Semi-continuous system for benthic diatom cultivation and marennine production

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ABSTRACT

The feasibility of culturing different blue *Haslea* species and strains in different types of photobioreactors (PBRs) was studied on the long-term (until 151 days). The different strains of blue *Haslea* were selected for their peculiarity to produce marennine-like blue pigments as a potential industrial high-value compound. The present study aims at assessing several factors in PBRs to obtain sustained blue pigment production in semi-continuous culture. Therefore, the effect of mixing, silicate concentration in the culture medium and type of light on marennine or marennine-like pigment production were investigated in parallel to the productivity of different *Haslea* strains and species. It was shown that the presence of mixing in semi-continuous PBR affected marennine production, cultures without any mixing achieving significantly higher marennine concentrations and productivities. Additionally, concentrations of silica from 45 to 75 µg L^{-1} in the culture medium produced higher marennine concentrations than that of 30 µg L^{-1}. There were no significant differences in marennine production between the LEDs mixing different color and fluorescent tubes in semi-continuous PBR, thus LED could be a great option from the sustainability standpoint. Marennine production in the standard conditions used for this work was largely different between species. *Haslea* sp. produced the lowest pigment yields comparatively to the three *H. ostrearia* strains showing similar marennine productivity over 14 mg L^{-1}. Preservation, until 155 days of marennine separated from culture supernatant and concentrated ("blue water") was increased at low temperature (4 °C) and absence of light. This study validates the efficiency of semi-continuous systems to support long-term marennine production. However, additional work is still needed to pinpoint other factors that can further reduce the costs and result in maximum yields of marennine for industrial applications.

Keywords: *Haslea ostrearia*, marennine, semi-continuous culture, photobioreactor

1. Introduction

Marine diatoms have increasingly attracted attention for several industrial purposes. This group of microalgae has the ability to produce various products with high aggregated value such as pigments, exopolysaccharides, fatty acids, proteins and many other high-value compounds [1–6]. These valuable compounds can potentially be used for various applications such as pharmacology, cosmetology, food additives, biofuels and aquaculture [7–14]. However, each strain of diatom and type of product requires appropriate bioprocess design in order to increase the production of enriched biomass and molecules of interest to an industrial scale [15]. Unlike other groups of microalgae, diatoms are sometimes more exigent to cultivate at large scale, particularly by their specific needs for silicate [16]. Indeed, for certain species, there is a lack of information regarding growth and metabolite production conditions, nutrient needs or responses to light. Some species of marine diatoms such as *Phaeodactylum tricornutum*, *Skeletonema costatum* and *Chaetoceros* sp., are already produced at large scale [17–19]. However, many other strains are relatively challenging to produce in a controlled and optimized large-scale production. Therefore, a well-designed bioprocess engineering approach should be established such as the design of culture systems (*ca*. photobioreactor engineering), light regime and optimization of growth medium to achieve larger biomass concentrations [15,20].

The pennate diatom *Haslea ostrearia* is a marine species that has long been known and has been studied both at laboratory- and mesocosm-scale [12,21–23]. This diatom has the peculiarity in synthesizing and excreting the water-soluble blue pigment marennine, which gives added value to the bivalve *Crassostrea gigas* in the French oyster industry [9,24]. This diatom is a cosmopolite species that has been observed in many low depth marine habitats, both in Northern and Southern hemispheres [9]. Moreover, other species from the same genus and with the ability to produce marennine-like pigments have been discovered in both Northern (like *H. karadagensis* and *H. provincialis*) and Southern hemisphere (*H. nusantara*) [9,25–27].

Most importantly, previous works revealed that marennine and possibly all marennine-like pigments present biological activities, such as antioxidant [28,29], antibacterial, antiviral and antiproliferative [13,30–32], which leads to potential application of these pigments to various fields. However, the production of *H. ostrearia* using conventional and also specific photobioreactors (PBRs) is very challenging due to its low biomass and the yield of excreted marennine (extracellular marennine, EMn, released in the medium) per cell [33,34]. Thus, different low volume PBRs have been implemented, such as immersed membrane PBRs [33], agar immobilization PBRs [34] or airlift type PBR using medium modification to obtain EMn yield until 15.7 mg L^{-1} [20]. However, further developments should be conducted to obtain better productivity, stability and feasibility for industrial purposes. To help development of marennine production, we considered that some important knowledges need to be obtain, like impact of silica concentration, type of light, *Haslea* strains used and preservation of EMn sampled for the PBR.

The present study mainly focuses on the assessment of EMn production by *H. ostrearia* with different designs of PBRs and medium adjustments. We used a semi-continuous system to represent a realistic condition for up-scaling purposes with different types of PBRs and the production of EMn was evaluated. Several experiments were also performed to optimize marennine-like pigment production by different *Haslea* strains and to answer the following questions: 1) Do the mixing of the culture and the silica concentration affect the yield of EMn in a long-term semi-continuous operation of a 10 L PBR ? 2) Do the type of light and strain of *Haslea* affect the yield of EMn in a long-term semi-continuous operation of a 30 L PBR ? 3) Do the temperature and the presence of light affect the long-term degradation of EMn produced?

2. Materials and methods

2.1 Experimental set up

All experiments were conducted at the Station aquicole de Pointe-au-Père (Université du Québec à Rimouski, 48°31 N; 68°28 W, Québec, Canada). A total of 5 experiments were performed using a semi-continuous mode of culture. The first experiment consisted of comparison between mixed and non-mixed culture of *H. ostrearia* with 10-L replicated semi-continuous system filled to 4-L. For the second experiments, the silicate concentration concentrations of silica (30, 45, 60, and 75 μ g L^{-1}) was tested with the same replicated semi-continuous system. The third experiment was oriented to test light fluorescent tubes T5-5000 K with LED system (24 blue and 48 white LEDs, 14 W, 6500 Ka) in replicated 30-L flat bottom square-shaped acrylic PBR prototype. During the fourth experiment, the similar 30-L flat bottom PBR were used in triplicate to test 4 different *Haslea* cultures composed of two strains of *H. ostrearia* sampled for different countries, one strain of *H. provincialis* and one identified species of *Haslea* from Mexico. Finally, the last experiment was not oriented on *Haslea* culture, but mostly on marennine produced to test the effect of temperature and light on their degradation.

2.2 Stock culture conditions

Four different blue *Haslea* strains were used in this work, namely, two *H. ostrearia* strains, one isolated from the Atlantic-Coast of France (Bourgneuf Bay, 46°58’27’’ N, 1°59’ 55’’ W) (NCC-CBB2), and the other from the Pacific-Coast of United States of America (San Juan Islands, Griffin Bay, Jackle’s Lagoon, 48°27’41’’ N, 122°59’19’’ W) (NCC-San Juan), *H. provincialis* from the Mediterranean Sea, France (Boulouris, 43°2’45’’ N, 6°47’42’’ E) (NCC-Provincialis), and *Haslea* sp. from the East-Coast of Mexico (Punta Nizuc, Cancún,

Yucatán, 21°02'09'' N; 86°46'39'' W), were obtained from the Nantes Culture Collection (NCC), Université de Nantes. The strains of the species *H. ostrearia* and *H. provincialis* were identified based upon general morphological dimensions in addition to features considered characteristic of the genus, for instance, the striation, the presence of external longitudinal strips over many areolae, with intervening continuous slits [25–27]. The strain collected in Cancún did not match with any already known species, and its description will be performed in future work. All strains were non axenic but grown in sterilized 500 mL Erlenmeyer flasks, containing 250 mL of sterilized Guillard F/2 medium at 19 °C ± 1 °C. Cultures were grown at an irradiance of 100 µmol photons m^{-2} s^{-1} provided by T5/5000 K fluorescent tubes in a 14h/10h light/dark cycle. Irradiance was measured with a Q201 quantum radiometer (Macam Photometrics Ltd., Livingston, Scotland). The cultures were transferred to 2.8 L Erlenmeyer flasks filled with 2 L seawater and were kept for 14 days. Each PBR was inoculated with approximately 2000 cells mL^{-1} from two gently homogenized and mixed 2.8 L Erlenmeyer flasks. Cells were estimated with the use of Nageotte counting chambers. Each experiment was realized in room-controlled temperature to maintain constant conditions (±0.5 ^{o}C) and light intensity regularly controlled using a Q201 quantum radiometer (Macam Photometrics Ltd., Livingston, Scotland).

2.3 Experiment I: long-term effect of mixing on extracellular marennine (EMn) production

The aim of this experiment was to determine if the mixing of *H. ostrearia* culture affects the production of EMn. As this species mostly behaves like a benthic diatom, it forms a biofilm at the flask bottom when unstirred, and mixing of the culture could disturb the biofilm, possibly enhancing exchanges with the water column, which could eventually influences EMn production. Therefore, two conditions were applied during this experiment: culture with mixing and culture without mixing, as described in Figure 1, also showing a picture of the culture system. Mixing was applied after EMn sampling each 3-4 days and consisted of manual and

147 gently mix until homogenization of the microalgae culture and biofilm. In both conditions, the
148 cultures of *H. ostrearia* (NCC-CBB2) were used in the semi-continuous system and maintained
149 in 4 L autoclaved Guillard F/2 medium [35] containing 45 µg L^{-1} silica in 10 L borosilicate
150 media storage bottles used as PBR (n = 3 for each treatment, Fig. 1) at a level of 200 µmol
151 photons m^{-2} s^{-1}, 14/10 h light/dark cycle, temperature of 20 °C and salinity of 28 ppm. Filtered
152 air (through 0.22 µm sterile syringe filter) was slightly supplied at the surface of water
153 (headspace) without mixing the microalgae culture to ensure a slow but constant water
154 movement in the tank and providing nutrient renewal to the cells and keeping a positive pressure
155 preventing contamination from the outside. Each 3-4 days during 150 days, sample of culture
156 medium was collected in each PBR, filtered through 0.22 µm syringe sterile filter to eliminate
157 potential pelagic cells and EMn concentration determined following the protocol from Prasetiya
158 et al. [12] (see section 2.2.6 “Estimation of EMn concentration”). Each week, 1 mL of culture
159 medium was sampled for bacterial concentration estimation after addition of 3 mL of
160 glutaraldehyde (0.1% v/v) to fix the sample and 25% of the water supernatant in each PBR were
161 gently removed and replaced by autoclaved F/2 medium with silica. For the non-mixed
162 treatment, F/2 medium was replaced with caution to avoid homogenization of culture and
163 biofilm. Despite the precautions to avoid any bacterial contamination, we consider that there is
164 some risk of bacterial development in the context of long-term culture of benthic diatoms. Thus,
165 we validate if sampling and/or mixing manipulations could facilitate bacterial development
166 analyzed using a CytoFLEX flow cytometer (Beckman Coulter, IN, USA) fitted with a 488 nm
167 laser operated at 15 mW under a flow rate of 60 µL per minute. Data were analyzed with the
168 Expo32 v.1.2b software (Beckman Coulter Inc., Fullerton, CA). Heterotrophic bacteria were
169 quantified in diluted samples stained with SYBR Green I nucleic acid bounder (Molecular
170 Probes Inc., OR, USA).
171

2.4 Experiment 2: long-term effect of silica concentration on extracellular marennine (EMn) production

A similar design of PBR as in Experiment 1 without mixing was applied using the culture of *H. ostrearia* (NCC-CBB2) in 4 L autoclaved F/2 medium [35] containing different concentrations of silica (30, 45, 60, and 75 μg L^{-1}) in 10 L borosilicate media storage bottles (n = 3 for each treatment) at a level of 200 μmol photons m^{-2} s^{-1}, 14/10 h light/dark cycle, temperature of 20 °C and salinity of 28 ppm. Filtered (0.22 μm) air was slightly supplied at the water surface (PBR headspace) without mixing the microalgae culture. EMn concentrations were determined every 3-4 days on cell-free culture water (syringe-filtered on 0.22 μm) using the Beer-Lambert law as explained further in the section 2.2.6 (Estimation of EMn concentration). When EMn concentrations were over 6 mg L^{-1}, 25% of the water supernatant was gently removed and replaced by autoclaved F/2 medium with silica. All operations were realized in a laminar flow hood to avoid bacterial contamination.

2.5 Experiment 3: long-term effect of the light source on extracellular marennine (EMn) production

This series of experiments were run with cultures of *H. ostrearia* (NCC-CBB2) in 30 L autoclaved Guillard F/2 medium [35] containing 45 μg L^{-1} of silica in a flat bottom square-shaped acrylic PBR prototype of our own design (details in Fig. 2) and 14/10 h light/dark cycle, temperature of 20 °C and salinity of 28 ppm. A total of 6 PBR were used, three for each treatment (n = 3), one with the light fluorescent tube T5-5000 K (n = 3) and the other with LED system (n = 3, 72 mixed blue (24) and white (48) LEDs, 14 W, 6500 Ka) at 200 μmol photons m^{-2} s^{-1}. Filtered (0.22 μm) air was slightly supplied at the surface of water without mixing the microalgae culture. EMn concentration was determined each 3-4 days on cell-free culture water

196 (syringe-filtered on 0.22 μm) using the Beer-Lambert law. When marennine concentrations
197 were over 6 mg L^{-1}, 25% of the water supernatant were gently removed and replaced by
198 autoclaved F/2 medium with silica.
199
200 *2.6 Experiment 4: Extracellular Marennine (EMn) production of different species or strains*
201 *of* Haslea

202 Experiments were run in autoclaved Guillard F/2 medium containing 45 μg L^{-1} of silica
203 in the same 30 L PBR prototype as in Experiment 3 (Fig. 2) and 14/10 h light/dark cycle (72
204 mixed blue and white LEDs, 14 W, 50.8 cm to 68.6 cm, 6500 Ka) at 200 μmol photons m^{-2} s^{-1},
205 temperature of 20 °C and salinity of 28 ppm. Filtered (0.22 μm) air was slightly supplied at the
206 surface of water (PBR headspace) without mixing the microalgae culture. EMn concentrations
207 were determined every week on cell-free culture water (syringe-filtered on 0.22 μm) using the
208 Beer-Lambert law. When EMn concentration were over 6 mg L^{-1}, 25% of the water supernatant
209 was gently removed and replaced by autoclaved F/2 medium with silica (45 μg L^{-1}). In this
210 experiment, 3 PBRs were used for each strain (n = 3) as mentioned in the section "Culture
211 conditions".
212
213 *2.7 Experiment 5: Effect of temperature and light on extracellular marennine (EMn)*
214 *degradation*

215 The experiment was performed to investigate the effect of temperature and the presence
216 of light on EMn degradation in a long-term storage (>150 d). Briefly, EMn was collected from
217 the CBB2 strain produced in flat bottom 30 L PBR filtered on 1 μm (SOE polypropylene
218 cartridge, Cole-Palmer). Afterwards, EMn was stored at different conditions (n = 3 for each
219 treatment): 1) at 20 °C with 200 μmol photons m^{-2} s^{-1} of light (treatment A), 2) at 20 °C in dark

(treatment B), and 3) at 4 °C in dark (treatment C). The degradation of EMn concentration in each treatment was evaluated for >150 d using a spectrophotometer (Cary 100 Bio UV-Visible, Agilent Technologies).

2.8 Estimation of extracellular marennine (EMn) concentration

Optical density was measured at 677 nm in a 10 cm cell using an Agilent Technologies spectrophotometer (Cary 100 Bio UV-Visible) and the specific extinction coefficient (ε677) for EMn of 12.13 L g^{-1} cm^{-1}, as stated in [36]. The concentration of EMn C (g L^{-1}) was calculated according to the following formula:

$$[C] = \frac{\mathbf{A}_{\lambda\,\mathbf{max}}}{\boldsymbol{\varepsilon}_{\lambda\,\mathbf{max}}\ \mathbf{x}\ \boldsymbol{l}}$$

Where $A_{\lambda max}$ is the absorbance at the peak wavelength in red region (677 nm), $\varepsilon_{\lambda max}$ is the specific extinction coefficient at the peak wavelength, and *l* is the cuvette path length.

2.2.5 Statistical analyses

All data were analyzed using the software SigmaPlot version 12.0 for Windows. Prior to statistical analyses, normality and homogeneity of data were checked using Shapiro-Wilk and Kolmogorov-Smirnov test, respectively. All statistical analyses were performed at a maximum significance level of 5% by two-way analyses of variance (ANOVA) for all experiments and data were log-transformed if necessary.

3. Results

3.1 Effect of mixing on extracellular marennine (EMn) production

In this experiment, 25% of the total volume of *H. ostrearia* culture was harvested every two weeks in both mixed and non-mixed treatments. The EMn concentration accumulated in these semi-continuous systems was measured at each harvesting time (Fig. 3A). It was found that during the majority of the experimental period (>150 d), the concentration of EMn was systematically higher after the first 40 days in the condition without mixing (with a peak concentration of 11.82 mg L^{-1}) than that of the mixed treatment (peak concentration of 7.07 mg L^{-1}). However, a statistical interaction effect between the "time" and "mixing" factors ($p < 0.001$, Table 1) as the difference between mixed and non-mixed treatments was not systematic during the first 40 days. Additionally, EMn productivity was also assessed for both treatments. It appeared that EMn productivity was not temporally stable and varied between a negative value up to 0.86 mg L^{-1} d^{-1}. Non-mixed cultures showed around 50% more productivity on average (0.86 ± 0.03 mg L^{-1} d^{-1}) than mixed cultures (0.43 ± 0.15 mg L^{-1} d^{-1}) (Fig. 3B). Moreover, EMn productivity was affected not only by the "time" factor, but also by the "mixing" condition without interaction effect between both factors (Table 1). Additionally, it was found that the bacterial density was not affected by the presence of mixing (Table 1). However, a significant decrease in bacterial density was observed for both treatments between week 4 and 8 to maintain stable values below 6 x 10^7 cell mL^{-1}.

3.2 Effect of silica concentration on extracellular marennine (EMn) production

We observed that low concentrations of silica in the medium affected negatively the EMn production in semi-continuous cultures of *H. ostrearia* (Fig. 4). An interaction effect between "time" and "concentration" (Table 2) were obtained with lower values when silica concentration was 30 µg L^{-1}. Higher and similar results were observed for all others silica concentrations between 45 to 75 µg L^{-1} (Fig. 4).

3.3 Influence of the type of light on extracellular marennine (EMn) production

Results showed that no effect was found between LED and tube-light types on the production of EMn in the semi-continuous system (Fig. 5). A "time" factor affected the EMn production (Table 3). Thus, LED lights resulted in similar EMn production compared to that of fluorescent tubes.

3.4 Differences between Haslea *species and strains on blue pigment production*

Results showed that species or strains of *Haslea* could differ in marennine and marennine-like pigment (MLP) productivity (Table 4, Fig. 6). Time productivity affected also EMn concentration obtained, without interaction effect (Table 4). The *Haslea* species from Cancun showed lower EMn production compared to all *H. ostrearia* strains originating from different areas. These strains showing similar EMn concentrations up to 14 mg L^{-1} in the 30-L flat bottom PBRs in semi-continuous operation without mixing (Fig. 6).

3.5 Effect of temperature and light on extracellular marennine (EMn) degradation

In this experiment, the effect of temperature and the presence of light (200 μmol photons m^{-2} s^{-1}) on degradation of EMn was investigated. It was found that both the temperature and light significantly affect EMn degradation without interaction between both factors (Table 5, Fig. 7). Important depletion in EMn concentration over time was observed when this pigment was stored at 20 °C in the presence of light (treatment A). Similar pattern was observed for 20 °C treatment in dark condition until 60 d of storage. After this period, the degradation of EMn was lower in dark condition. However, storage in cold (4 °C) and dark condition preserved EMn concentrate over more than 150 d, as only 11% of degradation have been observed during

293 experimentation (Fig. 7).

294

295 **4. Discussion**

296 The various biological activities displayed by EMn have increased the interest towards
297 it as a possible probiotic compound for aquaculture [8,9,13,25]. For potential industrial
298 applications, it has therefore become important to determine the most efficient method to
299 culture this diatom species. In the present study, a long-term semi-continuous system was
300 applied to different species and strains of blue *Haslea* to assess feasibility for the application of
301 this microalgae, particularly with regards to EMn produced, to the aquaculture industry.

302 Our results demonstrated that the production of EMn in a 10 L PBR with semi-
303 continuous operation is significantly influenced by the presence of mixing. At each medium
304 renewal period (14 d) and at the end of semi-continuous culture, EMn concentration measured
305 when no mixing was approximately 1-2x higher than that of the culture with mixing (Fig. 3). A
306 similar tendency was also displayed in EMn productivity over >155 d of experiment. These
307 results suggest that a better marennine production would be achieved in a culture without
308 mixing. Bacterial development in this semi-continuous system were rapidly stable after some
309 weeks without effect of mixing conditions. Furthermore, contrary to study of Fuentes et al. [37]
310 using other microalgae species, bacteria seem not to affect marennine productivity as the
311 bacterial density decrease observed between week 4 to 8 was not related to marennine
312 productivity changes. Thus, for the rest of the study, bacterial density was not considered as
313 problematic in this kind of semi-continuous PBR system. The obtained EMn productivity was
314 comparable to a previous study by Prasetiya et al. [12]. A lower EMn production in the culture
315 system was possibly related to the biology of *H. ostrearia*. Indeed, this pennate diatom species
316 has been considered a tychopelagic organism, with EMn supposedly mainly produced during
317 the benthic phase according to Robert [38], mostly based on observations made in oyster ponds,

318 closed environments almost similar to large-scale batch cultures. In natural open environments,
319 however, large blooms of blue *Haslea* and concomitant pigment releases have been observed,
320 in Corsica (France), Tasmania (A ustralia), North-Carolina (USA). *H. ostrearia* can form
321 biofilm on the bottom of culture flasks or the oyster ponds, which allows them to bloom and
322 release EMn as in batch mode [9,39]. Therefore, the presence of mixing presumably alters the
323 formation of *Haslea's* biofilm, which eventually perturbs EMn production. However, recent
324 study by Nghiem Xuan et al. [20] showed interesting EMn productivity in air-lift PBR,
325 suggesting that EMn could be produced adequately without bottom biofilm development.
326 Furthermore, *H. ostrearia* culture in semi-continuous mode in exponential phase of growth also
327 demonstrated the great importance of light level for EMn production [12]. Thus, the present
328 study may support the fact that omitting mixing in a semi-continuous culture system for a long
329 period of time is not a requirement condition for *H. ostrearia* cultivation, and that it can
330 minimize the cost and also the use of energy, which could be an advantage from a sustainability
331 point of view.

332 Apart from mixing, the growth and production of valuable compounds in microalgae,
333 like marennine as discussed in Gastineau et al. [9], can be influenced by the medium
334 composition [40–42]. Our results reveal that different concentrations of silica in the medium
335 with semi-continuous operation affected EMn production. Indeed, silica has a significant role
336 in limiting the growth of marine diatoms, including *H. ostrearia* [23]. Our study indicated that
337 the addition of silica over 45 μg L^{-1} resulted in EMn concentration higher than obtained when
338 using 30 μg L^{-1}. The highest EMn concentration was observed at the end of experiment (75
339 days, [EMn] = 8.7 mg L^{-1}). This EMn concentration was still lower than a previous study
340 conducted by Nghiem Xuan et al. [20], where the highest EMn concentration is ~16 mg L^{-1}.
341 Nevertheless, this concentration was obtained in a batch culture of *H. ostrearia* with the
342 concentration of silica in similar medium (F/2) was around 72 μg L^{-1}.

In the present study, we also investigated the type of light on EMn production in 30 L PBRs. Our results showed that the type of light from both LED mixing blue and white color (1:2) and fluorescent tubes did not affect EMn production. At a similar light intensity (200 µmol photons m^{-2} s^{-1}), the mean of EMn concentrations after 43 days was 10.8 and 10.5 mg L^{-1} in the LED and fluorescent semi-continuous PBR, respectively. This is in line with a previous study also using a semi-continuous system, with light intensity ranging from 100 to 500 µmol photons m^{-2} s^{-1} provided by fluorescent tubes, which demonstrated that EMn production increased with irradiance [12]. A previous study from Alego and Synder [43] revealed a similar trend to our results, where these authors showed no significant difference between the use of LED and fluorescent tubes on the growth of diatom *Chaetoceros calcitrans* as well as their chlorophyll production. As from the sustainability point of view, LEDs have lower operational costs than that of fluorescent tubes, with less energy waste as heat for equivalent light energy production (about 50% of the energy, according to the retailer's technical specifications between the two technologies). Therefore, the utilization of LEDs for *H. ostrearia* (or possibly other *Haslea* species) to produce marennine could be a promising strategy. If the type of light sources seems less crucial on EMn production than the light intensity, the quality of light spectrum provided, however, could have a more significant effect. Indeed, an enhancement of EMn production has been showed when *H. ostrearia* was cultured under blue light in comparison with any other light quality, the increase being more pronounced at limiting irradiance [44].

Until now, all studies dealing with marennine production have been realised on *H. ostrearia* collected from the same area in the North Atlantic Coast of France. Until now, no EMn productivity comparison between strains or species from different areas in the world have been realized. Recent works on blue *Haslea* using scanning electron microscopy (SEM) observation and molecular approaches have revealed an unsuspected biodiversity of this genus, illustrated by the description of several new species, *H. karadagensis* [25] detected in the Black

Sea, *H. provincialis* [26] in the Mediterranean Sea, and *H. nusantara* from the Southern hemisphere [27]. Gastineau et al. [9] also stated one undescribed species yet in the Canary Islands (*H. silbo sp*. ined.), and more are to be described. Furthermore, it has been shown that *H. ostrearia* cell size, which varies according to the life cycle as for other pennate diatoms (e.g., [10]), has also an influence on EMn production [44]. Our results revealed that the majority of clones or strains of Haslea showed very similar productivity pattern during the 91 d experiment. Difference was observed only with the *Haslea sp*. strain sampled in Cancun with lower EMn productivity in the culture conditions tested. Differences between species could be related to the temperature used. Absence of differences in EMn produced by *H. ostrearia* was observed between the strain CBB2 and San Juan, and also for pigment produced by *H. provincialis*, all strains collected in temperate waters, the Atlantic Coast of France, the Pacific Coast of USA, and the Mediterranean Sea, respectively. The strain from Cancun corresponds to the undescribed *Haslea sp*. that is phylogenetically close to *H. silbo sp*. ined., which thermal optimum is undoubtedly higher than that of other strains. Several studies have shown that the geographic locations or localities and also seasonal variations could affect the chemical composition and the production of bioactive compounds in algae [45,46]. However, in our study if the thermal optimum is respected, the EMn productivity seem not too much related to strain origin.

In this study, we determined the effect of the presence of light and temperature on EMn concentration to obtain the best method to preserve marennine extracted from cell culture, called blue water [31]. Our results revealed that the highest percentage of degradation in EMn concentration was observed at 20°C in the presence of light, where approximately 80% of the initial EMn concentration was reduced over 155 days of storage. In contrast, a low temperature combined with the absence of light reduced ca. 18% of the initial EMn concentration only. This result suggests that marennine should be stored at low temperatures in the absence of light.

Although no direct role for photosynthesis has been demonstrated for marennine, this observation is in line with the previous studies made on other natural pigments in photoautotroph organisms, which can be affected by room temperature and the presence of light [47–49]. For instance, the presence of light and temperature can significantly reduce the quality of several photopigments such as chlorophyll, anthocyanin, fucoxanthin and phycocyanin, demonstrating that most of these biomolecules are sensitive to one or both factors.

5. Conclusion

The present study demonstrates that semi-continuous system using flat panel PBR could be used over 6 months to produce up to 14 mg L^{-1} of extracellular marennine. Mixing, silicate concentration and light system have been optimized to decrease productivity costs. Furthermore, our results present remarkable stability for EMn productivity between *Haslea* strain or species sampled in different temperate areas when culture was maintained at 20°C. However, for more tropical strains, like species sampled in Cancun, this culture condition does not allow such high productivity. Moreover, EMn preservation method should be carefully considered in order to obtain the maximum benefit of EMn. All these results represent valuable information to develop the production and the storage of marennine or marennine-like pigments, which are necessary steps before any commercial operation of a blue *Haslea* and its pigment.

Statement of informed consent

No conflicts, informed consent, or human or animal rights are applicable to this study.

Author contributions

The conception and design of this study was performed by F. S. Prasetiya, M. Foret and R.

Tremblay. The acquisition and interpretation of data was conducted by F. S. Prasetiya, M. Foret, R. Gastineau and R. Tremblay. F. S. Prasetiya drafted the article. R. Tremblay, J. L. Mouget, J. S. Deschênes, and R. Gastineau reviewed it for significant intellectual content. R. Tremblay, J. L. Mouget, J. S. Deschênes, and R. Gastineau approved the final version of the article. R. Tremblay and J. L. Mouget were responsible for the funding acquisition.

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628 **Tables**

629

630 **Table 1**. Results of the two-way analysis of variance (ANOVA) on the interactive effects of
631 "time" and "mixing" on the marennine (EMn) concentration (mg L^{-1}), EMn productivity (mg
632 L^{-1} d^{-1}) and bacterial density (cell mL^{-1}). Significant differences at $P < 0.05$ are in bold and
633 indicated by *. DF = degrees of freedom, SS = Sum-of-squares, MS = Mean squares, F = F-ratio, P =
634 p-values.

Effect	DF	SS	MS	F	P
EMn concentration (mg L^{-1})					
Time	39	769.402	19.728	24.400	**<0.001***
Mixing	1	403.921	403.921	499.570	**<0.001***
Time x Mixing	39	105.391	2.702	3.342	**<0.001***
Residual	160	129.366	0.809		
EMn productivity (mg L^{-1} d^{-1})					
Time	31	771.365	24.883	2.892	**<0.001***
Mixing	1	287.964	287.964	33.472	**<0.001***
Time x Mixing	31	65.219	2.104	0.245	1.000
Residual	175	1505.525	8.603		
Bacterial density (cell mL^{-1})					
Time	7	4.634e+013	6.620e+013	6.494	**<0.0001***
Mixing	1	1.581e+013	1.581e+013	1.550	0.222
Time x Mixing	7	6.888e+013	9.840e+012	0.965	0.473
Residual	32	3.262e+014	1.019e+013		

635

636

637 **Table 2**. Results of the two-way analysis of variance (ANOVA) on the interactive effects of
638 "time" and "silicate" on the marennine (EMn) concentration (mg L^{-1}). Significant differences
639 at $P < 0.05$ are in bold and indicated by *. DF = degrees of freedom, SS = Sum-of-squares, MS =
640 Mean squares, F = F-ratio, P = p-values.

Effect	DF	SS	MS	F	P
Time	12	6.787	0.566	19.648	**<0.001***
Silicate	3	8.217	2.739	95.156	**<0.001***
Time x Silicate	36	2.422	0.0673	2.337	**0.003***
Residual	52	1.497	0.0288		

641

642

643 **Table 3**. Results of the two-way analysis of variance (ANOVA) on the interactive effects of
644 "time" and "light" on the marennine (EMn) concentration (mg L^{-1}). Significant differences at
645 $P < 0.05$ are in bold and indicated by *. DF = degrees of freedom, SS = Sum-of-squares, MS =
646 Mean squares, F = F-ratio, P = p-values.

Effect	**DF**	**SS**	**MS**	**F**	**P**
Time	13	649.618	49.971	44.798	**<0.001***
Light	1	0.410	0.410	0.368	0.547
Time x Light	13	16.629	1.279	1.147	0.342
Residual	56	62.466	1.115		

647

648 **Table 4**. Results of the two-way analysis of variance (ANOVA) on the interactive effects of
649 "time" and "strain" (or species) on the marennine (EMn) concentration (mg L^{-1}). Significant
650 differences at $P < 0.05$ are in bold and indicated by *. DF = degrees of freedom, SS = Sum-of-
651 squares, MS = Mean squares, F = F-ratio, P = p-values.

Effect	**DF**	**SS**	**MS**	**F**	**P**
Time	12	1786.054	148.838	31.479	**<0.001***
Strain	3	350.628	116.876	24.719	**<0.001***
Time x Strain	36	199.45	5.54	1.172	0.342
Residual	104	491.728	4.728		

652

653 **Table 5**. Results of the two-way analysis of variance (ANOVA) on the interactive effects of
654 "time" and "treatment" on the marennine (EMn) concentration (mg L^{-1}). Significant
655 differences at $P < 0.05$ are in bold and indicated by *. DF = degrees of freedom, SS = Sum-of-
656 squares, MS = Mean squares, F = F-ratio, P = p-values.

Effect	**DF**	**SS**	**MS**	**F**	**P**
Time	19	71.035	3.739	31.479	**<0.001***
Treatment	4	381.442	95.36	24.719	**<0.001***
Time x Treatment	36	199.45	5.54	1.215	0.44
Residual	175	42.748	0.244		

657

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Figure caption

Figure 1. Schematic design of 10 L photobioreactors (PBRs) to identify the effect of mixing on marennine (EMn) production by *Haslea ostrearia*.

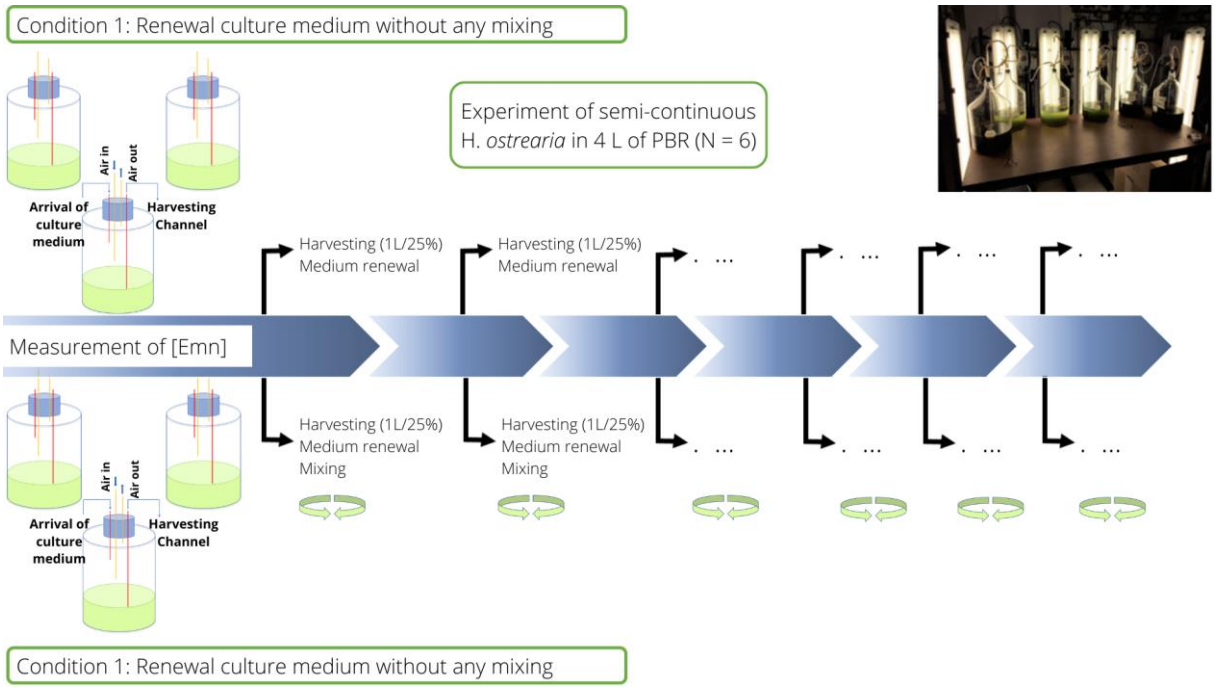
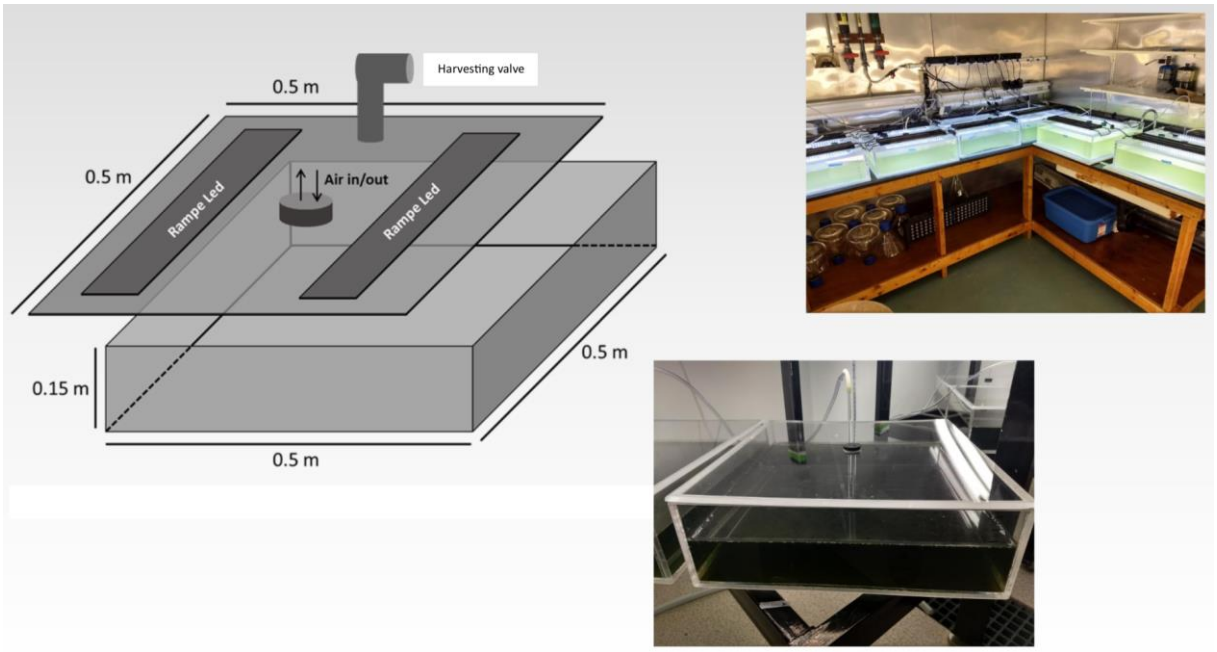
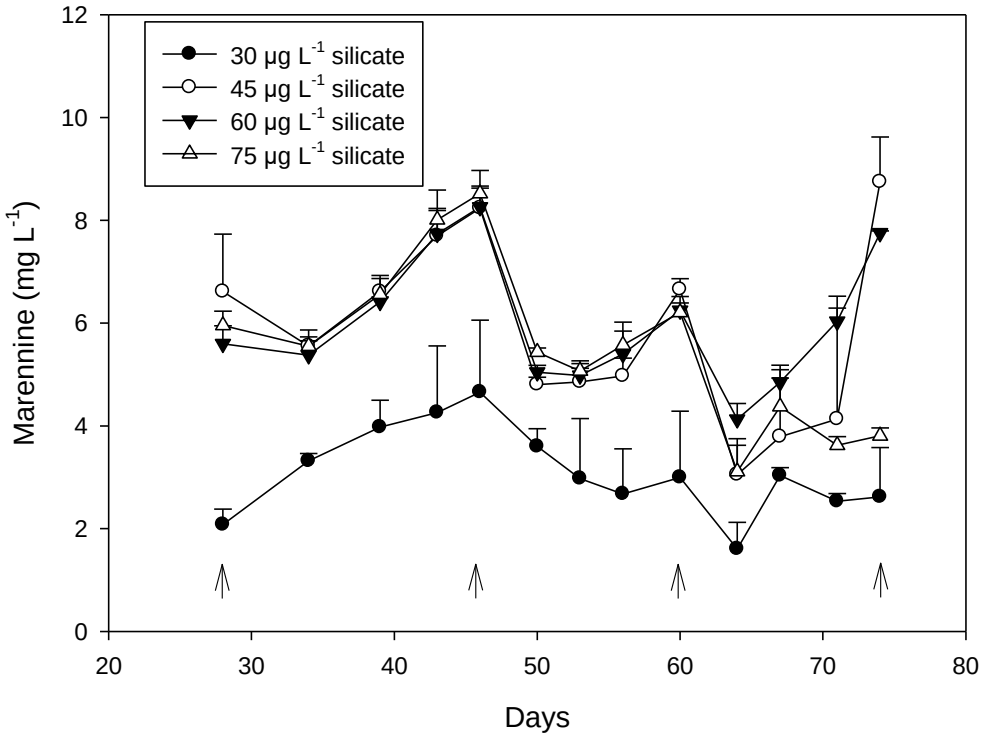


Figure 2. Scheme of prototype of 30 L photobioreactor (PBR) semi-continuous system for experiments 3 and 4.



671 **Figure 3.** Extracellular Marennine (EMn) accumulated concentration (A) and productivity (B)
672 in a semi-continuous system with 25% of culture volume harvested each two weeks in mixed
673 and non-mixed photobioreactor (PBR). Arrows indicate harvest time realized after the measure
674 of marennine concentration. Values are means ± Standard Error (n = 3). See Table 1 for
675 statistical results showing the interaction between time and mixing treatment for marennine
676 concentration and the individual time and mixing treatment effect for the productivity.
677
678 **Figure 4.** Extracellular Marennine (EMn) concentration (mg L^{-1}) accumulated in semi-
679 continuous system in relation to different silicate concentrations (30 – 75 µg L^{-1}) with 25% of
680 culture volume harvested when concentration was over 6 mg L^{-1}. Arrows indicate harvest time
681 realized after the measure of marennine concentration. Data points are mean ± Standard Error
682 (n = 3). See Table 2 for statistical results showing the interaction between time and silicate
683 concentration on marennine concentration.
684



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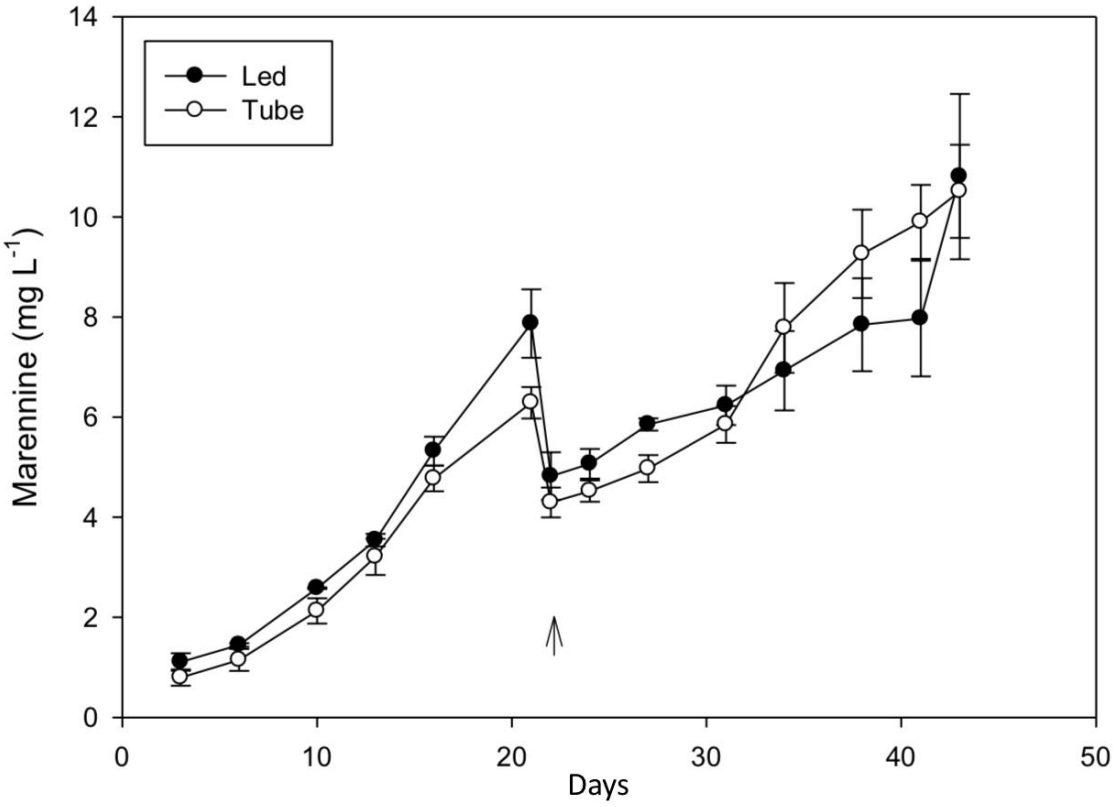
687 **Figure 5.** Extracellular Marennine (EMn) concentration (mg L^{-1}) accumulated in semi-
688 continuous system in relation to different light systems with 25% of culture volume harvested
689 when concentration was over 6 mg L^{-1}. Arrow indicate harvest time realized after the measure
690 of marennine concentration. Data points are mean ± Standard Error (n = 3). See Table 3 for
691 statistical results showing only the effect of time on marennine concentration.
692



693
694

695 **Figure 6.** Extracellular Marennine (EMn) concentration (mg L^{-1}) accumulated in a semi-
696 continuous system in relation to different *Haslea* strains and species, with 25% of culture
697 volume harvested when concentration was over 10 mg L^{-1}. Data points are mean ± Standard
698 Error (n = 3). See Table 4 for statistical results showing the effect of time, then the effect of
699 strains or species on marennine concentration.

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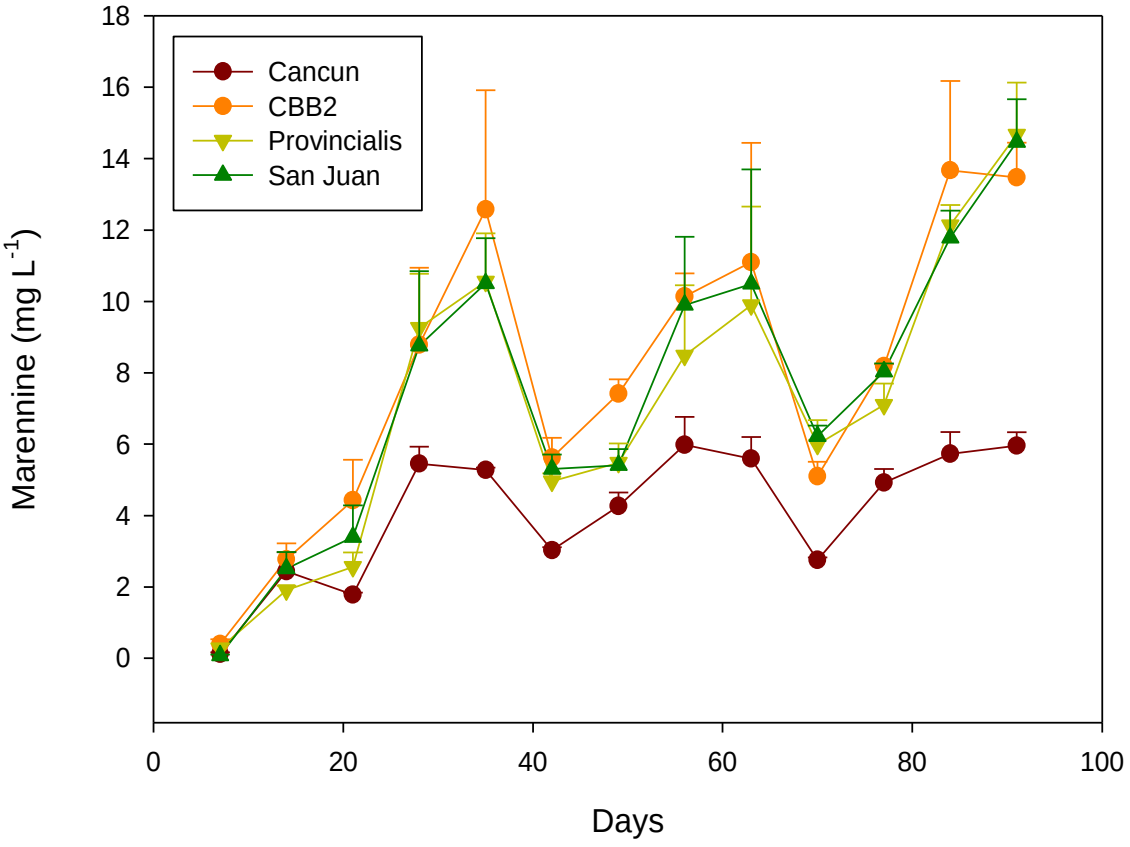

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702

703 **Figure 7.** Evolution of extracellular marennine (EMn) degradation with time in relation to
704 temperature and light exposure (4 and 20 °C, absence and presence of ambient light,
705 respectively) during 155 d of storage. Data points are mean ± Standard Error (n = 3). See Table
706 5 for statistical results showing the effect of time, then the treatment on marennine
707 concentration.

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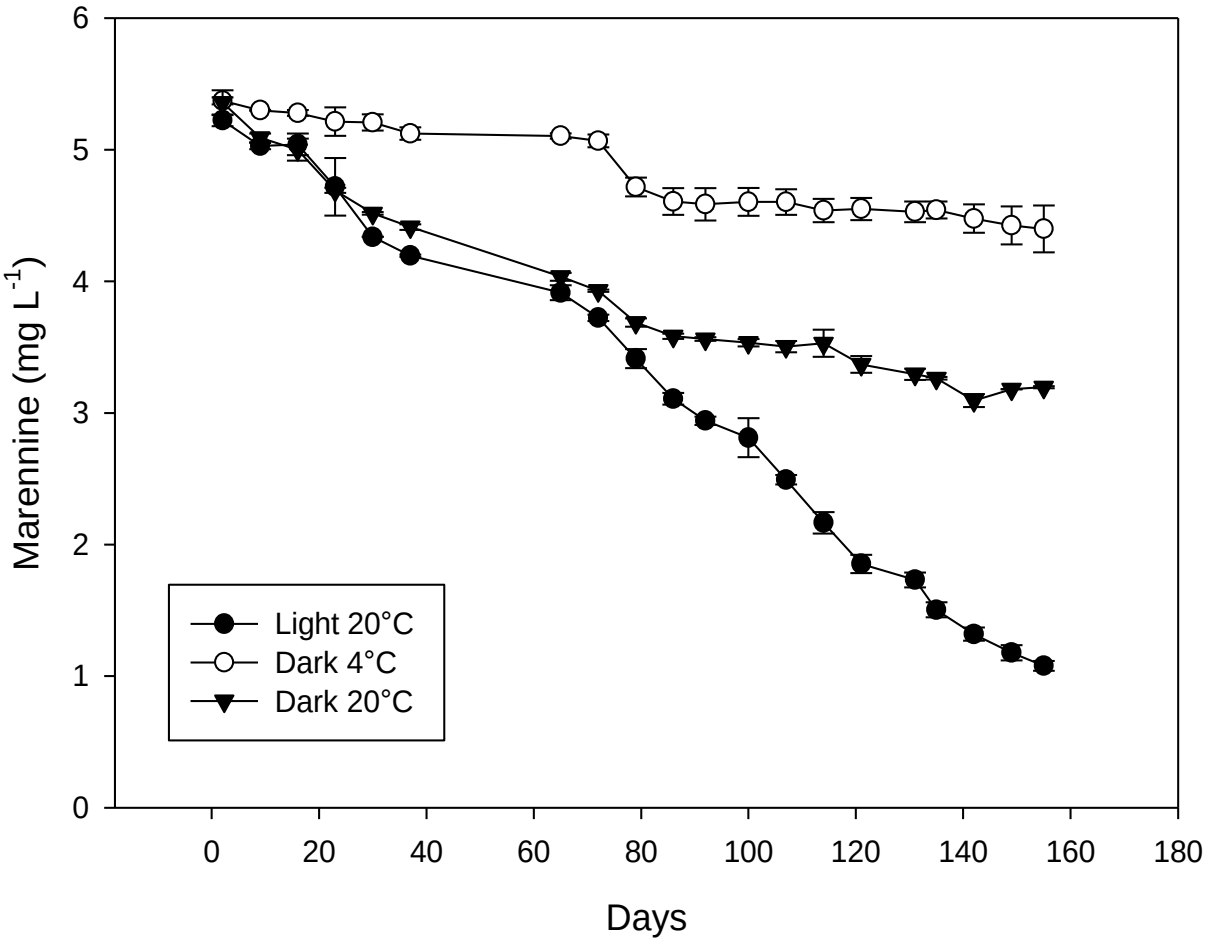

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