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Jaouen

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# Design of an artificial culture medium to optimize *Haslea ostrearia* biomass and marennine production

R. Nghiem Xuan<sup>a,c</sup>, I. Safitri<sup>d</sup>, J.L. Mouget<sup>a,b</sup>, J. Pruvost<sup>c,\*</sup>, V. Turpin<sup>a</sup>, P. Jaouen<sup>c</sup>

<sup>a</sup> Université de Nantes, MMS, EA 2160, Faculté des Sciences et des Techniques, 2 Rue de la Houssinière, BP, 92208, 44322, Nantes Cedex, France

<sup>b</sup> MMS (Mer Molécules Santé), Le Mans Université, Ave O. Messiaen, 72085, Le Mans, France

<sup>c</sup> GEPEA, Université de Nantes, CNRS, UMR6144, Bd de l'Université, CRTT, BP 406, 44602, Saint-Nazaire Cedex, France

<sup>d</sup> Marine Science Department, Faculty of Mathematics and Natural Sciences, Universitas Tanjungpura, 78124, Pontianak, Indonesia

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## A B S T R A C T

### Keywords:

*Haslea ostrearia*  
Marennine  
Culture medium  
Microalgae  
Photobioreactor

The diatom *Haslea ostrearia* was first studied by Gaillon in the year 1820 because of the greening phenomenon of oysters in western France. This microalga has the capacity to produce and excrete a blue pigment, called marennine, that has antioxidant, anti-bacterial and anti-viral properties, with possible industrial applications related to aquaculture and cosmetics. However, it is difficult to produce biomass in large concentrations in photobioreactors (*i.e.*, usually  $1 \text{ kg m}^{-3}$  dry weight or higher) due to stirring sensitivity, and also because diatoms have special requirements, such as a supply of silica. This work presents a design for a new, completely artificial, seawater medium named NX (*i.e.*, Nghiem Xuan) for optimizing cultivation of the diatom *H. ostrearia* in photobioreactors. NX takes account of the carbon and phosphorus sources in either organic or inorganic form, and the composition of the main elements (C, H, O, N, P, S, Si). Optimization of the calcium, magnesium and iron concentrations was found to be essential in order to achieve the highest productivities. The resulting NX medium was validated in an airlift photobioreactor. This led to productivities of  $4.9 \times 10^8 \text{ cell L}^{-1}$  (*ca.*  $500 \text{ mg L}^{-1}$  of biomass) and  $15.7 \text{ mg L}^{-1}$  of marennine, higher than has previously been reported in the literature.

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## 1. Introduction

Microalgae are increasingly becoming the subject of investigation for several industrial purposes. They have the potential to produce molecules for pharmacology, cosmetology, food, bio-based chemicals and biofuels [1]. Over 15,000 valuable chemically-determined compounds, such as antioxidants, carotenoids, fatty acids, polymers, peptides, enzymes, toxins and sterols [2], are produced by all the microalgae that have been identified to date, but only a few species are currently produced on an industrial scale: *Haematococcus* (Chlorophyceae), for example, which is produced for astaxanthin [3], and *Chlorella* (Chlorophyceae) and *Arthrospira platensis* (Cyanophyceae), which are produced worldwide for food and nutraceutical purposes [1,4,5]. Regarding diatoms, which represent the largest group of microalgae [6], applications mainly concern aquaculture [7]. Except for some species like *Phaeodactylum tricorutum*, which are already produced on a large scale [8], one of the biggest challenges is the difficulty in setting up a controlled and optimized large-scale production.

Issues linked to scaling up the cultivation of a given strain usually relate to the lack of information regarding growth and metabolite

production conditions, specific nutrient needs and responses to light, but also the need to establish adequate tools and methods for controlled and optimized scaling-up of production. This is generally supported by bioprocess engineering approaches, such as the design of culture systems, *i.e.*, photobioreactor engineering [9], but in some cases, a specific growth medium has to be specially designed to fulfill conditions of intensified production, which results in larger biomass concentrations (usually  $1 \text{ kg m}^{-3}$  dry-weight or higher). The composition of the medium used in flask culture must be adapted and chemical dissolution stability can be impaired due to the need for larger nutrient concentrations.

*H. ostrearia* is a marine diatom that has long been known [10] and mainly studied at laboratory scale. This diatom has the capacity to produce and excrete a blue pigment named marennine that has industrial potential [11]. There are two forms of marennine, which differ in their spectral characteristics: the intracellular marennine (IMn) synthesized in the cell, and the extracellular marennine (EMn) excreted in the culture medium [12]. In a culture, the relative abundance of the two forms was found to depend on the medium composition variability, and on abiotic and biotic factors [13–15].

The Mer-Molécules-Santé (MMS) laboratory has a long experience in culturing *H. ostrearia* at laboratory scale, and in recent decades hundreds of strains have been maintained for years in culture at Le Mans University and in the Nantes Culture Collection (NCC), using enriched natural seawater media, such as Guillard F/2 [16], or Provasoli-based medium ES1/3 modified by Robert [14]. *Haslea* strains have also been grown in artificial seawater (ASW), e.g. the diatom artificial medium (DAM) specifically designed for *Haslea* culture [17] (not used due to its composition complexity and cost), or the ASW proposed by Berges et al. [18], modified according to Mouget et al. [19], which results in production as good as with natural seawater.

Culturing *H. ostrearia* using conventional photobioreactors (PBRs) is a challenge, so specific PBRs have been implemented, such as immersed membrane PBRs and agar immobilization PBRs [20,21]. In both cases, the yield of excreted marennine (EMn) per cell remains low (around  $25 \text{ mg } 10^9 \text{ cell}^{-1} \text{ d}^{-1}$  for immersed membrane PBRs and  $5 \text{ mg } 10^9 \text{ cell}^{-1} \text{ d}^{-1}$  for agar immobilization PBRs). Despite some outdoor semi-pilot attempts [22], the scaling-up of production is still an objective for industrial applications.

Our work aims to develop an optimized medium for both biomass and EMn production in PBRs. A biomass concentration of  $1 \text{ g L}^{-1}$  in dry weight is targeted, based on the use of ES1/3, which has been identified as the best medium for EMn production [14]. The newly-designed artificial seawater culture medium NX (i.e. Nghiem Xuan) was finally tested in a continuous airlift PBR, and revealed the highest EMn yield for a conventional PBR in the published literature.

## 2. Materials and methods

### 2.1. Organism and culture maintenance

A strain of the diatom *H. ostrearia* (NCC 495) was obtained from the Nantes culture collection of the MMS laboratory and cultivated in natural seawater from Le Croisic (Loire-Atlantique, France), enriched with Guillard F/2 medium. The salinity was fixed at 28 PSU. Cultures of *H. ostrearia* were maintained in 250 mL Erlenmeyer flasks containing 100 mL medium with 10 % dilution after 4–5 days of culture. Light was supplied by white fluorescent tubes (F36W/840 T8) across the day/night cycle 14 h/10 h at  $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$  inside the culture chamber (SANYO MLR-350) at  $16^\circ \text{C}$ .

### 2.2. Culture media tested

Table 1 shows the composition of all the culture media that were compared for this study. All these media were made from natural seawater provided by the Croisic Océarium (Loire Atlantique), except the ASW medium [19], which is an artificial seawater. Seawater was filtered at  $0.7 \mu\text{m}$  then  $0.45 \mu\text{m}$  before being used as a medium. The medium was then sterilized in an autoclave at  $121^\circ \text{C}$  for 20 min. The average elemental composition of this water was estimated from a previous measurement of different seawater composition (data not shown).

The modified Provasoli ES1/3 medium is a natural, enriched seawater not supplied with copper, unlike Guillard F/2 medium, which is specifically for diatom culture [16]. The Conway natural seawater medium is a medium that is commonly used for marine microalgae culture [23].

Because of the precipitation of sodium bicarbonate with phosphate and silica sources during autoclave sterilization, these elements were added after the sterilization step by filtration at  $0.2 \mu\text{m}$  under a laminar flow hood.

### 2.3. Macro and micro nutrient concentration tests

The concentration of nutrients were tested in flat culture flasks of 50 ml volume (flasks for cell culture tissue, VWR collection, France).

**Table 1**

Elemental content comparison between the culture media tested.

Ions	Culture media composition ( $\text{mol L}^{-1}$ )				
	F/2 (Natural seawater)	ES1/3 (Natural seawater)	ASW (Artificial seawater)	Conway (Natural seawater)	25N100P (Natural seawater)
B	$4.2 \times 10^{-4}$	$4.5 \times 10^{-4}$	$3.7 \times 10^{-4}$	$5.4 \times 10^{-1}$	$4.5 \times 10^{-4}$
Br	$8.4 \times 10^{-4}$	$8.4 \times 10^{-4}$	$7.3 \times 10^{-4}$	$8.4 \times 10^{-4}$	$8.4 \times 10^{-4}$
C	$1.4 \times 10^{-4}$	$1.4 \times 10^{-4}$	$2.1 \times 10^{-3}$	$1.5 \times 10^{-2}$	$5.8 \times 10^{-2}$
Ca	$1.0 \times 10^{-2}$	$1.0 \times 10^{-2}$	$9.4 \times 10^{-3}$	$1.0 \times 10^{-2}$	$1.0 \times 10^{-2}$
Cl	$5.5 \times 10^{-1}$	1.0	$4.7 \times 10^{-1}$	$5.6 \times 10^{-1}$	1.0
Co	$4.2 \times 10^{-8}$	$5.8 \times 10^{-8}$	$5.8 \times 10^{-8}$	$8.4 \times 10^{-5}$	$5.8 \times 10^{-8}$
Cu	$3.9 \times 10^{-8}$	–	$3.9 \times 10^{-8}$	$8.0 \times 10^{-8}$	–
F	$6.8 \times 10^{-5}$	$6.8 \times 10^{-5}$	$6.7 \times 10^{-5}$	$6.8 \times 10^{-5}$	$6.8 \times 10^{-5}$
Fe	$1.2 \times 10^{-5}$	$6.3 \times 10^{-6}$	$6.6 \times 10^{-6}$	$4.8 \times 10^{-3}$	$6.3 \times 10^{-6}$
K	$1.0 \times 10^{-2}$	$1.0 \times 10^{-2}$	$8.8 \times 10^{-3}$	$1.0 \times 10^{-2}$	$1.0 \times 10^{-2}$
Mg	$5.3 \times 10^{-2}$	$5.3 \times 10^{-2}$	$4.1 \times 10^{-2}$	$5.3 \times 10^{-2}$	$5.3 \times 10^{-2}$
Mn	$9.1 \times 10^{-7}$	$2.3 \times 10^{-6}$	$2.4 \times 10^{-6}$	$1.8 \times 10^{-3}$	$2.3 \times 10^{-6}$
Mo	$5.4 \times 10^{-8}$	–	$6.6 \times 10^{-9}$	$5.1 \times 10^{-5}$	–
N	$8.8 \times 10^{-4}$	$3.0 \times 10^{-4}$	$5.5 \times 10^{-4}$	3.5	$1.1 \times 10^{-2}$
Na	$4.7 \times 10^{-1}$	$4.8 \times 10^{-1}$	$4.2 \times 10^{-1}$	$4.7 \times 10^{-1}$	$4.8 \times 10^{-1}$
Ni	–	–	$6.3 \times 10^{-9}$	–	–
P	$3.6 \times 10^{-5}$	$1.6 \times 10^{-5}$	$2.2 \times 10^{-5}$	$3.9 \times 10^{-1}$	$1.2 \times 10^{-3}$
S	$2.8 \times 10^{-2}$	$4.3 \times 10^{-2}$	$2.5 \times 10^{-2}$	$2.8 \times 10^{-2}$	$4.3 \times 10^{-2}$
Se	–	–	$1.0 \times 10^{-9}$	–	–
Si	$2.0 \times 10^{-4}$	$1.0 \times 10^{-4}$	$1.1 \times 10^{-4}$	$2.0 \times 10^{-4}$	$1.2 \times 10^{-3}$
Sr	$9.0 \times 10^{-5}$	$9.0 \times 10^{-5}$	$8.2 \times 10^{-5}$	$9.0 \times 10^{-5}$	$9.0 \times 10^{-5}$
Zn	$1.7 \times 10^{-7}$	$3.0 \times 10^{-7}$	$2.5 \times 10^{-7}$	$1.5 \times 10^{-4}$	$3.0 \times 10^{-7}$

The flat surface allows better illumination and light propagation conditions than in conventional Erlenmeyer flasks (i.e. curved surface). They can, therefore, be considered as a useful alternative for rapid but reliable screening of culture conditions, such as the impact of various nutrient concentrations on cell productivity.

Nutrients were divided into two categories, namely macronutrients (C, Ca, Cl, K, Mg, N, Na, P, S, Si), which represent the main elements involved in cells, macromolecules synthesis, and micronutrients, which are usually added at small concentration in the medium (B, Br, Co, Cu, F, Fe, Mn, Mo, Ni, Se, Sr, Zn). It is worth noting that the respective impact of each nutrients is difficult to distinguish, as each addition can involve a counter-ion and may modify the equilibrium of the medium, producing micro-precipitation, which may prevent the cells from accessing the nutrient [16,24].

To avoid chemical contamination with endogenous macro and micronutrient concentrations from the inoculum, the biomass was centrifuged at  $10,500g$  (Hettich Mikro 22R C1110, Marshal scientific, United states) for 10 min and the supernatant was removed. The cells were then rinsed with a sterile solution of  $28 \text{ g L}^{-1}$  NaCl at pH 7.8, prepared beforehand, and re-suspended in it to reach a mean cell density of  $3.6 \times 10^5 \text{ cell mL}^{-1}$ . This concentrated culture enabled starting tests in flasks at  $10^4 \text{ cell mL}^{-1}$  with  $500 \mu\text{L}$  inoculum, to prevent dilution of the medium composition. Tests were carried out under the same conditions as the inoculum culture (SANYO MLR-350 culture chamber, Richmond Scientific, UK). However, due to the large variation of irradiance distribution inside the culture chamber, flasks were placed horizontally in column stacks as shown in Fig. 1 to obtain similar light intensity on each flask.

The cell density was measured using a Nageotte counting chamber (Marienfeld, AQRA, India) and extracellular marennine was measured by spectrophotometry, as described by Pouvreau et al. [12].

### 2.4. General approach for investigating mixotrophic and autotrophic growth

The role of organic and inorganic carbon and phosphorus sources was investigated in particular. This consisted of cultivating *H. ostrearia* cells with different combinations of sodium acetate ( $\text{C}_2\text{H}_3\text{NaO}_2$  for organic carbon form), sodium bicarbonate ( $\text{NaHCO}_3$  for inorganic carbon



Fig. 1. Culture chamber SANYO MLR-350 (left) and culture flask positions inside the culture chamber (right).

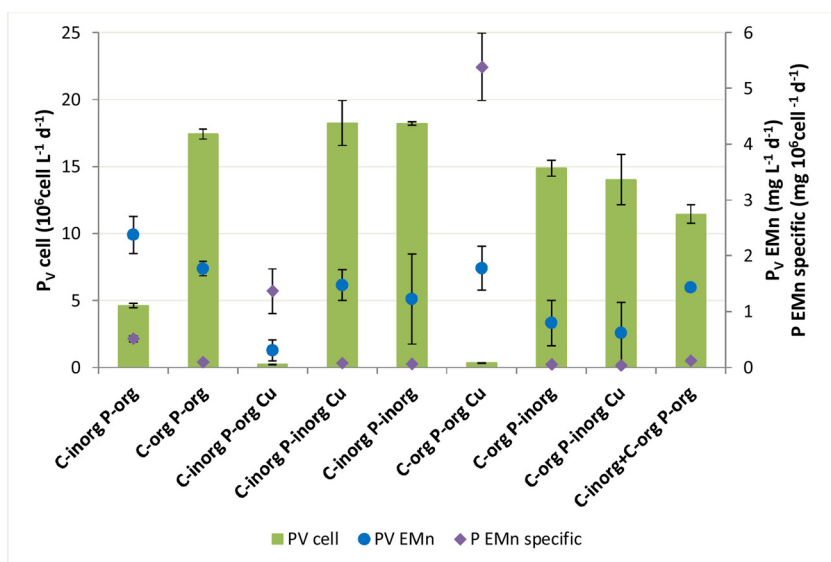


Fig. 2. Cell, Extracellular Marennine (EMn) and specific EMn productivity of *Haslea ostrearia* in batch culture after 7 days in natural seawater-enriched (ES 1/3) based medium with different sources of phosphorus and carbon. P EMn specific and Pv are, respectively, the specific EMn productivity and the volumetric productivity. Data is mean  $\pm$  95 % CI for n = 3.

form),  $\text{Na}_2\text{-}\beta$ -glycerophosphate (for organic phosphorus form) and sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$  for inorganic form) in the same molar concentrations as in the modified ES1/3 culture medium. The aim of this experiment (Fig. 2) was to check the capacity of *H. ostrearia* for growth under autotrophic or mixotrophic conditions and to determine which phosphorus and carbon forms are preferred for producing biomass and/or EMn.

Special attention was then given to investigating the effect of copper (Cu) on growth. Copper is an important micronutrient for the growth of microorganisms, such as microalgae, fungi and bacteria [25]. It can be lethal, however, in high concentrations. In addition, heterotrophic metabolism is highly sensitive to Cu concentrations because it can damage the respiratory chain (in *Escherichia coli*), according to Domek *et al.* [26,27]. The Cu is used by enzymes to drive diverse structures and biochemical reactions. The intracellular Cu used in the respiratory chain and the process of moving from  $\text{Cu}^+$  to  $\text{Cu}^{2+}$  allows the production of free hydroxyl radicals. These reactive species are responsible for the degradation of proteins, nucleic acids, lipids, etc, and the death of the microorganism at high copper concentrations [28]. The same phenomenon is observed for microalgae, with an impact on respiration and photosynthesis [29]. However, in the case of microalgae,

the respiration process is known to be more sensitive than photosynthesis to copper [30].

The presence or absence of Cu in the tested medium can, thus, reveal which metabolism the cell is forced to use, depending on the medium composition (photosynthesis or respiration process). As shown in Table 1, F/2 and ASW, the media possess the same amount of added Cu.

## 2.5. Material for assessing the new medium and validation of productivity

Validation of the growth medium on *H. ostrearia* growth and marennine productivity was carried out in a lab-scale airlift photobioreactor. This flat-panel PBR has been used for microalgae strain characterization, as described [23,31,32]. The volume was 1 L and the depth 0.03 m ( $a_{\text{Light}} = 33.3 \text{ m}^{-1}$ ). Mixing was done by gas injection at the bottom of the PBR. Temperature was regulated by cool air blowing into the stainless-steel back, and the pH was controlled by  $\text{CO}_2$  injection, supplied by an automated regulating valve.

**Table 2**

Preliminary results of biomass and Extracellular Marennine (EMn) productivities in different media. Data is mean  $\pm$  95 % CI for n = 3.

Medium	F/2	ES1/3	ASW	Conway
<b>P<sub>v</sub> Biomass</b> (10 <sup>6</sup> cell L <sup>-1</sup> d <sup>-1</sup> )	19.30 $\pm$ 11.02	4.38 $\pm$ 0.22	11.00 $\pm$ 1.74	0
<b>P<sub>specific</sub> EMn</b> (mg 10 <sup>6</sup> cell <sup>-1</sup> d <sup>-1</sup> )	0.26 $\pm$ 0.12	0.46 $\pm$ 0.10	0.27 $\pm$ 0.02	0

## 2.6. Statistics

The statistics were compiled using XLSTAT 2019 software (Addinsoft). Normality of the distribution law for the different samples was identified by the Shapiro-Wilk test to select the tests (T-test). Statistics was measured with triplicate data for all the experiments presented in this paper.

## 3. Results

### 3.1. Determination of key nutrients responsible for *H. ostrearia* growth

A series of preliminary experiments was conducted to compare *H. ostrearia* growth and EMn production in the different culture media to estimate which nutrient concentrations have the greatest impact. The strain was cultivated in batch conditions in the Sanyo culture chamber, under culture chamber conditions. The results of biomass and EMn productivity depending on culture medium are presented in Table 2.

The Conway medium was too concentrated and precipitated during the culture growth. This resulted in cell death. The three other media resulted in biomass and EMn production. However, the ASW and F/2 media were found to be more suitable for biomass production than the ES1/3 medium. It should be noted that ES1/3 medium was previously identified as the best medium for EMn production, mainly due to its stimulating effect on cells for EMn production rather than growth [14]. This tends to be confirmed by our results, as higher specific EMn productivity was achieved for this medium.

Macronutrient elementary concentrations (nitrogen, phosphorus, carbon, sulfate and silicon) are given in Table 1 for all the growth media. No significant differences were observed except for a lower nitrogen concentration in the ES1/3 medium. In addition, no copper was added to ES1/3, as it was with the F/2, ASW and Conway media, however, differences due to the presence of copper in different seawater supplies cannot be disregarded. Nitrogen concentrations remained proportional to phosphorus concentration in the three media, and a higher concentration of silica was also found in ES1/3 than in the other media. The main difference between the media was the supply of phosphorus, which was organic (Na<sub>2</sub>- $\beta$ -glycerophosphate) for the ES1/3 medium.

### 3.2. Effects of organic and inorganic sources of carbon and phosphorus on *H. ostrearia* growth

The various carbon and phosphorus sources (organic or inorganic, noted as org and inorg respectively), as well as the addition of Cu, were tested in flat flasks (Fig. 2). The composition of the conventional ES1/3 medium (C-inorg P-org) was shown to strongly limit cell productivity with only 4.6  $\times$  10<sup>6</sup> cell L<sup>-1</sup> d<sup>-1</sup>. Biomass productivity was also strongly inhibited when organic P sources were combined with the addition of Cu, with 2.2  $\times$  10<sup>5</sup> and 3.3  $\times$  10<sup>5</sup> cell L<sup>-1</sup> d<sup>-1</sup> for C-inorg P-org Cu and C-org P-org Cu respectively. However, when both P and C sources were in organic form, cell productivity was significantly higher with 17  $\times$  10<sup>6</sup> cell L<sup>-1</sup> d<sup>-1</sup>. Similar high productivity of was also observed when using only inorganic sources, with 18  $\times$  10<sup>6</sup> cell L<sup>-1</sup> d<sup>-1</sup> for both C-inorg P-inorg Cu and C-inorg P-inorg.

Concerning the specific EMn productivity results, carbon and

phosphorus sources also seemed to have a consistent impact. The first apparent observation emphasizes that all the culture media composed of organic phosphorus seemed to stimulate specific EMn production. In contrast, the culture media made with inorganic sources of phosphorus definitely resulted in low levels of specific EMn production. For growth media where photosynthesis was hypothesized to occur (C-inorg P-org Cu and C-org P-org Cu), low biomass with no significant differences (T.Test p = 0.424) and high specific EMn productivity was obtained, with respectively 1.37 and 5.38 mg 10<sup>6</sup> cell<sup>-1</sup> d<sup>-1</sup>. However, the medium (C-inorg P-org Cu) induced lower EMn productivity than (C-org P-org Cu), with respectively 0.30 and 1.78 mg L<sup>-1</sup> d<sup>-1</sup>. Comparing similar medium compositions with and without copper, the presence of copper was found to stimulate EMn synthesis in the presence of organic carbon, whereas the opposite was found with inorganic carbon, with EMn production significantly lower (T.Test p = 0.008).

These results lead to the assumption that *H. ostrearia* has difficulty consuming inorganic carbon (photosynthesis) in the presence of an organic source of phosphorus, and may, therefore, favor the organic source of carbon for growth, but this hypothesis cannot be demonstrated without further studies.

Fig. 3 presents a detailed analysis of the influence of copper and P organic sources. For a better clarity, each result was normalized against its control. P-org C-inorg and P-org C-org experiments results have been normalized against the same treatment with inorganic phosphorus (P-inorg C-inorg and P-inorg C-org). Concerning the Cu experimental results, these have been normalized against the same treatment without Copper addition (C-inorg P-org, C-inorg P-inorg, C-org P-org, C-org P-inorg). This emphasized the significant impact that organic phosphorus has on biomass productivity. Cell productivity lost 74.6 % vs the control with an inorganic carbon source, compared to cell productivity with an inorganic phosphorus medium, whereas, in the presence of organic carbon, the culture had 17.1 % higher productivity with organic phosphorus than with inorganic. As a result, it can be concluded that *H. ostrearia* presents significantly better growth in either a completely organic or inorganic medium (in terms of carbon and phosphorus), and presents significant difficulties with growth on organic phosphorus with inorganic carbon. Additionally, the organic phosphorus allows *H. ostrearia* to achieve a gain of 94.0 % and 122.9 % in EMn productivity when combined with an inorganic and an organic source of carbon, respectively.

Regarding EMn production, two typical media can be considered. C-inorg P-org medium offers a final EMn concentration of 18.0  $\pm$  0.2 mg L<sup>-1</sup> compared to 11.1  $\pm$  3.3 mg L<sup>-1</sup> for the C-inorg P-inorg medium. For the former, a small quantity of biomass was produced whilst producing a large quantity of EMn, whereas for the latter, a large amount of biomass was produced but with a small EMn specific productivity.

Concerning the impact of Cu on biomass and EMn productivity, Fig. 3 confirms the previous hypothesis on completely inorganic medium. Copper does not significantly impact biomass productivity when an inorganic P source is used (C-inorg P-inorg and C-org P-inorg) in contrast with an organic P source (C-inorg P-org and C-org P-org).

Regarding marennine production, the correlation of the presence of copper with the resulting productivity is unclear. Copper was found to only stimulate EMn productivity for a completely inorganic medium (carbon and phosphorus) with 20.4 % more EMn. However, the ES1/3 medium induced a loss of 87.2 % EMn productivity when copper was

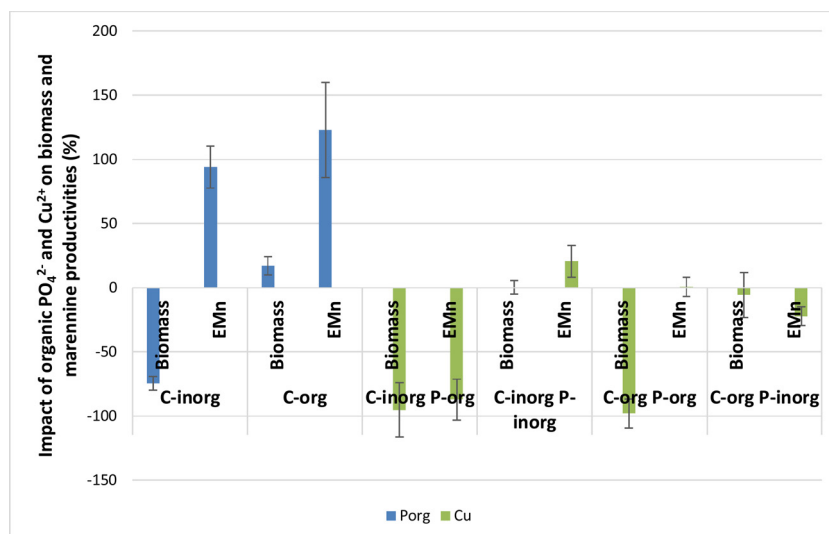


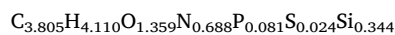
Fig. 3. Impact of organic phosphorus and copper on marennine and biomass productivities according to carbon and phosphorus sources in ES1/3 based medium. Data is mean  $\pm$  95 % CI for n = 3.

added, whereas (C-org P-org Cu) medium showed a constant specific EMn production (Figs. 2 and 3).

Because organic sources were not found to be mandatory for *H. ostrearia* growth, a fully mineral medium was retained as a basis for the remaining part of this study. At large scale, a medium such as this will indeed limit bacterial contamination.

### 3.3. Determination of inorganic C and P source concentration in natural seawater culture medium

A stoichiometry analysis based on biomass elemental composition (C, H O, N, P, S, Si) has been carried out with the aim of defining a medium that allows a biomass concentration of  $1 \text{ g L}^{-1}$  to be achieved without macronutrient limitation [33,34]. Failing to obtain significant biomass for analysis, the elemental composition of *H. ostrearia* has been estimated based on the elemental composition of *Chlorella vulgaris*, which was grown separately for the purpose (data not shown). Contrary to the *Chlorophyceae*, *H. ostrearia* is a member of the *Bacillariophyceae* and needs silicon for frustule synthesis. According to Turpin et al. [35], the macronutrient mass ratio C:N:P:Si, for *H. ostrearia* growth is 106:16:1:16. The cell, therefore, needs the same mass of silica as nitrogen. Based on this result, and the molecular weight of Si ( $28 \text{ g mol}^{-1}$ ) and N ( $14 \text{ g mol}^{-1}$ ), the following molar elemental composition formula of *H. ostrearia* is proposed:



The biomass molecular weight ( $M_{\text{biomass}} = 24.72 \text{ g mol}^{-1}$ ) and this formula allow us to theoretically determine the C, N, P, S, Si concentration needed to produce  $1 \text{ g L}^{-1}$  of biomass (for example, for  $1 \text{ g}$  of biomass, *H. ostrearia* needs  $7 \text{ mmol}$  of nitrogen). Based on this analysis, phosphorus and carbon concentrations in the ES1/3 medium were found to be underestimated compared to the nitrogen and silica available in the medium. The composition of the medium was, therefore, adjusted to propose a composition more equilibrated in N and P. By applying a 1.5 coefficient to ensure  $1 \text{ g L}^{-1}$  biomass was achieved without limitation, this led to 25-fold and 100-fold increases in N and P, respectively (noted 25N100 P) (Table 1).

In the first instance, micronutrient concentrations were considered to be in excess in the medium, and their original concentrations as defined in the ES 1/3 medium were, therefore, kept, but in completely inorganic source. However, the silica concentration (Table 1) was found to be impossible to achieve without the precipitation phenomenon. According to Fournier and Rowe [36], amorphous silica solubility is restricted to  $100\text{--}150 \text{ mg L}^{-1}$  ( $1.7\text{--}2.7 \text{ M}$ ) at  $25^\circ\text{C}$ , which could decrease to  $1\text{--}7 \text{ mg L}^{-1}$  ( $1.7 \times 10^{-5}$  to  $1.2 \times 10^{-4} \text{ M}$ ) with the presence of calcium, aluminium and iron cations. However, it may increase with dissolved inorganic matter. Because of this very low solubility, it was

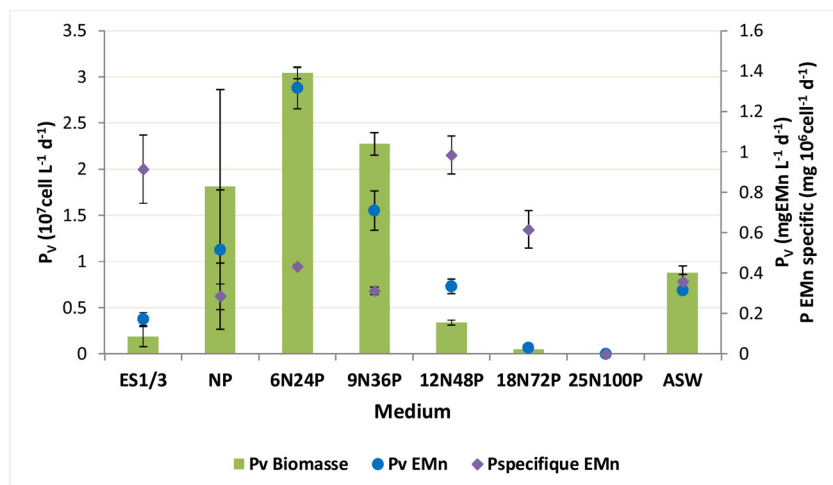


Fig. 4. Cell, EMn and specific EMn volumic productivities of *Haslea ostrearia* for various media (ES 1/3, artificial seawater (ASW) and ES 1/3 based medium with completely inorganic C and P (NP)). P EMn specific and Pv are, respectively, the specific EMn productivity and the volumetric productivity. Data is mean  $\pm$  95 % CI for n = 3.

decided to supply silica separately to the photobioreactor experiments.

A new set of experiments in flat flasks was designed to validate the new media proposed (Fig. 4), namely NP medium (which corresponds to a fully mineral medium but with the same N and P molar concentration as the previous ES1/3 medium shown in Table 1, but obtained from natural seawater with various enrichments in N and P up to 25N100P). Note that the period of growth for this experiment did not exceed 8 days.

According to Fig. 4, the NP medium was found to be much more efficient than ES1/3 in terms of both biomass and EMn. Although the ES1/3 medium allowed the best EMn specific productivity (Figs. 2 and 3), 6N24P allowed higher EMn concentration with  $1.32 \text{ mg L}^{-1}$  compared to  $0.17 \text{ mg L}^{-1}$  for ES1/3. The ASW medium allowed cell growth higher than the ES1/3 medium but was still inferior to NP, with respectively  $0.9 \times 10^7 \text{ cell L}^{-1} \text{ d}^{-1}$  and  $1.8 \times 10^7 \text{ cell L}^{-1} \text{ d}^{-1}$ .

The progressive increase in concentration of N and P resulted in an increase of biomass up to the 6N24P enrichment. This medium composition showed the best biomass and EMn production, of respectively  $3.0 \times 10^7 \text{ cell L}^{-1} \text{ d}^{-1}$  and  $1.3 \text{ mg L}^{-1} \text{ d}^{-1}$ . With higher enrichment (9N36P and more), therefore, a significant decrease in EMn and biomass productivity was observed, and the 18N72P medium ultimately led to cell death. These results could be explained by the silica and carbon concentration, which was the same for all media but not increased proportionally. The rationale was that Si concentration limited the strain growth at  $3.0 \times 10^7 \text{ cell L}^{-1} \text{ d}^{-1}$  (see below). A significant precipitation was observed from the 12N48P concentrations. Note that  $3.0 \times 10^7 \text{ cell L}^{-1} \text{ d}^{-1}$  also represents the maximum value of biomass production that is currently reported in the literature [19]. Medium stability could, thus, explain the actual low *H. ostrearia* biomass production, as precipitates are not biologically available for cell growth.

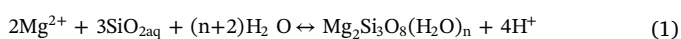
The 6N24P medium, which led to the best productivity, was used as a basis for further investigation of *H. ostrearia* macronutrient needs in PBR (see final section).

### 3.4. Component interaction and medium precipitation

Nguyen *et al.* [37,38] explained the precipitation/flocculation phenomenon of a seawater-based medium by the interaction between high concentrations of  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$  and  $\text{Mg}^{2+}$  ions. By using Visual MINTEQ V3 software (KTH, Sweden), which enables prediction of dissolution equilibrium, the percentage of chemical species of macronutrients (N, P, Si, C) was investigated for the 25N100P medium at different pH levels and at a fixed temperature ( $22^\circ\text{C}$ ) (Fig. 5).

Dissolution equilibrium predictions confirmed the interaction of the  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$  and  $\text{Mg}^{2+}$  ions. The seawater endogenous ions,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , were found to react with the macronutrient enrichment ions in the culture medium. In addition, the extent of precipitation increased with pH value. The magnesium precipitated with around 0.8 % of carbon and 20 % of phosphorus, as well as with the calcium with 0.4 % of carbon and 3.4 % of phosphorus at pH 7.8. Phosphorus precipitation with calcium and magnesium was emphasized, with an important loss of 23.35 % of the phosphate even at pH 7.8. Note that no significant precipitation was predicted with the nitrogen source.

Unfortunately, silica interaction could not be predicted with Visual MINTEQ, as the software was set up for freshwater analysis. However, silica interaction in seawater is a common matter for diatom culture and has been studied by Vollast *et al.* [39]. Their results confirm the precipitation of hydrated magnesium silica, called synthetic sepiolite, by the addition of metasilicate in seawater (Eq. (1)):



This equilibrated equation finds the solubility of amorphous silica to be 115 ppm [40] with the possibility of precipitation of other nutrients dependent on variations in temperature and pH.

The analysis shows that the precipitation phenomenon in natural

seawater could represent a significant limitation for *H. ostrearia* cultivation due to the high content of both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , as it limits medium enrichment and, therefore, the achievable biomass concentration and productivity. Reducing calcium and magnesium, as a possible solution to prevent precipitation, was investigated by designing an artificial medium.

### 3.5. Study of the impact of micronutrients on biomass and marennine production in artificial seawater medium

Based on the previous study (Fig. 4), it was assumed that a medium composition of nitrogen and phosphorus higher than 6N24P (inorganic) could be limited by silica concentration. The 6N24P (inorganic) composition was, therefore, chosen for micronutrient addition tests to prevent Si limitation during growth in artificial seawater. The design for the experiment, which consisted of a large set of 180 flat flasks (Fig. 6), provided useful information on the importance of each micronutrient for the optimization of both biomass and EMn production in artificial medium conditions.

The first objective was to decrease the magnesium and calcium concentration to reduce the occurrence of precipitation/flocculation, which was found to lead to culture death. The best productivity was obtained at a cell density of  $35.0 \pm 2.2 \times 10^6 \text{ cell L}^{-1} \text{ d}^{-1}$ , with a calcium concentration of ca.  $1.0 \times 10^{-3} \text{ M}$ , which is 9.4 times less than in the ASW medium. The best productivity for magnesium was found at  $6.4 \pm 0.4 \times 10^6 \text{ cell L}^{-1} \text{ d}^{-1}$  for a magnesium concentration of  $5.3 \times 10^{-2} \text{ M}$ , which is the same concentration as the ASW medium composition.

The second objective was to increase micronutrient concentration to ensure a high productivity with increasing concentrations of nitrogen, phosphorus and silica, thus, preventing growth limitation. The only micronutrient that led to the highest biomass productivity of  $25.8 \pm 4.0 \times 10^6 \text{ cell L}^{-1} \text{ d}^{-1}$  was iron, with a concentration of  $1.2 \times 10^{-4} \text{ M}$ . This concentration represents 10 times that in the ASW medium, and biomass productivity increased 5-fold. Zinc also seemed to increase biomass and EMn production when increased 100-fold, compared to ASW. In addition, 10 times less boron than in ASW increased biomass productivity, reaching  $6.9 \pm 0.9 \times 10^6 \text{ cell L}^{-1} \text{ d}^{-1}$ . Finally, a 10-fold increase in nickel nutrient ( $3.4 \times 10^{-8} \text{ M}$ ) led to higher EMn and biomass productivity, with  $0.61 \pm 0.04 \text{ mg L}^{-1} \text{ d}^{-1}$  and  $7.3 \pm 0.4 \times 10^6 \text{ cell L}^{-1} \text{ d}^{-1}$ , respectively. *H. ostrearia* seemed to tolerate the ASW range of concentrations for the other nutrients: bromine, cobalt, copper, fluorine, magnesium (as previously presented), manganese, molybdenum, potassium, sulfur, selenium and strontium (T.Test with  $p > 0.05$ ). We can note that conclusions were similar for both biomass and EMn, except for the molybdenum test, which seemed to show an inverse correlation. This was not investigated further in the present work.

These results enabled the design of the new artificial seawater NX medium based on the highest productivities previously obtained (Fig. 6).

### 3.6. Validation of the artificial seawater NX medium in a photobioreactor

Following verification that there was no visual precipitation, three recipes for culture medium were tested in a 1 L Airlift photobioreactor. The same conditions of light ( $300 \mu\text{mol m}^{-2}\text{s}^{-1}$ ), temperature ( $22^\circ\text{C}$ ), pH (7.8) and cell inoculation concentration were applied. However, to definitely prevent silica and carbonate precipitation in the artificial medium, the concentration of carbon and silicon added was equilibrated with the nitrogen and phosphorus concentration following the same approach based on stoichiometric growth, leading to 25N100P6Si media with a 10 mM of dissolved carbon concentration maintained constant for the entire culture duration with  $\text{CO}_2$  addition via the gas phase. Fig. 7 presents the results in terms of cell density and EMn concentration for batch cultures of 22 days.

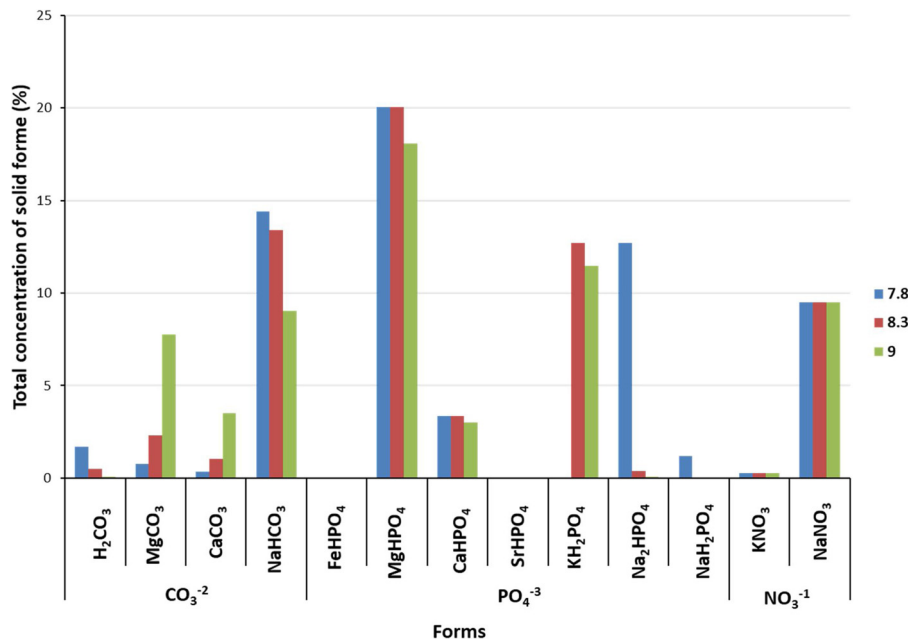


Fig. 5. Simulation of precipitated molecules (%) in the 25N100 P medium for 3 pH values (Visual MINTEQ software).

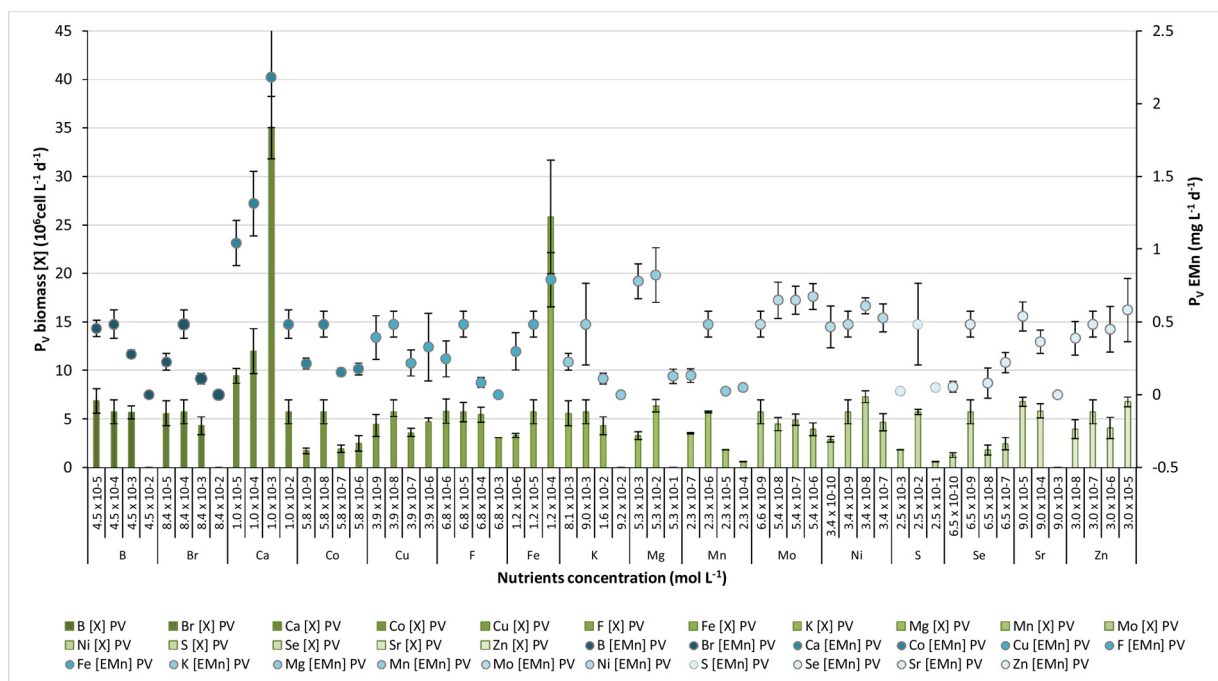


Fig. 6. Biomass [X] (bars) and EMn (circles) volumetric productivities (Pv) according to different macro and micro nutrient concentrations in artificial seawater. Data is mean  $\pm$  95 % CI for  $n = 3$ .

The three batches represented in Figs. 7 and 8 reveal the efficiency of the new artificial seawater culture medium NX. In terms of biomass, the ES1/3 medium limited cell concentrations at  $7.9 \times 10^7$  cell  $L^{-1}$ , whereas in the NX medium cultures reached  $4.9 \times 10^8$  cell  $L^{-1}$  (a 6.2-fold increase). The culture in natural seawater 25N100P6Si quickly decreased after 4 days, containing a whitish mineral precipitation. In terms of extracellular marennine, ES1/3 medium reached  $8.6$  mg  $L^{-1}$ , while NX allowed the culture to reach  $15.7$  mg  $L^{-1}$ . Both NX-25N100P6Si and natural seawater 25N100P6Si media received  $1.4$  g  $L^{-1}$  of sodium bicarbonate,  $1.2 \times 10^{-3}$  M of silica and  $1.2 \times 10^{-3}$  M of phosphorus at the beginning of the culture. As a result, better stability was observed for the artificial seawater NX than for natural

seawater, with negligible precipitation phenomena under the same conditions.

#### 4. Discussion

Our results demonstrate that *H. ostrearia* needs either a completely organic or completely inorganic medium to achieve the highest biomass and EMn concentrations. Using an organic medium could be an opportunity to cultivate *H. ostrearia* and to decrease the risk of culture medium precipitation, for example. According to Andersen [16],  $Na_2\text{-}\beta$ -glycerophosphate from Provasoli ES medium [41] could be used as a substrate by bacteria because of their heterotrophic nature. *H. ostrearia*



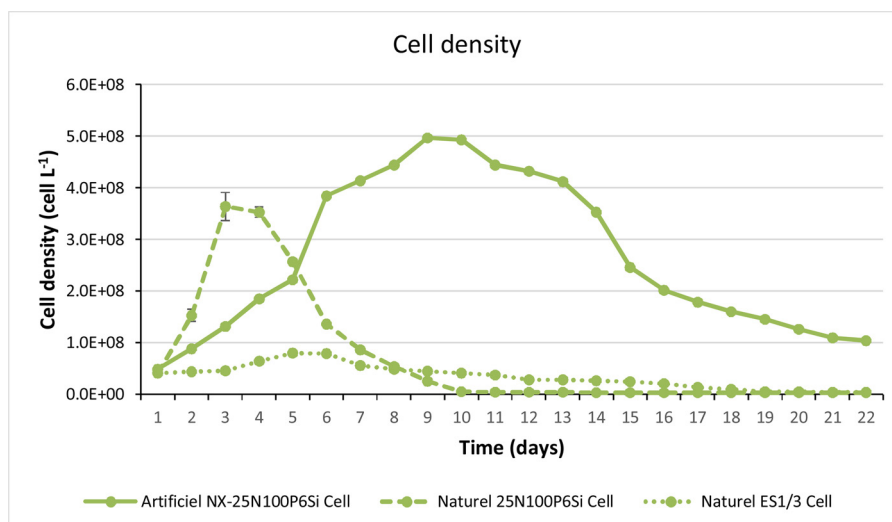


Fig. 7. Comparison of *Haslea ostrearia* cell density culture in new NX-25N100P6Si medium with two natural seawater-based media. Data is mean  $\pm$  95 % CI for n = 3.

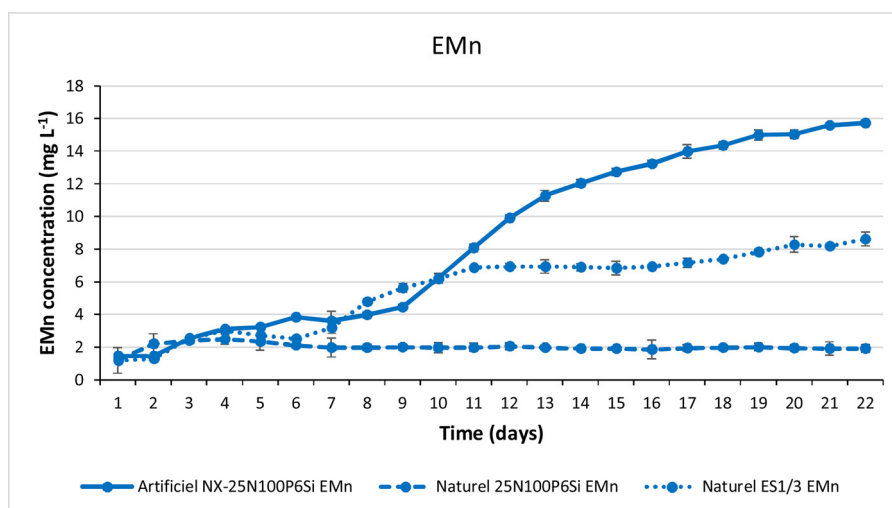


Fig. 8. Comparison of EMn concentration from *Haslea ostrearia* culture in new NX-25N100P6Si medium with two natural seawater-based media. Data is mean  $\pm$  95 % CI for n = 3.

has formerly been suspected of being a mixotrophic strain, able to use different (organic and inorganic) forms of nutrients [42]. However, no proof has been brought to affirm the mixotrophic nor the heterotrophic growth of the strain for EMn production purpose, although an organic carbon source could also be used by *H. ostrearia*, being found in its natural growing environment [45]. The results from this study indicate that the culture of *H. ostrearia* in a medium made with organic phosphorus and inorganic carbon may hypothetically influence *H. ostrearia* growth (Fig. 2) but also EMn production per cell (Fig. 2). Both media containing organic phosphorus and carbon sources, as well as inorganic phosphorus and carbon sources, lead to important yield of EMn and biomass. This could indicate a mixotrophic metabolism, which is known to provide a better yield of energy production than autotrophic metabolism [43,44], increasing then the biomass yield and the synthesis of complex compounds, such as marennine. Note that in addition to modifying the overall physiology of *H. ostrearia*, using an organic medium could increase the production costs.

If it is assumed that Cu could inhibit respiration and favor photosynthesis process, the hypothesis from the results (Figs. 2 and 3) is that *H. ostrearia* could display heterotrophy or mixotrophy in the presence of organic phosphorus. EMn overproduction could, therefore, be the result

of mixotrophy/heterotrophy metabolism or of a stress resulting from the organic source of phosphorus in the medium. The impact of copper on marennine production has already been observed by Minier et al. [46], who showed that copper toxicity varies according to the physiological stage of the microalgae, especially in relation to EMn synthesis. Marennine production could, therefore, play an important role in copper bio-availability in oyster ponds. It remains difficult to argue whether *H. ostrearia* is able to consume organic matter or not, although the presence of organic carbon seems to positively stimulate its growth. However, this study confirms the relevant impact of the phosphorus source on this species. *H. ostrearia* is able to produce significant biomass with completely organic or completely inorganic sources of carbon and phosphorus. However, under these conditions, *H. ostrearia* seems to favor the production of biomass rather than marennine.

With a mineral medium, the stoichiometric approach based on the elementary composition of biomass (CHONPSSi method) allowed the macronutrient composition to be equilibrated, which sustained high *H. ostrearia* growth. Although the ES1/3 medium showed higher specific EMn productivity ( $0.51 \pm 0.04 \text{ mg } 10^7 \text{ cell}^{-1} \text{ d}^{-1}$ ) than with the inorganic medium ( $0.07 \pm 0.01 \text{ mg } 10^7 \text{ cell}^{-1} \text{ d}^{-1}$ ) that was studied, the equilibrated medium 6N24 P was found to produce more biomass and

EMn ( $3.04 \pm 0.04 \times 10^7$  cell L<sup>-1</sup> d<sup>-1</sup> and  $1.31 \pm 0.07$  mg L<sup>-1</sup> d<sup>-1</sup>, respectively). However, in the case of this inorganic medium, a severe precipitation phenomenon was observed, which revealed significant limitation for scaling up *H. ostrearia* cultivation and confirmed possible interaction of the endogenous Ca<sup>2+</sup> and Mg<sup>2+</sup> ions from seawater with added PO<sub>4</sub><sup>2-</sup>, HCO<sub>3</sub><sup>-</sup> and SiO<sub>3</sub><sup>2-</sup> from the enrichment solution.

In order to increase the concentration of different nutrient sources in the medium while preventing precipitation, a new artificial medium adapted to *H. ostrearia* was designed. Following a detailed study of the nutrient impact on both biomass and EMn productivity, calcium concentration was found to be decreased 10-fold ( $1.0 \times 10^{-3}$  M), leading to higher productivities ( $35.0 \pm 2.2 \times 10^6$  cell L<sup>-1</sup> d<sup>-1</sup> and  $2.2 \pm 0.2$  mg<sub>EMn</sub> L<sup>-1</sup> d<sup>-1</sup>). However, a higher decrease in calcium concentration led to lower productivities ( $12.0 \pm 1.6 \times 10^6$  cell L<sup>-1</sup> d<sup>-1</sup> and  $1.3 \pm 0.2$  mg<sub>EMn</sub> L<sup>-1</sup> d<sup>-1</sup>). As a major cation in cell culture with magnesium and sodium, calcium is a required nutrient for eukaryotic cells [35]. Decreasing the calcium concentration does not necessarily mean that less calcium is needed for growth, but it was found to definitely decrease the precipitation phenomenon. However, *H. ostrearia* seems to need magnesium in the same concentration as in ASW ( $5.3 \times 10^{-2}$  M), since decreasing the magnesium concentration led to lower productivity than for ASW Mg concentration (Fig. 6).

Surprisingly, by increasing the iron concentration 10-fold compared to the ASW medium ( $1.2 \times 10^{-4}$  M), a 5-fold increase in biomass productivity was observed ( $25.8 \pm 4.0 \times 10^6$  cell L<sup>-1</sup> d<sup>-1</sup> and  $0.8 \pm 0.1$  mg<sub>EMn</sub> L<sup>-1</sup> d<sup>-1</sup>). This could be explained by the importance of iron in the iron-sulfur cluster and enzymes structure, present in ferredoxin and cytochromes, which are responsible for photosynthesis [34]. However, it should be noted that this iron concentration was the highest tested in this study, since precipitation appeared over  $1.2 \times 10^{-4}$  M. Thus, *H. ostrearia* might tolerate a higher iron concentration.

Zinc testing also showed better productivities when 100 times more zinc was added than in the ASW medium. Zinc plays a vital role in maintaining the integrity of ribosomes, as a cofactor for enzyme and protein folding [34,47], and seems to be a major requirement for *H. ostrearia*.

Nickel also yields better productivities when increased by a factor of 10. However, nickel ion is used as a cofactor by different enzymes, mainly as a substitute for other metals in phytoplankton [34]. These results could also be explained by a lack of other nutrients (Fe, Zn, Mg).

It was also shown that the other micronutrients were present in sufficient concentrations in the ASW medium, and these concentrations were the same in the NX medium. However, compared to other nutrients, increasing molybdenum concentrations showed better EMn production and lower biomass production than in low concentrations. No hypothesis could be confirmed, but according to Merchant and Helmann [34], Mo is responsible for the activity of nitrogenase and some other enzymes as a cofactor, and low concentrations may be stressful for marennine production.

After the new artificial medium NX recipe was designed, its efficiency was tested in batch mode using photobioreactors. NX medium achieved concentrations of  $4.9 \times 10^8$  cell L<sup>-1</sup> ( $\pm 1.1 \times 10^4$ ) and  $15.7 \pm 0.1$  mg L<sup>-1</sup> EMn using a conventional agitated photobioreactor, compared to  $8.0 \times 10^7$  cell L<sup>-1</sup> ( $\pm 8.8 \times 10^3$ ) and  $8.62 \pm 0.04$  mg L<sup>-1</sup> EMn for the ES1/3 medium. Biomass production was, therefore, increased 5.7-fold and marennine yield was almost twice as high. This emphasizes the interest of the NX medium for growing *H. ostrearia*. However, it was observed that *H. ostrearia* started to overproduce EMn when the biomass reached a maximum concentration of  $4.9 \times 10^8$  cell L<sup>-1</sup> (around day 9). This phenomenon could be attributed to cell lysis due to nutrient deficiency/limitation, and merits further investigation. As a benthic diatom, *H. ostrearia* has a particular physiology that could be influenced when maintained in suspension in a PBR, as in this study. Marennine, which presents a significant bioactivity [11], could also inhibit the growth of *H. ostrearia*

cells.

It should be noted that the NX medium did not allow 1 g L<sup>-1</sup> of biomass (equivalent to ca.  $8.0 \times 10^8$  cells L<sup>-1</sup>) to be achieved. Since this limit was obtained through stoichiometric analysis by setting macronutrient requirements, it could be explained by a possible limitation of certain micronutrients. But considering the previously-described design strategy, this would be surprising as they were provided in excess. A particular physiological response could be the explanation: overproduction of EMn only seems to appear from day 9 (Fig. 7), which also corresponds with higher cell concentration. EMn concentration, therefore, only increases when biomass starts to decrease. EMn overproduction or accumulation could be due to cell death, or conversely cell death could be due to EMn overproduction, as EMn is also suspected as presenting an inhibitory effect for growth [11,19,48]. This phenomenon could possibly have occurred here, given the large EMn concentration that was achieved, but investigation of the possible inhibitory effect of EMn on *H. ostrearia* growth was not within the scope of this study and remains to be further investigated.

## 5. Conclusion

An artificial growth medium was designed for the cultivation of *H. ostrearia* in PBRs. It enables the growth of *H. ostrearia* to be stimulated with equilibrated nutrient composition, while preventing the formation of precipitate under normal growth conditions. This allows the largest reported productivity values, to our knowledge, to be achieved for the production of both biomass and marennine.

Additionally, the NX medium combined with the airlift PBR was found to be effective for growing *H. ostrearia* cells in suspension. This would be a great advantage in comparison with immobilized-cell PBRs, which could also be used for growing these benthic species, but would certainly increase the precipitation phenomenon due to low mass transfer without a stirring system. This condition leads to a pH and temperature gradient within the PBR, which is closely linked to the calcium and magnesium precipitation phenomenon [49–51].

The stability of the NX culture medium remains to be optimized, as precipitation occurred at high pH and temperature levels (data not shown). For exploitation under industrial conditions, pH or temperature regulation should be efficient and the medium composition could be simplified by reducing or omitting costly elements. The same procedure (*i.e.*, testing the effect of each micronutrient on growth) as described in this study could be applied for the purpose.

In this study, *H. ostrearia* was able to grow on both carbon and phosphorus organic sources. This could be further investigated, as mixotrophic culture remains a possible solution for optimizing biomass and EMn production, but may also reduce the precipitation phenomenon. Finally, working without natural seawater will facilitate research by preventing precipitates and, therefore, controlling the nutrient intake by cells. Seawater would be preferable on a very large scale (for economic reasons), but would limit biomass and marennine production due to the endogenous calcium and magnesium concentration, unless adequate medium renewal or an enrichment strategy, such as fed-batch, were applied.

## Statement of informed consent

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

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## Author contributions

The conception and design of this study was carried out by R. Nghiem Xuan. The acquisition and interpretation of data was done by R. Nghiem Xuan, I. Safitri, J. L. Mouget, J. Pruvost, V. Turpin and P. Jaouen. R. Nghiem Xuan drafted the article. J. L. Mouget, J. Pruvost, V. Turpin and P. Jaouen reviewed it for significant intellectual content. J. L. Mouget, J. Pruvost, V. Turpin and P. Jaouen approved the final version.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.algal.2019.101653>.

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#### Further reading

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