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Allelopathy Effect of the Blue Diatom *HasleaOstrearia* (Gaillon) Simonsen: Growth Inhibition in Aquaculture Relevant Microalgae

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ABSTRACT

We are well known that the marine pennate diatom *Hasleaostrearia* (Gaillon) Simonsen produces the blue-green pigment called marennine. Marennine was identified as a polyphenolic compound. This compound is capable to inhibit the growth of herpes simplex virus type 1 (HSV-1) and several bacteria, including pathogens as *Vibrio* sp. The aim of this study was to determine how strong the allelophatic effect of marennine is generated by*H. ostrearia* and identifying its minimal concentration that inhibits the growth of other microalgae. The experiment consisted of two phases of research activities. The first phase was biovolumecomparaison between *H. ostrearia* and microalgae suitable for aquaculture tested to take into account the differences in size of the microalgae tested(*Tetraselmissuecica, Chaetocerosgracilis, Skeletonemacostatum, Pavlovalutheri*and*Isochrysisgalbana (T-Iso*) in term of *H. ostrearia* biomass and production of marennine. The second phase was the bioactivity tests, which is include growth kinetics, allelophatic effects and concentration of marennine in the culture medium, measured with spectrophotometer. The results of this study showed that *H. ostrearia* could inhibit several species of microalgae currently used in aquaculture (*T. suecica, C. gracilis* and *S. costatum*). The statistical analysis (One way ANOVA) showed that there are significant differences (P < 0, 05) between treatments andwhen co-cultured with *H. ostrearia*, these microalgae exhibited a significant growth inhibition. The highest inhibition value was 97.77% (on the treatement*H. ostrearia+C. gracilis*). In this treatment, the concentration of marennine in the culture was 5.35 mg L^{-1}. According to the results, we can conclude that the supernatant of *H. ostrearia* with marennine acts as an allelochemical. The minimal concentration is 0.23 mg L^{-1} and the highest is 5.35 mg L^{-1}. The treatments *H. ostrearia*with*P. lutheri* and *T-Iso* did not performed any inhibition.

Key words: *H. ostrearia*, marennine, microalgae and Allelopathic effect.

INTRODUCTION

One of the diatom potential to be developed is *Hasleaostrearia*. This species is known as a diatom that is able to produce a blue-green pigment, the so-called marennine. This pigment is water soluble, and according to Pouvreau*et al.* (2006), it could have a polyphenolic nature. *Hasleaostrearia* cells are often found in the cultivation of Oysters (*Crassostreagigas*) both on the body and in oyster ponds. In addition, the presence of these particular diatoms is causing greening of the oyster's gills.

Pouvreau*et al.*(2006) proposed that the marennine produced by *H. ostrearia* is an allelophaticsubstances from the class of polyphenols. The presence of blue-green pigment had an impact on other microalgae, in particular microalgae used as feed of *C. gigas*, eg *Skeletonema costatum* and *Tetrasel missuecica*. Another study conducted by Gastineau *et al.,* (2012) showed that, *H. ostrearia* is able to inhibit the development of herpes simplex virus type 1 (HSV-1) and several bacterial pathogens.

The existence of *H. ostrearia* in oyster ponds, and the biological activities of marennine, raises the question of a possible allelochemical effect in the ecosystem of oyster ponds. Marennine produced by *H. ostrearia* and released in the seawater, could act as an allelochemical substance, through the competition for space (growth and development).

Allelopathic test of an allelochemical (often in form of terpenoids, alkaloids and phenolic compounds) can be done with the test of growth and competition within the same container. The present study is focused on the assessement of the allelopathic effect of *H. ostrearia* in conditions of microalgal co-cultures. In this case, the source of allelopathic substances will be taken throughout the body of *H. ostrearia* and culture media. Growth kinetics and allelopathic tests are done on several microalgae species commonly used in aquaculture as feed of *C. gigas*, or in hatcheries.

The scientific question to answer is whether the presence of *H. ostrearia*in the culture environment will affecting the population of other microalgae. If the effect is observed, then how strong is the allelophatic effect produced by *H. ostrearia*

on the growth of other microalgae and how much is the concentration of marennine in the culture, where these concentrations showed growth inhibition of other microalgae. Thus the aim of this research is to determine how strong the allelophatic effect of marennine generated by *H. ostrearia* is, and identify its minimal concentration that inhibits the growth of other microalgae (through measurement level of inhibition), by way of spectrophotometry (absorbance value).

MATERIAL AND METHOD

Research Materials

The main experimental species used in this study is *Hasleaostrearia* from on the Atlantic Coast of France, in Marennes-Oléron Bay and Bourgneuf Bay. The other species for allelopathy study are *Isochysis galbana (T-Iso*CCAP 927*), Tetraselmis suecica* CCMP 904*, Chaetoceros gracilis* UTEX*, Skletonema costatum* and *Pavlova lutheri*.

Methods

The study consisted of two phases such as:

The first phase was to measure the biovolume of each microalgal species, *H. ostrearia* and other microalgae relevant for aquaculture. The rationale is to measure and to compare the cell volume of *H. ostrearia* with other microalgae, and to express possible allelopathic effects in function of the volume ratios and on a per cell basis. The second phase was the bioactivity measurement which includes growth kinetics, allelophatic effects and concentration of marennine in the culture medium, measured with a spectrophotometer (Absorbance value).

Microalgae Culture

Microalgae were grown in sterilized conical flasks 500 ml (by autoclaving), containing medium of artificial seawater (ASW) (Mouget *et al*., 2009). The flasks were placed in a room with temperature controlled at 16 ± 1 °C, an irradiance of 100 µmol photons m^{-2} s^{-1} (quanta meter), and a photoperiod of 14h / 10h light / dark except *I. galbana (T-Iso)* grown at 21 ± 1 °C and a photoperiod of 18h / 6h because originally from Hawaii Institute of Marine Biology. The light was provided by Philips TLD 36 watt/965 Fluorescent tubes.

Measurement of Biovolume Microalgae

This method refers to the method of Hillebrand *et al*. (1999). Microalgae were grown in ASW, and biovolume were then calculated when the exponential phase was reached. First, number of existing microalga was counted. Microalga was then placed on Nageotte/Neubauer counting slide, depending on the species. Afterwards volume of each microalga was calculated. Observations were made using a light microscope (Axiostar) with magnification of 200, 400 and 1,000 X, directly connected to the camera and computer. ImageJ software was used to measure the length, width and height of microalga, while the calculation of volume and data collection was done by using Microsoft excel 2010.

Growth Kinetics

This test is performed to determine the optimum time of diatom growth. In this research, experiments were conducted in a glass tube containing the culture of *I. galbana (T-Iso*CCAP 927, *T. suecica*CCMP 904*, C. gracillis*UTEX*, S. costatum*and *P. lutheri*in the filtered *Artificial Seawater* medium (ASW) (Millipore 0.22 µm). Afterwards, samples were incubated at 16°C for 14 days less than 100 µmol photons m^{-2} s^{-1} in the 14/10h light-dark phase except *I. galbana* stored at 21 ± 1 °C and photoperiod of 18h/6h. The number of cells was counted each day using a Nageotte/Neubauer counting slide. The tests were conducted in triplicate.

To analyze the growth of microalgae, the growth kinetics data were analyzed by growth curve based on data obtained per unit of time.From these data, we can calculate the generation time (time products/double) and the relative growth of different types of microalgae culture results. The formula for the generation time is:

$$G = \left\{ \frac{t\log 2}{\log b - \log B} \right\}$$

Where:

G = the generated time
b = concentration of microalgae at the end of the observation
B = concentration of microalgae at the beginning of the observation

While the growth rate formula (Dauta *et al*., 1990) is as follows:

$$\mu = \left\{ \frac{\ln Nt - \ln No}{t} \right\}$$

Where:

Nt = number of cells after a period of time t (peak)
No = number of cells in the inoculated at time = 0
µ = growth rate (unit/day)

Allelopathic Test

This test is conducted to determine the allelopathic effects of *H. ostrearia* on the other microalgae. In this research, experiments were performed in a sterile Erlenmeyer containing culture *I. galbana, T. suecica, C. gracillis, S.costatum*and *P. lutheri* in the filtered ASW (Millipore 0.22 µm). Then, *H. ostrearia*was added into each culture. Furthermore, the sample incubated at 16 °C for 14 days less than 100 µmol photons m^{-2} s^{-1} in the light-dark phase of 14/10 hours except *I. galbana* grown at 21 ± 1 °C and photoperiod of 18h/6h.

The number of cells was counted each day using nageotte/Neubauer slide. The experiments were performed using a comparison of biovolume between *H.ostrearia* and others microalgae. This ratio is obtained from the results of previous biovolume measurements. The tests were conducted in the three replicates. At the end of the experiment (10 days), cell densities were calculated using Nageotte / slide Neubauer counting. The percent growth rate (G%) was calculated using the formula below:

$$G\% = \left\{ \frac{\left(Nf_{(mn)} - Ni\right)}{\left(Nf_{(0)} - Ni\right)} \right\} x100$$

Where:

$Nf_{(mn)}$ = number of cells with treatment
$Nf_{(0)}$ = number of control cells
Ni = exponential phase (5 x 10^3 cells)

To determine the level of inhibition was calculated by the formula below:

$$I\% = 1 - G\%$$

Quantitative Determination of Extracellular Marennine in the Culture

To determine the concentration of extracellular marennine contained in each sample, dose/concentration of marennine in each *H. ostrearia* culture was calculated during the treatment. The dose was measured at the end of the incubation period (t = 10 days). For the target microalgae, the cultures were filtered by using milipore, to obtain the supernatant (extracellular marennine for dose measurements). Then, the absorbance of the supernatant was measured by spectrophotometry (used Spekol 1,100 and wavelength range 350-750 nm), and the concentration determined according to Robert et al. (2002) use the specific extinction coefficient for EMn (Blue Water) (ε λmax = 17.2 L $g^{-1}cm^{-1}$), Aλmax (Absorbance λmax) and Length (cm) of cuve. The concentration of extracellular marennine (Blue Water) was calculated using the formula below:

$$[C] = \left\{ \frac{A\lambda max}{\varepsilon\lambda max.L} \right\}$$

Data Analysis

The experimental design used in this study was completely randomized design (CRD) with nine treatments and three replications. Allelophatic test results were analyzed statistically by ANOVA test (one-way) using SPSS 16.0 for Windows software. Discrepancy observed was further tested with HSD *Tuckey* post hoc test multicomparaison.

RESULT AND DISCUSSION

Biovolume

From biovolume calculation, it showed that volume average (mean ± SE, n=35) of *H. ostrearia* is 1,662.52 ± 45.48 μ m^3cells^{-1}, while for another microalga is *T. suecica* (387.37 ± 17.02 μm^3 $cells^{-1}$), *C. gracilis* (93.15 ± 5.63 μm^3 $cells^{-1}$), *I. galbana* (58.59 ± 3.59 μm^3cells^{-1}), *S. costatum* (41.89 ± 3.12 μm^3 $cells^{-1}$) and *P. lutheri* (26.06 ± 2.03 μm^3 $cells^{-1}$). The volume obtained for each microalga, was then compared to the biovolume of *H.ostrearia* as a reference for the growth kinetics test and test allelopathic. Results of the comparisons are as follows:

Table 1. Comparison biovolume some microalgae with *H. ostrearia*

	H.ostrearia	***T.suecica***	***C.gracilis***	***I.galbana***	***S.costatum***	***P.lutheri***
Bv	8.3126	1.937	0.466	0.293	0.2094	0.13
Co	1	4	18	28	37	64
No	14.27	130.92	73.21	602.17	135.42	392.17
Ni	0.5	2	9	14	18.5	32
Vo	5.5	2.3	18.5	3,5	20.5	12.5

Where:

BV = biovolume of microalgae in the exponential phase (Ni:5,000 cell) [x 10^6 μm^3]
Co = Comparison of the biovolume
No = concentration of the cell in the 14th day (exponential phase) [x 10^4 μm^3cells^{-1}]
Ni concentration of the cell must be set (in exponential phase) [x 10^4 μm^3cells^{-1}]
Vo = volume must be set (mL)

Table 1 show that *H. ostrearia* has the greatest volume as compared to the other microalgae tested, so the biovolume measurements can be comparable with the number of cells that are in the culture.

Growth Kinetics

Based on the observations was made from the cell concentration in the cultures of *H. ostrearia, Isochrysis galbana (T.Iso* CCAP 927*), Tetrasel missuecica* CCMP 904*, Chaetoceros gracillis* UTEX*, Skletonema costatum* and *Pavlovalutheri*. After 14 days of growth, it can be seen that the daily growth of some microalgae population is still increasing (Table 2).

The highest *H. ostrearia* density occurred on day 14th as much as 14.27 x 10^4 cell mL^{-1}. The highest density of *T. suecica* also on day 14th, as much as 130.92 x 10^4 cell mL^{-1}, on the last day of the observation, whereas for *C. gracilis* is as much as 98.79 x 10^4 cell mL^{-1} on day 10th. *I. Galbana (T-Iso)* density occurred in day 14th is 602.17 x 10^4 cells mL^1, *S. costatum* 135.42 x 10^4 and *P. lutheri* much as 392.17 x 10^4. The growth curves are presented in Figure. 1.

Based on observations of cell population growth in Tables 2 and 3, it is shown that the highest relative growth rate is those of *T. suecica* and *I. galbana*, with an average relative growth rate is 0.29 and 0.27, respectively. This means that both microalgae can rapidly acclimate to a new environment and culture. It can be seen from the value of k (relative growth rate) which is high enough. According to Dauta *et al*. (1990), high value of relative growth indicates that microalgae have ability to adapt to the new environment and culture, and suggests that the algae had short adaptation and grow rapidly.

Tabel 2. The microalgae density during culture period

Species	Time (Days) (mean ± S.E; n=3)						
	T_O	T_4	T_6	T_8	T_{10}	T_{12}	T_{14}
H.ostrearia[x10^4]	1 ± 0	0.5 ± 0.05	5.06 ± 0.04	9.21 ± 0.04	11.85 ± 0.03	13.175 ± 0.02	14.27 ± 0.05
T.suecica[x10^4]	3 ± 0	38.42 ± 1.83	77 ± 0.7	101.42 ± 0.46	121.21 ± 0.87	123.38 ± 0.47	130.92 ± 0.28
C.gracilis[x10^4]	9 ± 0	16.08 ± 0.84	40.46 ± 0.21	90 ± 0.49	98.79 ± 0.39	87.71 ± 0.57	73.21 ± 0.65
I.galbana[x10^4]	14 ± 0	196.42 ± 1.51	384.5 ± 3.14	460 ± 1.63	502.67 ± 3.3	536.67 ± 3.45	602.17 ± 3.96
S.costatum[x10^4]	18.5 ± 0	49.46 ± 1.74	85.29 ± 1.04	96.08 ± 0.39	100.96 ± 1.45	121.21 ± 1.06	135.42 ± 2.87
P.lutheri[x10^4]	32 ± 0	133.42 ± 1.38	195.79 ± 1.27	257.50 ± 1.05	305.33 ± 2.86	330.17 ± 4.96	392.17 ± 2.12

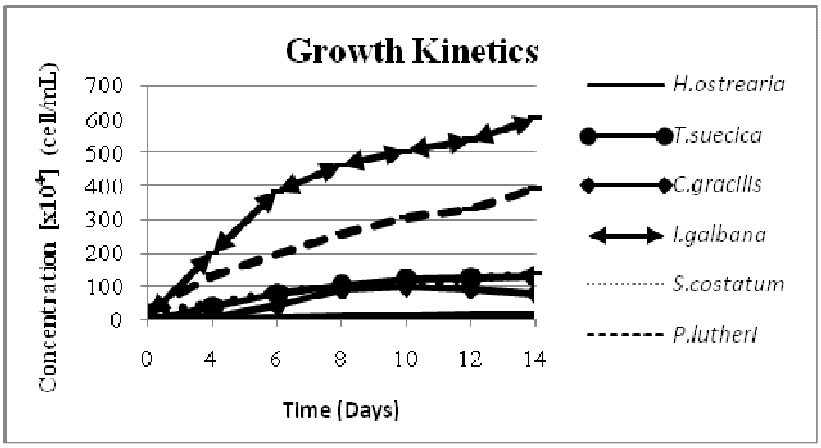

Figure 1. Growth curves for the different microalgae experessed in number of cells per unit volume.

Tabel 3. Generation time, Growth Rate and Equation rate of microalgae population growth

Species	Generated Time (Days)	Growth Rate (d^{-1})	Equation Rate of Population Growth
H.ostrearia	2.89	0.24	y = 1.1588x – 1.1469
T.suecica	2.32	0.29	y = 9.7532x + 9.8107
C.gracilis	4.05	0.17	y = 6.3766x + 10.13
I.galbana	2.58	0.27	y = 42.092x + 60.498
S.costatum	4.87	0.14	y = 8.2929x + 22.729
P.lutheri	3.87	0.18	y = 25.63x + 37.483

Observations in the growth curves of populations of the 7 algal species for the experimental period (14 days) can be viewed in Figures 1. For instance, based on the growth curves it is observed for the given experimetal period (14 days), that *T. suecica* has exponential phase / log phase with a sharp increase in the growth rate from 0 to 10 days to increase the density from 3,000 cells mL^{-1} to 121.21 x 10^6 cells mL^{-1}. This is indicated by the results from calculation of doubling time (generation time) of the six species population growth of algae, which means generation of the lowest time is the shortest time it takes one (generation) population to grow to 2-fold or the next generation. Growth of *T. suecica* population in this phase showed the shortest doubling time (generation time) as much as 2.32 days as compared to other microalgae. This shows that *T. suecica* needs a short time to grow 2-fold on the next generation.

Differences in the growth rate in some microalgae resulted in differences in the length of the logarithmic phase, and in the biomass producted. Based on these results, cultures with low growth rate will have longer exponential phase. This is caused by the lower growth rate of cultures will reach saturation point microalgae slower than other cultures.

Table 3 shows the growth characteristics of the 6 species of microalgae used as microalgae tests or target organisms for *Haslea*allelopathy effect. These characterisctics includes the generation time, relative growth, and the time to reach the maximum density. At the time of peak population, the populations of microalgae achieve the highest cell density. Hence, Table 3 shows that the times to reach peak population

for each microalga vary with species from 8 to 10 days and the shortest was for *I. galbana* which is on the 8th days, but the biomass (as estimated by the biovolume, see Fig. 2) is low. Based on this result, it can only be inferred that for our culture conditions, the species of microalgae that has the fastest harvesting time is *I. galbana*.

The determination of a growth curve can be done in several ways, such as by direct calculation of the number of cells using Nageotte / Neubauer counting device (it gives the number of cells in cultures using quantitatively limited volumes), dry weight calculation (it needs more biomass), different molecular markers (more or less complicated to measure as routine, chlorophyll *a*, carbon, ATP, etc.), or calculation and measurement by cell volume (Mayer *et al.,*2007). Based on biovolume measurements cell, the growth curve of microalgae can be seen in Figure 2.

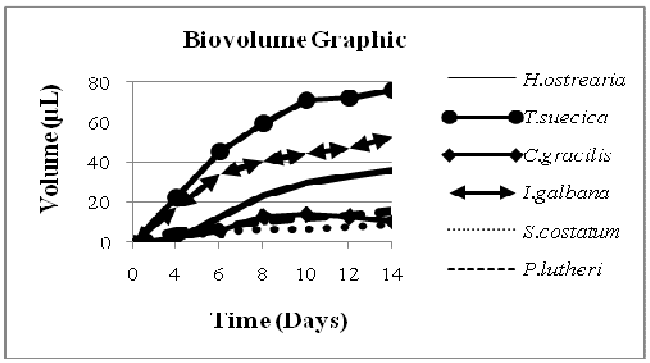

Figure 2. Growth curves based on the cell volume (µL)

Figure 2 shows that the six cultures weren't through acclimation phase, as explained above (growth conditions unchanged during inoculation, inoculum in log phase. Cells were placed in the same growth medium and the same environment as the medium and the previous environment, thus they do not require adaptation time (Fogg, 1975).

Allelopathic Test with other Microalgae

*H. ostrearia*allelopathic test consisted in the co-culture of *H. Ostrearia* plus one of the following aquaculture relevant microalgae: *T. suecica, C. gracilis, I. galbana* (*T-Iso*), *S. costatum*and *P. lutheri*. As presented in Table 2, the M_0 treatment (monoclonal cultures, controls) showed that the concentration of microalgae growth (mean ± SE) for each species is 11.85 x 10^4 ± 0.03 cells mL^{-1} at t = 10 for control *H. ostrearia*; 121.21 x 10^4 ± 0.87 cells mL^{-1} at t=10 for *T. suecica*; 98.79 x 10^4 ± 0.39 cells mL^{-1} for *C. gracilis*, 502.67 x 10^4 ± 3.3 cells mL^{-1} for *I. galbana* (*T-Iso*); 100.96 x 10^4 ± 1.45 cells mL^{-1} for *S. costatum* at t=10 and 305.33 x 10^4 ± 2.86 cells mL^{-1} for *P. lutheri*.

For treatment M_1 (*T. suecica* CCMP 904 + *Hasleaostrearia*) concentration of *T. suecia* was 31.42 x 10^4 ± 3.78 cell mL^{-1} and for *H. ostrearia* at 8.08 x 10^4 ± 0.84 cells mL^{-1}. Treatment M_2 (*C.gracillis* UTEX + *Hasleaostrearia*) was 2.69 x 10^4 ± 0.09 cell mL^{-1} for *C.gracilis* and 20.33 x 10^4 ± 0.18 cell mL^{-1} for *H.ostrearia*. In treatment M_3 (*I. Galbana* (*T-Iso*) + *Hasleaostrearia*) performed 2 times with different temperatures, A is placed on treatment temperature of 16 °C and B placed on the 21 °C, at a temperature of 16 °C the concentration of *T-Iiso* was 48.2 x 10^4 ± 5.71 cells mL^{-1} and for *H. ostrearia* at 8.21 x 10^4 ± 2.11 cell mL^{-1}, whereas when placed at a temperature of 21 °C *T.iso* concentration of 261.22 x 10^4 ± 6.35 cell mL^{-1} and 2.64 x 10^4 ± 0.55 cell mL^{-1} to *H. ostrearia*.

For treatment M_4 (*Hasleaostrearia* + *S. costatum*), cells concentrations were 2.11 x 10^4 ± 0.59 cell mL^{-1} for *S.costatum* and 18.44 x 10^4 ± 1.71 cell mL^{-1} for *H. ostrearia*. On treatment M_5 (*P. lutheri* + *Hasleaostrearia*) average concentration *P. lutheri* was of 217.78 x 10^4 ± 3.15 cell mL^{-1}, whereas for *H. ostrearia* it was 4.02 x 10^4 ± 1.25 cells mL^{-1}.

Based on the calculation of the biovolume of microalga in the test medium (ASW), treatment Mo (control) showed that the volume of microalgae (mean±SE) on each species is 29.55 ± 0.03 µL at t=10 for control *H. ostrearia*; 70.43 ± 0.87 µL at t=10 for *T. suecica*, 13.8 ± 0.39 µL for *C. gracilis;* 44.18 ± 3.3 µL for *I. galbana* (*T-Iso*); 6.29 ± 1.45 for *S. costatum* µL at t=10 and 11.93 ± 2.86 µL for *P. lutheri*.

Allelopathic test with the treatment M_1 showed that the total volume of microalgae grown in test medium was 25.15 ± 0.84 µL for *H. ostrearia* while *T. suecica* was 18.26 ± 3.78 µL. Allelopathic test with treatment M_2 showed that the microalgae volume on the culture medium for *H.ostrearia* was 50.7 ± 0.18 µL and 0.39 ± 0.09 µL for *C.gracilis*, which declined, because of the allelochemical effects generated by *H.ostrearia*.

Allelopathic test in the treatment M_3 showed that the biovolume for the diatoms grown at 16 °C was 20.47 ± 2.1µL for *H. ostrearia* and 4.24 ± 5.71 µL for *T-Iso*. When the two species were grown at a temperature of 21 ^{0}C, the volume average was 6.88 ± 0.55 µL for *H. ostrearia* and 22.96 ± 6.35 µL for *T-Iso*.

Allelopathic test for treatment M_4 showed that the average volume of *H. ostrearia* was 46.16 ± 1.71 µL, while for *S. costatum* it was 0.13 ± 0.39 µL. In this treatment, almost the entire culture was dominated by *H. ostrearia*. Treatment M_5 showed that the average volume of *H. ostrearia* was 10.025 ± 1.25 µL and 8.51 ± 3.15 µL for *P. lutheri*.

Based on the results above, it is shown that in terms of biovolume, *H. ostrearia* dominated each culture except for the culture of *T-Iso* (at temperature 21 ^{0}C) and *P. lutheri* that seems to be dominant when the two species were co-cultured. According to the growth rate and inhibition level, it has been shown that *H. ostrearia*isable to inhibit the growth of other microalgae, when they are co-cultured. The overall inhibition level on the microalgae tested, whether it results from the blue water or nutrient competition, was 74.38% ± 3.13; 97.77% ± 0.09; 90.5% ± 1.14; 98.4% ± 0.59 and 28.72% ± 1.03 for *T. suecica*, *C. gracilis*, *I. galbana* (16 ^{0}C), *S. costatum* and *P.*

lutheri, respectively. However, when co-cultured with *C. gracilis, S. costatum* and *C. calcitrans*, no inhibition observed for *H. ostrearia*.

Table 4. Growth Rate (%) and Inhibitions

Treatment	Growth Rate (%) Mean±S.E	Inhibition (%) mean±S.E
T. suecica (+H. ostrearia)	25.62 ± 3.13	74.38 ± 3.13
C. gracilis (+H. ostrearia)	2.23 ± 0.09	97.77 ± 0.09
I. galbana-16 °C *(+H. ostrearia)*	9.5 ± 1.14	90.5 ± 1.14
I. galbana-21 °C *(+H. ostrearia)*	54.77 ± 2.53	45.23 ± 2.53
S. costatum (+H. ostrearia)	1.6 ± 0.59	98.4 ± 0.59
P. lutheri (+H. ostrearia)	71.28 ± 1.03	28.72 ± 1.03

One way ANOVA test showed that the significant value of 0.09 for the test of homogeneity of variances which means the data is homogeneous. Thus, the assumption of homogeneity of variance has been met so that ONE WAY ANOVA test can be continued. Seen from the ANOVA table that the calculated F value = 996.09, which is where the value is greater than the value of F table = 1.84 (based on the F distribution tables) so it can be concluded that we can reject H_0. With a significance value of 5%, we can conclude that the same, ie, reject H_0. It can be seen from the significant value = 0.000. This value is smaller than the significance level = 0.05 level. So, allelopathy treatment (*H. ostrearia* against other microalgae) influenced the growth of microalgae.

To determine the main factors in growth inhibition of microalgae, further HSD Tukey test was used. HSD Tukey test showed that culture treatment of *S. costatum* then *C. gracilis* the strongest influence, respectively. For the treatment of *T. suecica* and *I. galbana* grown at 16 °C, results indicated that the effect of treatment gave a moderate level. This result was also confirmed by the inhibition that occurs only about 74.38% and 90.5% (Table 4).

HSD Tukey test showed that the allelopathic effect against *P. lutheri* and *I. galbana* (placed at a temperature of 21 °C) did not show significant differences, but the treatment can inhibit the growth of the microalgae as much as 28.72% and 45.23% for *P. lutheri* and *I. galbana* (at a temperature of 21 °C), respectively. On the other side, statistical analysis showed that the growth of *H.ostrearia* was also affected by this treatment (when combined with the *P. Lutheri* and *I. galbana* (*T.iso* 21 ^{0}C).

Calculating concentration of ExtracellularMarennine (EMn) on the Culture

The values of marennine concentration in the blue water at the end of the allelopatthy test varied with the co-cultured species. Filtered samples using Miliphore indicates that the highest blue water concentration was observed on M_2 (*H. ostrearia* + *C. gracilis*) is 5.35 mg L^{-1}. On the other side, the lowest concentration found at M_5 (*H. ostrearia* + *P. lutheri*) which is equal to 0.47 mg L^{-1}. Table of marennine concentration on each treatment is provided as follows.

Table 5. Extracellular Marennine Concentration After allelopathic Test

Treatment	Absorbance	Concentration (mg L^{-1})
H.ostrearia + T.suecica	0.19	2.19
H.ostrearia + C.gracilis	0.46	5.35
H.ostrearia + T.iso 16 °C	0.28	3.26
H.ostrearia + T.iso 21 °C	0.14	1.63
H.ostrearia + S.costatum	0.28	3.26
H.ostrearia + P.lutheri	0.04	0.47

In Table 5 it is shown that the treatments *H. ostrearia* + *C. gracilis* and *H. ostrearia* + *S. costatum* resulted in concentrations as high as 5.35 mg L^{-1} and 3.26 mg L^{-1}, respectively. *H. ostrearia* + *T-Iso* (16 °C) indeed have a particularly high concentration value (ie 3.26 mg L^{-1}). However, *T-Iso* was still present in these cultures, so the results of optical density could be the combination between pigments of *H. ostrearia* and *T-Iso*.

The aim of this study was to dertermine the influence of *H. ostrearia* and marennine on the growth of different species of aquaculture-relevant microalgae, when they are co-cultured with the blue diatom. Our results showed that the influence of *H. ostrearia*is species dependent. Indeed, the growth of *T. suecica, C. gracilis*and *S. costatum* was slowered down or inhibited when these species were co-cultured with *H. ostrearia*, whereas *P. Lutheri*, and *T-Iso* seemed almost indifferent. There are several hypotheses that may explain this phenomenon. For example, the overall effect of co-culturing *H. ostrearia* with another microalgae could be due to the competition for space's occupation, for which the larger volume of *H. ostrearia* may give it an advantage, although, smaller cells usually have higher growth rates, in comparison with larger cells. However, all the species whose development was inhibited were planktonic species, whereas *H. ostrearia* is tychopelagic, and no correlation between inhibition and cell size can be observed. According to another hypothesis, the

influence observed could reflect competition relationships for nutrients and energy, which allows algae to respirate and photosynthesis. Although at the beginning of the treatment, the biovolume for each species was equivalent, at the time of its growth, all of four microalgae were hampered by *H. ostrearia*. The influence of extracellular marennine released by *H. ostrearia* in response to competition for space could thus be a defense strategy. Our results are in accordance with Pouvreau *et al*., (2007) which demonstrated that *H.ostrearia* exerts an allelopathic effect against *S. costatum* and other microalgae.

Harper *et al*., (2001) states that marine microalgae are generally able to develop a self defense system by producing a chemical compounds (chemical defense). Chemical compounds produced by marine microalgae is useful to defend themselves against predators, space competition (to grow and feeding) to prevent ultraviolet exposure. In this study, the secretion of extracellular marennine by *H. ostrearia* could possibly be considered as a response to space competition. It seems that all four microalgae are affected by the presence of these compounds.

Secondary metabolites produced by an organism (in this case *H. ostrearia* who produces extracellular marennine) are usually used to protect themselves from other organisms by inhibiting or killing them. The purpose of the formation of secondary metabolites is something that remains largely unknown, but many experts argue that a secondary metabolite is a detoxification product of toxic metabolites and can not be removed by the organism itself (Mannito, 1981).

Marine microalgae are believed to have the ability to produce bioactive compounds, such as antibiotics, toxins and antitoxins, antitumor and antimicrobial agents. Research conducted by Gastineau *et al*., (2012, 2013) confirmed that blue diatoms (*H. ostrearia*–like diatoms) have such capabilities, thanks to marennine like piments. Molecular compositions of compounds produced depend on the fertility of marine environment, cell defense mechanism against alteration from outside its natural environment (Leone *et al*., 2007).

The treatment M_3 (*H. Ostrearia+I. galbana* (*T-Iso*) also demonstrated that microalgae require specific environments to grow optimally. As an example in this case, when we put the treatment on temperature of 16 °C, *H. ostrearia* successfully grows, while the growth of *T-Iso* was depressed (inhibited). In contrast, *T-Iso* grows well when placed at a temperature of 21 °C but *H. ostrearia*experienced an inhibition. According to Gastineau *et al*., (2013) *H.ostrearia* would grow optimally at temperature of 16 °C and *I.galbana* (T.iso) at temperature of 21 °C (Espinoza *et al*., 2002; Van Bergeijk *et al*., 2010).

High concentration of *H. ostrearia* was found in T_4 (beginning of culture). This could be possible because *H.ostrearia* stressed which allowed them to divide rapidly. This result is consistent with the statement of Weckstrom and Korhola (2001) which stated that temperature has a strong influence on physiological function of microalgae. High temperatures will affect the metabolism, increase cell doubling rate, respiration and affect the movement of diatoms due to the changes in the viscosity of their cytoplasm in the raphe. This is also supported by Campbell *et al*. (2004), which stated that temperature influence the stability of communities, removal some species of diatom and changes in resource availability. Often these factors create a succession of certain diatom species and increase biodiversity.

Treatment M_5 (*H. ostrearia+P. lutheri*), *P. lutheri* grow normally with a slight decreased in number. This is probably due to the competition for nutrients available in the media, which should be shared between the two organisms. However, it doesn't mean that *H. ostrearia* (by way of extracellular marennine) is not effective. Its influence is likely to occur but to a very small extent (inhibition = 28.72% ± 1.03).

Resistancy of certain microalgae against the secondary metabolite of *H. ostrearia* is probably due to the structure of the body layer of microalgae exposed, impermeable nature of microalgal cells and the enzyme toxin produced by the microalgae itself, so the effect of allelopathy of *H. ostrearia* was not so tangible.In general, the allelopatic effect of *H. ostrearia* was higher than the durability of *C. gracilis, S. costatum*and *T. suecica*. However, in some cultures, it seems that the allelopathic power decreased over time for instance in T_{10} for treatment M_3 (*H. ostrearia* + *I. galbana* (*T-Iso* at temperature 21 °C) and the treatment M_6 (*H. ostrearia* + *P. lutheri*). Declining allelopathic power indicates that there has been changement or adjustment of bioactive substances which exist in *H. ostrearia*. Decomposition of dead microalgae in culture may also possibly eliminate toxicity of the secondary metabolites.

Gastineau *et al*.,(2012) explained that marennine produced by *H. ostrearia* able to deliver growth inhibition during the process of mitotic (embryo). This is possible because of the phenolic compounds contained in marennine. It causes lasting cell division cycle. So the generated cell production slightly. In the other hand, according to the research of Pouvreau *et al*. (2007) flavonoids (in this case phenol) can inhibit cell membrane active transport (Na^+ and K^+) so causing the cell wall lysis (rupture). However, until now unknown which the phase inhibited.Einhellig (1995) asserts that the phenolic compounds and their derivatives can affect several important processes such as cell division, mineral absorption, balance, respiration, photosynthesis, protein synthesis and chlorophyll content in microalgae. In addition, growth inhibition may also be due to nutrient mobilization barriers. This could be possible because of the large body of *H. ostrearia* which allowed them to absorb more nutrients as compared to other microalgae, although given its large size, its growth rate is lower than those of smaller cells.

From the overall treatment, allelopathic is not influenced by the light level and the agitation which carried out a better nutrient utilization. According to Mouget *et al*., (2005), *H. ostrearia* and other microalgae grow normally with white light. Agitation before algal counting was not really affect the quantity and allelopathic of culture. Furthermore, temperature, illumination and medium culture were inequal quantity, so that the result should be mostly the effect allelopathy of *H. ostrearia*.

CONCLUSION

H. ostrearia has an allelopathiceffect, which are suitable for aquaculture. The allelopathy power generated greatly varies with the species of microalgae that coexist with *H. ostrearia*. Among the species relevant for aquaculture, *C. gracilis* was the most sensitive but *P. Lutheri* and *T-Iso* being quite insensitive. This result can give some guidance for the potential of valorisation of *H. ostrearia* and its pigment marennine in

aquaculture. The concentrations of marennine which have been shown to inhibit the growth of microalgae ranged from 0.47 mg L^{-1} to 5.35 mg L^{-1}. However measurement with HPLC-PDA is suggested to obtain more precise values of marennine concentration, and to confirm that marennine is the main/only chemical involved in the allelopathic effect caused by a supernatant of *H. ostrearia* culture.

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