Optimization of diatom *Hasleao strearia* cultivation in different mediums and nutrients

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Abstract. *Hasleaostrearia* is a marine diatom that can feed oysters. This study aimed to cultivate *H.ostrearia* in the following media: seawater (SW), seawater filtered by activated carbon (SW+CA), and drilling water (DW). The three-water media were supplemented with two types of nutrients, Walne-Conway+silica (CS) and ES-1/3 Provasoli (P), leading to a six experiment series. The cultures were grown in 200 mL and 400 mL respectively. The dilution cycle was every 3 - 4 day for 200 mL volume and every 4 - 5 days for the 400 mL volume for 20 - 40 days or until the cultures showed a decrease in growth. The microalgae exhibited a better growth in SW and SW+CA CS, whereas DW showed a diminishing number of cells in both the CS and P medium. The highest value of Emn in the 200 mL volume presented in the SW CS medium (1.94 mg L^{-1}) and in the SW+CA CS medium (8.16 mg L^{-1}), while the lowest value of Emn was found in the SW+CA CS medium (0.42 mg L^{-1}). Imn was apparent in the SW P medium (0.56 mg L^{-1}). In conclusion, both SW CS and SW+CA CS showed a better performance in growth. The seawater was more stable during the experiment in both the 200 mL and 400 mL volumes.

1. Introduction

Hasleaostrearia is one of the benthic diatoms that is distributed worldwide. It was found for the first time in the Bourgneuf Bay region of the Atlantic coast and Marennes Oléron, France. This species produces a blue-green pigment called Marennine. This microalga is the only species within the Bacillariophyceae that can produce this type of pigment, which is accumulated in the apical areas [1] of the cells, namely the Intracellular marennine (Imn) and which is released into the medium of the Extracellular marennine (Emn) [2].

This marine pennate diatom can be found in seawater for a few weeks a year. Once bivalves have filtered this species, they turn green. The specific coloration is deposited on both gills and on the labial tentacles of the organism, and sometimes on the intestine [3][4][5][6]. The greening phenomenon is because H. osteraria's presence increases the oyster's price by twenty percent in the market, but its culture for feeding purposes in aquaculture is quite difficult. Moreover, this pigment could inhibit bacterial pathogen growth (prophylactic effect) and it also displays interesting properties as an antiproliferative (against human solid tumors) as well as allelopathic activities [7, 8, 9, 10].

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The objective of this study was to cultivate *H.ostrearia* in the following media: seawater (SW), seawater filtered by activated carbon (SW+CA) and drilling water (DW). The three-water media were supplemented with two types of nutrients; Walne-Conway+silica (CS) and ES-1/3 Provasoli (P), leading to a six experiment series. The semi-continuous mode condition was executed using in short-term cultures to gain an understanding of the optimal growth condition of the living cells and to determine the production of Emn and Imn regarding their pigment concentration.

2. Materials and Methods

2.1 *Culture conditions*

The strain of *H. ostrearia* used in this work was obtained from the Nantes Culture Collection (NCC) type Ho n°10. The species was cultured in sterilized 250 mL and 500 mL Erlenmeyer flasks containing a 200 mL volume and 400 mL volume respectively, in laboratory scale containing Walne-Conway+Silice or Provasoli nutrients [11] [12]. The diatom was cultured at an irradiance of 182.5 µmol photons m^{-2} s^{-1} (DampProof LED IP65 Osram, 55 W) at ±21 °C with a photoperiod of 14:10 h for the light/dark cycle. The irradiance was measured using an Amprobe LM-120 (converted online www.egc.com) and Li-cor 250 A.

2.2 *Biometry*

The cells size was observed and measured before, during and at the end of the experiment (dilution cycle 8^{th} and 12^{th}) by using an inverted-microscope (Leica) and ImageJ tools correspondingly. Several cells were observed and measured, and then the average was estimated as the size of the cells (Figure 1).

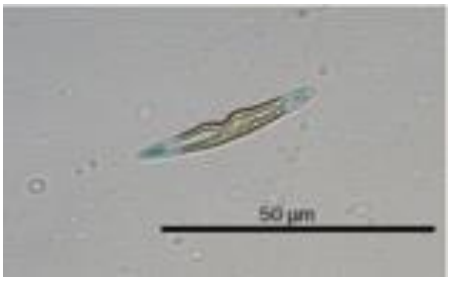

Figure 1. *Hasleaostrearia* species (Personal documentation, 2018)

2.3 *Laboratory experiment*

The culture was grown in several media (Seawater, seawater filtered by activated carbon, and drilling water) with Conway+Silice (CS) or Provasoli (P) as the nutrients (composition of the chemical compounds of both media - CS and P – has been provided in Annex 2). The 200 mL and 400 mL volumes were tested in the laboratory (Figure 2). During the exponential growth, the cultures were transferred directly into the fresh medium. The dilution cycle was every 3 - 4 days for the 200 mL volume and every 4 - 5 days for the 400 mL volume during the 20 - 40 days or until the cultures showed a decrease in growth. All of the cultures in the experiment were replicated three times.

Figure 2. Culture setup of *H. ostrearia*

2.4 *Cell density of H.ostrearia*

The culture of the algae was setup in a semi-continuous mode to maintain the growth in the exponential phase at a low cell density. This mode is effective at avoiding nutrients and light competition during the culture. The initial cells were 10.000 cell mL^{-1} for the 200 mL volume and 20.000 cells mL^{-1} for the 400 mL volume. *H.ostrearia* was first acclimated for one week to gain enough of a cell concentration for the experiment. It was then maintained sustainably in the exponential phase through the dilution with a fresh medium every 3 - 4 days for the 200 mL volume and every 4 - 5 days for the 400 mL volume. Almost 75% and 80% of the culture's total volume was discarded in the 200 and 400 mL volume, correspondingly. The cell concentration was determined approximately for 5 - 12 dilution cycles or 19 - 40 days, depending on the water resource, nutrient, and volume of the cultures.

For each cell counting day, the samples in the Erlenmeyer flasks were gently stirred for around 15 minutes to homogenize the culture and to avoid aggregates in the bottom of the flask. Furthermore, the cell growth was observed by using an inverted-microscope (x200 magnification) and by counting the cell densities with a Nageotte hemocytometer. The growth rate and generation time were calculated in the following equations (1) and (2) [7] [13].

$$x = \frac{\ln N2 - \ln N1}{d2 - d1} \quad \ldots\ldots\ldots (1)$$

where x (day^{-1}) is the growth rate, N1 and N2 represents the cell density at the start and the end of each growth period, and d1 and d2 show the measurement time.

$$G = \frac{t \log 2}{\log b - \log B} \quad \ldots\ldots\ldots (2)$$

where G is the generation time (time $generation^{-1}$), t is the culture time, and b and B are the concentration of the microalgae at the end and at the beginning of the observation respectively.

2.5 *Estimation of the marennine concentration (Emn and Imn)*

The concentration of the extracellular marennine (Emn) and intracellular marennine (Imn) was measured at the end of a given culture period (3 - 4 days or 4 - 5 days) for the 200 mL and 400 mL volume serially. The 45 mL samples were centrifuged (15-minute, 3000 g) to separate the supernatant and pellets for better pigment analyses. The amount of marennine released into the medium during the algae growth (supernatant) was measured directly with a spectrophotometer (Thermo scientific Evolution 220 UV-Visible Spectrophotometer) and was determined as Emn. The pigment accumulated in the apical cells (pellets) was determined as Imn. To estimate the Imn, 4 mL of a 5 mM $Na2HPO_4$/ KH_2PO4 hypoosmotic buffer (pH 8) was added to the algal pellets, stirred gently for 30 minutes in the dark and followed by centrifugation [14] [15]. The cell pigment concentration was determined spectrophotometrically and using a specific extinction coefficient as described in [15]. The concentration (C) of Emn and Imn was calculated according to the following formula (equation 3).

$$[C] = \frac{A\,\lambda max}{\varepsilon\lambda\,max\,.\,l} \quad \ldots\ldots\ldots (3)$$

where$A\lambda_{max}$ is the absorbance of the solution at the peak wavelength in the red region (670 nm), $\varepsilon\lambda_{max}$ is the specific extinction coefficient (17.2), and l is the cuvette path length (1 cm).

2.6 *Statistical analyses*

The cell density and pigment analyses obtained from the cultures grown in SW CS, SW+CA CS, P CS 200 mL volume, SW and SW CS 400 mL were formulated using Microsoft Office Excel 2007 and SPSS 19.0.

3. Result and Discussion

3.1 Biometry

The cell size at the start of the experiment was 40 µm and this was re-measured at the 8th dilution and at the end of the experiment (11th dilution). There was no size difference between the beginning (40 µm) and the end of the experiments (38 µm).

3.2 Growth kinetics of H.ostrearia

Several different types of culture media and nutrients were tested related to *H. ostrearia*. Figures 3 and 4 show the growth curves of the cultures within a 200 mL volume carried out in CS and P media respectively. Moreover, Figure 5 presents the cell growth curves within the 400 mL volume with the CS medium. The cultured with the 400 mL P medium was tested, however in the first dilution cycle, the growth curves exhibited a diminution in cell density (initial 20 x 10^3 cell mL^{-1}, and in the 1st dilution, it was 11.35 x 10^3 cell mL-1).

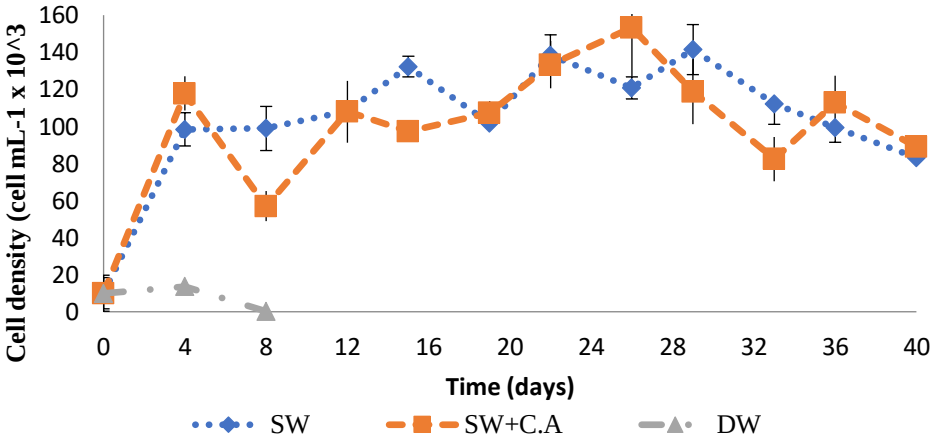

Figure 3. Growth curves of *H.ostrearia* cultivated in the semi-continuous exponential phase maintained for 40 days in the 200 mL Conway+silica (CS) nutrient with 3 different water resources (Seawater (SW), seawater filtered by activated carbon (SW+CA), and drilling water (DW)). Mean ± Standard error.

For the eleven cycles presented in Figure 3, the microalgae exhibited a better growth in both the SW and SW+CA CS media. Generally, there was no significant difference in the growth phase, except that the cultures within the SW medium showed a more stable level of performance than the cultures with SW+CA. The experiment with the SW CS medium showed a similar augmentation in each exponential phase (3 - 4 days) and statistically, there was no significant difference with the exception of the fifth, sixth, eighth and eleventh transfers (Student's *t*. test). The cultures with SW+CA CS presented unstable growth. Unlike the SW and SW+CA CS mediums, the DW CS medium displayed decay in the cell multiplication. The decreasing cell number showed directly in the first dilution cycle. It is possible that the DW medium was not providing the nutrition required for *H. ostrearia*'s growth.

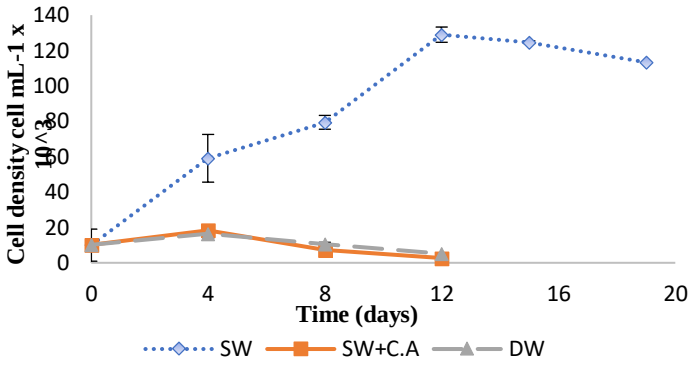

Figure 4. Growth curves of *H.ostrearia* cultivated in the semi-continuous mode exponential phase maintained for 20 days in the 200 mL ES-1/3 Provasoli (P) nutrient with 3 different water resources (Seawater (SW), seawater filtered by activated carbon (SW+CA), and drilling water (DW)). Mean ± Standard error.

Another culture was taken to verify the growth performance of *H.ostrearia*. Three different water resources were added to the P nutrient (Figure 4). The curves exhibited an obvious difference showing cell increase. Only the SW P medium presented an augmentation of growth, while the SW+CA and DW P media showed there to be decay in the cultures.

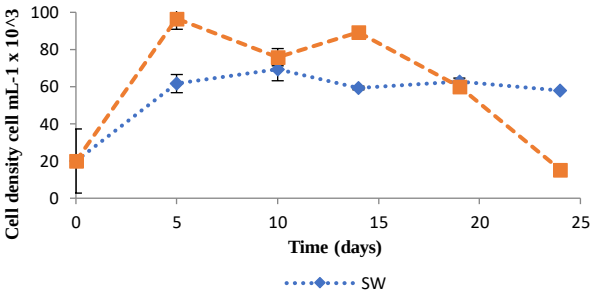

Figure 5. Growth curves of *H.ostrearia* cultivated in the semi-continuous mode exponential phase maintained for 25 days in the 400 mL Conway+silica nutrient with 2 different water resources (Seawater (SW) and seawater filtered by activated carbon (SW+CA). Mean ± Standard error.

Cultures with 400 mL volume were carried out after an enhancement culture with the SW and SW+CA CS mediums in the 200 mL volume (Figure 5). The 25 days of testing showed there to be an increase in cell growth even though the performance was not as good as in the 200 mL volume. *H.ostrearia* is a benthic species which prefers to stay in the bottom of the flask; the augmentation of the volume influenced their growth and as well as the necessity for nutrients and light.

3.3 Concentration of extracellular marennine (Emn) and intracellular marennine (Imn)

An observation of the pigment concentration was carried out on *H.ostrearia*. Similar to the cell concentration measurement, the concentration was determined at the end of each dilution cycle (Figure 6). During the eleven cycles of 200 mL in volume, the values for Emn from both SW CS and SW+CA revealed there to be no significance difference. However, for Imn, there was a clear distinction of value. Imn showed there to be a higher grade of concentration. The maximum value of Emn SW CS during the cultures was 1.94±0.19 mg L^{-1} (mean±SE, *n*=3) and the lowest was 0.58 ±0.19 mg L^{-1}, while the maximum value of Emn SW+CA CS during the test was 1.45±0.26 mg L^{-1} (mean±SE, *n*=3) and the lowest was 0.42±0.26 mg L^{-1}. Furthermore, the highest value of Imn SW CS during the growth was 5.76±0.11 mg L^{-1} (mean±SE, *n*=3) and the lowest was 1.05 ±0.11 mg L^{-1}, whereas the highest

value of Imn SW+CA CS during the experiment was 8.16±0.15 mg L^{-1} (mean±SE, *n*=3) and the lowest was 1.22 ±0.15 mg L^{-1}.

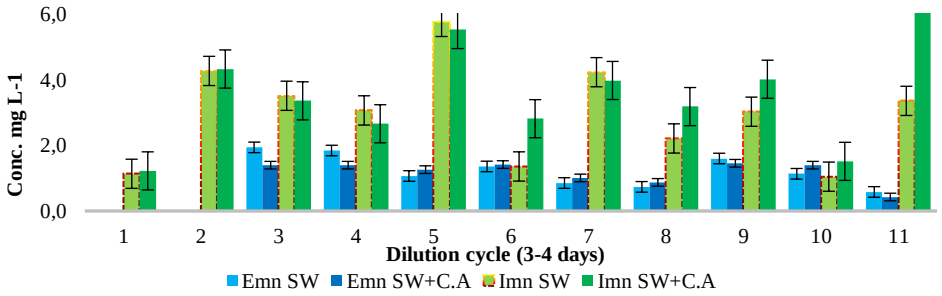

Figure 6. Concentration of Extracellular marennine (Emn) and Intracellular marennine (Imn) mg L^{-1} in a semi-continuous mode culture of *H.ostrearia* obtained from 200 mL Conway+Silice nutrient with Seawater (SW) and seawater filtered by activated carbon (SW+CA) water resources. Means ± standard error (n=3).

An equal measurement was undertaken for the Emn and Imn pigment analyses with the P medium as part of a shorter dilution cycle (Figure 7). In line with the cell density concentration, the Emn and Imn concentration within the P medium showed a lesser value compared to the CS medium. The maximum value of Emn SW P during the cultures was 1.94±0.19 mg L^{-1} (mean±SE, *n*=3) and the lowest was 0.48 ±0.19 mg L^{-1}, whereas the highest value of Imn SW P during the experiment was 5.27±0.27 mg L^{-1} (mean±SE, *n*=3) and the lowest was 0.56 ±0.27 mg L^{-1}.

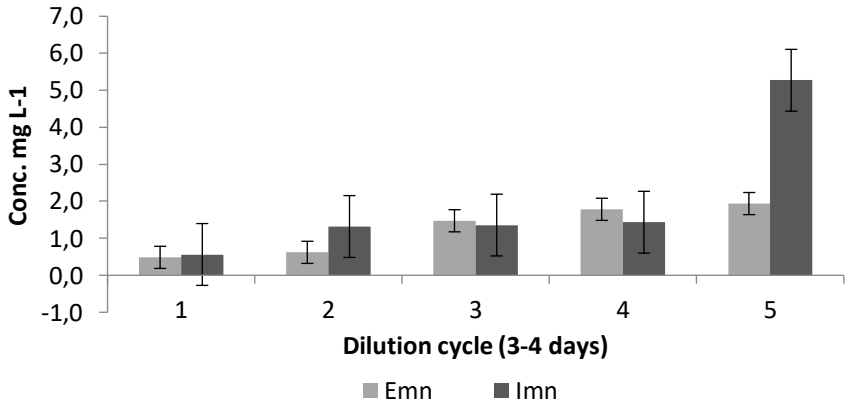

Figure 7. Concentration of Extracellular marennine (Emn) and Intracellular marennine (Imn) mg L^{-1} in semi-continuous culture of *H.ostrearia* obtained from 200 mL ES-1/3 Provasoli nutrient with Seawater (SW). Means ± standard error (n=3).

The test within the 400 mL volume led to a maximum value of Emn SW CS during the culture of 1.03±0.15 mg L^{-1} (mean±SE, *n*=3), and the lowest was 0.23 ±0.15 mg L^{-1}. The maximum value of Emn SW+CA CS during the test was 1.12±0.09 mg L^{-1} (mean±SE, *n*=3) and the lowest was 0.06±0.09 mg L^{-1} (Figure 8). Moreover, the highest value of Imn SW CS during the growth phase was 6.34±0.15 mg L^{-1} (mean±SE, *n*=3) and the lowest was 1.80 ±0.15 mg L^{-1}, whereas the highest value of Imn SW+CA CS during the experiment was 6.01±0.06 mg L^{-1} (mean±SE, *n*=3) and the lowest was 1.57 ±0.06 mg L^{-1}.

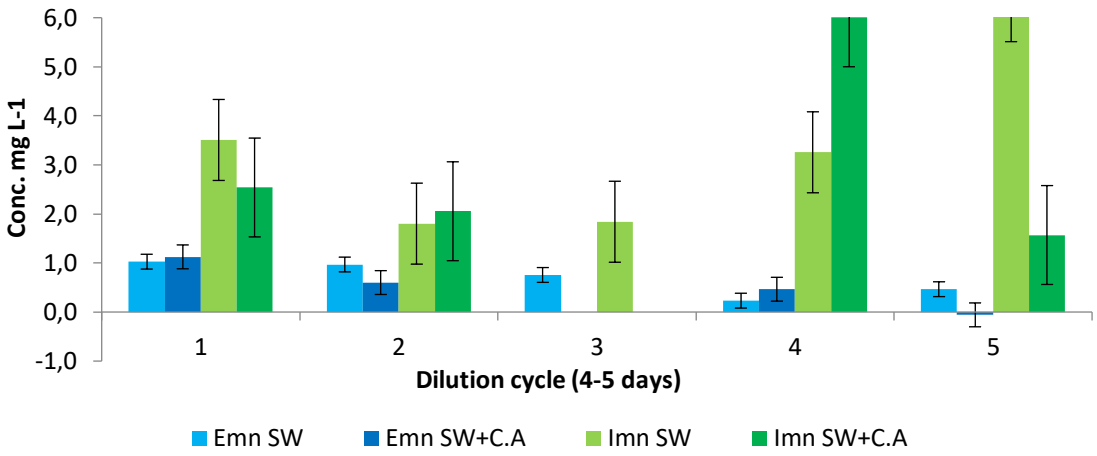

Figure 8. Concentration of Extracellular marennine (Emn) and Intracellular marennine (Imn) mg L^{-1} in semi-continuous culture of *H.ostrearia* obtained from 400 mL Conway+Silice nutrient with Seawater (SW) and seawater filtered by activated carbon (SW+CA) water resources. Means ± standard error (n=3).

3.4 Growth kinetics in the function of the different media and nutrients

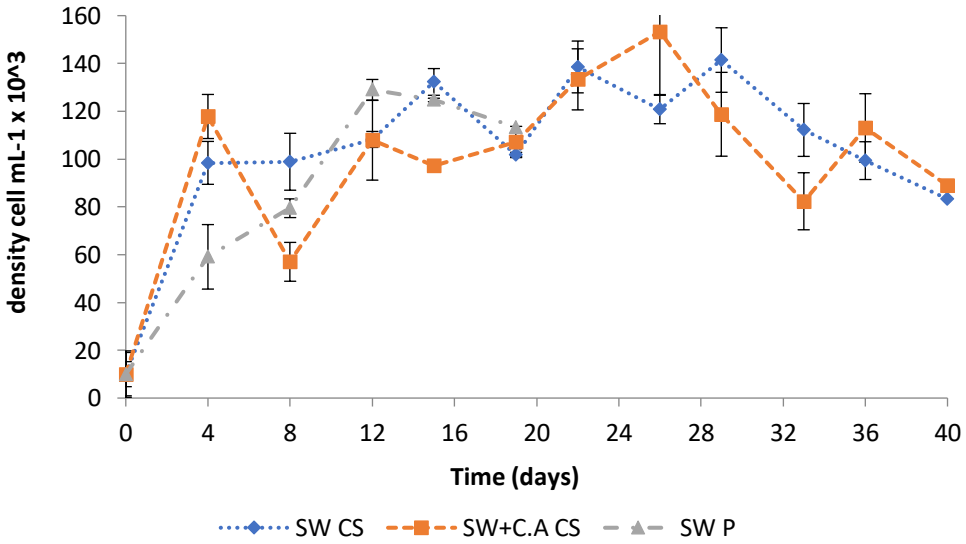

Figure 9. Growth curves of *H.ostrearia* cultivated in the semi-continuous exponential phase maintained for 20-40 days in the 200 mL Conway+silica with 2 different water resources (Seawater (SW CS), seawater filtered by activated carbon (SW+CA CS)), and Seawater ES-1/3 Provasoli medium (SW P). Mean ± Standard error.

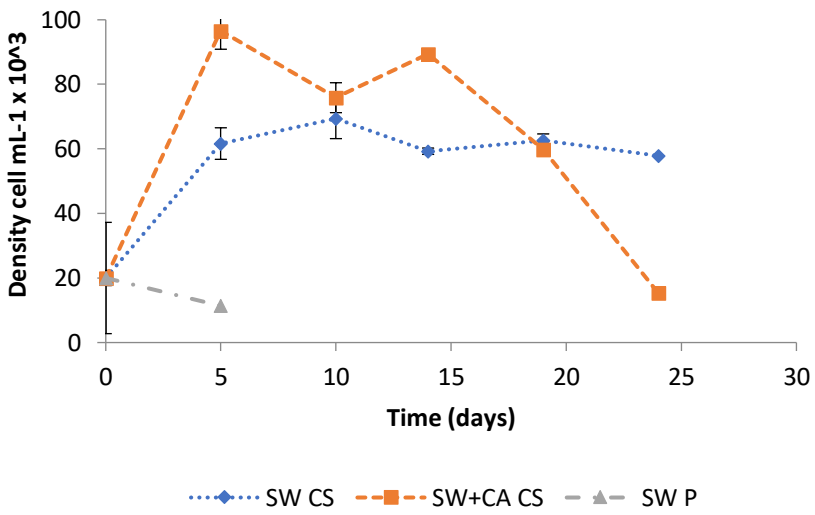

Figure 10. Growth curves of *H.ostrearia* cultivated in semi-continuous mode exponential phase maintained for 5-25 days in the 400 mL Conway+silica with 2 different water resources (Seawater (SW CS), seawater filtered by activated carbon (SW+CA CS)), and Seawater ES-1/3 Provasoli medium (SW P). Mean ± Standard error.

As described above, this secondment was intended to find and verify the best growth of *H.ostrearia* by testing several water resources and nutrients. The comparison has been shown in Figure 9 for the 200 mL volume and Figure 10 for the 400 mL volume. The results clearly show that there was no significant difference in the growth curves for both SW and SW CA CS nutrients, conversely to the SW P nutrient. The culture growth with the P nutrient was slower than the others. As well as in the 400 mL volume, the enhancement of the cell numbers was not good enough compared to the 200 ml volume, followed by the decrease in curves in SW+CA CS at the end of the experiment.

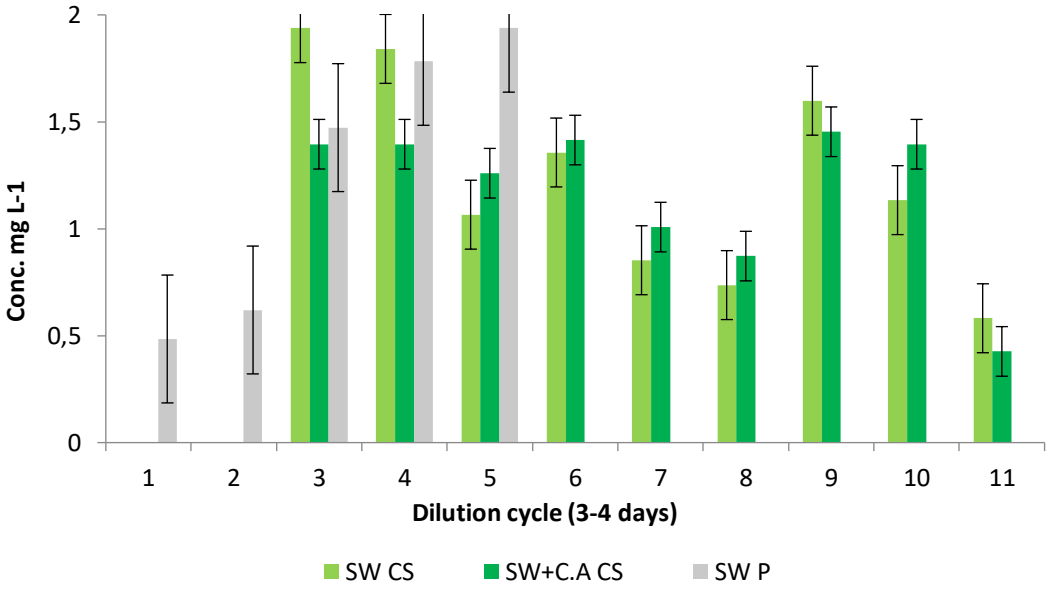

Figure 11. Concentration of Extracellular marennine (Emn) mg L^{-1} in the semi-continuous culture of *H.ostrearia* maintained for a 5 - 11 dilution cycle in the 200 mL Conway+silica with 2 different water resources (Seawater (SW CS), seawater

filtered by activated carbon (SW+CA CS)), and Seawater ES-1/3 Provasoli medium (SW P). Mean ± Standard error.

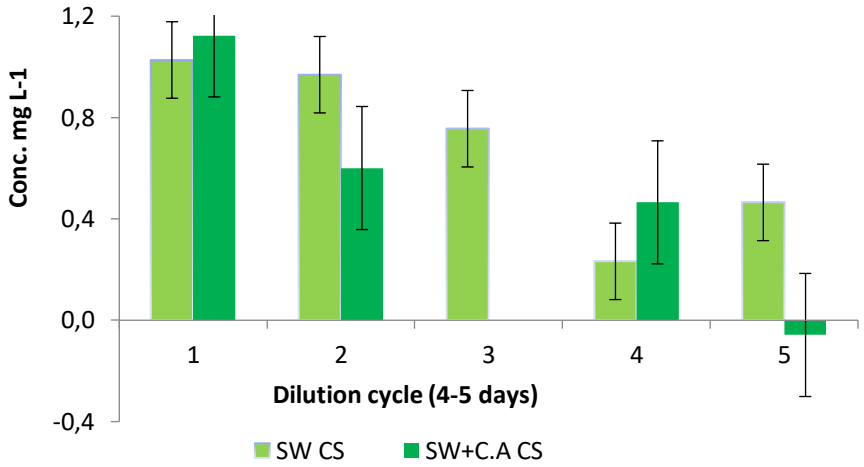

Figure 12. Concentration of Extracellular marennine (Emn) mg L^{-1} in a semi-continuous culture of *H.ostrearia* maintained for 5-11 dilution cycle in the 400 mL Conway+silica nutrient with 2 different water resources (Seawater (SW CS) and Seawater filtered by Carbon active (SW+CA CS)). Mean ± Standard error.

In addition, to complete the comparison, the Emn and Imn concentration has been presented in Figures 11 - 14 for SW CS, SW+CA CS, and SW P both in the 200 mL and 400 mL volumes. The highest value of Emn within the 200 mL volume presented in the SW CS medium (1.94 mg L^{-1}) and Imn was remarkable in the SW+CA CS medium (8.16 mg L^{-1}). The lowest value of Emn was found in the SW+CA CS medium (0.42 mg L^{-1}) and for Imn, it was in the SW P medium (0.56 mg L^{-1}). The experiment produced ±10 L supernatants for both SW CS and SW+CA CS, and ±4 L supernatants from the SW P medium (Annex 4). The supernatants were conserved directly in a dark room at a temperature of 4 °C.

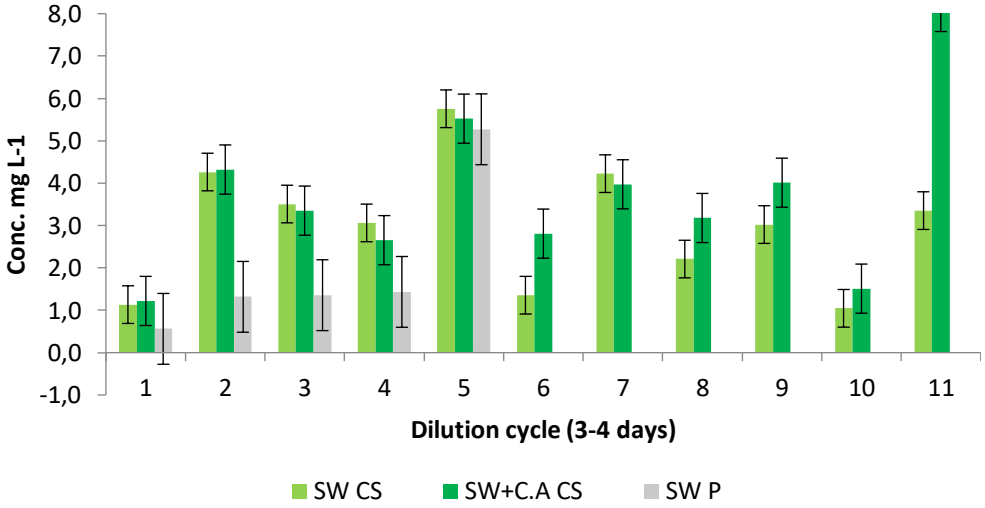

Figure 13. Concentration of Intracellular marennine (Emn) mg L^{-1} in the semi-continuous culture of *H.ostrearia* maintained for the 5 - 11 dilution cycle in the 200 mL Conway+silica with 2 different water resources (Seawater (SW CS), seawater

filtered by activated carbon (SW+CA CS)), and Seawater ES-1/3 Provasoli media (SW P). Mean ± Standard error.

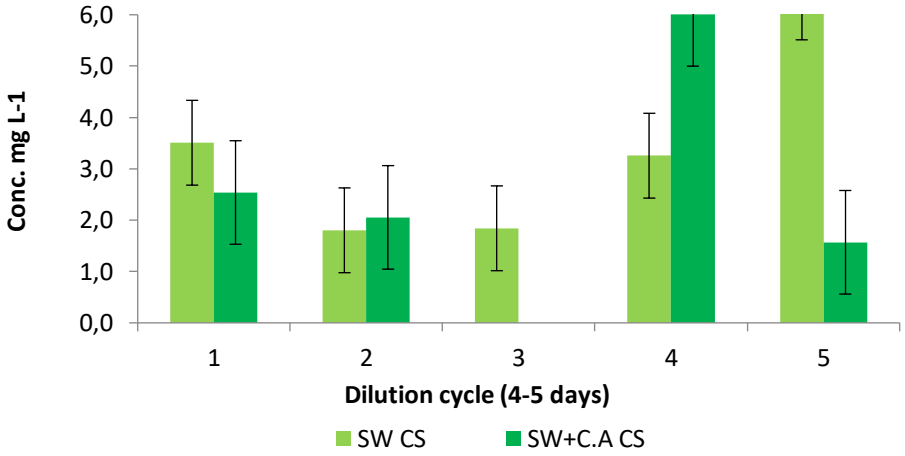

Figure 14. Concentration of Intracellular marennine (Emn) mg L^{-1} in the semi-continuous culture of *H.ostrearia* maintained for 5 - 11 dilution cycle in the 400 mL Conway+silica with 2 different water resources (Seawater (SW CS) and seawater filtered by activated carbon (SW+CA CS)). Mean ± Standard error.

3.5 Growth rate and generation time

During the cultures, the growth rate of the microalgae was quite stable. The growth rate averages of the cultures with SW CS, SW+CA CS, and SW P in the 200 mL volume were 0.05, 0.18, and 0.12. The other culture within a 400 mL volume showed an average growth rate of 0.04 for SW CS and -0.01 for SW+CA CS, explained in this case because the culture tended to decrease. A significant relationship was observed between the growth rate and the cell density of the cultures. Pearson's correlation indicated that the growth rate had a positive correlation with cell density in SW CS 200 mL (r=0.784, sig (2-tailled 0.012)), but not with the other parameters. Otherwise, a negative significant correlation was show between the growth rate and Emn SW P 200 mL (r=0.894, sig (2-tailled 0.041)). It was explained by [9] that a higher decrease in growth rate resulted in a higher release of Emn into the culture medium. The generation time of the species cultured in 200 mL within the SW CS, SW+CA CS, and P media was 9.19, -0.33, and -9.35-hour generation^{-1}, respectively. The negative value proved that there was a diminution in species multiplication.

4 Conclusion

In conclusion, based on the several different types of nutrient and water resource used in this study, both seawater and seawater filtered with activated carbon with the Conway+Silice nutrient showed a better performance in growth. Seawater was more stable during the experiment, both in the 200 mL or 400 mL volume. In a future study, seawater with the Conway+Silice medium could be used to grow *Hasleaostrearia* and to try out a larger volume in a semi-large scale (volume > 50 L).

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