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## Does culture supernatant of *Haslea ostrearia* containing marennine affect short-term physiological traits in the adult blue mussel *Mytilus edulis*?

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### ABSTRACT

Marennine, the blue-green pigment excreted by the pennate diatom *Haslea ostrearia* has potential for applications as a natural antimicrobial agent in bivalve aquaculture. However, utilization of this pigment should be assessed further prior to its application in bivalve hatcheries. The present study aims to investigate the effect of the culture supernatant of *H. ostrearia* containing marennine (Blue Water, BW) on physiological rates of adult mussel *Mytilus edulis*. The hypothesis tested in this study was that BW could alter clearance and respiration rates of adult *M. edulis* as indicative physiological traits. Furthermore, the effect of BW on veligers and pediveligers larvae was assessed in physiological experiments to determine whether the fixation of marennine by the larvae can influence larviphagy in this species. Our results showed that BW significantly lowered CR by 43% and reduced the oxygen consumption by 30% in adult mussels, as compared to control. In larviphagy experiments, mussels ingested veliger and pediveliger larvae without discrimination between greened or non-greened larvae. Moreover, fixation of BW in bivalve gills at 3 mg L<sup>-1</sup> affected the physiological rate but not intensity of larviphagy in adult *M. edulis*. This study thus provides an insight into the necessity to determine the potential effect of marennine on adult bivalve for the future utilization of this pigment in bivalve hatcheries.

### 1. Introduction

Bivalves play a significant role in energy and nutrient flux between benthic and pelagic communities in the aquatic environment (Dame, 1993, 2011). They are able to control the abundance of primary producers, zooplankton and larval stages of other marine species, including bivalves themselves (Fréchette and Bourget, 1985; Prins et al., 1991; Coen et al., 2007). As food and oxygen are obtained by filtering water across their gills in bivalve suspension-feeders, filtration and respiration are considered useful tools to measure their physiological state (Widdows and Johnson, 1988). These two physiological indicators are frequently used to determine bivalve's response to the presence of dissolved chemicals and particulate matters in water. To date, many studies have shown that biotoxin, especially from toxic dinoflagellates, can alter bivalve behavior, physiology and cellular activity (Bardouil et al., 1993; Bricelj et al., 1996, 2005; Hégaret et al., 2007, 2011; Navarro and Contreras, 2010). Nevertheless, studies on the effects of

non-toxic organic compounds on bivalves are less documented.

Marennine is a water-soluble blue pigment that is excreted by the non-toxic diatom *Haslea ostrearia* (Gaillon) Simonsen. This non-toxic organic compound can be fixed preferentially on the gills and labial palps of bivalves and is responsible for the greening phenomenon of Pacific oysters *Crassostrea gigas* on the French Atlantic Coast (Gastineau et al., 2014; Prasetya, 2015). This natural greening phenomenon contributes to the economic value of cultured oysters due to the organoleptic and essential fatty acids modification in green oysters (Piveteau, 1999; Prasetya et al., 2017a). Most importantly, marennine has antibacterial properties and could act as antibacterial and natural protective agent against pathogens in aquaculture (Gastineau et al., 2012, 2014; Turcotte et al., 2016). In the mussel *Mytilus edulis* and the scallop *Placopecten magellanicus*, Turcotte et al. (2016) revealed that culture supernatant containing marennine (hereafter named as blue water, BW) can increase the survival of their larvae when they are exposed to *Vibrio splendidus*. Consequently, marennine could be

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potentially applied in shellfish aquaculture as natural antimicrobial agent and immunostimulant if the concentration is maintained below the toxicity level (Falaise et al., 2019). However, before the development of this potential new antibacterial agent in commercial hatchery, knowledge on their effect on other ontogenic stages is necessary. Recent study from Prasetya et al. (2017a) demonstrated that marennine affects behavior, physiology and biochemical traits of juvenile blue mussel *Mytilus edulis* and the American oyster *Crassostrea virginica*. However, no evidence was documented on the effect of this compound on adult bivalves.

The selected biological model in the present study was the blue mussel *M. edulis* that is spread and cultivated worldwide. The main hypothesis of the present study was that marennine could alter clearance and respiration rates of adult *M. edulis* as indicative physiological traits. Additionally, a possible effect of marennine on larvae was studied in physiological experiments to investigate whether marennine could influence larviphagy in this species. Previous studies have revealed that most bivalve perform cannibalism by ingesting veliger larvae from their species. Thus, *M. edulis*, *C. virginica* and *C. gigas*, lead to larval death, as the filtered larvae are rejected in pseudofaeces or directly ingested (Tamburri and Zimmer-Faust, 1996; Lehane and Davenport, 2004; Tamburri et al., 2007; Troost et al., 2008). An increased filtration pressure could therefore reduce the number of bivalve larvae. We hypothesize that the greening of larvae by marennine may perturb larval recognition by the adult mussels and increase the palatability as resulting green larvae could be confounded with microalgae and thus stimulate filtration in mussels.

## 2. Materials and methods

### 2.1. *Haslea ostrearia* culture

The cultures of *H. ostrearia* (NCC-136) were obtained from the Nantes Culture Collection (NCC), Université de Nantes, France. The stock cultures were maintained in autoclaved F/2 medium at 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Furthermore, pilot-scale cultures were conducted in two 100 L flat bottom circular photobioreactors (PBRs) at irradiance 180  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 14/10 h light/dark cycle, temperature of 20 °C and salinity of 28 ppt. Marennine concentration was estimated on cell-free culture water (syringe-filtered on 0.20  $\mu\text{m}$ , Sterile-R) using the Beer-Lambert law as described in Prasetya et al. (2016). The optical density was measured at 677 nm in a 10 mm cuvette using a spectrophotometer (Cary 100 Bio UV-vis, Agilent Technologies), as well as the specific extinction coefficient for extracellular marennine ( $\epsilon_{677} = 12.131 \text{ g}^{-1} \text{ cm}^{-1}$ ), as stated in Pouvreau et al. (2007). To achieve optimum advantage provided by marennine in aquaculture, feasibility in using this pigment needs to be taken into account. The utilization of purified marennine obtained according to the anion-exchange chromatography method (Pouvreau et al., 2006) seem not be the best option, since it is a costly and time-consuming process. Therefore, in this study we used the supernatant containing the marennine (blue water, BW) concentrated 100 times (from 100 L to 1 L) using two ultrafiltration units (30 and 3 kDa) as described in Turcotte et al. (2016).

### 2.2. Animals and diet preparation

Adult *M. edulis* were obtained in June 2013 from the Havre-aux-Maisons lagoon (Iles-de-la-Madeleine, QC, Canada, 47°25' N, 61°50' W), where a pure *M. edulis* population is observed (Myrand et al., 2009). Introduction and transfer permits from Fisheries and Oceans Canada were obtained and our study did not involve endangered or protected species. Animals were transported to the Station Aquicole de Pointe-aux-Pères (Institut des Sciences de la Mer, Rimouski, Canada). Upon arrival, 40 individuals of adult mussels (mean shell length =  $63 \pm 1.8 \text{ mm}$ ) were numbered with bee tags, and were acclimated to

laboratory conditions for 30 days prior to physiological experiment. Another 40 adults were laboratory acclimated in similar condition for larval production. Animals were placed in a 300 L maintenance tank with light aeration, salinity of 29 ppt, temperature of 16 °C and natural photoperiod. Furthermore, animals were continuously fed with microalgae strains from the National Center of Marine Algae and Microbiota (Bigelow Laboratory for Ocean Sciences, MA, USA). The species were *Pavlova lutheri* (CCMP 459), *Tisochrysis lutea* (CCMP 1324) and *Chaetoceros muelleri* (CCMP 1316) at 1:1:1 equivalent volume and a daily ration of  $7 \times 10^8$  cells animal  $\text{d}^{-1}$  (3% dry weight algae/dry weight mussel per day).

### 2.3. Larval production

Spawning was induced by thermal shock from 12 to 25 °C. When gametes began to be released, adult mussels were transferred to 18 °C filtered (1  $\mu\text{m}$ ) seawater to improve oocyte or sperm release. A total of 40 different individual parents were used to generate gametes in a pool-cross design to produce one random larval family. Gametes were mixed at a ratio of 10 spermatozooids oocyte<sup>-1</sup> and fertilized eggs were then transferred to 180 L tanks at densities of  $30 \times 10^6$  eggs tank<sup>-1</sup> and left undisturbed at 18 °C for 48 h in filtered (1  $\mu\text{m}$ ) and UV-pretreated seawater.

D-larvae were collected by filtering the water on a 20  $\mu\text{m}$  mesh screen and transferred at densities of 10 larvae  $\text{mL}^{-1}$  into 60 L rearing tanks. Larvae were fed every 2–3 days with a mixture of the 3 microalgae species as mentioned above at 60 cells  $\mu\text{L}^{-1}$  (1:1:1), cultured with treated seawater, photoperiod and temperature as explained earlier. Before each feeding period, water in each tank was entirely renewed. Microalgae were harvested in the late exponential growth phase approximately from 6 to 10 days after flask inoculation in order to feed the larvae.

### 2.4. Greening and physiological measures

Mussel adults (20 in.) and larvae (54000 in.) were greened in BW (equivalent to 3  $\text{mg L}^{-1}$  of marennine) for 16-h and fed with a mixture (3% of mean mussel dry weight) of three hatcheries microalgae as mentioned in the section “Animals and diet preparation”. Adult mussels and larvae fed only with the microalgal mixture constituted the controls.

Physiological measurements were performed only on adult mussels over two days on individuals randomly sampled. Twelve chambers filled with 1  $\mu\text{m}$ -filtered and UV treated seawater were used concurrently, which allowed us to measure 10 animals (5 greened and 5 non-greened) and two controls (empty shell). Mussels that remained closed in the chamber during the measurement were excluded from physiological analysis. Physiological rates were also standardized to mass-specific rate equivalent to individuals of 1 g dry mass using weight exponents as described in Bayne et al. (1987).

Clearance rate (CR), which could be defined as the volume of water removed of suspended particles per unit time, was measured using a static system as described in Prasetya et al. (2017a). Briefly, adult mussels were placed in 1.3 L metabolic chambers 1 h prior to measurements, then fed with the mixture of the three microalgae species as mentioned above at an initial concentration of 100000 cells  $\text{mL}^{-1}$  (1:1:1 in dry weight). Circulation was generated by fine bubble aeration around the wall of the metabolic chamber in order to reduce the re-suspension of feces. Food particles were counted every 10 min during 60 min of measurement using a particle counter machine (Beckman Coulter Counter Z2, Mississauga, ON, Canada). The instrument was installed with a 70  $\mu\text{m}$  aperture tube and was adjusted to measure particle size in the range 3.5–8  $\mu\text{m}$ , the size of the three microalgae used in this study and known to be efficiently retained by *M. edulis* (Barillé et al., 1993; Ward and Shumway, 2004). The utmost difference between two consecutive measurements was used to calculate CR as described in

Gilek et al. (1992) and then expressed as one liter of water cleared from microalgae  $\text{h}^{-1} \text{g}^{-1}$  tissue dry weight after standardization to mass-specific rate equivalent (Tremblay et al., 1998).

Respiration or oxygen consumption was measured on the same individuals in 0.85 L metabolic chambers following Prasetya et al., 2017a. Briefly, the rate of oxygen uptake was determined by sealing the metabolic chambers and measuring the decrease in dissolved oxygen using an YSI (5331) polarographic electrode coupled to a YSI micro-oxygenometer (Yellow Springs, OH, USA). Afterwards, the output signal was monitored continuously on a chart recorder until there was a 20% decrease in oxygen saturation. Respiration was then expressed as  $\text{ml O}_2 \text{h}^{-1} \text{g}^{-1}$  tissue dry weight on standardized mass-specific rate equivalent.

### 2.5. Larviphagy interaction experiment

Experiments were also conducted within the physiological experiments on control and BW exposed adult mussels to estimate if the presence of greened larvae modifies their CR values on microalgae. Briefly, two size ranges of larvae were used, veligers ( $160.1 \pm 2.7 \mu\text{m}$ ) and pediveligers ( $274.8 \pm 2.1 \mu\text{m}$ ) of 8 and 18 days post-fertilization, called “small” and “large” larvae, respectively. These two sizes of larvae were then subdivided into “greened larvae (GL)” and “non-greened larvae (NGL)”. After the greening larvae treatment (60 000 larvae by size) in BW for 16-h as described above, larvae at the surface were siphoned on  $35 \mu\text{m}$  mesh screen to obtain only live larvae, and then recovered in 500 mL to estimate the concentrations. Afterwards, the larviphagy experiment was performed in the same 1.3 L metabolic chambers and condition described for clearance measures by offering high larval density (around  $100 \text{ larvae mL}^{-1}$ ) of GL or NGL. Mussels exposed only to microalgae (without larvae) were used as control.

### 2.6. Data analysis

Univariate variables in physiological experiments were compared using a *t*-test to assess the effect of BW on CR and respiration. The homoscedasticity was graphically assessed and normality tested by a Saphiro-Wilk test. Data were transformed into square root when necessary. Additionally, the two-way ANOVA was performed to determine whether there were any differences in CR between the two factors (BW concentration and type of larvae). Statistical analysis on CR was also performed when the diet was shifted from larvae to microalgae only. All statistical analyses were conducted at a significance level of  $\alpha = 0.05$  and performed using Graphpad Prism 5 for Mac OS X software.

## 3. Results

### 3.1. Effect of BW on physiological traits of *M. edulis*

A significant difference of clearance rate (CR) in adult *M. edulis* was observed (*t*-test<sub>2, 38</sub> = 2.649; *P* = 0.008) with CR 43% lowered for mussels exposed to BW comparatively to control (Fig. 1).

The BW also significantly lowered the oxygen consumption in adult *M. edulis* (*t*-test<sub>2, 38</sub> = 2.649; *P* = 0.005). The adult mussels exposed to  $3 \text{ mg L}^{-1}$  of BW had 30% lower oxygen consumption than that of control (Fig. 2).

### 3.2. Effect of BW on CR in presence of larviphagy

Larvae from both size ranges appeared to be healthy with mortality in the rearing tanks less than 15% until pedivelger stage, presence of abundant food in the digestive system, and larvae largely active with their velum. After the greening treatment, larvae showed a blue light ring around the shell with similar behavior than non-greened larvae (Fig. 3). CR estimated only on the monoalgal diet (control) showed similar values than the first experiment (Fig. 1). The two-way ANOVA

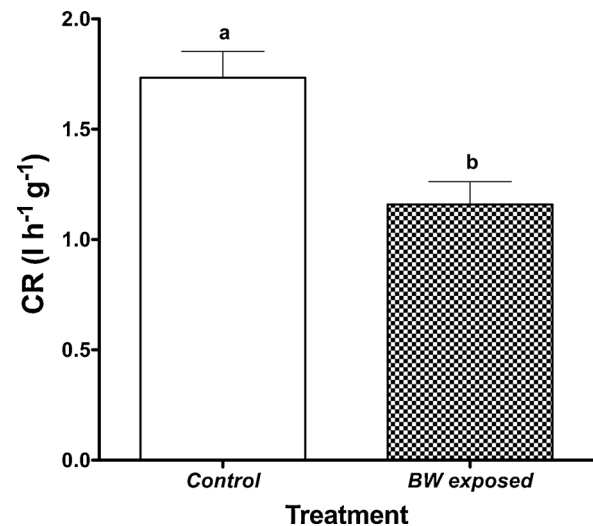


Fig. 1. Clearance rate (CR,  $\text{l h}^{-1} \text{g}^{-1}$  dry weight, mean  $\pm$  standard error,  $n = 10$  per condition) of adult mussels *M. edulis* exposed or not to BW containing  $3 \text{ mg L}^{-1}$  of marennine. Different lower case letters indicate significant difference.

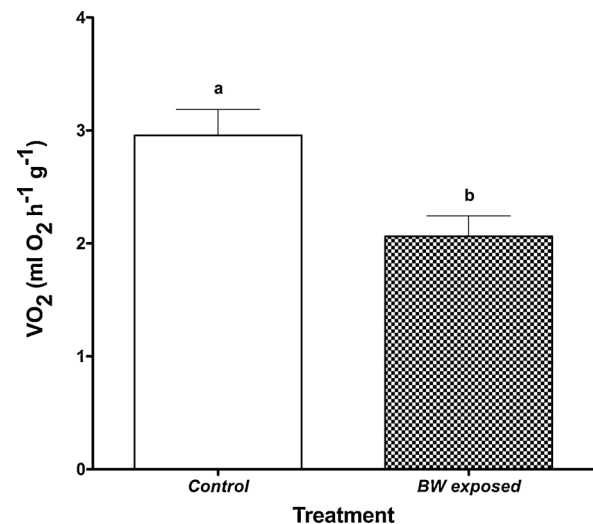


Fig. 2. Oxygen consumption ( $\text{VO}_2$ ,  $\text{ml O}_2 \text{h}^{-1} \text{g}^{-1}$  dry weight, mean  $\pm$  standard error,  $n = 10$  per condition) of adult mussels *M. edulis* exposed or not to BW containing  $3 \text{ mg L}^{-1}$  of marennine. Different lower case letters indicate significant difference.

revealed that significant difference in CR was only due to BW without interaction effect (Table 1). Regardless the size of the larvae given, it was found that the CR of mussels did not change significantly when they fed either on greened or on non-greened larvae (SGL and SNGL; LGL and LNGL), suggesting that the greening of larvae by BW did not affect the CR in presence of larviphagy (Fig. 4). Additionally, the intensity of CR in presence of larviphagy was similar between veligers and pediveligers larvae (Table 1).

## 4. Discussion

The present work aimed to determine the consequence of greening by BW (supernatant of *Haslea ostrearia* culture containing marennine) on physiological rate of adult mussels *M. edulis* and to determine whether the greening of adults and larvae could affect the CR in presence of larviphagy. To our knowledge, no other study has reported the short-term effect of BW containing marennine on the adult mussel *M. edulis* and also the possible consequence of the presence of greened

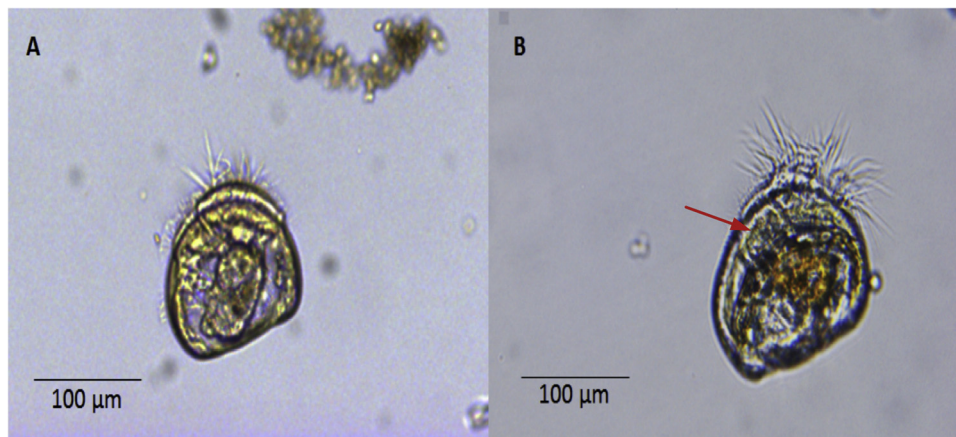


Fig. 3. Veliger larvae control (A) and exposed to the BW containing marennine with a blue light ring around the shell (B).

**Table 1**  
Factorial ANOVA analysis for mussel's clearance rates obtained from different treatments.

Factors	df	SS	MS	F	p
Interaction	3	0.302	0.101	0.298	0.8267
[BW marennine]	1	1.892	1.892	5.595	<b>0.0242</b>
Type of diet	3	0.607	0.202	0.598	0.6208
Residual	32	10.82	0.338		

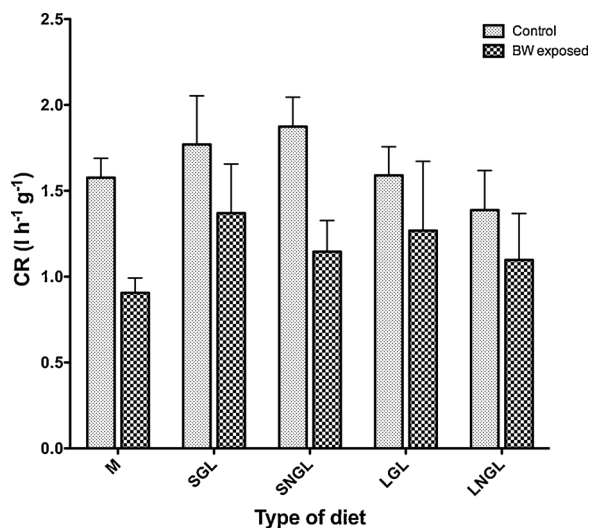


Fig. 4. Mean clearance rates (CR ( $l\ h^{-1}\ g^{-1}$  dry weight)) of adult mussels *M. edulis* exposed or not to BW in relation to different types of diet (M: only microalgae, SGL: with presence of small greened larvae, SNGL: with presence of small non-greened larvae, LGL: with presence of large greened larvae, LNGL: with presence of large non greened larvae). Values are mean  $\pm$  standard error of mean based on 10 individuals per concentration.

larvae on the CR of adults. In this study, it was found that the greening by BW marennine affects the physiological rates of adults *M. edulis*. However, our hypothesis that the greening of larvae may perturb larval recognition by the adult mussels increasing their palatability and stimulating their clearance rate does not seem to be confirmed. We observe a trend of increasing CR in presence of veliger or pediveliger greened larvae, but the variability between individuals outweighs significant differences. As the CR in control (mussels exposed to only microalgae without larvae) had similar values than other treatments for non-greened adult mussels, we suggest that 1 h of CR measurement was too short or the larviphagy rate was too high to measure a potential

impact of larvae on adult mussels CR.

#### 4.1. Physiological responses to marennine

Unlike the effect of natural toxic substances produced by dinoflagellates (Hégaret et al., 2007; Navarro and Contreras, 2010; Jauffrais et al., 2012; Basti et al., 2013, 2015; Navarro et al., 2018), the studies of the effect of natural non-toxic substances such as marennine are scarce. The present study provides evidence that the clearance rates of adult *M. edulis* were significantly reduced when they were exposed to BW, in accordance with Prasetya et al. (2017a), who observed that juveniles of *M. edulis* had a clearance rate reduced by 56% in response to marennine. The CR reduction by BW on adults was up to 43%. However, distinction should be made due to different types of marennine, concentration and also the stage in the life cycle of *M. edulis* used in the experiment. Indeed, the marennine solution used by Prasetya et al. (2017a) was the purified form and the effect was observed at  $2\ mg\ L^{-1}$  on the stage of juveniles, which are considered more sensitive than the adults. In line with this finding, Gastineau et al. (2017) observed that adult oysters *Crassostrea gigas* that were exposed to the BW  $5\ mg\ L^{-1}$  had approximately 50% lower CR in comparison with control. These results suggest that marennine is not only able to change coloration in bivalve oysters and mussels but also affect their physiological rate.

Our results show, the accumulation of marennine present in the tissues of adult mussels affects the consumption of oxygen. This result is in coherence with Prasetya et al. (2017a) who observed significant effect in consumption of oxygen on juvenile of American oysters *Crassostrea virginica* following a 16 h exposure to  $2\ mg\ L^{-1}$  of purified marennine. Nevertheless, the same authors found similar trends on reduction of oxygen uptake on the juvenile of *M. edulis* at similar concentration of marennine ( $2\ mg\ L^{-1}$ ) but this effect was not significant. Such inhibition of oxygen uptake and clearance rate has been previously documented in studies involving toxic and non-toxic algae (Bricelj et al., 1996; Li et al., 2002; Navarro and Contreras, 2010; Navarro et al., 2014, 2018). From the result of our study, the blue water solution containing marennine appears to have inhibitory effect on the mechanism of oxygen uptake and filtration activity on gills of bivalves, presumably due to the interaction between this pigment and cilia organs. Thus in reaction to an exposure to marennine, bivalves *M. edulis* and *C. virginica* adapt behaviorally by closing their valves and decreasing their CR for certain period of time, and biochemically, for instance, by change in the unsaturation of their cell membrane phospholipids in gills (Prasetya et al., 2017a).

#### 4.2. Consequence of greening to larviphagy

Significant differences were observed in filtration activity for both

treatments (control vs BW) when the adult mussels *M. edulis* fed on microalgae. However, no significant difference due to the presence of larvae (greened or not) was observed. Thus, this result invalidated our hypothesis that greened larvae by marennine could change the palatability of adults mussels, increasing the CR on microalgae. However, no estimation of direct larviphagy (number of larvae ingested by adult mussels) has been obtained and potential selectivity of greened larvae could be possible.

Vulnerability of larvae to larviphagy by the adults in bivalve species could be explained by the relationship between the bivalve's gills area and the size of the larvae themselves. The adult mussels' gills may retain any particles above their specific size limit, which ranged from 2 to 7 µm (Møhlenberg and Riisgård, 1978). In our study, the larvae of *M. edulis* used were at the stage of D-larvae (8 and 18 days after fertilization) with size ranged from 100 to 200 µm. Therefore, these larvae could mostly be retained on adult *M. edulis* gills (Hendriks et al., 2005; Sprung, 1984; Strohmeier et al., 2012). However, bivalve larvae could avoid being filtered by the filter feeders due to two possibilities. First, by detecting the hydro-mechanical stimuli in the inhalant flow field as described in Jakobsen (2001) and Kingsford et al. (2002). Second, by detecting the filter feeders chemically (Fitt and Coon, 1992; Tamburri and Zimmer-Faust, 1996). In this study, fixation of marennine by the gills of *M. edulis* could perhaps facilitate detection of this animal by the mussel larvae by chemical sensors. However, further studies should be conducted to address this possibility. Previous studies revealed that size and biochemical properties affect the feeding behavior of bivalves, where the preference could fall to the either smaller particles, higher biochemical content or a combination between them (Beninger et al., 2008; Bougrier et al., 1997; Mafra et al., 2009; Prasetya et al., 2017b; Shumway et al., 1985). As Turcotte et al. (2016) have demonstrated that greened larvae accumulated more energetic lipids, this biochemical modification could increase the palatability of adult mussels on greened larvae. Thus, it could be possible that larviphagy of greened larvae could be increased without increase of microalgae ingestion.

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None.

## Declaration of Competing Interest

None.

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