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# Effect of Extracellular Marennine produced by *Haslea ostrearia* on the Blood Clams *Tegillarca granosa*

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## Abstract

Marennine is a blue-green pigment produced by diatoms of the *Haslea* genus, one of which is *Haslea ostrearia*. This marennine pigment is water-soluble and confirmed to contain polyphenols and glycosides. There are two forms of marennine pigment: the intracellular form of marennine (IMn) and the extracellular form of marennine (EMn). Marennine pigments exhibit various biological activities such as antibacterial, antiviral, antioxidant, allelopathic, and inhibiting the growth of other diatoms. With this biological activity, marennine can be used in various fields, one of which is aquaculture. This research aims to determine the level of toxicity and analyze the effect of exposure to the extracellular marennine produced by *H. ostrearia* on the juvenile blood clam *Tegillarca granosa*. By using the toxicity test method, Bluewater which is a supernatant of *H. ostrearia* (BW) was tested on juveniles of commercially important bivalve species, the blood clams *Tegillarca granosa,* with three treatments, namely control (0 mg/L), treatment A (0.25 mg/L) and treatment B (0.5 mg/L of BW). The observation parameters of this study were $LC_{50}$-72 h with one-way ANOVA analysis. The results showed that the BW produced by *H. ostrearia* increased the survival of juvenile blood clams *T. granosa* up to 27.7% in treatment B (0.5 mg/L) compared to control within 72 h. The one-way ANOVA analysis revealed that the control and treatment B (0.5 mg/L) were significantly different. This study shows that there is potential to develop the use of marennine in shellfish aquaculture.

## INTRODUCTION

Marennine pigments or blue-green pigments are produced by diatoms of the *Haslea* genus. Marennine pigment can make shells green (greening effect) on the gills, labial palp, and digestive system in oysters such as *Crassostrea gigas* and *Ostrea edulis* (Gastineau *et al*., 2012). Then, Prasetiya *et al*. (2015) confirmed that

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marennine impacted the behavior of both *Mytilus edulis* and *Crassostrea virginica* having significant but moderate effects on the valve opening activities. Even marennine was non-toxic compound, has similar inhibitory effects on the growth *M. edulis* and *C. virginica.* They found that marennine alters the unsaturation index of membrane phospholipids in the gills of both species *M. edulis* and *C. virginica.*

Prasetiya *et al*. (2020) confirmed that the supernatant of the diatom *H. ostrearia* culture reduces the clearance rate, oxygen consumption, and condition index of bivalves. The alteration in these physiological parameters appeared to be species- and age-specific. These findings improve our understanding of marennine and its possible effect on bivalves.

According to Pouvreau *et al*. (2006), There are two forms of marennine pigments, namely the intracellular form of marennine (IMn), which accumulates in the cytoplasm to the apical axis of the cell, and the extracellular form of marennine (EMn), which is released by cells into the external environment. Gastineau *et al*. (2014) confirmed that pure marennine samples contain polyphenols and glycosides, with several glycosidic bonds.

*H. ostrearia* can be found in France, has recently been cultivated in Indonesia, and of course undergoes an adaptation process that can change its biological activity. Marennine pigment has the potential as an antioxidant (Pouvreau *et al*., 2008; Gastineau *et al*., 2014; Prasetiya *et al*., 2021), antibacterial and antiviral (Gastineau *et al*., 2012; 2014; Prasetiya *et al*., 2020), and has an allelopathic effect on other microorganisms (Pouvreau *et al*., 2007; Prasetiya *et al*. 2016). Therefore, an assessment of marennine related to its potential application should be conducted to achieve the optimum benefit.

The Blood clams *Tegillarca granosa* is an invertebrate animal that is classified into the kingdom Animalia, phylum Mollusca, class Bivalvia, order Arcida, family Arcidae, Genus *Tegillarca* (Linnaeus, 1758). Like other bivalves, this species is considered as a filter feeder, where they filter their food particles (such as phytoplankton and zooplankton) using their gill. Moreover, *T. granosa* is considered a commercially important species to be developed as a source of protein and minerals to fulfill the food needs of the Indonesian people (Sahara, 2011). Until today, no studies have been found about the potential of marennine application on this bivalve species. The possibility of having marennine to increase the market value of *T. granosa* like in the case of *C. gigas* in France remains to be studied. However, several assessments should be conducted, especially the effect of marennine on this bivalve species.

Toxicity tests could be performed to detect the toxic effect of a substance on a biological system. A toxicity test is a preliminary biological test to detect bioactive compounds of a substance, both natural and synthetic. The concentration that causes the death of the test organism as much as 50%, is called the $LC_{50}$. The mortality depends on the amount of concentration and the length of the test time (Dey and Harborne, 1991).

Permatasari *et al*. (2018), demonstrated that the *H. ostrearia* supernatant culture (hereafter called blue water, or BW) adapted in Indonesia can inhibit the pathogenic bacteria that are often found in aquaculture activities such as *Vibrio harveyi* and *Staphylococcus aureus*. This result confirmed that the semi-purified extracellular marennine can be advantageous in aquaculture due to its inhibition of the growth of pathogenic organisms.

Considering the potential use of the Haslea genus of microalgae, which produces marennine pigment, and the allelopathic properties of marennine pigment, which can influence the growth of organisms, it is important to know the level of toxicity of the marennine pigment so that its use in the field of mariculture can be carried out optimally. Until now, knowledge regarding the effects of the

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extracellular pigment marennine on other organisms is still very limited. Therefore, this research aims to determine the effect of exposure to the extracellular pigment marennine produced by *H. ostrearia* on the biota of juvenile blood cockles *T. granosa*. In this research, the pigment used is BW because it is more practical and easier to obtain.

## METHODOLOGY

### Ethical Approval

The juvenile blood clams *T. granosa* used in this study were well cared for and carried out first so that the blood clams could adapt to the artificial environment. Acclimatization is carried out for 7 – 14 days to adapt the biota to new environmental conditions (Rohmani, 2014). The acclimatization process for *T. granosa* was carried out in an aquarium containing water sea for approximately 7 (seven) days using aeration and feeding by AquaPharm Coral Food Sea Plankton with around 4000 – 6000 cells/mL once a day. This method is based on experience in research that has been through more than 10 times to adjust to the environment.

### Place and Time

This research was conducted in September 2020 – March 2021. The research was conducted at the Aquaculture Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran, West Java, Indonesia. Fresh juvenile blood clams of *T. granosa* were obtained from the supplier in Bandung, West Java, Indonesia, and confirmed that juvenile blood clams were from the Java Sea along the Tegal – Cirebon waters. The BW was obtained from the Laboratory of Microbiology and Molecular Biotechnology, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran, West Java, Indonesia.

### Research Materials

The device needed in this research is an Air pump (Amara Q-6, China) for aeration on acclimatization and testing blood clams, Aquarium, and the material needed is Artificial Sea Water (Monster Laut, Indonesia), Juvenile blood clams *T. granosa* (from Cirebon, Indonesia), and nutrients food/concentrated plankton for feeds the blood clams (Coral Food Sea Plankton AquaPharm), and BW (from West Java, Indonesia)

### Research Design

The method used in this research is a laboratory-scale experimental method, namely the marennine toxicity test on juvenile *T. granosa* shellfish biota using the Brine Shrimp Lethality Test (BSLT) method (Prasetiya *et al.*, 2017), with the research parameter observed being the lethal concentration value ($LC_{50}$). The data obtained were analyzed using one-way analysis of variance (ANOVA), with the significant difference criterion used being a confidence level of 95% ($p < 0.05$).

The marennine used was obtained from the Molecular Microbiology and Biotechnology Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. The extracellular pigment that was used was *H. ostrearia* supernatant culture at concentrations of 0 mg/L (control), 0.25 mg/L (treatment A), and 0.5 mg/L (treatment B). A total of 10 blood clams were placed in each container containing 800 mL of filtered seawater. Additionally, the observations were conducted every 24 hours for 72 hours.

### Work Procedure

The research procedure included estimating the concentration of extracellular marennine, acclimatization of juvenile blood clams, preparing marennine with different concentrations, toxicity test ($LC_{50}$-72 h), observation, data processing, and data analysis. The research parameters observed were lethal concentration values ($LC_{50}$) in testing the toxicity of the extracellular pigment marennine in juvenile shellfish *T. granosa* (Prasetiya *et al.*, 2017).

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Determination of the concentration of marennine in a solution using the spectrophotometer method at a wavelength of 660 nm with absorbance results Then the absorbance results are entered into the formula for estimating the concentration of extracellular pigment marennine [EMn] in g/L following Prasetiya *et al*. (2016), which can be calculated using the following formula:

$$[C] = \frac{A\lambda_{max}}{\varepsilon\lambda_{max} \times l}$$

Where :

C = concentration of analyte (g $mL^{-1}$)
$A_{\lambda max}$ = absorbance value at $\lambda_{max}$
$\varepsilon_{\lambda max}$ = marennine absorption coefficient (12.13 L $g^{-1}cm^{-1}$)
l = width of the cuvette (1 cm)

The acclimatization was carried out before testing BW on the animals. Acclimatization is the adaptation stage of organisms to the diluent water used for toxicity tests. In this study, acclimatization was carried out for 7 – 14 days to adapt the biota to the new environmental conditions (Rohmani, 2014).

The acclimatization process of *T. granosa* was carried out in an aquarium container containing 5 L of seawater for approximately 7 days with aerated and was fed 4000 – 6000 cells $mL^{-1}$ every day. Under controlled environmental conditions (room temperature around 26˚C, salinity 22 – 30 ppt). The freshwater was added when the salinity started to rise, to maintain the salinity in the aquarium. The clam's shell must be cleaned before entering the aquarium, and the seawater in the aquarium has been aerated for the day before.

Such as the research designs that have been made, the concentration used was 0 mg/L (control); 0.25 mg/L (treatment A), and 0.5 mg/L (treatment B). In preparing marennine with different concentrations, the following dilution formula is required (Gunawan *et al*., 2004):

$V_1 \times N_1 = V_2 \times N_2$

Where:

$V_1$ = Initial volume
$N_1$ = Initial concentration
$V_2$ = Final volume
$N_2$ = Final concentration

Then the calculation of the BW concentration of 0.25 mg/L in 800 mL of seawater was 114.94 mL BW and 685.06 mL seawater, while the concentration of BW 0.5 mg/L in 800 mL of seawater was 229.89 mL BW and 570.11 mL seawater.

After being calculated, it is ready in a container with an aerator for 2 h before the juvenile blood clams are placed into the container. Every container containing 800 ml of seawater is set with 10 blood clams. Then, observations were made for 72 h (three days) and took care of as much as 1000-2000 cells/ml during the test. If any dead blood clams are in the following day, separate the dead clam's shell from the test container. This is to avoid the impacts of clam carcasses. The parameter in this study is $LC_{50}$-72 h, which can be calculated manually based on Finney's probit table or using direct calculations with the help of GraphPad Prism software for Windows. In this study, the data were calculated by GraphPad Prism software for getting a value of $LC_{50}$ every 24 h for 72 h.

## Data Analysis

Data were analyzed using SPSS 25 for Windows. All statistical analyses were performed at a maximum significance level of 5 % by one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD).

## RESULTS AND DISCUSSION

The results of observations on the toxicity test of the BW *H. ostrearia* against *T. granosa* can be seen in Table 1.

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Table 1. Observation data.

| Death rate | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Concentration | Control | | | 0.25 mg/L | | | 0.5 mg/L | | |
| Repeat/Time | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 24 hours | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 hours | 2 | 2 | 2 | 0 | 1 | 1 | 0 | 1 | 0 |
| 72 hours | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 0 | 1 |
| Survival Rate (%) | 63.3% | | | 83.3% | | | 90% | | |

From the observations made, it was shown that the administration of BW *H. ostrearia* affected the survival of *T. granosa* where the mortality value of mussels in the control was higher than the two treatments (0.25 mg/L and 0.5 mg/L). It can also be seen from the color of the water during observation on the third day, that the color of the water in the control tends to be cloudier than the two treatments which can be seen in Figure 1.

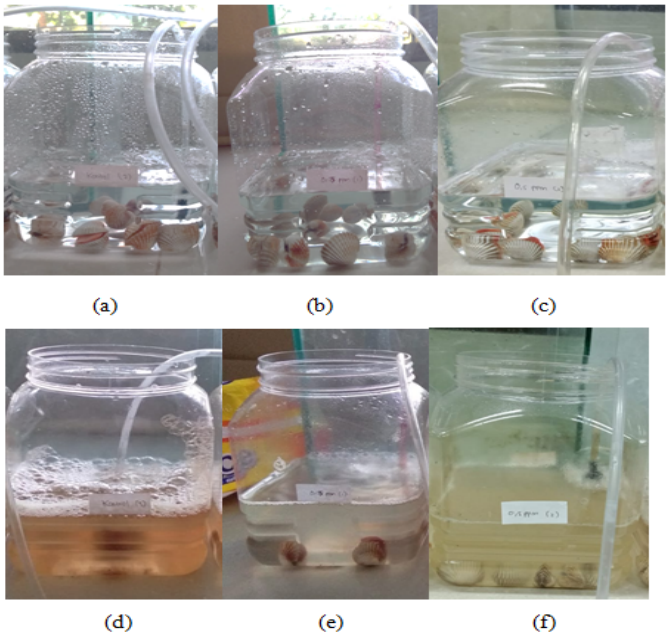


Figure 1. *T. granosa* conditions.
Description : Condition of blood clams on the first day ((a) control, (b) 0.25 mg/L and (c) 0.5 mg/L), and conditions on the third day ((d) control, (e) 0.25 mg/L and (f) 0.5 mg/L)

Then the data is processed using GraphPad Prism software to get the $LC_{50}$ value from every 24 hours of observation. $LC_{50}$ result at 24 hours is 0 mg/L. While the $LC_{50}$ at 48 hours was 0,1664 mg/L and 72 hours was 0,4994 mg/L. The graphic of $LC_{50}$ can be seen in Figure 2.

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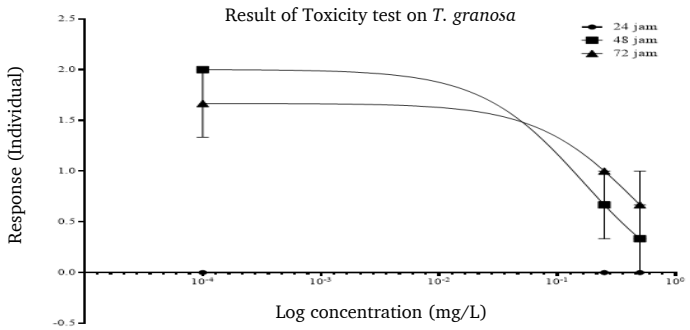


Figure 3. Response of *T. granosa*.

In contrast to the BW toxicity test in Prasetiya *et al*. (2020), which stated that BW from *H. ostrearia* had a toxic effect on *A. salina*. In this test, blood clam showed a fairly good survival when added BW. This is shown in the survival rate which increases with the high concentration of BW given.

Ramasamy and Balasubramanian (2012) found 12 bioactive components from *T. granosa* extract, one of which is phenol compounds. It's assumed that blood clams absorb/consume phenolic compounds in the environment, so in this test, the BW gave the ability to survive longer than the control (without BW). Considering that *T. granosa* is a filter feeder, mussels filter their food in the form of particles and organic matter suspended in water using hollow gills (Sahara, 2011).

Gastineau *et al*. (2014) confirmed that marennine contained polyphenolic compounds and glycosides. Where the glycosidic bonds contained in marennine are quite numerous so there may be polysaccharide compounds in it. If it's associated with the needs of blood clams, according to Sinardi *et al*. (2013) stated that the chitin content in clam shells ranges from 14-35%. Chitin (a polysaccharide compound) is the main component of the exoskeleton of invertebrates, crustaceans, and insects where this component functions as a supporting and protective component (Pratiwi, 2014). So, it is assumed that *T. granosa* also needs polysaccharides as supporting and protective components in their shell.

Based on the results of the one-way ANOVA calculation, the value of $p = 0.034$ with a 95% confidence level ($p < 0.05$). So, there is a significant difference between the three treatments. Tukey's post-test showed that the mortality in the control and treatment B (0.5 mg/L) was significantly different.

## CONCLUSION

The extracellular marennine in the form of BW affects the survival rate of blood clams *T. Granosa*. The highest $LC_{50}$ value of BW for this bivalve species was obtained at 0.1664 ppm after 48 h of exposure, whereas the highest mortality (27.7%) was obtained in the control. Juvenile blood clams experienced an increase in survival at the highest concentration (0.5 ppm) with a survival rate of 90%.

## CONFLICT OF INTEREST

There is no conflict of interest among all authors upon writing and publishing the manuscript.

## AUTHOR CONTRIBUTION

The authors confirm their contribution to the paper as follows: Eri Bachtiar (Concept, Funding acquisition, Review & editing), Ajeng Dinda Lestari (Investigation, Formal analysis, Writing original draft, Review & editing), Sri Astuty (Methodology, Review & editing), Sunarto (Concept, Funding acquisition, Project administration, and Fiddy Semba Prasetiya (Concept, Resources, Supervision, Review & editing).

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