica (France) and **B)** on sediments in the Beaufort Strait, North Carolina (United States).



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[Colors should be used for this figure]

Figure 2. Exposure of the mussel *Mytilus edulis* embryos to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 48 h period. **A)** Percentage of embryos that reached the D-larval stage (grey bars) and percentage of mortality (black bars). **B)** Length of the larvae that reached the *D* stage (µm). Values are means ± SE (n=3). Error bars with different lower case letters are significantly different.

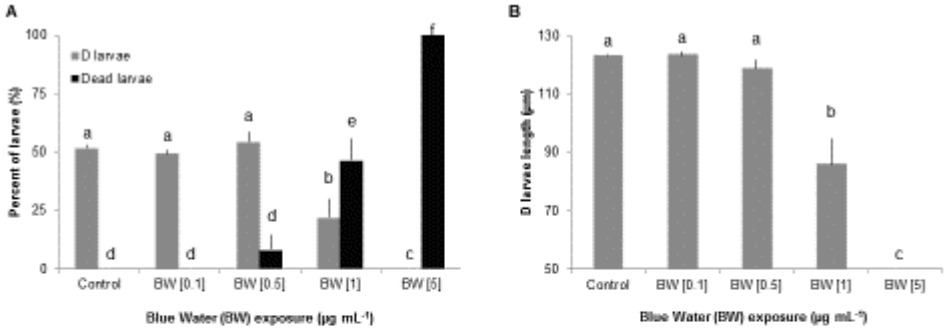

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Figure 3. Exposure of the mussel *Mytilus edulis* D-larvae to different concentrations (µg mL^{-1}) of Blue Water (BW). **A)** Percentage of swimming D-larvae exposed during 24 h (light grey bars) and 72 h (dark grey bars) to BW. Values are means ± SE (n=6). Error bars with different lower case letters are significantly different. **B)** Observation of D-larvae of the control condition (left picture) and exposed to 5 µg mL^{-1} of BW (right picture) after 24 h under light invert microscopy. D-larvae exposed to 5 µg mL^{-1} of BW were totally motionless with marennine agglutinated on the velum (arrow). Scale bars: 100µm.

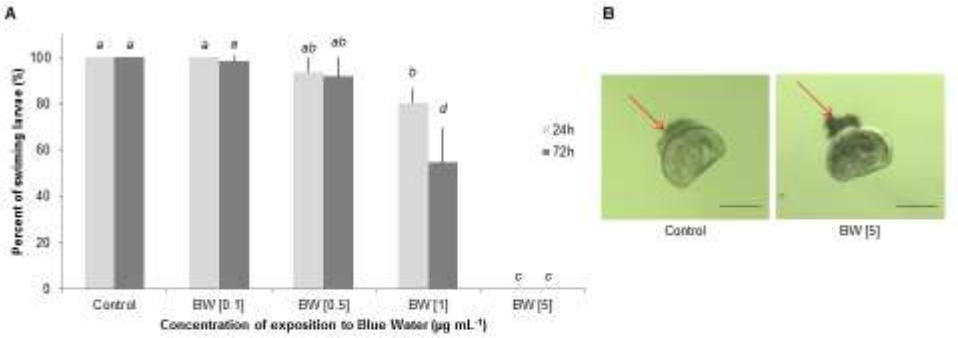

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Figure 4. Mussel *Mytilus edulis* veliger larvae exposed to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 5 d period. **A)** Percentage of veliger larvae mortality and **B)** veliger larvae mean length after BW exposure. Values are means ± SE (n=3). Error bars with different lower case letters are significantly different.

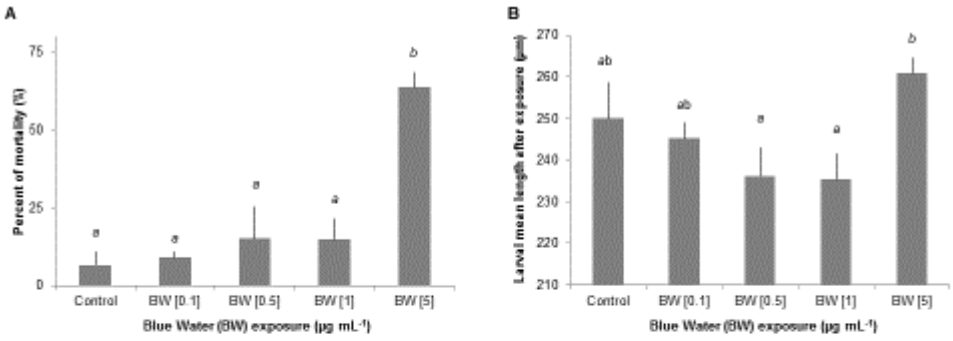

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Figure 5. Percentage of mortality of the barnacle *Chthamalus bisinuatus* larvae exposed to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 9 d period. Results are means ± SE (n=4).

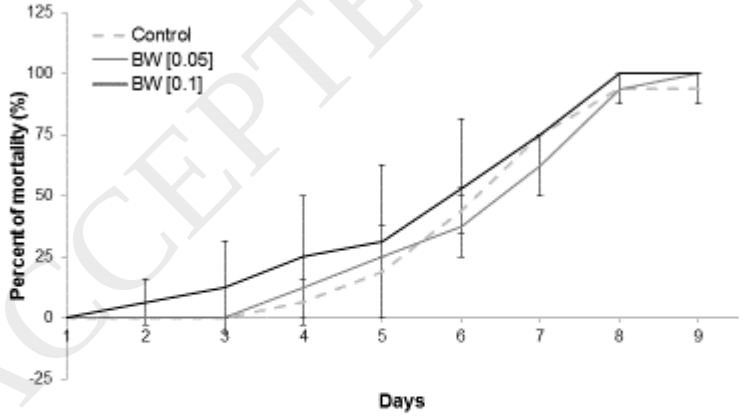

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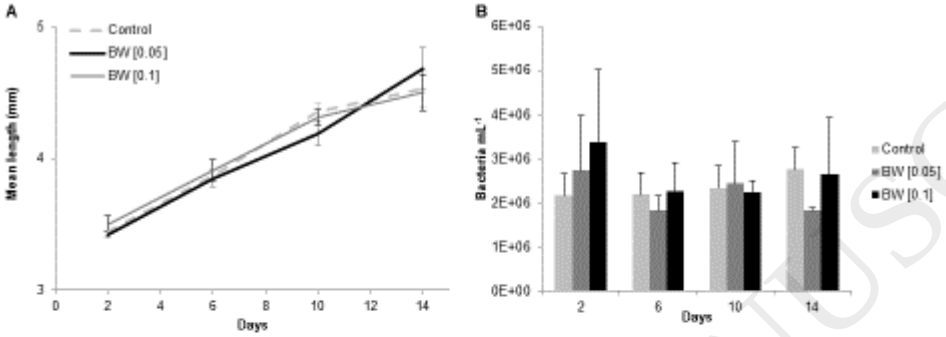
Figure 6. Winter flounder *Pseudopleuronectes americanus* larvae exposed to different concentration (µg mL^{-1}) of Blue Water (BW) over a 14 d period. **A)** Mean lengths (mm) of *P. americanus* larvae. **B)** Bacterial load (bacteria mL^{-1}) in larval tanks and **C)** larval energy reserves: percentages of triglyceride (TAG), sterol (ST), acetone mobile polar lipids (AMPL), and phospholipids (PL) and TAG/ST ratio. Results are means ± SE (n=3).



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BW concentration (µg mL^{-1})	Total lipids (µg larva^{-1})	%TAG	%ST	%AMPL	%PL	TAG/ST
Control	3.9 ± 0.7	0 ± 0	11.7 ± 1.8	13.4 ± 10.6	71.8 ± 10.5	0 ± 0
0.05	3.3 ± 0.1	0.3 ± 0.3	13.2 ± 3.3	12.0 ± 17.0	70.8 ± 21.4	0.02 ± 0.03
0.1	4.6 ± 0.7	0.6 ± 0.6	12.4 ± 0.9	6.7 ± 6.3	77.2 ± 5.7	0.05 ± 0.01

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Figure 7. Sea urchin *Sphaerechinus granularis* eggs exposed to different concentrations (µg mL^{-1}) of *Haslea ostrearia* Concentrated Supernatant (CS) before fertilization. **A)** Fertilization rate and **B)** observation under phase contrast (left panels) and fluorescence (right panels) microscopy (Gx400) of *S. granularis* eggs of the control condition (upper raw) and exposed to CS at 50 µg mL^{-1} (lower raw) 2 h post exposure to CS. Eggs were fixed in a DNA fluorescent stain (Hoechst fixative) to observe cell nuclei of the control dividing egg and spermatozoa agglutinated around the unfertilized egg exposed to 50 µg mL^{-1} of CS. Values in A) are means ± SE (n=3). Error bars with different lower case letters are significantly different. Scale bars in B): 25 µm.

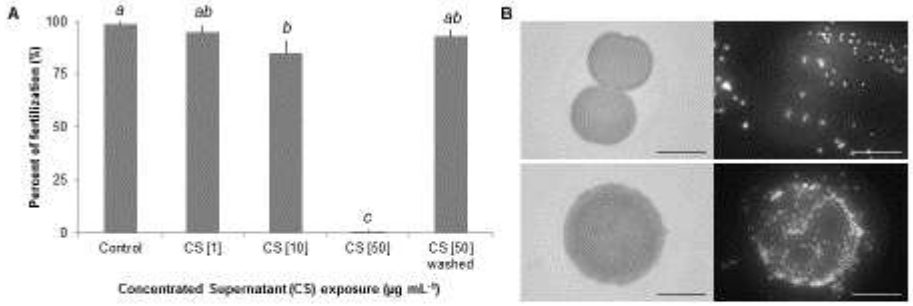

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Figure 8. Exposure of newly fertilized sea urchin *Sphaerechinus granularis* eggs to different concentrations (µg mL^{-1}) of concentrated supernatant (CS) of *Haslea ostrearia* culture. **A)** Kinetic of first cleavage of *S. granularis* eggs exposed to the CS 10 min post fertilization. **B)** Observation of *S. granularis* embryos under phase contrast microscopy 8 h post exposure to CS (Gx200) and **C)** 48 h (top raw) and 72 h (lower raw) post exposure to the CS (Gx400). Embryos of the "unwashed" conditions were still incubated in the CS while embryos of the "washed" condition were rinsed 6 h post exposure to the CS. The kinetics of first cleavage presented in A) is representative of 3 replicate experiments. Views in B) and C) are representative of the observed stages, scale bars: 50 µm.

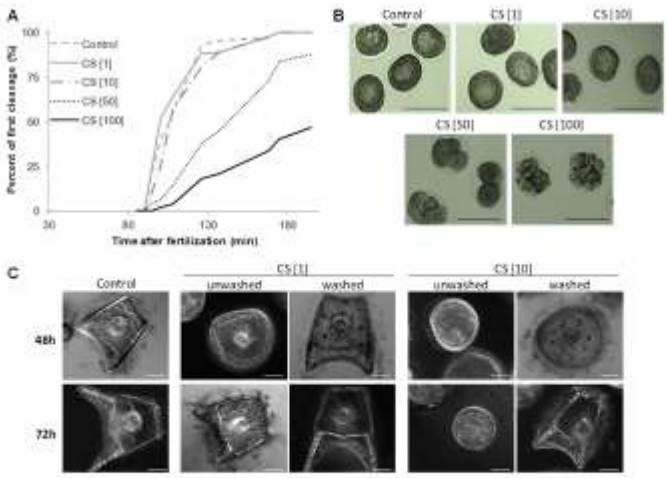

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Figure 9. Relative growth inhibition of the bacteria *Vibrio chagasii*, *Vibrio crassostreae* and *Vibrio sp.* exposed to 10 µg mL^{-1} of Blue Water (BW) and 10 µg mL^{-1} of Concentrated Supernatant (CS) over a 24 h period. Values are means ± SE (n=3).

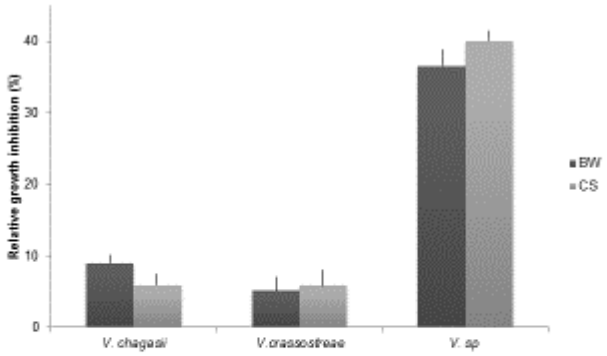

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Figure 10. Percentage of viable oyster *Crassostrea gigas* hemocytes exposed to different concentrations (µg mL^{1-}) of purified Extracellular Marennine (EMn). Values are means and SE (n=6).

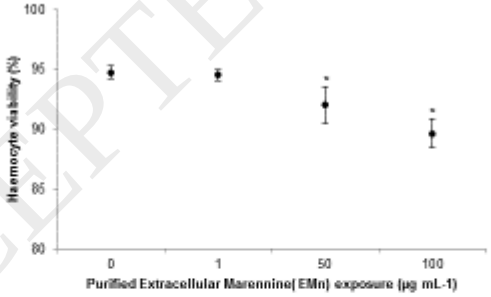

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Table 1. Summary of the marennine based solutions and marine organisms used in the present study

Marennine based solutions	Production process	UV-visible spectra	Method of quantification	Marine organisms tested and studied effect
Blue Water **(BW)**	Ultrafiltration of *H. ostrearia* culture medium (3-30kDa)		ε_{677}=12.13 L g^{-1} cm^{-1} (Pouvreau et al. 2006c)	Mussel *M. edulis,* barnacle *C. bisinuatus* and winter flounder *P. americanus*: Larval survival and/or development
				Bacterial species of the *Vibrio* genus: Bacterial growth
Concentrated Supernatant **(CS)**	Innovative process being patented		ε_{669}=17.2 L g^{-1} cm^{-1} (Robert et al. 2002)	Bacterial species of the *Vibrio* genus: Bacterial growth
			ε_{677}=12.13 L g^{-1} cm^{-1} (Pouvreau et al. 2006c)	Sea urchin *S. granularis*: Fertilization, early embryonic development
Purified Extracellular Marennine **(EMn)**	- Ultrafiltration of *H. ostrearia* culture medium (3-30kDa) - Anion-Exchange chromatography - Dialysis & freeze-drying (Pouvreau et al. 2006c)	Adapted from Pouvreau et al. (2006)	ε_{677}=12.13 L g^{-1} cm^{-1} (Pouvreau et al. 2006c)	Preliminary assays with EMn on the sea urchins *S. granularis* and *P. lividus*: Fertilization Oyster *C. gigas* haemocytes: Cytotoxicity
			Weighing of the purified EMn dried powder	Fish gill cell line: Cytotoxicity

Table 2. Summary of the marennine based solutions effects on the marine organisms tested

Marennine based solutions	Marine organisms tested	Summary of the observed effects
Blue Water **(BW)**	Mussel *M. edulis*	Higher mortality and developmental delay for embryos from 1 µg mL^{-1} Higher mortality for veliger larvae from 5 µg mL^{-1} Decreased of motility for D-larvae from 1 µg mL^{-1} No observed effects at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
	Barnacle *C. bisinuatus*	No observed effect on larval survival at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
	Winter flounder *P. americanus*	No observed effect on larval and development at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
Concentrated Supernatant **(CS)**	Bacterial species of the genus *Vibrio*	Growth inhibition at 10 µg mL^{-1} Similar effects observed with BW solutions and CS solutions
	Sea urchin *S. granularis*	Decreased of the fertilization rate from 10 µg mL^{-1} and delay of the embryonic development from 1 µg mL^{-1} Reversible effects after rinsing the eggs and/or the embryos Similar effects on fertilization observed with CS solutions ad EMn solutions on *S. granularis* and *P. lividus*
Purified Extracellular Marennine **(EMn)**	Sea urchins *S. granularis* and *P. lividus*	
	Fish gill cell line Rtgill-W1	No observed effect on cell viability from 0 to 54 µg mL^{-1}

	Oyster C. *gigas* haemocytes	No observed effect up to 1 µg mL^{-1}