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**Greening phenomenon in bivalve by marennine
produced from *Haslea ostrearia* and its consequences on
bivalve's integrated response**

Fiddy Semba Prasetya

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Thèse de Doctorat

Fiddy SEMBA PRASETIYA

*Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université du Maine
sous le label de L'Université Nantes Angers Le Mans*

École doctorale : *Végétale Environnement Nutrition Agro-alimentaire Mer (VENAM)*

Discipline : *BIOLOGIE DES ORGANISMES*

Unité de recherche : *MER MOLÉCULE ET SANTÉ (MMS) – EA n°2160, Université du Maine,
UFR Sciences et Techniques, Avenue Olivier Messiaen 72085 Le Mans Cedex 9*

Soutenue le 27 Novembre 2015

Greening phenomenon in bivalve by marennine produced from *Haslea ostrearia* and its consequences on bivalve's integrated response

JURY

Rapporteurs : **Tatiana VALLAEYS**, *professeur*, Université de Montpellier 2
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Titre de thèse

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Title of thesis

Greening phenomenon in bivalve by marennine produced from *Haslea ostrearia* and its consequences on bivalve's integrated response

Résumé

Le but de ce travail de doctorat est d'évaluer la possibilité d'utiliser *Haslea ostrearia* et la marennine en ostréiculture. Les objectifs de ce projet sont : (1) l'évaluation du comportement alimentaire de l'huître creuse *Crassostrea gigas* sur des cellules d'*H. ostrearia* de différentes tailles et les conséquences potentielles sur les populations algales; (2) la caractérisation du verdissement par la marennine et ses conséquences sur la physiologie de *C. gigas*. (3) les conséquences du verdissement sur les traits comportementaux, physiologiques et biochimiques de plusieurs espèces de bivalves; (4) l'utilisation conjuguée d'*H. ostrearia* avec d'autres microalgues d'importance en aquaculture.

Nos résultats suggèrent que la taille des cellules affecte considérablement le processus de sélection d'*H. ostrearia* par l'huître. Cette étude démontre également que la forme extracellulaire de la marennine contribue significativement au verdissement dans les mucocytes des branchies. Mis à part le verdissement des organes palléaux des bivalves, une concentration modérée de marennine (2 mg L⁻¹) affecte les performances comportementales, physiologiques et biochimiques des bivalves. Néanmoins, ces effets pourraient être compensés par ses activités biologiques comme agent antibactérien naturel et source d'alimentation mixte d'algues en conchyliculture.

Mots clés

Haslea ostrearia, marennine, bivalves, conchyliculture

Summary

This Ph.D. thesis focuses on several assessments to achieve the optimum benefit of utilization of marennine in the field of aquaculture. The study covers: (1) the assessment in feeding behavior of the Pacific oyster *Crassostrea gigas* on different sizes of *Haslea ostrearia* and its ecological consequence; (2) the characterization of the greening by marennine and its consequences on some physiological traits of on *C. gigas*. (3) the consequence of greening by marennine on behavioral, physiological and biochemical traits of bivalves; (4) the utilization of *H. ostrearia* and marennine in a combination diet with other microalgae relevant to aquaculture.

Our results suggest that cell size impacts considerably the selection process of *H. ostrearia* by oyster. This study also demonstrates that the extracellular form of marennine contributes significantly to the greening in the mucocytes of the gills. Apart from greening the pallial organs of bivalves, marennine (2 mg L⁻¹) affects the behavioural, physiological and biochemical performance. Nevertheless, these effects can be compensated for its biological activities such as natural antibacterial agent and use as a mixed algal diet for bivalve aquaculture.

Mots clés

Haslea ostrearia, marennine, bivalves, aquaculture

DEDICATIONS

A humble work dedicated to my parents,
The love of my life, Wiwin Natalia and Adrian Semba Prasetya

“The best of people are those who bring most benefit to the rest of mankind.”

- Anonymous -

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RÉSUMÉ

Le phénomène du verdissement des huîtres a été décrit dès le XVIIème siècle. Il a rapidement été démontré que la diatomée *Haslea ostrearia* était responsable de ce phénomène du fait de sa capacité à synthétiser un pigment bleu-vert nommé marennine. Ce phénomène est avantageux pour l'industrie ostréicole française, la coloration verte des branchies des huîtres par la marennine augmentant leur prix de vente. Par ailleurs, il a été suggéré que ce pigment pourrait être utilisé en tant qu'agent antipathogène naturel en éclosion et en ostréiculture, en raison de ses activités antibactériennes et antivirales démontrées *in vitro*. Néanmoins, cette proposition requiert de plus amples investigations qui ont déterminé mon sujet de thèse.

Dans le cadre de mon doctorat, plusieurs études ont été effectuées pour évaluer la possibilité d'utiliser *H. ostrearia* et la marennine en ostréiculture. Ces études ont porté sur : (1) L'évaluation du comportement alimentaire de l'huître creuse *Crassostrea gigas* sur des cellules d'*H. ostrearia* de différentes tailles et les conséquences potentielles sur les populations algales; (2) La caractérisation du verdissement par la marennine et ses conséquences sur la physiologie de *C. gigas*. (3) Les conséquences du verdissement sur les traits comportementaux, physiologiques et biochimiques de plusieurs espèces de bivalves; (4) L'utilisation conjuguée d'*H. ostrearia* avec d'autres microalgues pertinentes en aquaculture dans le régime alimentaire.

Premièrement, nous avons cherché à déterminer si la taille des cellules d'*H. ostrearia* peut affecter leur sélection au niveau des organes palléaux de *C. gigas*. Ce travail a montré que *C. gigas* rejette "préférentiellement" les grandes cellules d'*H. ostrearia*. Comme conséquence, le processus de tri pourrait avoir une certaine importance écologique, à la fois dans les milieux naturels et en aquaculture, en particulier dans les claires. Ainsi, notre étude suggère que le rejet préférentiel des cellules de grande taille dans les pseudofèces pourrait favoriser le développement et / ou la maintenance des sous-

populations de grande taille d'*H. ostrearia*, les cellules rejetées dans les pseudofèces étant douées de reviviscence.

Deuxièmement, des expériences complémentaires ont été effectuées pour mieux comprendre le mécanisme du verdissement ainsi que son influence sur le comportement alimentaire de *C. gigas*. Nos résultats ont montré que le verdissement des organes palléaux d'huîtres a été majoritairement causé par la forme extracellulaire du pigment, tandis que la contribution de la forme intracellulaire ingérée en même temps que les cellules d'*H. ostrearia* reste minoritaire. En outre, le verdissement par la marennine a été identifié dans les mucocytes des branchies, qui semblent être le site de fixation de ce pigment. Enfin, le verdissement peut diminuer de manière significative le taux de filtration et le taux d'ingestion, tandis qu'aucune différence n'a été observée en termes de production de pseudofèces chez l'huître.

Troisièmement, nous avons évalué l'effet de la marennine sur les traits comportementaux, physiologiques et biochimiques de deux espèces de bivalves commercialement importants, la moule bleue *Mytilus edulis* et l'huître américaine *Crassostrea virginica*. Notre étude a montré que la concentration en marennine dans les branchies des deux espèces est positivement corrélée à la concentration de marennine dissoute dans le milieu. Une réponse comportementale a été détectée en présence de la concentration de marennine la plus élevée (2 mg L^{-1}), les deux espèces présentant un degré d'ouverture de valve réduite par rapport aux groupes témoins. La marennine à 2 mg L^{-1} a diminué de manière significative le potentiel de croissance (SFG) de 58% et 85% chez *M. edulis* et *C. virginica* respectivement. Le processus du verdissement a eu chez *M. edulis* un effet sur la concentration en acides gras totaux contenus dans la glande digestive, ce qui suggère que la marennine a un impact sur l'accumulation de réserves d'énergie chez ce bivalve. En ce qui concerne les lipides polaires, le verdissement a augmenté la concentration en acides gras insaturés dans les branchies de *C. virginica*. Cette augmentation peut indiquer un mécanisme de régulation antagoniste des effets de la marennine.

Enfin, nous avons évalué l'impact de l'effet allélopathique d'*H. ostrearia* en co-cultures, de façon à identifier les espèces d'algues résistantes ou sensibles à la marennine

d'être utilisées conjointement en aquaculture. Nos résultats ont montré que l'inhibition de la croissance des microalgues en présence d'*H. ostrearia* et de la marennine variait selon les espèces. *S. costatum*, *C. calcitrans* et *T. lutea* étaient significativement plus sensibles, tandis que *T. suecica* et *P. tricorutum* semblaient être plus résistantes. Cette étude a confirmé que la marennine libérée dans le milieu de culture agit comme un composé allélochimique, pouvant expliquer la domination occasionnelle d'*H. ostrearia* et la diminution de population d'espèces algales sensibles dans les claires, mais également qu'il existe des espèces indifférentes déjà largement utilisées en aquaculture.

Cette étude de doctorat permet de mieux comprendre le mécanisme du verdissement des bivalves causés par la marennine, ainsi que ses conséquences biologiques sur les individus. En outre, notre étude fournit des informations sur l'utilisation potentielle de la marennine comme agent anti pathogène naturel en conchyliculture. Néanmoins, des études plus approfondies devraient être effectuées en particulier sur l'efficacité de ce pigment par rapport aux autres anti pathogènes naturels.

ABSTRACT

Oyster greening phenomenon was firstly described in the seventeenth century and was considered as a natural phenomenon. Nevertheless, recent findings have confirmed that the diatom *Haslea ostrearia* was responsible for the greening as this diatom able to synthesize the blue-green pigment designated as marennine. This phenomenon has been advantageous for the French oyster industry since the green coloration in oysters' pallial organs by marennine can increase their market price. Moreover, apart from marennine' coloring action, it is hypothesized that this pigment could potentially be used as natural antipathogen sources in hatchery and oyster farming, due to their antibacterial and antiviral activities, which has been demonstrated *in vitro* at the laboratory scale. Nonetheless, several assessments need to be conducted to achieve the optimum benefit of utilization of marennine in the field of aquaculture.

In the context of my Ph.D. study, several studies have been conducted to assess the possibility of utilization of *H. ostrearia* and marennine in the field of oyster aquaculture. Thus, this Ph.D. thesis focuses on: (1) The assessment in feeding behavior of the Pacific oyster *Crassostrea gigas* on different sizes of *H. ostrearia* and its ecological consequence; (2) The consequence of greening by marennine on behavioral, physiological and biochemical traits of bivalve; (3) The utilization of *H. ostrearia* and marennine in combination diet with other microalgae relevant to aquaculture. (4) The characterization of the greening by marennine and its consequences on physiological traits of on *C. gigas*.

Firstly, we would like to determine if cell size might affect algal selection on pallial organs of *C. gigas*. This work showed that *C. gigas* selectively rejects larger cells of *H. ostrearia*. As consequence, the sorting process could have some ecological significance, both in natural environments and in aquaculture, especially in oyster ponds. In addition, our study suggests that the preferential rejection of large-size cells in pseudofaeces could favour the development and/or the maintenance of sub-populations of large-size *Haslea*, given that *H. ostrearia* cells rejected in pseudofaeces have a revival capacity that is not altered by pre-ingestive processing.

Secondly, complementary experiments were conducted to understand better the mechanism of greening and its influence on feeding behavior of *C. gigas*. Our findings showed that the greening in pallial organs of oyster was significantly caused by the extracellular form of pigment, compared to the intracellular form in *Haslea*'s cell. In addition, greening by marennine was identified in the mucocyte of the gills, indicate that mucocyte as the site of marennine fixation. Moreover, greening by marennine significantly decreased the clearance rate (CR) as a consequence of its fixation on oyster gills. However, the CR of oyster recovers to normal state after 7 days of exposure, suggesting that oyster is able to depurate the effect of marennine.

Thirdly, we would like to evaluate the effect of marennine on the behavioral, physiological and biochemical traits of two commercially important bivalve species, the blue mussel *Mytilus edulis* and the eastern oyster *Crassostrea virginica*. Our study showed that the concentration of marennine found on the gills of both species was positively correlated to the concentration of dissolved marennine in the water medium. However, a behavioral response was detected at the higher marennine concentration (2.0 mg L^{-1}), with both species displaying curtailed valve opening compared to control groups. Marennine at 2.0 mg L^{-1} significantly decreased scope for growth by 58% and 85% for *M. edulis* and *C. virginica*, respectively. The greening process had effect on total fatty acids contained in the digestive gland but only restricted to mussel, suggesting that marennine interferes the accumulation of energy reserves in this bivalve. In addition, with respect to polar lipids, greening by marennine effectively increased the concentration of unsaturated fatty acids in the gills of *C. virginica* only. This increase may indicate a regulatory mechanism counteracting marennine.

Lastly, we would like to assess the allelopathic effect of *H. ostrearia* in realistic conditions of microalgae co-cultures, to identify which algal species relevant for aquaculture are either sensitive or resistant to *H. ostrearia* and its supernatant containing marennine. Our findings showed that inhibition of growth of microalgae due to the presence of *H. ostrearia* and marennine was species-dependent. *S. costatum*, *C. calcitrans* and *T. lutea* were significantly more sensitive, whereas *T. suecica* and *P. tricorntum* appeared to be more resistant. This study confirmed that marennine released into the

culture medium can act as an allelochemical compound, thus explaining the dominance of *H. ostrearia* and the loss of sensitive algae in oyster ponds, but also that some species are insensitive, which allows co-culturing and use in a mixed algal diet in aquaculture.

This Ph.D. study provides a better insight about the greening mechanism of bivalves caused by marennine, as well as its biological consequences on bivalves. Moreover, our study provides information about the assessment on the putative utilization of marennine as natural antipathogen agent particularly in bivalve aquaculture. Nevertheless, further studies should be carried out, particularly on the efficiency of marennine compared to other natural antipathogen.

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1 CHAPTER I: GENERAL INTRODUCTION

1.1 Shellfish culture development worldwide: problems and challenges

With the current demographic issue of expanding world population, it is recognized that the use of marine resources derived from capture fishing nearly reached its maximum (FAO 2014). Therefore, aquaculture is one of the alternatives to facilitate the harvesting of seafood and may rapidly become a necessity to support market demand.

One of the major parts in today's aquaculture is conchyliculture, meaning the breeding of shellfishes. Indeed, bivalve aquaculture represents 22.8% of world aquaculture production with approximately 11.5% of the global total revenue in 2012 (FAO 2014). Production from this sector has increased rapidly over the last decades, from 1 million tons in 1950 to 15.2 million tons in 2012, placing the bivalve's culture on the second rank in the global aquaculture production with 7 million tons on the world market (FAO 2014).

Bivalves present several advantages for aquaculture. For instance, they are filter-feeding animal that feeds directly in the aquatic environment. In contrary to fish aquaculture there is no need to provide additional food source, therefore during the growing phase at the sea, the cost of food is nearly absent. In addition, the rearing techniques used are relatively simple and inexpensive in terms of labor.

In France, conchyliculture has existed since the middle ages, explaining the current wide diversity of breeding techniques (Lucas 2012). Ostreiculture is one of the major types of shellfish culture in France. The native *Ostrea edulis* was the first species to be cultured since the 17th century using former salt marshes (evaporation ponds for salt production) in the Marennes region (Grelon 1978). Juvenile oysters were placed in ponds ("claires") for approximately 4 or 5 years before the sale. At the turn of the 19th century, the flat oyster culture was well developed, especially in the Bay of Arcachon with the maximum production of 20,000 metric tons per year (Buestel et al. 2009). However, production of flat oyster was interrupted by the massive unexplained mortalities after 1920, which led to their extinction in both the Arcachon and Marennes region. During this period of time, the Portuguese cupped oyster *Crassostrea angulata* replaced the endemic flat oysters until its

production gradually decreased nearly to the point of extinction in France due to the iridovirus outbreak in 1996 (Buestel et al. 2009). Subsequently, massive introductions of the Pacific oyster *Crassostrea gigas* were conducted as a resurrection plan to overcome the crisis. This species was well established and demonstrated fast growth and thus production increased rapidly. Today, France is considered as the major oyster producer in Europe with the total production of 116,000 metric tonnes (FAO 2008).

The development of aquaculture, as well as the problems and challenges found in this, quite varies depending on the country. To date, eutrophication of the coastal environment due to aquaculture activity is one of the crucial challenges in applying sustainable aquaculture. Bivalve suspension feeders produce faeces and pseudofaeces, with high organic content through their biodeposition process. The potential impact of biodeposition associated with suspended bivalve aquaculture on benthic biogeochemical and biological parameters has been numerously studied. The first study of the impact of shellfish farming on the ecosystem was carried out by Dahlbäck and Gunnarsson (1981). These authors noted a link between increased sedimentation rates, accumulation of organic matter and greater sulfate reduction and sulphide accumulation under shellfish farm of *Mytilus edulis* in Sweden relative to the control site. In addition to this, (Chamberlain et al. 2001) it was observed that suspended mussel farm activity in southwest Ireland resulted in elevated levels of organic carbon which reduced macrobenthic infaunal diversity within a radius of 40 m around the farm. However, most studies on organic enrichment of seabed and alteration on benthic communities due to shellfish farming have concluded that the effect is much less than that caused by finfish farming (e.g. salmon) (Crawford et al. 2003). Recent studies show that alteration on the benthic environment and benthic communities could be influenced by type and intensity of farming (Mckindsey et al. 2011).

Another significant issue faced by shellfish culture is the loss of stock through disease or pathogen infections. There are several examples of the negative impact of disease outbreaks and mass mortality on the production and trade. To date, bivalve summer mortality was mostly reported not only in France (e.g. Thau lagoon and Marennes-Oléron Bay, in French Mediterranean coast and Atlantic coast, respectively) (Pernet et al. 2010; Dégremont 2011), but also in Ireland and the Channel Islands (Lynch et al. 2011; Martenot

et al. 2011) as well as several cases in Australia and New Zealand (Zippel and Kaspar, unpublished results). Specifically in France, *C. gigas*' mortality affects any stage of production, including larvae, juveniles or adults, regardless of the farming technique. Recent findings suggest that both herpesvirus OsHV-1 and *Vibrio splendidus* are strongly associated with oyster bivalve mass mortality (Dégremont 2011).

To avoid detrimental economic losses due to disease outbreak, antimicrobial drugs (hereafter named, AMDs) are commonly supplied to the animals as food additives, immersions or injections (Rico et al. 2013). In addition, AMDs can also be applied as prophylactic (by preventing diseases before they occur), therapeutics (by treating sick animals or growth booster) (Rico et al. 2013). Nonetheless, utilization of AMDs is becoming more restricted since many studies observed the side effects for both environment and public health safety. For instance, the drawbacks of using massive amounts of antibiotics have resulted in the development of resistant bacteria strains (Miranda and Zemelman 2002; Seyfried et al. 2010) and the presence of residual AMDs in the muscle of commercialized fish, which subsequently has potential consequences on human health (Cabello 2006b). Vaccination may be viewed as the most effective option to control fish and shellfish diseases, but yet, it remains considerably expensive and pathogen-specific (Sakai 1999; Evans et al. 2004; Harikrishnan et al. 2011). Therefore, some of the proposed solutions are the use of prospective natural antibiotics (e.g. plant, macro- and microalgae extracts) (Lee et al. 2009; Citarasu 2010; Sasidharan et al. 2010; Gastineau et al. 2012b; Gastineau et al. 2012c; Fleurence et al. 2012; García-Bueno et al. 2014; Reverter et al. 2014) or probiotics (beneficial microbial strains) to support sustainable aquaculture practices (Mohapatra et al. 2013). Moreover, recent trends show that there is an increasing awareness in consuming organic and environmentally friendly food. Thus, the limitation or replacement of chemical products in aquaculture by using natural treatments could enhance the consumption of aquaculture products.

Recently, several studies demonstrate that microalgae produce secondary metabolites that inhibit deleterious pathogens in aquaculture (Desbois et al. 2008, 2009; Vo et al. 2011; Molina-Cárdenas et al. 2014). Among microalgae, *H. ostrearia* is a pennate diatom able to synthesize and excrete water-soluble blue pigment marennine, which could

potentially be used in aquaculture as natural antipathogen (Gastineau et al. 2012c, 2014c). The purified form of marennine significantly inhibits the growth of bacteria (e.g *Vibrio aesturianus* and *V. splendidus*) that contributed to the summer mortality events in France (Gastineau et al. 2012c). Both antibacterial and antiviral activities observed *in vitro*, could lead to the potential development of *H. ostrearia* and marennine with maximum benefit to the oyster industry, and more widely to aquaculture. **Therefore, it's essential to understand the interaction in ecophysiological context between the bivalves and *H. ostrearia* as well as its marennine pigment.** Within the framework of my Ph.D., several experiments were performed to understand this interaction and more specific objective of the study will be explained on the later section (part: research objectives).

1.2 Oyster's greening phenomenon by *Haslea ostrearia* and marennine

Greening of oysters is a natural phenomenon that was first described by Sprat (1669). He observed that oysters from the Tolesbury ponds presented a green coloration on their gills. Several hypotheses were made to explain this greening. For instance, the role of grass, plants and mosses grown on the shores of the ponds that could be ingested by oyster were hypothesized as the cause of greening. Another hypothesis was the presence of metallic ions or the presence of pollutants as zinc or copper in the pond's water or sediment that can contribute to gills coloration in the oyster. Afterwards, first experimental work was conducted by Gaillon (1820) who observed that the greening in oyster might be related to the presence of microscopic organism. However, this hypothesis was still in debate until Bory de Saint Vincent (1824) argued that the greening was caused by the diatom named *Navicula ostrearia* in "psychodiaire" kingdom, which contained all organisms whose position is unclear neither animal or plant. Afterwards, Simonsen (1974) with scanning electron microscopy facilities confirmed this result and also changed the "blue navicula" from the genus *Navicula* to *Haslea*. More recently, it has been experimentally confirmed that *H. ostrearia* is consumed and ingested by oysters (Barillé et al. 1994a; Piveteau 1999;

Cognie et al. 2001a). This diatom is able to synthesize the water-soluble blue pigment known as marennine (Lankester 1886), derived from the Marennes-Oléron area. Indeed, the greening occurs when *H. ostrearia* proliferates, becoming dominant and releasing marennine into the seawater. As a consequence, the oyster ponds become dark-green and the oysters, through their feeding, fix the pigment and their gills turn green (Fig. 1-1). In addition, the role of marennine in the oyster greening process has also been demonstrated through laboratory experiment using either the culture supernatant of *H. ostrearia* (Ranson 1927; Neuville and Daste 1979), or a solution of purified marennine (Pouvreau 2006).

To date, “green-oyster” is mainly produced on the Atlantic Coast of France, e.g. Marennes-Oléron Bay and Bourgneuf Bay, as two of the most important sites of shellfish culture. In their production, greening is carried out during the refining process, in which the oysters are placed in particular ponds or ‘*claires*’ containing microalgae, to mature and fatten oyster. On the economic perspective, the green oysters are advantageous for the producers’ income due to their higher price comparatively to ordinary oyster. The price of green oysters is approximately 20% higher than the other due to the unpredictability of greening phenomenon and the organoleptic modification on their taste (Piveteau 1999). Most importantly, recent studies have shown that the purified forms of marennine have antioxidant properties (Pouvreau et al. 2008), anti-pathogen properties (Gastineau et al. 2012b, c) and could have an effect in outcompeting other diatoms (e.g *Haslea crucigera*, *Nitzschia closterium* and *Skeletonema costatum*) in oyster ponds (Pouvreau et al. 2007a).

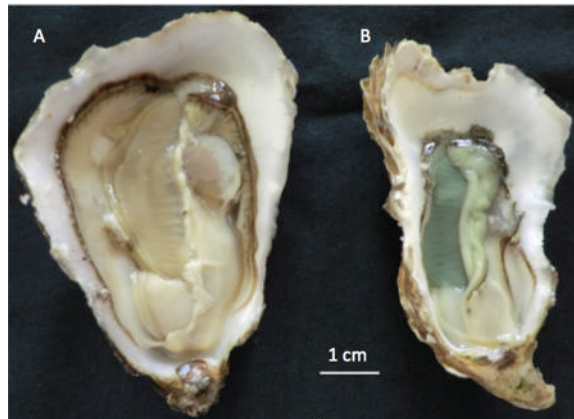


Figure 1-1. Pacific oyster *C. gigas* in control (A) and with marennine exposure (B)

For decades, literature has shown that the natural greening phenomenon has not only been restricted in French oyster ponds but was also described in Great-Britain (Sprat 1669), Denmark (Petersen 1916) and the United-States (Ryder 1884; Mitchell and Barney 1918). Suggesting that *Haslea* genus with ‘marennine-like’ pigment is distributed elsewhere. Indeed, recent finding has shown that other species from the genus of *Haslea*, apart from the one in France, are able to color bivalve’s gills and have also demonstrated some of the biological activities (Gastineau et al. 2012b). Thus, any studies related to this phenomenon will contribute significantly to the valorization of the pigment in many fields, but mainly in shellfish aquaculture.

1.3 *Haslea ostrearia*: Biological and Ecological Characteristics

Diatoms are unicellular, eukaryotic organisms characterized by their yellow-brown pigmentation and their siliceous cell walls forming two valves fitting together, namely epitheca and hypotheca (Round et al. 1990). Thus, they are classified as algae, division Bacillariophyta. Among the diatoms, *H. ostrearia* has a peculiarity in producing blue pigment so-called marennine. This diatom species has a fusiform siliceous frustule characterized by bilateral symmetry form as pennate, from the family of Bacillariophyceae

(Fig. 1-2). Furthermore, *H. osteraria* has two raphes and central furrow which is the character of diatom from the family of Naviculaceae. Several studies have observed the ultrastructure of the frustule by scanning electron microscopy (SEM) (Neuville and Daste 1979). Previous observation revealed that dimension of *H. osteraria* can vary between 60 to 120 μm for its trans-apical axis (length) and 6 to 12 μm on its anti-apical axis (depth) (Robert 1983a). In addition, another *in vitro* observation showed that the maximum and minimum length of *Haslea's* cell could reach 140 μm and 36 μm , respectively (Neuville and Daste 1978). In contrast, another study showed that cultured cell could be maintained until a minimum size of 17 μm , from the maximum size of 138 μm of initial cell's size (Davidovich et al. 2009).

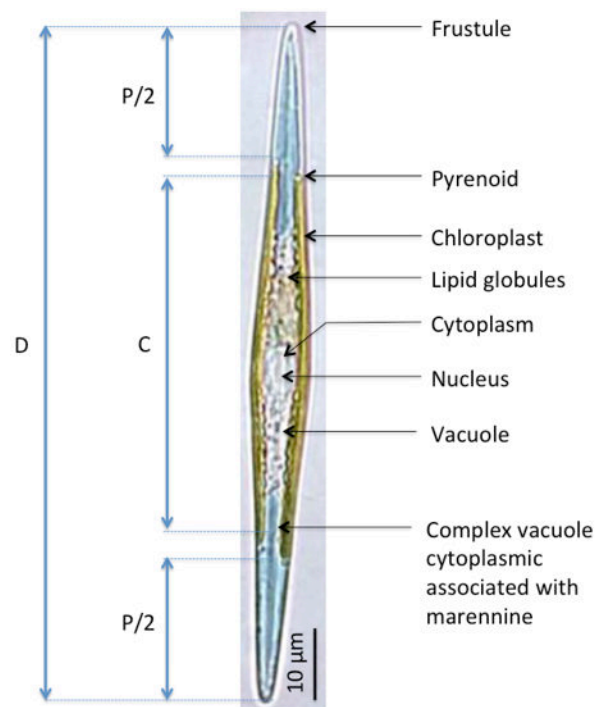


Figure 1-2. Scheme of *H. osteraria* on pigmented state, taken from the front view of biometric calculation (adapted from (Gastineau et al. 2014a)). D: Dimension of transapical axis; C: Size of chloroplasts; P/2: Dimension of the apical pigmented area.

Haslea's cell has a central nucleus whose visibility depends on the cell's stage of development. Robert et al. (1983) described that the anterior view shows the two parietal chloroplasts, adjacent to the nucleus. Furthermore, two vacuoles are distributed along the axis on both sides of the core whereas the cytoplasm is reduced around the core along the vacuoles and chloroplasts, as the widest at the apex of the cell (Nassiri et al. 1998). The presence of lipids is evident in the cytoplasm depending of the cell's growth stage. The marennine is located at the cytoplasmic vesicular complex, which accumulates mainly on the apex of the cell (Nassiri et al. 1998). The same authors also demonstrated that the vesicles involved in pigment production cycle.

The complete taxonomy of *H. ostrearia* is showed as follows (Simonsen 1974):

Division	: Bacillariophyta
Class	: Bacillariophyceae
Sub-class	: Bacillariophycidae
Ordo	: Naviculales
Sub-ordo	: Naviculineae
Family	: Naviculaceae
Genus	: <i>Haslea</i>
Species	: <i>Haslea ostrearia</i>

Like many other diatoms, *H. ostrearia* undergoes the mitotic cell division. When mitosis occurs, one of the two valves of the frustule from the parent cell is received by each daughter cell. This inherited valve is used as the outer valve, and concomitantly, the daughter cell synthesizes a new inner valve. Therefore, one of the daughter cells is identical in size to the parental cell, and the other is smaller. This phenomenon leads to a reduction in the average cell size of the population and to its vanishment, known as MacDonald and Pfitzer's rule (Pfitzer 1869; Macdonald 1869) (Fig. 1-3A). In *H. ostrearia*, the cell's size reduction in apical length as a consequence of binary division can be around 4 μm per month (Davidovich et al. 2009). Nevertheless, this vegetative reproduction can be counteracted in the natural environment by a phase of sexual reproduction. This phase occurs when zygotes turn into auxospores, which expand and form initial cells that recover

the maximum specific cell size. Unlike the centric diatoms that are oogamous, producing small motile male gametes and large non-motile female gametes (Sims et al. 2006), the pennate diatom is aplanogamous which means it does not release flagellate gametes (Chepurnov et al. 2004) (Fig. 1-3B).

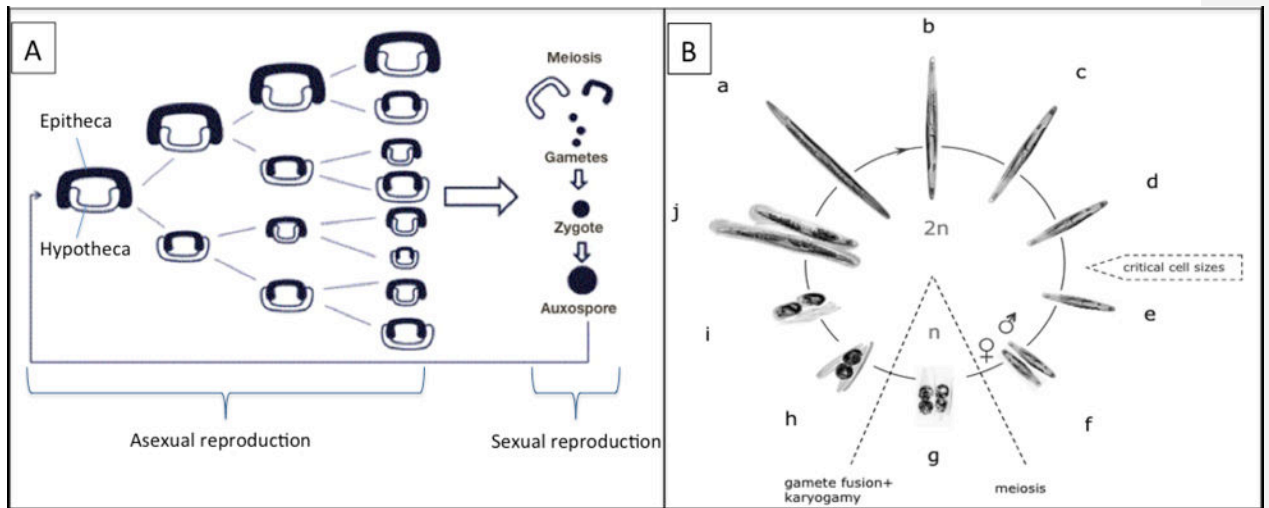


Figure 1-3. Reproduction in centric (A) and pennate diatom (B). A: MacDonal-Pfitzer scheme for cell size reduction, and the restitution of large cell size via auxosporulation, for a generic centric diatom. B: Diagrammatic representation of life cycle in pennate diatom *Haslea ostrearia*. B_a) an initial cell, (B_b-B_e) vegetative cells passing through mitotic cycles, (B_f) pairing of gametangia, (B_g) gametogenesis, (B_h) zygotes, (B_i-B_j) formation of auxospore; n and 2n, haplontic and diplontic phases (cell length range: e.g. 20-95 μm). Adapted from Haberman (<http://www.seagrant.umn.edu>) and (Gastineau et al. 2014b).

Sexual reproduction in *H. ostrearia* can be triggered by short photoperiod at low irradiance conditions and sexual compatibility between pairs of clones (Neuville and Daste 1979; Davidovich et al. 2009; Mouget et al. 2009). Additionally, the cardinal points, which is the size threshold of *H. ostrearia* to be able to conduct sexual reproduction, has been successfully determined at approximately 68 μm (Davidovich et al. 2009). Same authors observed that the largest *Haslea*'s cell produced by the auxosporulation from this threshold size was around 140 μm. Another factor that could provoke the auxosporulation is the light

quality, such as the presence of the red part of Photosynthetically Active Radiation (PAR) spectra (Mouget et al. 2009).

Haslea ostrearia is considered as thychopelagic species as it exists in benthic, planktonic and occasionally in both epiphytic (associated with plants) and epilithic (associated with stone and other hard substrates), living in both warm and tropical waters (Round et al. 1990). Some studies have revealed that *H. ostrearia* is capable of tolerating a wide range of salinity and is compatible to withstand high ultraviolet exposure, such as that in the artificial seawater oyster ponds (*claire*) characterized by their low turbidity and shallow depth (Neuville and Daste 1978; Mouget et al. 1999a). In addition, this microalgae is also able to adapt to high irradiance level (Mouget et al. 1999a) and change in light quality (Mouget et al. 2004), which may explain the seasonal dominance of this species in the ponds. Several authors also revealed that *H. ostrearia* possesses defense mechanisms against stress caused by ultraviolet at similar exposures to those experienced in oyster ponds (Rech et al. 2005). Despite its nature as an obligate photoautotroph, *H. ostrearia* could either exhibit photoheterotrophy or photomixotrophy. The former means that *H. ostrearia* depends on light for most of its energy and principally on organic compounds from the environment for its carbon. Whereas the latter means that *H. ostrearia* can also use a mix of different sources of energy such as nitrogen and carbon organic substances, instead of having a single trophic mode (Neuville and Daste 1978; Robert et al. 1982). Similar to other diatoms, two pathways for inorganic carbon assimilation exist in *H. ostrearia*: First, the Calvin-Benson cycle that is associated to the RuBPC and the PEPCK (Tremblin and Robert 2001) and second, the β -carboxylation pathway that is associated to anhydrase carbonic activity, (Rech et al. 2008). The first pathways are strongly correlated with the pigment accumulation in the cell as the carbon fixation rate and the enzyme activity of RuBPC and PEPCK decreased concomitantly with the accumulation of marennine (Tremblin and Robert 2001). Whereas the second strongly depends on the irradiance during the growth as the carbonic anhydrase activity is higher under low irradiance (Rech et al. 2008).

Biochemical composition of *H. ostrearia* has been previously described in Robert et al. (1983). The authors demonstrated that the carbohydrate and proteins are stable during

the ageing of the cultures with the average of 50 and 200 mg 10⁻⁹ cells, respectively. However, lipid composition can vary as a function of the culture phase (exponential or stationary), which range between 60 and 200 mg 10⁻⁹ cells (Robert 1983b). At this range of concentration, proportions of fatty acids, glycolipids and neutral lipids are similar to those of other diatoms (Groth-Nard 1994). Nevertheless, Joux-Arab et al. (2000) demonstrated that there is no significant difference in lipid content on the different size of *H. ostrearia*. The most important lipids in plastid membranes such as galactolipids are similar to other pennate diatoms, indicated by more C18/C16 forms of both mono- and digalactosyldiacylglycerols (MGDG and DGDG, respectively), yet significantly different from the centric diatoms, which are mainly composed C20/C16 (Dodson et al. 2013). In *H. ostrearia*, the fatty acids (FAs) composition and their position of stereospecific numbering (*sn*) can be influenced by temperature. For instance, at 20°C, the eicosapentaenoic acid (EPA; C20:5) at *sn*-1 position and C16 FAs at *sn*-2 position of MGDG and DGDG are abundant. However, at 30°C, no EPA or other C20 FAs are observed, but rather higher percentages of C18 FAs at *sn*-1 (Dodson et al. 2014).

For many decades, *H. ostrearia* was believed to be the only diatom that is able to synthesize blue pigment on its apical axis. Nevertheless, recent findings showed that another species of the genus *Haslea* with ‘marennine-like’ pigment have been discovered worldwide. For instance, *Haslea karadagensis*, which is native to the shores of the Natural Reserve of Karadag in Ukraine, is characterized by the blue-grey apices (Gastineau et al. 2012a). Another species with ‘marennine-like’ pigment was also discovered from La Gomera (Canary Islands) and from Boulouris (French Mediterranean Sea). Revealed to be new species, they were named as *Haslea silbo* sp. Inedit and *Haslea provincialis* sp. Inedit, respectively (Gastineau 2011; Gastineau et al. 2014c). More recently, another species has been newly discovered from the tropic region at Seribu Islands (Java Sea, Indonesia), suggesting that the genus *Haslea* is cosmopolite. Distribution of *Haslea ostrearia* and the occurrence of the greening phenomenon in oyster are described in Fig. 1-4.



Figure 1-4. Distribution of the blue diatom *H. ostrearia* according to the literature. Each black dot indicates a site where the presence of *H. ostrearia* was assessed from observation of diatom with blue apices, or deduced from the occurrence of green oysters. Map is adapted and modified from (Gastineau et al. 2014a).

1.4 The nature of marennine and its function as potentially developed natural anti-pathogen in aquaculture

To date, many studies have been conducted on the valorization of marennine pigment for various purposes. However, the exact molecular structure of this pigment remains controversial as different hypotheses were proposed for its chemical nature, yet none have been established. For instance, a metallic salt (Lankester 1886; Mitchell and Barney 1918), carotenoid (Ranson 1937), chlorophyll degradation product (Moreau 1967; Neuville and Daste 1978; Robert 1983b), anthocyanin (Neuville and Daste 1972) or phycobilin-like molecule (Ryder 1884). Until then, a method was developed by Pouvreau et al. (2006a) to extract and to purify marennine by using ultrafiltration and anion-exchange chromatography (Fig. 1-5). Thus, according to its biophysical and chemical characteristics, marennine is hypothesized as a polyphenolic compound. It exists in two forms: the

intracellular form (IMn), which accumulates in the cytoplasm to the apical axis of the cell (Fig. 1-2), and the extracellular form (EMn) that is released by the cell to the external environment (Ranson 1927; Neuville and Daste 1978). These two forms can be distinguished by their spectral characteristics (UV-visible spectrophotometry, Raman spectroscopy) and their molecular weight where the extracellular form is much lighter than the intracellular one (0.9 and 10.7 kDa, respectively) (Pouvreau et al. 2006a).

The observation of the ultrastructure of blue cells demonstrated the abundance of vesicles with 5 μm of diameter in the cytoplasm along with the accumulation of marennine, which suggests that this pigment is synthesized or stored in these vesicles (Nassiri et al. 1998). Nevertheless, similar to its nature, the determination of marennine's production remains controversial as well. For instance, several authors suggest that the glucosamine-type biogenic substance controls the production of this pigment (Ranson 1927; Ranson 1937; Moreau 1970). On the other side, another authors suggest that the pigment production is influenced either by the excess of certain minerals or as a consequence of vitamins, nitrate or iron deficit in the presence of light (Neuville and Daste 1978; Robert et al. 1983). Light is claimed to be one of the factors that regulates the synthesis of marennine. This was first demonstrated by Moreau (1970) which shows that low irradiance favors pigment production. In contrast, other authors claim that the increase of pigment synthesis is associated with the increase of the photoperiod, light intensity or type of light (Neuville and Daste 1978; Tremblin and Robert 2001; Mouget et al. 2005). In spite of this, the hypotheses that the synthesis of marennine as a response to deficiencies or light stress is not fully admitted by other authors (Mouget et al. 1999a; Robert et al. 2002).

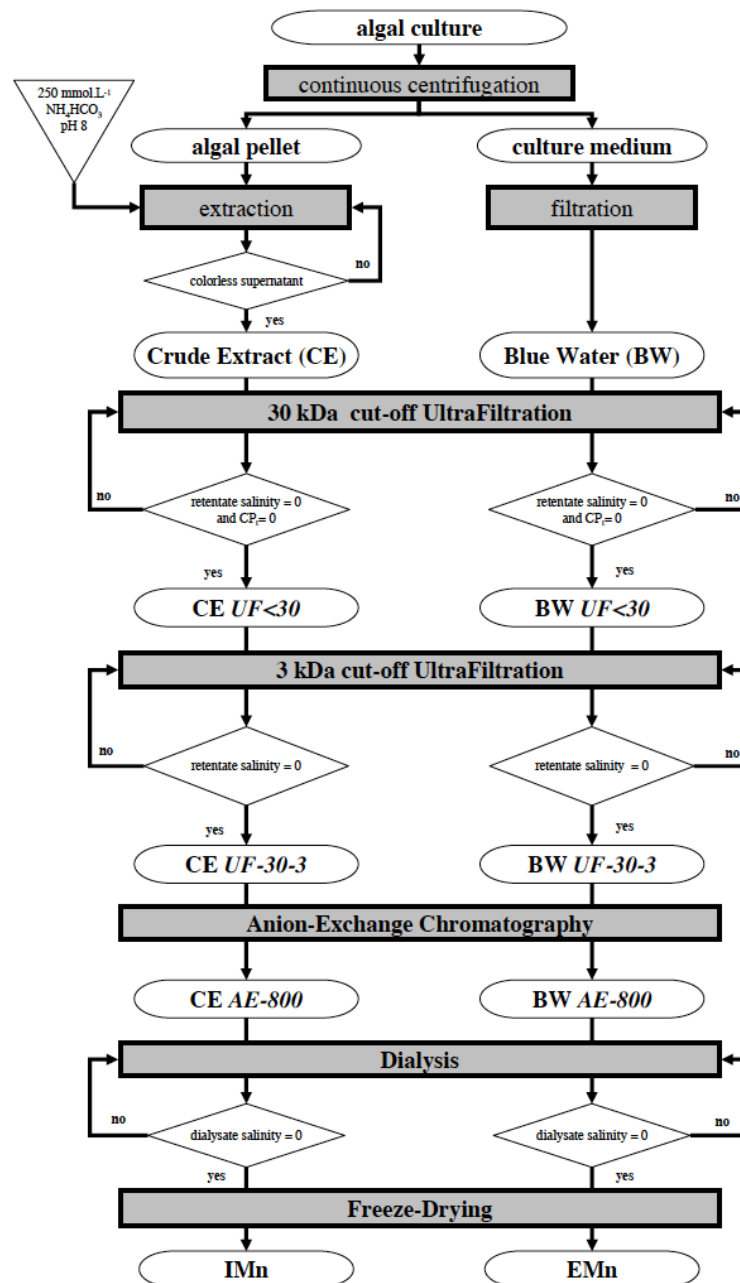


Figure 1-5. Schematic representation of extraction and purification process of intra- and extracellular marennine. Crude Extract (CE); BlueWater (BW); UF < 30 (permeate from 30 kDa cut-off UltraFiltration); UF-30-3 (retentate from 3 kDa cut-off UltraFiltration); AE-800 (fractions eluted at 800mmolL⁻¹ by Anion-Exchange chromatography); Pure Intracellular Marennine (IMn); Pure Extracellular Marennine (EMn); Marennine concentration in the permeate at the time t (CP_t). Adapted from (Pouvreau et al. 2006a).

Recent findings show that *H. ostrearia* present several biological activities. From the ecological perspective, marennine plays a significant role in photosynthesis, acting as a physical barrier by modifying light quality and intensity as it passes through the water column (Mouget et al. 1999a; Tremblin et al. 2000). Chemically, marennine contributes to the domination of *H. ostrearia* in oyster ponds through allelopathic interaction, by outcompeting other species (Pouvreau et al. 2007a). The unpurified marennine or ‘crude extract’ displays antiviral, antibacterial and antiproliferative activities (Bergé et al. 1999; Carbonnelle et al. 1999; Turcotte 2013 in press.). On the other hand, purified marennine demonstrates antibacterial and antiviral properties against detrimental pathogen in aquaculture, such as *Vibrio aesturianus* and *V. splendidus* (Gastineau et al. 2012c). Therefore, marennine could be considered a potentially valuable natural antipathogen to overcome the disease problem in the field of aquaculture. Nevertheless, several studies need to be conducted to achieve this goal, such as its effect on cultured species, improvement on extraction and purification method and the assessment on the nature of marennine itself.

1.5 Filter feeding bivalves and their role in ecosystem

Bivalves, including oysters (Family Ostreidae), mussels (Family Mytiloidea) as well as scallops (Family Pectinidae), obtain their food through filter feeding as a fundamental component of pelagic-benthic coupling. This process is considerably essential in order to understand the potential role of bivalves in the aquatic ecosystem. Filter-feeding can be defined as a type of suspension feeding in which particles are removed from water current by filtering or feeding upon particles suspended in water (Wong et al. 2003). Moreover, filter feeding in bivalves involves the filtration of seston from the water column that can be divided into organic and inorganic fractions. Bacteria, phytoplankton, organic detritus, micro/mesozooplankton can be categorized as organic fractions. Whereas inorganic fractions can include mineral, silt, sand or grains.

Indeed, various influences of bivalve suspension feeder on aquatic ecosystems have been well described in many studies (Berg and Newell 1986; Newell 1988, 2004; Dame 1993, 2011; Newell and Shumway 1993). For instance, bivalves are able to deplete and to modify the phytoplankton composition in coastal oceans by filtering large volumes of water and by selectively feeding on them (Prins et al. 1998). More importantly, from an aquaculture perspective, their ability to exploit naturally occurring phytoplankton at the base of the food chain is advantageous, thus the need for external feed input can be avoided (Crawford et al. 2003; Hamoutene et al. 2015). Therefore, bivalve aquaculture is favorable because, instead of resulting additional nutrient loading, they provide a transfer of nutrients from water column particles to benthic sediments in biodeposits, a rapid nutrient cycling when dissolved inorganic nutrients are released into the overlying water, and a removal of a portion of those nutrients when shellfish are harvested (Dumbauld et al. 2009). A simplified conceptual diagram on the mode of bivalve benthic-pelagic interaction in bivalve culture ecosystem is provided in Fig. 1-6 (Langdon and Newell 1996; Cognie 2001). In this model, if the problem of interaction between bivalve suspension feeders and microalgae is often considered from the trophic point of view, microalgae is the vital component since they are the main source of food for the bivalves (Asmus and Asmus 1991). In addition, the possibility of feedback mechanisms between the populations of benthic suspension feeders and populations of the primary level is also taken into account. This feedback mechanism may facilitate the nutrient enrichment in the pelagic compartment by relying on the dissolved excretion of bivalves and mineralization of their biodeposit (Asmus and Asmus 1991; Newell 2004). Additionally, the nutrient regeneration by bivalves throughout dissolved excretion can later be used by the microalgae (Prins and Smaal 1990, 1994).

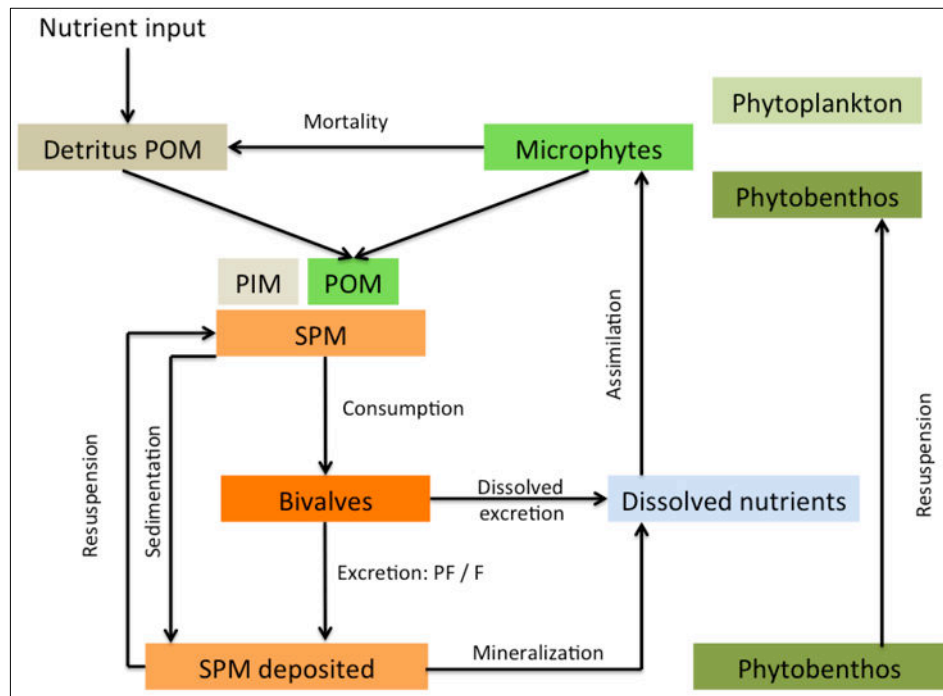


Figure 1-6. Simplified diagram describing the relation between bivalves suspension feeder and suspended particulate matter (SPM) in a model shellfish culture ecosystem (adapted from (Cognie 2001)). POM and PIM are the particulate organic matter and particulate inorganic matter, respectively.

Further explanation regarding bivalve-suspension particulate matter interaction and filter feeding process are briefly described in the section below (part: mechanism of filter feeding in bivalve).

1.5.1 *Crassostrea gigas*, *Crassostrea virginica* and *Mytilus edulis* as species model

1.5.1.1 *Crassostrea gigas*

The Pacific oyster *Crassostrea gigas* (Thunberg 1793) is a bivalve species native to the region of Japan and it has been introduced worldwide for aquaculture purposes (Orensanz et al. 2001). Taxonomical status of *C. gigas* is well established as follows (www.marinespecies.org):

Phylum : Mollusca
Class : Bivalvia
Subclass : Pteriomorpha
Order : Ostreoida
Family : Ostreidae
Genus : *Crassostrea*
Species : *Crassostrea gigas*

A distinction between bivalves species can be made based on phenotypic characters or simply distinguished by their shell morphology. Oyster *C. gigas* has an irregular elongated shell that is variable in size. The upper valve is moderately convex, unlike the lower valve that is quite deep and cup shaped. One of these valves is usually cemented to a hard substratum. Both valves present radial ribs, which start from the umbo. The valves usually whitish in color, and sometimes display purple streaks and spots. Full morphological description on the shell of *C. gigas* is well described in World Register of Marine Species (WoRMS).

1.5.1.2 Crassostrea virginica

The American Eastern oyster *Crassostrea virginica* (Gmelin, 1791) is the keystone species in most estuaries along the Atlantic and Gulf coasts. This species belongs to the same family of Ostreidae, than the Pacific oyster *C. gigas*. The Eastern oysters' distribution ranges from the gulf of St. Lawrence (Canada) to the Gulf of Mexico, the Carribean and the coasts of Brazil as well as Argentina. This species has been introduced to British Columbia (Canada), the west coast of the United States, Hawaii, Australia, Japan and the United Kingdom (Haye 2006; FAO 2006).

Morphologically, *C. virginica* is almost similar to *C. gigas*. Its valves are asymmetrical with the left valve generally thicker and more deeply cupped than the right. No gap is present between the two halves when the valves are closed. Eastern oyster settles

on the left valve leaving the right valve always on top (Galtsoff 1964). Variation in the shell shape and thickness may differ depending on the type of environment in which the oyster grows. For instance, on hard substrates, umbones are curved and pointing toward the posterior and shells are thicker. Whereas, in silty environments or on reefs, umbones grow generally straight, but shells are more fragile than those growing on hard substrates (Stanley and Sellers 1986).

1.5.1.3 Mytilus edulis

Mytilus edulis is commonly known as the edible blue mussel or blue mussel. The native region of this species is difficult to identify due to the presence of similar species and subspecies. Nevertheless, the blue mussel' native distribution is thought to span the North of Atlantic from Cape Hatteras, North Carolina to Newfoundland, Iceland, northern Norway and southern White Sea to the Bay of Biscay at the French-Spanish border. Additionally, this species has been introduced to various places including West of Canada, Chile, Australia and New Zealand. The taxonomy of blue mussel is described as follow (Turgeon et al. 1998):

Phylum	: Mollusca
Class	: Bivalvia
Subclass	: Pteriomorpha
Order	: Mytiloida
Family	: Mytilidae
Genus	: <i>Mytilus</i>
Species	: <i>Mytilus edulis</i>

The two shell valves of *M. edulis* are similar in size, and are roughly triangular in shape. Two valves are hinged together by means of a ligament. The interior part of the shell is a white pearl color with a broad border of purple or sometimes dark blue, named the

pallial line. The foot part of mussel secretes a byssus, which is a bundle of tough threads of tanned protein. The mussel uses these threads as mooring lines to attach itself to the substrate and to other mussels.

1.5.2 General anatomy in relation with the context of study

1.5.2.1 Gills in bivalves suspension feeder

In general, gills first appear at the pediveliger larval stage. Their structural and functional complexity increases until the juvenile stage has been reached. The development of gills has led to a new insight of the evolution on gills' structure, including the phylogenetic relationship among the bivalve taxa (Cannuel and Beninger 2006; Beninger and Decottignies 2008; Cannuel et al. 2009). Mussels *M. edulis* for instance, have the simplest gill structure which is the homorhabdic filibranch type (Fig. 1-7a). This homorhabdic means that the lamellibranch gill filaments are arranged in a flat, uniform series. Scallops have a similar gill type but the gill filaments are differentiated into principal and ordinary filaments, thus the gill type is termed heterorhabdic filibranch (Fig. 1-7b).

In contrast, for the pseudolamellibranch type such as the oysters, the surface area of the gill filaments is increased by folds of plicae (Figure 1-7b and d). In the context of my PhD study, the particle processing in bivalves will be specifically discussed only for the two types of gills: homorhabdic filibranch (the blue mussel *M. edulis*), plicate heterorhabdic pseudolamellibranch (the Eastern oyster *C. virginica* and the Pacific oyster *C. gigas*).

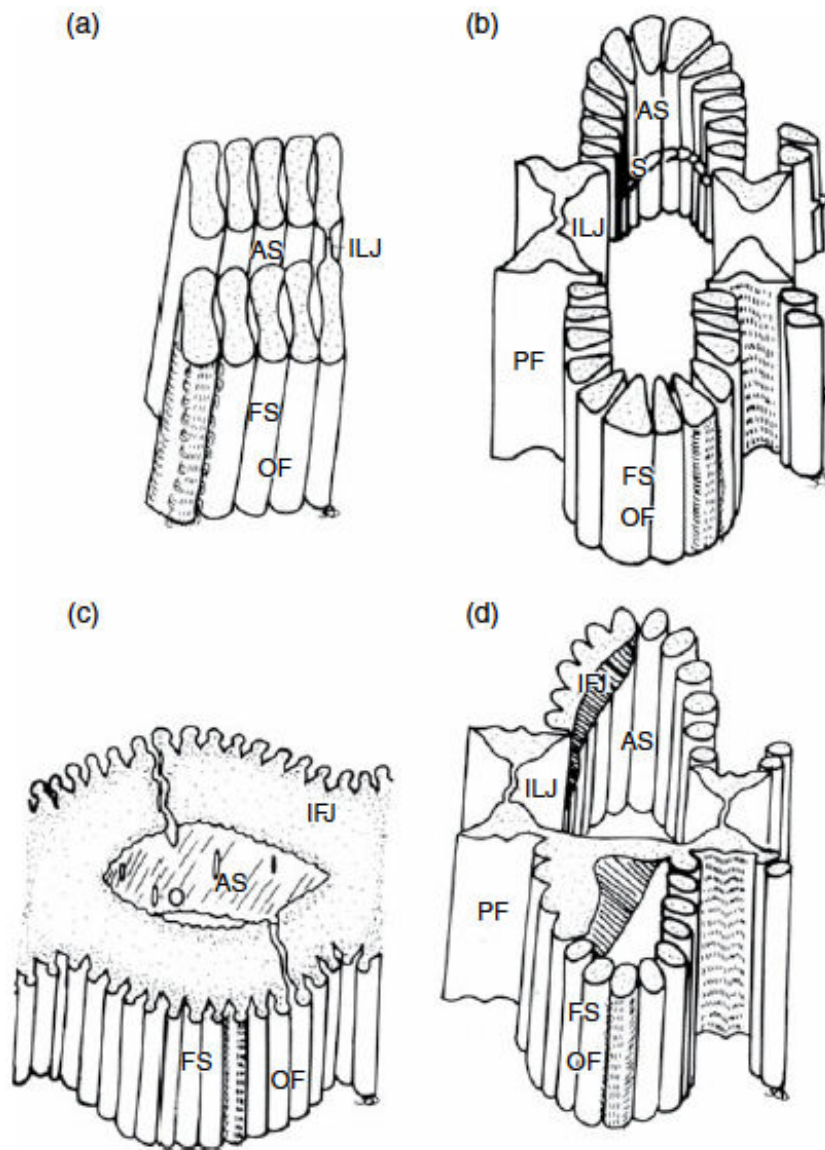


Figure 1-7. Transverse sections through the demibranchs of the principal gill types in different groups of bivalve. (a) Homorhabdic filibranch (e.g. Mussels). Ordinary filaments (OF) connected by interlamellar junctions (ILJ). (b) Heterorhabdic filibranch (e.g. Scallops). Principal filaments (PF) and OF, joined by ILJ and ciliated spurs (S). (c) Homorhabdic eulamellibranch (e.g. Clams). OF joined by interfilament junctions (IFJ). O: ostia. (d) Heterorhabdic pseudolamellibranch (e.g. Oysters). Principal filaments and ordinary filaments, joined by ILJ and IFJ. AS, abfrontal surface; FS, frontal surface. Pictures are derived from (Dufour and Beninger 2001).

1.5.2.1.1 Cilia on bivalve's gills and their role

It has long been known that the cilia on bivalve's gills are differentiated and functionally specialized (Dufour and Beninger 2001). These cilia create water current across the gills and capture the suspended particles in the water column and then transport them to the mouth and labial palps. To date, several types of cilia have been well recognized. In this subsection, different types of cilia are described with the example from one of the species model used in my thesis (e.g. *C. gigas*).

1.5.2.1.2 Lateral cilia

Lateral cilia originated from each side median part of the filaments. They create the beating water current across the gill. This current is directed from the inhalant to the exhalant cavity through the ostia (Fig. 1-8b).

1.5.2.1.3 Latero-frontal cilia

Latero-frontal cirri are situated on both sides of the interfilament space (Fig. 1-8b). These cilia beat in the opposite direction of the lateral cilia (Atkins 1937a). Latero-frontal cirri that are formed by the complex assemblage of individual cilia with feather shape have a significant role in particle capture (Riisgård 1988; Beninger et al. 1997b).

1.5.2.1.4 Cilia pro-latero-frontal

These cilia are around 5 μm in size and are arranged in a band adjacent to the end face of latero-frontal cilia of all filaments (Atkins 1937a) (Fig. 1-8b).

1.5.2.1.5 Frontal Cilia

On the ordinary filaments, the frontal cilia can be divided into two types (Atkins 1937a; Barillé et al. 1994a): 1) The median frontal cilia, which form a wide ciliary channel at the center of frontal region in each filament and beating towards the ventral tract located at the end of the gills; 2) The marginal frontal cilia, which are more spaced simple cilia that frame median ciliary pathway on both side of median frontal cilia. They are beating dorsally to the dorsal tracts (Fig. 1-8a and b).

1.5.2.2 Gill's membrane lipid as stress biomarkers

The membrane lipid bilayer structured a barrier for cells and the inside's subcellular organelles. Therefore, its physical characteristics are a keystone to describe the membrane structure and function (Parish 2013). Membrane fluidity is strongly correlated with the lateral movement of the constituent lipids and embedded proteins. Recent studies have revealed that the ectotherms, such as bivalves, can counteract the effect of reduced temperature or increase pressure by remodeling their membrane phospholipid, a process known as homeoviscous adaptation (Hulbert 2007; Pernet et al. 2007, 2008). This biochemical adaptation can be achieved by the changing of FAs, especially those which constitute polar lipids (PL) in the gills (Parish 2013). For instance, hard clams in Atlantic Canada increased the level of unsaturation of FAs in their gills when the temperature decreased (Parent et al. 2008). A similar result is also given by Hall et al. (2002) who observed a strong relationship between fluidity and PUFA 20:5 ω 3 in gill membranes of *P. magellanicus* following a 10°C temperature decrease. Remodelling membrane can be an adaptation response to stress due to temperature rising, as already observed in mussels and oysters (Pernet et al. 2007, 2008, 2010). Further explanation on gill's lipid membrane remodeling in relation to marennine exposure will be discussed in chapter 3.

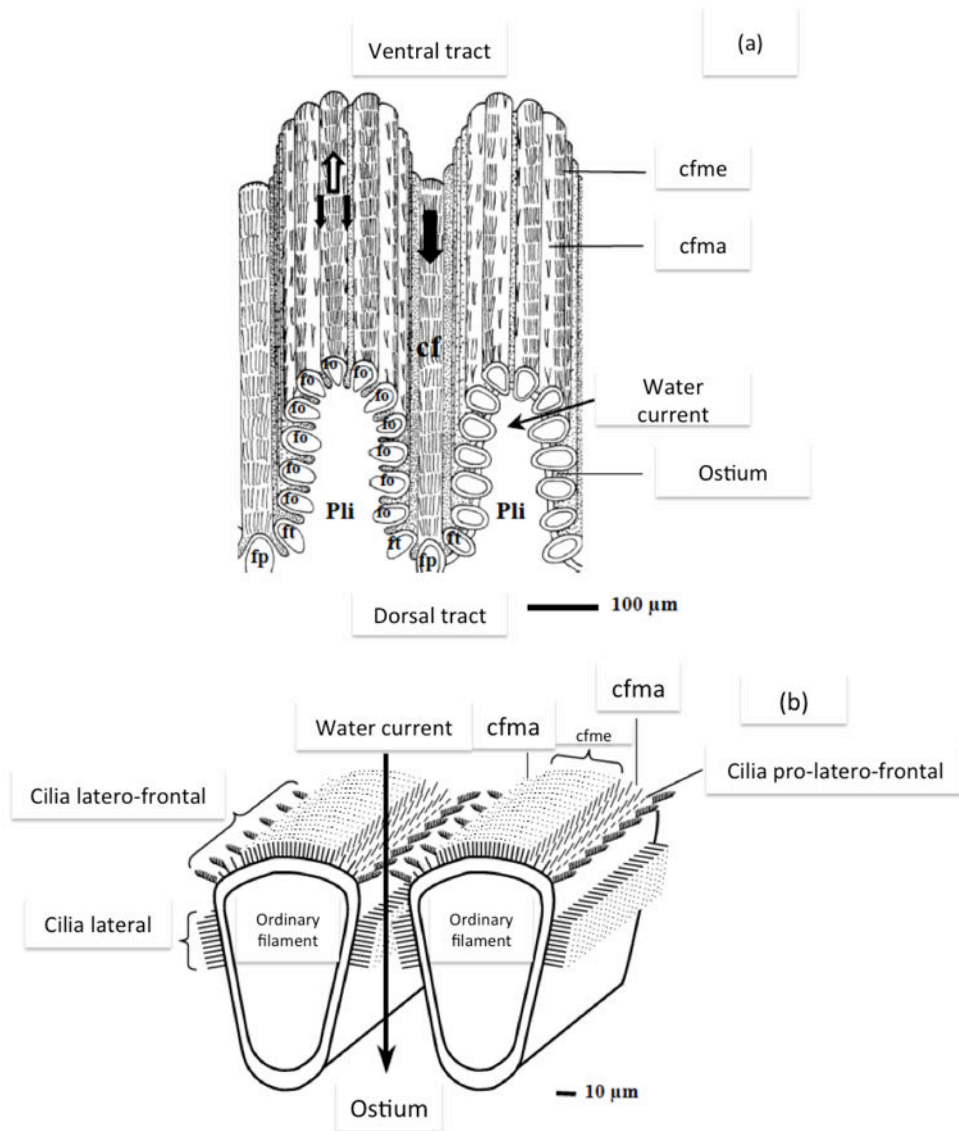


Figure 1-8. (a) Scheme of two plicated gills in oyster *C. gigas* and *C. virginica*, containing ordinary (fo) and principal filaments (fp). (b) Scheme of two ordinary filaments from the frontal view. cf: cilia frontal; cfma= cilia frontal marginal; cfme = cilia frontal median; fo, fp, ft = ordinary filament, principal filament, transition filament; Black thick arrow indicates ciliary current towards dorsal tracts; White thick arrow indicates ciliary current towards ventral tracts. Pictures adapted from Cognie 2001.

1.5.2.3 Labial palps

In an oyster, two pairs of labial palps are situated at the antero-ventral end of oyster's body, and slightly below the oral hood mantle (Fig. 1-9). Each pair of palps consists of one internal and external palp in which each of them has a smooth and ridge face, provided with the tracts and ridges inclined towards the mouth. The trough formed by the fusion between one external palp with its internal equivalent is called the oral-lateral trough and connects the mouth to the dorsal tracts via gill-palps basal junctions (Ward et al. 1994). Each of these two junctions accepts the material from one internal and one external dorsal tract. The material from the median dorsal groove is directed either to one or to the other two junctions. The mantle is fused at its antero-ventral end to form a hood over the anterior part that protects the mouth from direct contact with mantle cavity. Moreover, the two external palps converge above the mouth where they form the outer lip, while the two inner palps are reunited under the mouth of an inner lip (Fig. 1-9).

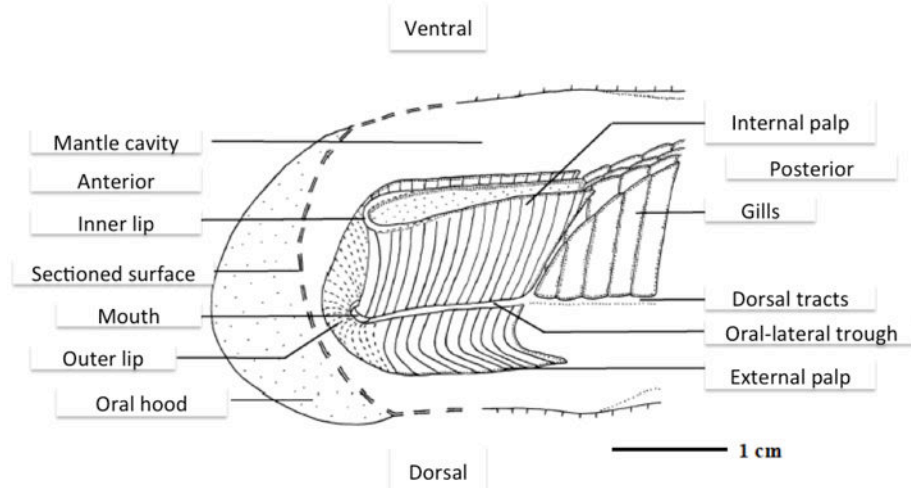


Figure 1-9. Anatomy of labial palps and the mouth area of oyster *C. gigas* and *C. virginica*. Picture adapted from Cognie (2001) and Galtsoff (1964).

In mussels, the inner surface of each palp faces the gill, and is folded into numerous ridges that carry ciliary tracts and mucocytes. The outer surfaces of the palps are smooth, and between the inner and outer surfaces, there is muscular-connective tissue. In mussels,

for instance, *Mytilus chilensis*, the palps may reach posteriorly for about one-third of the muscular-connective tissue. Different from oysters, the homorhabdic filibranch gills in mussels capture particles moving ventrally into the ventral gill particle groove, regardless of the particle concentrations. Then, they proceed anteriorly embedded in a viscous mucus cord towards the labial palps (Beninger and St-Jean 1997). Unlike the dorsal tract in oysters, the dorsal tract of mussels is not involved in particle accumulation and transportation. Thus, no selection takes place on the gills due to the simplicity of unplicated gills in mussels (Ward et al. 1998a). The outer and inner palps enclose the anterior gill region (Fig. 1-10); the outer demibranch is applied to the ridged surface of the outer palp and the inner demibranch to the ridged inner palp surface.

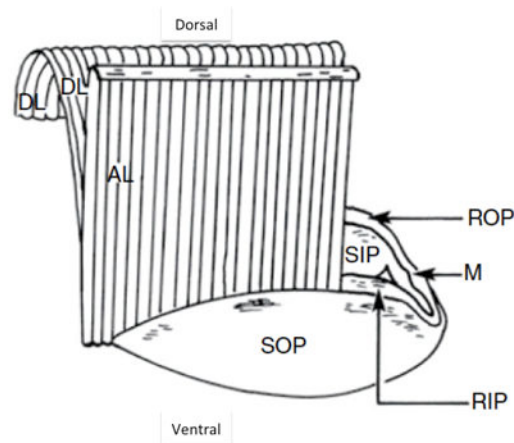


Figure 1-10. Scheme of the gill, labial palps and mouth in *Mytilus edulis*. AL = ascending lamella of gill; DL = descending lamella; M = mouth; RIP = ridged surface of inner palp; ROP = ridged surface of outer palp; SIP = smooth surface of inner palp; SOP = smooth surface of outer palp. Picture is adapted from Beninger and St-Jean (1997).

In general, labial palps have a main function of continually remove material from the food tracts on the gills to avoid gill saturation (clogging). Palps can act as a transit place when particles that are going to be processed are abundant. They hold the filtered material away from the mouth and deposit it as pseudofaeces, thus the animal can continue to filter and ingest at an optimum rate (Gosling 2015). The rejected materials in the form of pseudofaeces are carried along rejection tracts on the mantle to the inhalant opening.

Afterward, the pseudofaeces are regularly ejected through this opening. When the ingestive capacity is not exceeded or not yet overly saturated, particles from the gill move along acceptance tracts on the labial palps towards the mouth.

1.5.2.4 Stomach and digestive glands

When particles reach the mouth, they are transported by ciliary movement in the mouth that leads them into the oesophagus. This ciliary movement helps to drive the material to the stomach. This mechanism can be achieved by the release of acid- and neutral-mucopolysaccharide in mucocyte from cilia epithelium, which directly communicates mouth with stomach (Beninger et al. 1991). Bivalves generally have a large and oval-shaped stomach that lies completely embedded in the digestive gland (Fig. 1-11). It has been known that the stomach in bivalves is very complex, but typically the stomach in the species model used has been described (Purchon 1977). For instance, in Lamellibranch, the stomach has a sac form in which the oesophagus penetrates anteriorly and after the intestine comes out, accompanied by the crystalline style. This crystalline style, which is a semi-transparent gelatinous rod, originates in a style sac at the posterior extremity of the stomach. Furthermore, this style projects forward and dorsally across the cavity of the stomach to rest against the gastric shield, a thickened area of the stomach wall. A study revealed that the length of style is correlated with shell length, c.a. 50-60% of shell length (Alyakrinskaya 2001). The same author also demonstrated that the length of the style varies depending on the season, for instance, in spring when the food intake is high it has the maximum length.

The digestive gland is brown or black and consists of tubules that connect to the stomach by several ciliated ducts. This part is the main site of extra- and intracellular digestion, involving a wide variety of digestive enzymes such as esterases and acid-alkaline phosphatases. Moreover, the digestive gland plays a significant role in the storage of metabolic reserves, with a typical storage of large amount of neutral lipids (NL). This storage can be used as an energy source during the process of gametogenesis (Pernet et al.

2010) and during periods of physiological stress (Pernet et al. 2007, 2010; Rocchetta et al. 2014; Fokina et al. 2014, 2015). Moreover, fatty acids (FAs) in digestive glands can also be used as trophic markers (FATM) in the pelagic-benthic environment (Bergé and Barnathan 2005; Parrish 2013). The FATM concept is based on the observation that marine primary producers deposit certain FA patterns that may be conservatively transferred through aquatic food webs. And for this reasons, bivalves can be recognized as primary consumers. There are some specific FAs, groups of FAs and FA ratios that serve as FATM such as diatoms, flagellates, macroalgae and bacteria. For instance, high concentrations of 20:5(n-3) and 16:1(n-7), a ratio of 16:1(n-7)/16:0 close to 1 and the presence of C16 polyunsaturated fatty acids (PUFAs), mainly 16:4(n-1), are diatom markers (Kelly and Scheibling 2012). In contrast, FA composition of cyanobacteria is generally dominated by 16:0 and 14:0, with lower concentrations of 18:0, 16:1n-9, and 18:1n-9. In addition, FA biosynthesis in heterotrophic bacteria produces odd-numbered (*e.g.* 15:0, 17:0) and branched FAs (*e.g.* 15:1, 17:1), and iso- and anteiso-branched saturated fatty acids (SFAs) (Dalsgaard et al. 2003). These FAs, along with 16:1n-7 and 18:1n-7, are typically dominant in bacterial FA composition and are used as tracers for the contribution of heterotrophic bacteria to sediments. On the other hand, principal indicators of dinoflagellates are 18:4(n-3) and 22:6(n-6), while 18:2(n-6), 20:4(n-6) and 22:6(n-3) are indicative of heterotrophic flagellates and ciliates (Bergé and Barnathan 2005; Kelly and Scheibling 2012). Fatty acids 22:6(n-3) and 18:1(n-9) are abundant in lipids of zooplankton and thus provide an indicator of carnivorous organisms (Silina and Zhukova 2007a, b). Further explanation concerning the effect of environmental stress on NL as the energetic reserve and also on FAs in NL as FATM in bivalve will be linked to chapter 4.

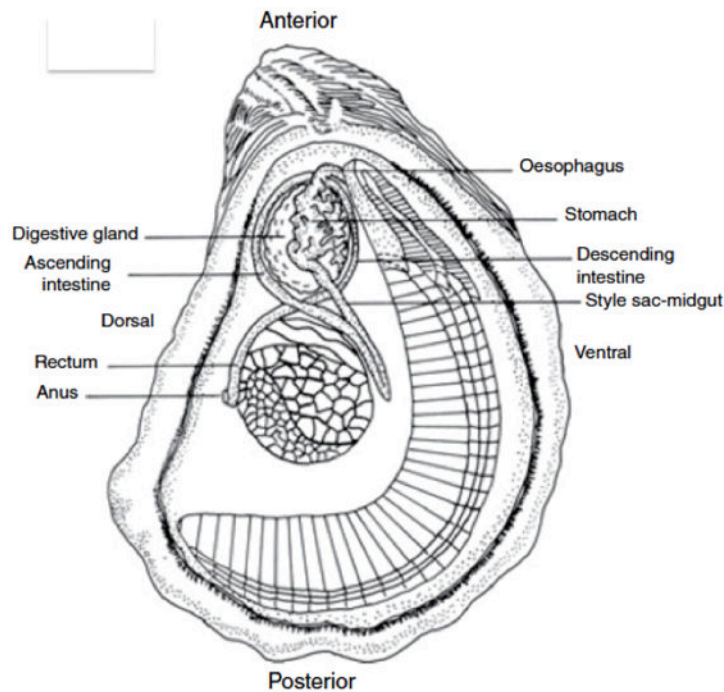


Figure 1-11. The digestive system of Eastern oyster *C. virginica*. Picture is adapted from Gosling (2015).

1.5.3 Mechanisms of suspension feeding in bivalve

1.5.3.1 Filtration and Clearance rate

As suspension feeders, bivalves filter the water to obtain organic and inorganic matter from the particles suspended in water. Two processes are recognized: 1) the filtration or pumping rate (FR), defined as the volume of water passing through the gills in a unit of time; 2) the clearance rate (CR), defined as the number of particles cleared from a certain amount of volume of water per unit of time. Thus, all particles presented to the gill area are cleared from suspension (Bayne et al. 1993; Riisgård 2001). Several definitions have been made on the definition of CR. For instance, Bayne et al. (1987), Macdonald and Ward (1994), and Bacon et al. (1998) defined CR as the volume of water cleared of particles $>2 \mu\text{m}$ per unit of time. Moreover, CR could be defined as the volume of water

cleared of all 100% efficiently-retained particles per unit of time (Petersen et al. 2004). To date, CR can be measured by using several methods, such as the static, flow-through and the biodeposition method. In the context of my Ph.D. study, only two methods are used in CR estimation: the static and flow-through methods.

Estimation of CR by the static method is based on measuring of the clearance of particles in suspension in a closed chamber. In this method, CR can be measured by monitoring the reduction in the particle concentration as a function of time with the following equation (Coughlan 1969):

$$CR = (V/t) \ln (C_o / C_i) \quad (1)$$

Where V is the volume of water and t is the incubation time. C_o and C_i are the initial and final particle concentrations in the closed chambers. This static method has been widely applied in many laboratories. However, several shortcomings associated with the closed system also have been in debate. For instance, the accumulation of excretory products, reduction in oxygen concentration and the reduction of particle concentration, can alter normal filtration behavior (Riisgård 2001).

Shortcomings in the static method have been overcome by using the second method, which is the flow-through chamber (FTC) method. In this method, CR can be estimated by using this following equation:

$$CR = Fl \times ((C_i - C_o) / C_i) \quad (2)$$

Where Fl is the flow rate (volume of water flowing through the chamber per unit of time); C_i and C_o are the particle concentrations at inlet and outlet of the chamber, respectively. However, several assumptions need to be established: 1) is that the particles must be 100% efficiently retained by the gills to reduce underestimation of CR measurement (Riisgård 2001; Filgueira et al. 2006). 2) The suspension of particles is assumed to pass the bivalves' inhalant opening once, to ensure that the particle concentration around the opening remains constant.

FR and CR can be varied within an individual, species, and population. This variation can be due to the endogenous and exogenous factors, which are correlated to each other (Bayne et al. 1987, 1993). One of the endogenous factors that can influence the CR is the bivalve size. As larger gills are associated with larger individuals, amounts of water can be facilitated and thus offer a larger area to capture suspended particles. Previous studies have revealed that gross gill area is positively correlated with FR and CR (Meyhöfer 1985; Meyhöfer et al. 1985). In contrast, Winter (1973) observed that reduction in the rate of increased filtration activity in larger animals. This can be due to the reduced activity of cilia in old specimen's gills, because the relative growth rate of the gill also decreases, as a bivalve grows either larger or heavier (Foster-Smith 1975). Therefore, filtration activity of bivalves associated with its length and tissue weight, can be standardized by using the allometric equation (Bayne 1976; Cranford et al. 2011):

$$FR = aW^b \quad \text{or} \quad FR = aL^b \quad (3)$$

Where W is the dry body weight (g); L is the shell length (mm); a and b are the constant and a weight (or length) exponent, respectively. Positive relationships between FR (or CR) and shell weight (or length) have been demonstrated for many species of bivalves (Bayne, 1976; James et al., 2001). In contrast, the weight (or length) exponent (b) generally decreases as the animal's size increases (Cranford et al. 2011). This could be explained by the relationship between the gill area, or possibly the area of the gill ostia, and the dry weight (or length) of the animal (Foster-Smith 1975; Bayne 1976). Further explanation regarding the standardization of FR and CR and their relationship with bivalve's shell length and tissue weight is discussed in (Cranford et al. 2011). Another factor that can influence FR and CR is the physiological status (Denis et al. 1999). So, it is not surprising that larger bivalves have a lower CR comparatively to the smaller one when its reproductive state is active. As gametes development and ripening demand a lot of energy (Bayne et al. 1999) that energy is moved into reproduction.

The exogenous factors that mostly affects a bivalve's FR and CR are particle or food concentration, quality and size (Bayne L et al. 1993; Defosse and Hawkins 1997;

Ward and Shumway 2004). For instance, FR and CR of *M. edulis* increase, when particle concentration increases until particle concentration (*Phaedactylum tricorutum*) reaches around $700 \mu\text{L}^{-1}$ (Foster-Smith 1975). In contrast, another study from Riisgård (2001) showed that the same species fed on *Rhodomonas baltica* filtered maximally at lower cell concentration, which is around 3–10 cells μL^{-1} . In addition, the particle quality factor, FR and CR in *M. chilensis* can be higher when the organic and chlorophyll content is also high (Velasco and Navarro 2002). A similar case is also exhibited in *C. gigas* where a decrease in organic matter in the food results in the decrease in the CR (Ward et al. 1998b).

Other exogenous factors that can affect bivalve CR are the temperature, salinity, current regime and the presence of biotoxin as well as chemical pollutants. The effect of temperature on bivalve's CR has been well studied (Bayne 1976; Kittner and Riisgård 2005; Pernet et al. 2007; Comeau et al. 2012). A study conducted by Comeau et al. (2008) on *C. virginica* showed 50% at 9°C to 100% reduction in CR at 0°C. While same authors observed CR ranged from 100% at 19°C to 17% at 0° (Fig. 1-12). Several studies have shown that the salinity can influence the CR. For instance, higher CR was observed in the Chilean scallop *Argopecten purpuratus* at a higher range of salinity (27-30 psu) comparatively to lower one (18-24 psu) (Navarro and Gonzalez 1998). Furthermore, another study on the effect of different salinities (10, 15, 25 and 35 psu) on CR was conducted by Mcfarland et al. (2013) on the Eastern oyster *C. virginica* and the green mussel *Perna viridis*. These authors observed that at salinities of 25 and 35 psu, green mussel CR were approximately double that of oysters. In addition, unlike the oysters, green mussel CR decreased by an order of magnitude at salinities of 10 and 15 psu.

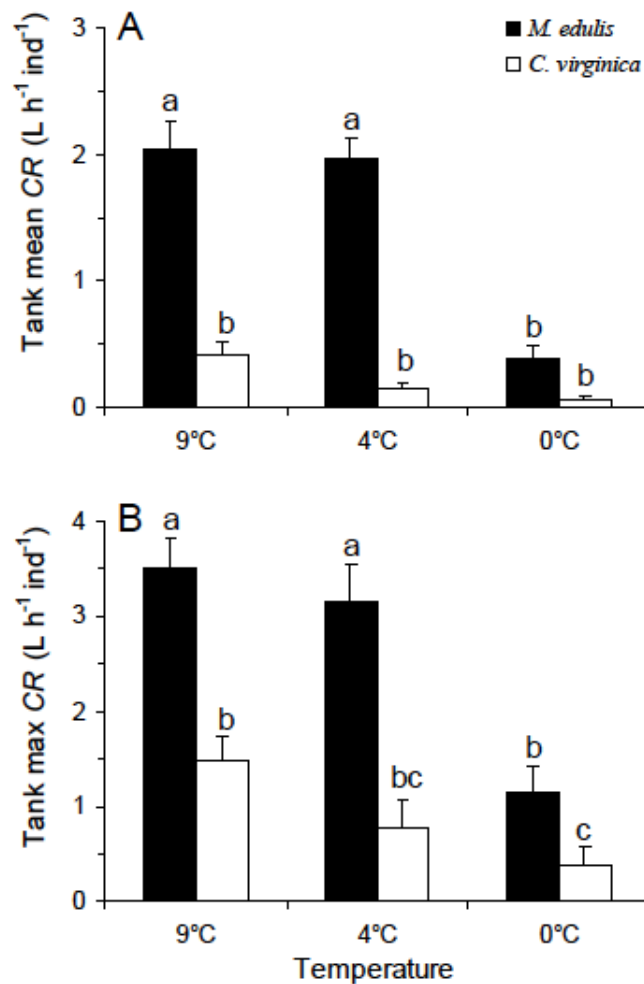


Figure 1-12. (A) Mean and (B) maximum clearance rate (CR) as a function of species (*Mytilus edulis*, *Crassostrea virginica*) and temperature (0, 4 and 9°C). Error bars represent one standard error of the mean. Different letters indicate significant differences ($p < 0.05$). Picture is adapted from Comeau et al. (2008).

More recently, Riisgård et al. (2013, 2014) studied the effect of salinity on CR of *M. edulis* that was collected from two different sites (the brackish Great Belt-Denmark and the low-saline Central Baltic Sea). The authors observed that the lack of Great Belt mussels to completely adjust to 5 psu, in contrast to the ease of Baltic Sea mussels to adjust back and forth between 6.5 and 20 psu, suggests that different genotypes of mussels in those sites may play a significant role in CR-salinity adaptation. On the other side, current water flow

can also influence the CR in bivalves. For example, Sobral and Widdows (2000) observed a maximum CR of 2.5 L h^{-1} per individual at current velocities up to 8 cm s^{-1} in the clam *Ruditapes decussatus*. However, this CR declined with increasing current speed, especially above 17 cm s^{-1} . Moreover, the effect of flow speed on CR can vary both between and even within species. For instance, on the infaunal cockle *Cerastoderma edule*, CR was not significantly affected by changes in current speed between 5 and 35 cm s^{-1} (Widdows and Navarro 2007).

Other factors that can affect the CR in bivalves are the presence of pollutants such as metals, biotoxins and chemical exudates from microalgae. Generally, negative impacts on bivalves' CR due to the presence of pollutants and metals have been observed in many studies (Mubiana and Blust 2007; Fernández et al. 2010; Tsangaris et al. 2010; Montes et al. 2012). Other studies showed that the biotoxin from microalgae such as dinoflagellates could alter bivalve's CR. For example, Navarro and Contreras (2010) showed that CR of *M. chilensis* exposed to toxic dinoflagellate *Alexandrium catanella* was significantly lower than those of controls. However, CR increased on the third day of the experiment suggesting that the bivalves possess a depuration mechanism (Fig. 1-13). Another study showed no effect of toxic alga *Karenia brevis* on clam *Mercenaria mercenaria* (Echevarria et al. 2012). In addition, responses of bivalve species' CR to the different biotoxins of harmful algae were species-specific, and this can also be influenced by the characteristics of the toxic microalgae (Hégaret et al. 2007). Most of the studies came up with the negative impact of biotoxin on CR, but most of the toxins used were the Paralytic Shellfish Toxin (PST) type. Indeed, non-toxic chemical exudates from the microalgae can also influence the CR in bivalves but the study on this is still scarce. In contrast to the biotoxin, chemical exudates from the diatom *Chaetoceros muelleri* increased the CR of scallop *Placopecten magellanicus* (Ward 1992). In the context of my Ph.D., the influence of marennine as a non-PST substance will be studied on different bivalve species. The part of this work will be presented in Chapter 4.

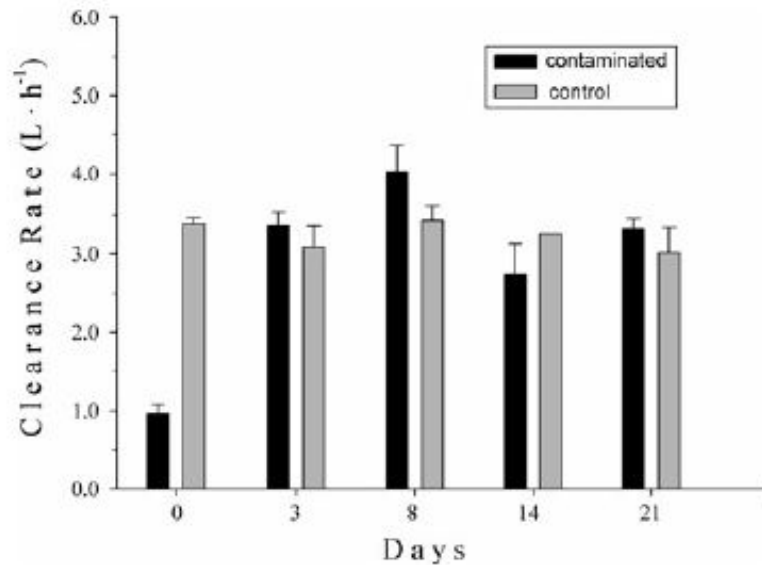


Figure 1-13. Clearance rate of *M. chilensis* exposed to a contaminated and a control diet. Values are means \pm SE. Pictures adapted from Navarro and Contreras (2010).

1.5.3.2 Particle retention

Retention or the particle capture can be defined as the process by which a suspended particle in the current water is flowing through the gill and diverted to the end of the frontal surface of filaments. This mechanism is previously suggested as the consequence of the presence of cilia latero-frontal, which play a significant role in capturing the particles (Atkins 1937b). This idea is supported by additional studies which showed that the efficiencies in capturing particles (RE) vary between bivalve species in function of the size of their latero-frontal cilia (Møhlenberg and Riisgård 1978; Jørgensen et al. 1984; Riisgård 1988). Also, it was observed that RE could be higher in small particles for the bivalve species with a large size of latero-frontal cilia comparatively to those with small or nonexistent cilia (Silverman et al. 1995, 1997).

Early studies of particle retention by bivalves showed that particles below 1 μm to 2 μm were passed through the gills, while particles larger than 3 μm were normally retained at a higher percentage (Jørgensen 1990). Later on, literature suggested that the RE may be either species or habitat specific. Most of the studies suggest that RE increases with particle

size to an upper threshold and that at the start of this threshold ($>7 \mu\text{m}$), the RE becomes species specific (Ward and Shumway 2004). Indeed, similar to filtration, RE is influenced by endogenous (bivalve size) and exogenous factors which are previously described in the section above.

1.5.3.3 Particle transport and selection: pre-ingestive and post-ingestive selection

Captured particles are transported with conveyor-belt style, to the frontal surface of the gills by the mucociliary process. Afterward, they are further transported on the ventral or dorsal ciliated tract of the gills which functioning appears to be species specific (Ward and Shumway 2004). For instance, in *M. edulis*, particles are transported in the ventral tract to the labial palps and fewer particles are transported along the dorsal tracts regardless to their concentration (Ward 1996). On the other hand, in *C. gigas* and *C. virginica*, due to their plicated gills form, the particles are transported via both the dorsal tract and ventral tract towards the labial palps (Beninger and St-Jean 1997; Ward et al. 1998a, b).

Once captured by the gills, particles will be sorted by the pre-ingestive selection mechanism, a process where the gills can preferentially reject particles in the form of pseudofaeces while preferentially ingesting the others (Newell and Jordan 1983). This selection can be influenced by macroscopic factors (*e.g.* gill and filament types) and microscopic factors (*e.g.* sensory detection and decisional behavior by individual or groups of cilia) (Beninger et al. 2004; 2008a, b). Generally, in heterorhabdic gill type, either filibranch (scallop) or pseudolamellibranch (oyster) gills are able to select particle based on its quality (Ward et al. 1998a; Cognie et al. 2003a; Beninger et al. 2004, 2008a, b). For instance, in *C. virginica* and *C. gigas*, particles in both gill's ventral and dorsal tract are transported to the labial palps. Yet, those in the ventral tract are of lesser trophic importance and thus more likely to be rejected as pseudofaeces prior to ingestion but may be re-sorted on the palps (Ward et al. 1997, 1998a). Indeed, in pseudolamellibranch, labial palps are involved in the selection process by being the only organs that can sort particles greater in size than the opening of the principal oyster filament (Cognie et al. 2003a).

Selection by labial palps was also confirmed by Beninger et al. (2008b) who observed that these organs perform particle selection but are restricted to its concentration. In contrast, bivalves with homorhabdic (one type of filament) gills are not capable of particle selection on the gill. Video endoscopy experiments have demonstrated that the sorting in the blue mussel *M. edulis* only occurs on the labial palps (Beninger and St-Jean 1997; Ward et al. 1998a).

The mechanism of selection is at least partially influenced by interactions between lectins, a group of carbohydrate-binding proteins, in the gill mucus and food particles (Pales Espinosa et al. 2009). To date, same authors observed specific microalgal-binding lectins in the mucus produced by feeding organs in the mussel *M. edulis* (Pales Espinosa et al. 2010a, b) and in the oyster *C. virginica* (Pales Espinosa et al. 2009). More recently, Rosa et al. (2013) suggest that the physico-chemical surface properties of particles (e.g. charge and wettability), may also contribute to the particle selection process.

In bivalves, production of pseudofeces is integral to the selectivity process that allows rejection of less nutritious particles. Thus, this mechanism improves the overall content of material being ingested and assimilated (e.g. to compensate for the poor quality seston in the natural environment) and prevents particle overloading during the ingestion processes (Beninger et al. 1991; Macdonald and Ward 1994; Bacon et al. 1998). Three mechanisms of pseudofeces rejection have been grouped at four main bivalve gill systems (Beninger et al. 1997a; Beninger and St-Jean 1997). This includes most bivalves with homorhabdic filibranch gills and all bivalves with eulamellibranch gills that have pallial transport of pseudofeces with vertical elevation achieved via composite cilia (Beninger et al. 1997a, b; Beninger and St-Jean 1997).

Table 1-1. Summary of particle processing on the pallial organs of different gill types. Cited and modified from Beninger in Gosling (2015).

Gill type	Species	Function		
		Gill	Labial palp	Mantle
Homorhabdic filibranch	<i>Mytilus edulis</i>	Indiscriminate transport, ventral tract only	Qualitative selection and pseudofaeces rejection; ingestion volume control	Pseudofaeces rejection
Heterorhabdic filibranch	<i>Placopecten magellanicus,</i> <i>Pecten maximus</i>	Preferential positive selection (dorsal tracts); ingestion volume control and preferential negative selection (ventral tracts)	Qualitative selection and pseudofaeces rejection	No rejection function; pseudofaeces expelled from ventral margin by shell adduction
Eulamellibranch	<i>Spisula solidissima, Mya arenaria</i>	Indiscriminate transport; ventral tracts only	Qualitative selection and pseudofaeces rejection; ingestion volume control	Pseudofaeces rejection
Pseudolamellibranch	<i>Crassostrea virginica,</i> <i>Crassostrea gigas, Ostrea edulis</i>	Preferential positive selection for particles smaller than principal filament tracts (dorsal tracts); ingestion volume control and initial negative selection (ventral tracts)	Qualitative selection and pseudofaeces rejection; ingestion volume control	Pseudofaeces rejection

In the Pseudolamellibranch, the mechanism of pallial transport is similar but vertical elevation is achieved via mantle ridges (Beninger and Veniot 1999). In contrast, for the scallops with heterorhabdic filibranch gills, pseudofeces is rejected via valve pulsing (or clapping) (Beninger et al. 1999). The rate of pseudofeces production for most bivalves increases with increasing particle concentration (Shumway et al. 1985; Bayne et al. 1993; Bacon et al. 1998). However, the ingestion and rejection of material on the gills depends on both particle concentration and gut fullness. When the gill is overloaded with particles or when the ingestive capacity is exceeded, more material is rejected (Ward et al. 1998a). A brief summary of the selection process at different groups of the gill is given in Table I-1.

Studies concerning the post-ingestive selection are still scarce. An early study by Shumway et al. (1985) showed that the flagellate *Chroomonas salina* is preferentially digested over the two other microalgae (*Phaedactylum tricornutum* and *Prorocentrum minimum*) in *C. virginica*, suggesting the post-ingestive selection in this bivalve. Same authors also demonstrated that selection was most likely based on qualitative factors since the phytoplankton species used were of similar size. More recent study confirms that the post-ingestive selection can be based on the particle quality as the one who lives and has higher organic content resulting in an increase in digestive efficiency (Brillant and MacDonald 2002, 2003).

1.5.3.4 Scope for Growth (SFG) and Dynamic Energy Budget (DEB) as energy budgeting model in bivalve

It is necessary to make growth prediction tools in bivalve species considering their important economic role in aquaculture. To date, two main approaches have been applied to model bivalve growth: the Scope for Growth (SFG) and the Dynamic Energy Budget (DEB). The SFG model is based on an energy balance, where energy available to growth and reproduction is calculated from the difference between energy absorbed from the food and the energy lost due to respiration and excretion (Smaal and Widdows 1994). A Positive value in SFG means that the organism has energy available for growth and reproduction that is manifest as an increase in body weight. In contrast, a negative balance in SFG will

result in a decrease body weight as a consequence of the utilization of reserves. DEB models allow describing the individual in terms of two state variables, structural body and reserves (van der Meer 2006). This describes the energy flow through organisms from assimilation to allocation to growth, reproduction and maintenance. Thus, in DEB, all energetic processes in an organism are functions of its state (e.g. age, size, sex, nutritional status, etc.) and the environment (food density, temperature, etc.) (Nisbet et al. 2000).

Indeed, distinctions can be made between SFG and DEB models. The conceptual difference between the two models is that SFG models assume assimilated energy is immediately available for respiration and excretion while the remainder is used for growth or stored as reserves. Thus, SFG models assume that energy from catabolism is lost. However, this assumption is not entirely correct since the energy from catabolism that has been reinvested in the anabolic process of growth actually stays in the organism instead of being subsequently lost so that the energy is still in balance. On the other side, DEB models assume that the assimilated energy is directly stored in reserves, which in turn are utilized to sustain other metabolic processes (Nisbet et al. 2000; van der Meer 2006). Additionally, an important assumption in the DEB model is the κ -rule where a fixed proportion of κ of the available energy is allocated to somatic maintenance (as the priority) and growth, whereas the remaining $1-\kappa$ is allocated to maturation and reproduction for juveniles and adults, respectively (Fig. 1-14) (Nisbet et al. 2000; Barillé et al. 2011).

Indeed both SFG and DEB models have advantages and disadvantages. The SFG model has been widely used due to its simplicity to empirically estimate growth through the measurement of organism physiological processes, which are relatively easy to measure. However, this model violates the energy conservation rules by presenting the energy imbalance assumption. This disadvantage is not present in the DEB model, which assumes common physiological processes across species and life stages. Nevertheless, the shortcoming of DEB is to estimate the basic parameter sets for different species (van der Meer 2006; Pouvreau et al. 2006b). In spite of the disadvantages of these models, SFG and DEB have been successfully applied in estimating individual bivalve growth (SFG: (Grant and Bacher 1998; Hawkins et al. 2002; Brigolin et al. 2009). More recently, Filgueira et al. (2011) observed that both models performed well in *M. edulis* in different ecosystems by

adjusting the half-saturation coefficient of food ingestion function term, X_k , as a common parameter in both models related to feeding behavior. The authors suggest that the connection of two models via calibration of X_k indicates the importance of feeding behavior and local trophic conditions for bivalve growth performance.

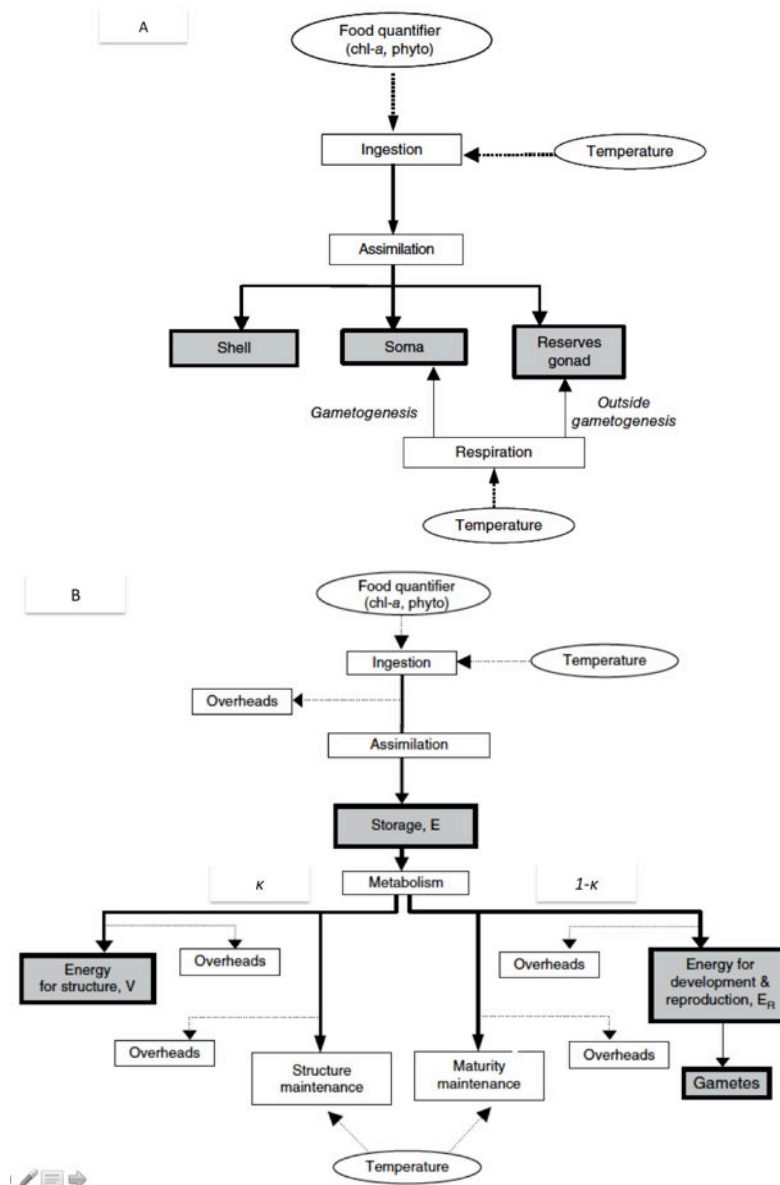


Figure 1-14. Scheme of the scope for growth (SFG) and dynamic energy budget (DEB) models for the Pacific oyster *Crassostrea gigas*. Forcing variables are shown in the ellipsis while state variables are in the grey boxes. Picture was modified from Barillé et al. (2011).

For decades, bivalves have been used as a biological indicator of environmental stress (Smaal and Widdows 1994; Widdows et al. 2002; Dame 2011). Among the physiological parameters in bivalves, scope for growth (SFG), is considered to be an advantageous tool to measure the impact of environmental stress such as, salinity, temperature and the presence of pollution as well as toxic substances from microalgae (Widdows and Johnson 1988; Widdows et al. 2002; Pernet et al. 2007, 2008; Navarro and Contreras 2010). For instance, a decrease in SFG was observed in blue mussel *M. edulis* exposed to oil and copper contaminants (Widdows and Johnson 1988; Smaal and Widdows 1994; Widdows et al. 2002; Mubiana and Blust 2007). A similar pattern was also observed for bivalves that were exposed to toxic dinoflagellate species where the SFG is significantly decline due to the presence of toxic substance in their diet (Li et al. 2002; Hégaret et al. 2007; Navarro and Contreras 2010; Manfrin et al. 2012; Navarro et al. 2014). However, to our knowledge, only a few studies have observed some of the effects of non-toxic organic compounds (non-PST) on cellular responses of bivalves but not on their physiological traits (Hégaret and Wikfors 2005a, b; Ford et al. 2008). Therefore, it is interesting to assess whether a non-PST compound, the pigment marennine, synthesized by the pennate diatom *H. ostrearia* (Gaillon) Simonsen, known as a non-toxic species, can affect bivalves, other than greening gills. Integrated response of species model to marennine will be further explained in chapter 4.

1.6 Research objectives

The main objectives of my Ph.D. research are: 1) to determine the role of size in pre-ingestive selection process in oyster *C. gigas* as a consequence of the presence of *H. ostrearia* in artificial seawater ponds (chapter 2); 2) To understand the mechanism of the greening phenomenon in bivalves by marennine and its interaction with bivalve's pallial organs (chapter 3). 3) To determine the consequence of greening by marennine on the integrative response of two bivalve *M. edulis* and *C. virginica* (chapter 4); 4) To understand

the allelopathic interaction between *Haslea ostrearia* and other microalgae species relevant in aquaculture, and to assess the feasibility of co-culture of *H. ostrearia* and other microalgae to get the maximum benefit of biological activities of marennine in the field of aquaculture (chapter 5).

Chapter 2: Role of size in pre-ingestive selection of *Haslea ostrearia* in *Crassostrea gigas*

To date, many studies have been conducted regarding the mechanism of pre-ingestive selection in bivalve, involving the pallial organs and ciliation and their interaction with the particles. However, the determinant factor influencing pre-ingestive selection in bivalve remains unclear. Among all factors proposed, earlier studies demonstrated that particle size was the first criterion to determine food selection (Atkins 1937b) yet its role remains inconsistent. Several studies repeatedly reported that there is no role of size in pre-ingestive selection (Newell and Jordan 1983; Newell 1988; Chretiennot-Dinet et al. 1991; Macdonald and Ward 1994; Bougrier et al. 1997). In contrast, other studies have displayed that bivalves can sort particles based on their size (Ballantine and Morton 1956; Miura and Yamashiro 1990; Cognie et al. 2003b; Mafra et al. 2009a). In this chapter, different sizes of *H. ostrearia* with identical biochemical properties, as a consequence of its asexual reproduction in artificial oyster ponds, were used to determine the effect of size on the mechanism of pre-ingestive selection. This study is considerably important due to recent discoveries on biological activities from marennine, a water-soluble blue pigment produced by *H. ostrearia*, particularly as an antipathogen in aquaculture. Therefore, understanding the mechanisms of the pre-ingestive selection of this microalga in oysters will give an idea on its uptake of marennine. The experiment regarding this study was held at the laboratory of MMS, Université de Nantes, France, by using a flow through chamber system. Furthermore, *in vivo* observations were conducted by using video-endoscopy and scanning electron microscopy. The results from this study have been submitted in Journal of Marine Biology.

Chapter 3: Greening of bivalve by *Haslea ostrearia*

The greening phenomenon of oysters has long been a known phenomenon in France that was achieved by refining the oyster in artificial seawater ponds containing microalgae (Sprat 1669; Gaillon 1820). The first greening experiment was conducted by placing the 'normal' oyster during 24 to 36 h into the green medium obtained from the artificial ponds that contain high-density *H. ostrearia*. In addition, greening can also be achieved by using green medium from the ponds without the presence of the cell (Ranson 1927). Greening experiments were subsequently conducted by placing the oysters into the axenic culture medium of *H. ostrearia* in which was added an aqueous extract of the cells of this same culture during 10 h (Neuville and Daste 1972). This study implied that the greening was affected by the direct adsorption of marennine pigment from the medium at the gills of oysters. More recently, as the method of extraction and purification of marennine developed, Pouvreau (2006) performed another experiment by placing the oysters into the pigment with the purified form. He observed that both intra- and extracellular purified form of marennine (IMn and EMn, respectively) were able to color the gills of the bivalve. Furthermore, within the similar exposure time, the latter showed stronger ability in coloring the gills comparatively to the other, suggesting the EMn is the main form of pigment that binds within the gills. Nevertheless, comprehension of the cellular mechanisms involved in the greening on bivalve gill's tissue has not been determined and remains unclear.

In this chapter, several questions were proposed to give a better insight in understanding the mechanism of bivalve greening by marennine and its consequence on bivalve's feeding behavior. First, can the greening only be realized by the presence of *H. ostrearia* and its EMn or by simply using the culture supernatant of *H. ostrearia* or crude extract without the presence of the cells? Secondly, can the greening be realized solely by the mechanism of ingestion of the cell of *H. ostrearia* without the presence of their extracellular form? Thirdly, can the fixation of marennine on the gills only be realized by bivalve's active filtration or it can be achieved passively. Fourthly, where exactly is marennine fixed in the gills? Lastly, does the marennine fixation affect the mechanism of filtration in bivalves?

The experiment concerning this study was conducted in the laboratory of MMS Université du Maine and Université de Nantes, France as well as ISMER-UQAR, Canada. Results from this study will be presented in the short communication Journal of Aquaculture.

Chapter 4: Consequence of greening by marennine on the integrative response of bivalve

Recent studies have demonstrated that purified marennine presents biological activities, such as allelopathic, antioxidant, antiproliferative, antimicrobial and antiviral properties (Pouvreau et al. 2007b, 2008; Gastineau et al. 2012b, c, 2014c). In addition, several species producing “marennine-like” pigment from the same genus *Haslea* were recently discovered elsewhere in the world (Gastineau et al. 2012b, 2014c), which leads to the potential development of this pigment in the field of aquaculture worldwide, as well as for shellfish culture in particular. However, the effect of marennine itself on bivalves has never been documented. An earlier study by Piveteau (1999) demonstrated that the oysters fed with *H. ostrearia* in artificial seawater ponds grow slower compared to those fed with *S. costatum* in a long period of time (e.g. 8 weeks). Yet, the reason for the delay of growth in oysters remains unclear either due to the poor quality of *Haslea* given or by the biological activity from the marennine released into the ponds.

In this chapter, the impact of excreted and purified marennine on the behavioral, physiological and biochemical characteristics of two commercially important bivalves species (the eastern oyster *C. virginica* and the blue mussel *M. edulis*) was studied. Here, several hypotheses were tested to answer the following questions. First, in short-term, does the presence of extracellular marennine (EMn) in the water column affect valve activity of bivalves? Secondly, does the tissues greened by EMn have a long term affect by influencing the physiological (e.g. clearance rates, oxygen consumption, absorption efficiency and scope for growth) and biochemical characteristics (composition of fatty acids in bivalve’s tissue) of bivalves? To answer these questions, an experiment was conducted in the Research Station Aquicole de Pointe-aux-Pères from Institut des Sciences

de la Mer (ISMER)-l'Université du Québec à Rimouski, Canada. Valve activity, as the short-term response, was measured *in vivo* by using Hall sensor. In addition, the physiological rate was measured by using the static chamber at the station while biochemical analysis was performed in ISMER. The result from this study has been submitted to Journal of Aquaculture.

Chapter 5: Allelopathic effect of *Haslea ostrearia* on other microalgae relevant to aquaculture

The pennate diatom *Haslea ostrearia* can co-occur with other phytoplankton in artificial oyster ponds. This diatom has the particular feature of synthesizing and excreting the water-soluble blue pigment marennine, which gives added value to the bivalve *Crassostrea gigas* in French oyster industry. An earlier study revealed that marennine accumulated in the cell of *H. ostrearia* was positively correlated with nutrient deficiency and light intensity (Neuville and Daste 1978; Robert et al. 1983; Mouget et al. 1999, 2004, 2005; Rech et al. 2008). Additionally, previous studies have presumed that the presence of *H. ostrearia* and marennine can affect the co-occurring phytoplankton in oyster ponds through allelopathic interaction (Moreau 1970; Neuville and Daste 1978; Turpin et al. 1999). Until then, Pouvreau et al. (2007) confirmed this allelopathic interaction by using the purified form of marennine.

In this chapter, the allelopathic effect of *H. ostrearia* and marennine produced in realistic conditions by microalgae co-culturing methods was assessed to determine the sensitivity of algal species that are typically used in aquaculture and possible diet combinations are discussed. In addition, microalgae co-cultures were maintained with the semi-continuous mode in order to avoid possible bias from nutrient and light limitations. The experiment concerning this study was conducted at the laboratory of MMS Université du Maine, France. The result from this study was accepted in Journal of Applied Phycology.

2 CHAPTER 2: ROLE OF SIZE IN PRE-INGESTIVE SELECTION OF *HASLEA OSTREARIA* IN *CRASSOSTREA GIGAS*

SUMMARY

The first article, entitled “Passive selection by the oyster *Crassostrea gigas* on the blue diatom *Haslea ostrearia*”, was co-authored by myself and by Priscilla Decottignies, Laurent Barillé, Romain Gastineau, Boris Jacquette, Amandine Figiel, Michèle Morançais, Réjean Tremblay, Jean-Luc Mouget, and Bruno Cognie. This article will be submitted in the Journal of Marine Biology in July 2015. As the first author, my contribution to this article was the execution of experiments, laboratory and data analysis as well as writing for the manuscript preparation. Bruno Cognie provided the original idea and assisted in the method development. All of the co-authors participated in this work. Results of this work was presented as poster presentation in the International Elsevier conference of Aquaculture 2013: To the next 40 years of sustainable global aquaculture in Las Palmas, Gran Canaria, 3 to 7 November 2013, and national workshop of BiogenOuest in St. Malo, France, 3 April 2014.

Passive selection by the oyster *Crassostrea gigas* on the blue diatom *Haslea ostrearia*

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Abstract

Recently, the blue pigment "marennine" produced by the pennate diatom *Haslea ostrearia* has revealed promising biological activities, in particular on pathogens of the oyster *Crassostrea gigas*. However, preingestive selection is identified as a feeding mechanism of oysters that may influence their uptake of microalgal metabolites. Therefore, we examined the role of microalgal cell size in the selective feeding of pacific oysters. Individual flow-through chambers were used to deliver to oysters mixtures containing *H. ostrearia* of varying cell length. Inflow, outflow and pseudofaeces samples were collected from the individual chambers during oyster feeding, while video endoscopy was used to sample material at the dorsal and ventral particle tracts. Visual counts of *H. ostrearia* cells were done on these samples and showed that the pseudofaeces contained larger cells than

ambient medium. In contrast, the proportions in pseudofaeces were identical to those in ventral tracts. In addition, video-endoscopy observations and imaging by scanning electron microscopy of gill and labial palps revealed that only those larger *H. ostreria* orientated dorso-ventrally could enter the principal filaments and then access to dorsal acceptance tract. These results show that for particle with only one axis that can exceed the width of the principal filaments, the selection at oysters' gill is passive and based on particle size. Given the crucial role of cell size in the life cycle of diatoms, the impact of this process on their population dynamic should be studied over a larger range of species.

Keywords: *Crassostrea gigas*, *Haslea ostrearia*, marennine, microalgal cell size, oyster, preingestive selection

2.1 Introduction

Suspension-feeding bivalves constitute the dominant trophic group in estuarine ecosystems, and their feeding mode may affect deeply nutrient recycling, seston dynamic and benthic food web (Prins et al. 1991; Asmus and Asmus 1991; Dame 1993; Ward and Shumway 2004). In these environments, suspension-feeders have to cope with broad fluctuations in both the quantity and quality of suspended particulate matter (Armstrong 1958; Berg and Newell 1986; Fegley et al. 1992; Barillé et al. 1997). It is thus not surprising to find an abundant literature concerning the response of suspension-feeding bivalves to seston fluctuations, in particular concerning physiological variables related to feeding, as clearance rate, retention efficiencies and pre-ingestive selection (Jørgensen 1990; Riisgård 2001; Riisgård and Larsen 2001; Ward and Shumway 2004).

Recent progress has been made on the understanding of the pallial organs and ciliation involved in pre-ingestive selection (Beninger and St-Jean 1997; Beninger et al. 1997; Ward et al. 1997; Cognie et al. 2003). Despite numerous studies, the criterion upon which bivalves rely on sorting particles remains unknown. Many particle characteristics have been identified as selection cues, namely size (Hughes 1975; Shumway et al. 1985; Defosse and Hawkins 1997), organic content (Bacon et al. 1998; Beninger et al. 2008a) or

surface properties (Ward and Targett 1989; Pales Espinosa et al. 2007, 2010a; Beninger et al. 2008b; Rosa et al. 2013).

Particle size was the first criterion used by early researchers examining food selection (Yonge 1926; Atkins 1937b) but its role remains relatively inconsistent, and no influence of particle size on pre-ingestive selection was repeatedly reported (Newell and Jordan 1983; Newell 1988; Chretiennot-Dinet et al. 1991; Macdonald and Ward 1994; Bougrier et al. 1997). On the opposite, numerous studies have demonstrated that bivalves can sort particles against their size (Ballantine and Morton 1956; Miura and Yamashiro 1990; Cognie et al. 2003; Mafra et al. 2009a). It may be argued, however, that the particles involved in most of these studies differed in more than one of their properties. For example, Hughes (1975) observed a size-dependent selection of particles by *Abra alba*, but noted a relation between particle size and food value. Cognie et al. (2001) rejected the role of particle size in selection by *Crassostrea gigas*, but used different algal species. To our knowledge, very few studies have unambiguously supported the idea that bivalves could use size criterion to discriminate particles. A preferential size-dependent rejection of larger particles was observed in *C. virginica* (Tamburri and Zimmer-Faust 1996) and in *Mytilus edulis*, *Ruditapes philippinarum* and *Tapes decussatus* (Defosse and Hawkins 1997). Nevertheless, the particles used in these studies were artificial: polystyrene or borosilicate glass particles for the former, and silica particles for the latter. However, more recently, using contrasting sized clones of a toxic diatom, Mafra *et al.* (2009b) have indisputably demonstrated the role of size in the selection performed on *C. virginica* gills.

A well-known feature of diatom biology is the MacDonald-Pfitzer rule (Pfitzer 1869; Macdonald 1869; Round et al. 1990). During vegetative growth and mitotic divisions, one of the daughter cell is smaller than the mother cell, and the mean cell size of a population decreases, a phenomenon that usually leads to the loss of monoclonal cultures in the laboratory. This phenomenon, unique to diatoms and especially crucial in most pennate species, can be compensated for when a critical threshold size is reached, and sexual reproduction and auxosporulation can occur. In diatoms, auxosporulation results in initial cells, which restore cells with specific maximum length (Amato 2010; Gastineau et al. 2014b). As a consequence of these biological traits, cells of a same species, but of different

size, are often found together in the natural environment (Mann 1988; Potapova and Snoeijis 1997; Mann et al. 1999). This phenomenon is expected to affect the kinetics of diatom selection and diatom metabolite uptake by bivalves through its impact on their feeding processes, namely clearance rate, pre-ingestive selection and absorption efficiency. Indeed, Mafra et al. (2009b) have recently demonstrated that the ability of *C. virginica* to sort particles against their size could affect its uptake of the neurotoxin domoic acid from *Pseudo-nitzschia multiseriis*. Studying this specific size-dependent sorting mechanism would be of crucial interest in understanding and modelling the transfer of a bioproduct from algal cells to suspension-feeding bivalves. The marine diatom *Haslea ostrearia* is known to produce marennine, a polyphenolic water-soluble blue pigment (Pouvreau et al. 2006a), responsible for the "greening" of the tissues of numerous marine invertebrates and in particular the gills of bivalves (Ranson 1927; Robert 1983; Turpin et al. 2001; Gastineau et al. 2014a). It has also been shown that *H. ostrearia* can be used as feed for the oyster *C. gigas* (Barillé et al 1994). Although *H. ostrearia* has long been recognized as the only greening agent of cultured oysters in France, other species from the same genus and producing "marennine-like" pigments were discovered elsewhere in the world (Gastineau et al. 2012a, 2014b, 2015). These authors have also revealed that marennine-like pigments have antibacterial and antifungal activities, in particular against pathogens of the oyster *C. gigas* (Gastineau et al. 2012b, 2012c).

In the present work, cultures of *H. ostrearia* differing according to their mean cell length were used to feed the oyster *C. gigas*, to investigate if cell size may affect algal selection on pallial organs (gills and labial palps). For feeding experiments, different methods were used, endoscope-directed *in vivo* sampling and naturally occurring particles (diatom cells) of different sizes, and Scanning Electron Microscopy (SEM) to provide images of the close relation between the microalgal clones and the structures and ciliation of pallial organs involved in the sorting processes. Different clones of *H. ostrearia* being used, the ecological implications of oyster selection on some biological traits of this diatom species, *e.g.*, the reproductive cycle and population structure, will be discussed.

2.2 Materials and methods

2.2.1 Algal culture

Haslea ostrearia populations used in this study were obtained from the Nantes Culture Collection [NCC; temperature: 14°C; light/dark cycle: 14/10 h; light intensity: 100 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$; ES1/3 medium, Provasoli (1968)], Faculté des Sciences et des Techniques, Université de Nantes (France). The three strains presented similar biochemical composition (Joux-Arab et al. 2000). Sixty-liter mass cultures were performed in polyethylene bags filled with underground seawater (Cognie et al. 2001b) supplemented with an enrichment solution (nitrogen, phosphorus, and silicon) (Turpin et al. 2001a). The different populations were then mixed at equivalent concentrations (around 3×10^6 cells L^{-1}) and offered to the bivalves.

2.2.2 Oyster sampling and maintenance

Adult oysters of *Crassostrea gigas* were collected in the intertidal zone of Bourgneuf Bay (France) (46-47° N, 1-2° W). After immediate transfer to the laboratory and removal of shell epibionts, they were placed for two minutes in a 0.1% hypochlorite solution to eliminate parasitic polychetes of the genus *Polydora*. The oysters were then thoroughly washed, maintained in filtered (Millipore 0.45 μm) seawater and oxygenated.

2.2.3 Ecophysiological experiments

Two series of experiments were run on different periods, one with oysters feeding on three algal cell sizes (condition A), the other, on two algal cell sizes (condition B). After 24 hours of acclimation prior to experimental conditions (A or B, Table 2-1), oysters were randomly chosen and placed in a flow-through experimental system, as described in Barillé et al. (2003). The mean flow rate for each individual tray was 10 L h^{-1} at 16°C. Two trays

containing an empty shell were used as sedimentation controls. After one hour of oyster acclimation, the collection of seawater samples at the outflow of the experimental trays was performed every 15 minutes, during one hour. Pseudofaeces were collected at the end of the observations. The samples (outflow seawater and pseudofaeces) were fixed with acetic Lugol's solution and cell counts were performed by means of light microscopy using "Nageotte" type hematimetric units. For each sample, a minimum of 300 cells was counted.

Table 2-1. Experimental conditions (mean \pm SE).

	POM (mg.L ⁻¹)	Cell concentration (10 ⁶ cells.L ⁻¹)	Cell size (length in μ m)
Condition A	2.5 \pm 0.1	3.1 \pm 0.2	Ho50: 50.2 \pm 1.4 Ho75: 74.9 \pm 2.2 Ho95: 95.2 \pm 1.8
Condition B	1.9 \pm 0.1	3.0 \pm 0.3	Ho-small: 37.5 \pm 5.2 Ho-large: 99.4 \pm 2.2

Note: Ho = *H. ostrearia*

2.2.3.1 Sample preparation for Scanning Electron Microscopy (SEM)

Oysters intended for SEM observations were placed in flow-through trays and fed with the same mixture as in experimental condition A (Table 2-1). Once pseudofaeces appeared, the oysters were collected, shucked and immediately fixed in a 2.5% hypertonic glutaraldehyde solution in sodium cacodylate buffer 0.1 M (Beninger et al. 1995). The collection-fixation step was performed within 30 s to limit stress-related mucus production and preserve the functional stage of the animal. After fixation (for at least 48 h), individuals were partially dehydrated in successive alcohol baths at increasing concentrations (up to 70%). At this stage, the oysters were dissected using a dissection microscope and microsurgical instruments. The samples were subsequently totally dehydrated in a 100% anhydrous alcohol bath and dried with CO₂ using a critical point

apparatus. The samples were then plated with gold-palladium alloy and observed under a scanning electron microscope with field effect (JEOL 5400). To estimate the tissue contraction of the samples during SEM preparation, additional measurements were conducted with a dissecting microscope on live individuals still on the half shell.

2.2.3.2 Video endoscopy directed sampling

This sampling was conducted concomitantly to ecophysiological measurements described above, following the procedure described in Cognie et al. (2003). At least 24 h before the sampling, a small aperture was milled in the shells to prevent damage of the optical insertion tube (OIT) when the oyster valves closed. Specimens were placed in flow-through trays and fed the same mixture as in experimental condition A (Table 2-2). Sampling was performed every 15 min in both dorsal and ventral particle grooves (using a micropipette) and at the inflow and outflow of the trays. After one hour of observation, pseudofaeces were recovered. The samples (outflow seawater, pseudofaeces, and ventral and dorsal grooves samples) were treated and analyzed as described above (section 2.2.3).

2.2.4 Data analysis

All statistical analysis were performed by using XLstat software. The percentages of the different cell sizes were compared, in the different samples, using the Kruskal-Wallis test (Conover 1999). Hypotheses tested in the experimental condition B are described in Table 2-2.

Table 2-2. Experimental hypotheses tested in condition B

H0	Proportions not significantly different at sampling sites	No selection by the pallial organs
H1	Proportions significantly different at sampling sites	Selection by the pallial organs
H1a	Proportions significantly different in water and at ventral and/or dorsal tracts	Selection at gill
H1b	Proportions significantly different in ventral tracts and pseudofaeces	Selection at labial palps
H1c	H1a and H1b accepted	Selection at gill and labial palps

2.3 Results

2.3.1 Condition A: oysters' feeding on three algal cell sizes + SEM observations

The heterorhabdic gill of *C. gigas* is composed of plicae and troughs corresponding respectively to the locations of ordinary and principal filaments (Fig. 2-1A and B). The latter were difficult to observe due to their position deep between each plica and the contraction effect induced by the preparation for SEM study. *In vivo* observations showed that the plicae were 200 to 250 μm wide, and the ordinary filaments between 35 and 40 μm . In addition, ordinary filaments located in the apical part of the plicae (or apical filaments) were approximately 45 μm wide. The same measurements, when performed using SEM, showed that plicae were only 120 to 140 μm wide (Fig. 2-1A), ordinary filaments 18 to 26 μm wide (Fig. 2-1B) and apical filaments 25 and 30 μm wide (Fig. 2-1A and D). Principal filaments were no longer visible and too deeply positioned in troughs to allow measurements (Fig. 2-1B and C). Thus, due to preparation for SEM, the dimensions of contracted gill plicae correspond to *ca.* 50% of that observed *in vivo*.

Cells of the three distinct algal populations were always observed mixed, whether free or in aggregates of mucus, regardless of the structure involved. No cell population was isolated in a ciliary tract. *Haslea* cells were observed on the gill surface without preferential orientation (Fig. 2-1B, C, and D). However, the cells distinguished at the bottom of the principal filaments were always orientated according to the longitudinal axis of the gill filaments (\forall Fig. 2-1E). *Haslea* cells transported dorsoventrally along the gill

surface reached the ventral grooves (Fig. 2-1E). They were then transported toward the anterior part of the pallial cavity in mucous aggregates (not shown), before being processed by the ridged inner surfaces of the labial palps. Aggregates of mucus and cells of the three *H. ostrearia* populations were observed on these surfaces (Fig. 2-1F, G, and H).

A dimensional gap between the size of the three *H. ostrearia* populations and that of the gill and labial palps structures was apparent. A Ho95 cell overlapped more than two apical filaments (Fig. 2-1D) and more than three ordinary filaments (*Fig. 2-1E). Regarding labial palps, cells of the smallest population were equivalent in size to the width of a plica.

SEM studies of the structures and ultrastructures of the pallial organs involved in *C. gigas* feeding were associated with ecophysiological measurements. The mixture of the three *H. ostrearia* populations represented a food ration of 2.5 mg L⁻¹ of total seston containing an organic fraction of around 50% (Table 2-1). Microscopic measurements of frustule lengths showed that the three *H. ostrearia* populations were significantly different in size (Table 2-1; ANOVA, P < 0.001).

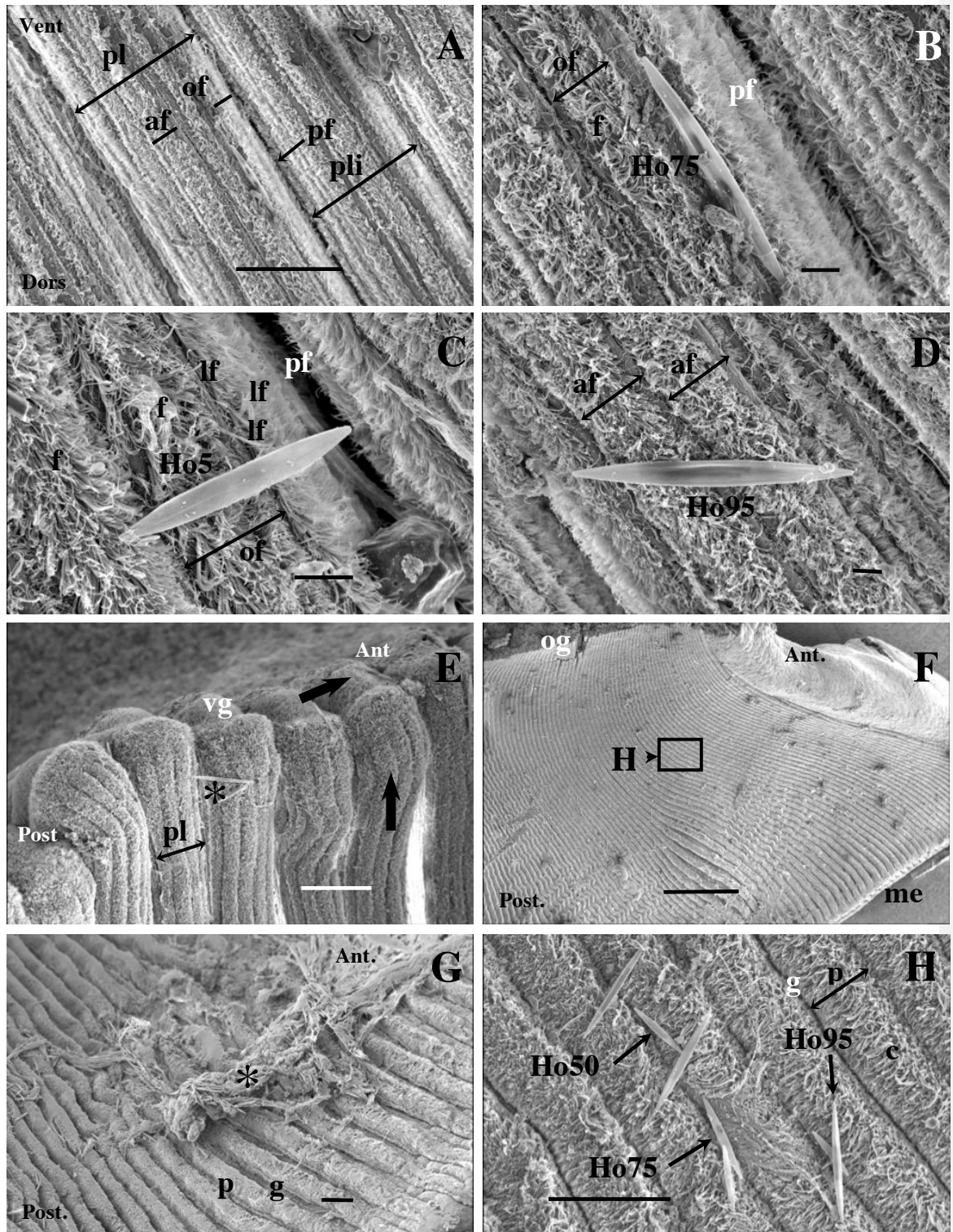


Figure 2-1. Scanning electron microscopy studies of the gill and labial palps of *Crassostrea gigas* fed with three different-sized populations of *Haslea ostrearia*

Ant., anterior part; Post., posterior part; Vent., ventral part; Dors., dorsal part; of, ordinary filament; pf, principal filament; vg, ventral groove; f, frontal cilia; p, plicae; g, grooves; c, cilia.

A: Frontal view of the gill showing differentiation between the principal filaments (pf) and the plicae constituted of ordinary (of) and apical (af) filaments. Scale bar, 100 μm . **B:** Detail of plicae for a *Haslea ostrearia* cell (length, 75 μm) in the main direction of the filaments. Scale bar, 10 μm . **C:** Ciliation of an ordinary filament conveying a *Haslea ostrearia* cell (length, 50 μm). lf, laterofrontal cirri; f, frontal cilia. Scale bar, 10 μm . **D:** Detail of plicae showing a *Haslea ostrearia* cell (length, 95 μm) lying across more than one apical filament (af). Scale bar, 10 μm . **E:** Side view of the gill and ventral groove (vg). Arrows indicate main particle transport. Scale bar, 100 μm . **F:** Ridged surface of a labial palp. og, oral groove; me, marginal edge. H, area corresponding to figures H. Scale bar, 1 mm. **G:** Detail of a ridged labial palp surface covered with an aggregate of mucus (*) containing the three cell sizes of *Haslea ostrearia*. Scale bar, 100 μm . **H:** Detail of the ridged labial palp surface, with cells of the three different-sized populations of *Haslea ostrearia*. Scale bar, 100 μm .

The proportions of the three populations were the same at the inflow and outflow of individual tanks (Table 2-3; χ^2 test, $p > 0.05$), indicating that they were retained with the same efficiency on gills. The proportions of the three populations at the inflow, however, were significantly different from those in the pseudofaeces (Table 2-3; χ^2 test, $p \leq 0.05$), indicating preferential pre-ingestive rejection of the two larger cell populations.

Table 2-3. Comparison of the proportions of the three *Haslea ostrearia* populations at sampling sites in condition A

	Mean proportions of the three populations (%)			χ^2 test (vs inflow)	
	Ho50	Ho75	Ho95	χ^2 observed	p
Inflow	40	31	29	-	-
Outflow	38	32	30	0.09	N.S.
Pseudofaeces	21	40	39	8.95	*

Note : *, $p \leq 0.05$; N.S., not significant.

2.3.2 Condition B: oysters' feeding on two algal cell sizes + video-endoscopy directed sampling

The mixture of the two *H. ostrearia* populations represented a food ration of 1.9 mg L⁻¹ of total seston containing an organic fraction of around 50% (Table 2-1). Biometry applied to frustule lengths showed that the two *H. ostrearia* populations were significantly different (ANOVA, $p < 0.001$). During the video-endoscopy directed sampling oysters showed no perturbation of their feeding behavior.

The proportions of the two size populations were the same in the inflow and outflow of individual tanks (Fig. 2-2), indicating that both populations were retained with the same efficiency on gills ($p > 0.05$). The Kruskal-Wallis test, however, clearly leads to rejection of null hypothesis H_0 and acceptance of H_1 : selection occurred on the pallial organs.

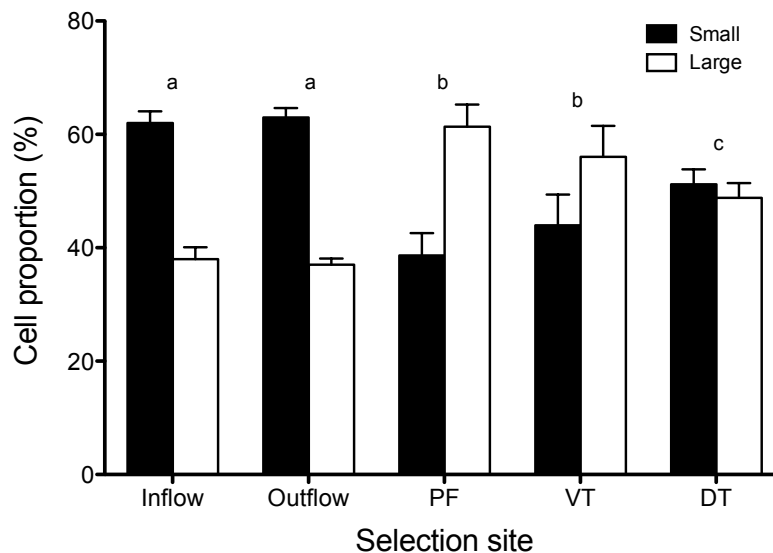


Figure 2-2. Mean percentages of *Haslea ostrearia* small (black bars) and large cells (white bars) in each sampling sites: inflow, outflow, pseudofaeces, dorsal tract and ventral tract. Error bars represent the 95 % confidence interval of the mean. Error bars with different lower case letters are significantly different.

A significant difference was observed between the proportions of the two *H. ostrearia* populations in inflow, ventral grooves, and dorsal tracts, thus allowing acceptance of *H1a*: selection occurred on the gills ($p < 0.05$). There was no significant difference between the proportion of cells in the pseudofaeces and at ventral grooves. The experimental hypothesis may, therefore, be rejected: *H1b* and *H1c*: no selection was performed by the labial palps.

2.4 Discussion

In the present study, cells were retained with the same efficiency when oysters were fed with mixed suspensions containing *H. ostrearia* of varying cell length. Previous studies demonstrated that in bivalves, retention efficiency varies with particle size and concentration. In *C. gigas*, Barillé et al. (1993) showed that particles above 6 μm ESD (equivalent spherical diameter) were retained with 100 % efficiency, at the total seston concentration used in our study (1.9 or 2.5 mg L^{-1}). This maximal retention efficiency threshold is slightly under the size of the smallest *H. ostrearia* cells fed to the oysters (Ho-small, 6.3 ± 0.1 mean ESD \pm SE), indicating that the cells of the three populations were retained with 100 % efficiency.

From both experiments, we demonstrated that the size of *H. ostrearia* cells is an essential criterion influencing the pre-ingestive selection in *C. gigas*. Oysters preferentially rejected in pseudofaeces the larger *H. ostrearia* cells (Ho-large or Ho75 and Ho95) when offered in mixed suspensions with the smaller ones (Ho-small or Ho50). In oysters, pre-ingestive sorting ability has been related to the presence of antagonistic ciliary tracts on the surface of the ordinary filaments composing plicae (Atkins 1937b; Ribelin and Collier 1977; Ward et al. 1994, 1998b). Particles transported by the median frontal cilia of ordinary filaments are directed towards the ventral grooves in which they are conveyed within an aggregate of mucus for a further preferential rejection. Conversely, particles transported by the marginal frontal cilia on both sides of the median frontal cilia are

directed towards the dorsal grooves and then conveyed to the mouth as a mucus suspension. Figures 2-1B-E show the dimensional relation between the size of the three *H. ostrearia* populations and that of the gill structures likely to perform particle sorting. Thus, for large cells with a naviculoid shape, such as those used in our study, bidirectional particle transport by ordinary filaments would seem difficult to achieve, even if they were directed according to the longitudinal axis of the filaments.

Another mechanism that could account for selection at gill level relates to the differentiation of heterorhabdic gills into principal and ordinary filaments (Atkins 1937b; Ward et al. 1994, 1998a; Beninger and St-Jean 1997). In bivalves possessing such a gill type, pre-ingestive sorting may be performed using principal filaments for the material to be ingested and ordinary filaments for the material to be rejected. Our SEM and video-endoscopy observations showed that the cells of the larger populations were present in the troughs containing principal filaments but were always orientated dorso-ventrally. These qualitative data confirm that passive selection related to particle size may operate on the gill, as suggested by Ward and Shumway (2004) and previously observed in *C. virginica* by Mafra et al. (2009b).

Particles transported in dorsal grooves and those conveyed with mucus aggregates in ventral grooves are directed towards the ridged surfaces of the labial palps (Ward 1996). In bivalves with homorhabdic gills, the labial palps are considered to be the main site for sorting and regulating the quantity of particles ingested. In bivalves with heterorhabdic gills, the palps were supposed to play a secondary role in these two functions and to serve mainly to reject the non-ingested material as pseudofaeces (Beninger and St-Jean 1997; Ward et al. 1998a). The differentiation of heterorhabdic gills into two types of filaments allows these two functions to be performed before the material reaches the palps. In addition, recent studies demonstrated that labial palps may also sort algal cells on the basis of their chemical properties (Cognie et al. 2003; Beninger et al. 2008a, b; Mafra et al. 2009b). In the present study, using *H. ostrearia* cells of contrasting size, we observed selection at oyster gills but not at labial palps, suggesting that particle size did not affect in the same way the selective ability of these two pallial organs.

State of knowledge concerning size criterion in selection on oyster pallial organs may be clarified and summarized in Table 2-4. Particle dimensions (length, width, height) should be considered as a physical constraint affecting access to principal filaments (PF) and consequently selective ability at the oyster gill. Particles with all dimensions smaller than the PF width can access freely to principal filaments troughs and their selection may occur at gill and/or labial palps. On the opposite, particles with all dimensions greater than PF width are unable to enter principal filaments and selection at gill is not possible. For particles with only one dimension greater than PF width, the access to principal filaments is limited to particles dorso-ventrally orientated. The selection at gill is passive or mechanical and a secondary selection at labial palps is possible.

Table 2-4. Role of particle size in selection at oyster pallial organs.

Particle dimensions	Access to principal filaments	Selection	
		At gill	At labial palps
All axes < PF width	Free	Possible	Possible
All axes > PF width	Not possible	Not possible	Possible
1 axis > PF width	Limited	Passive	Possible

Note : PF = principal filament

2.5 Conclusion

The demonstration that *C. gigas* selectively rejects larger cells of *H. ostrearia* could have some ecological significance, both in natural environments and in aquaculture, especially in oyster ponds. Indeed, in estuarine and coastal waters, oysters feed selectively on pennate diatoms, which constitute an important food source in intertidal areas with large mud flats (Cognie et al. 2001b). Considering the stock of cultivated and wild oysters, the sorting process evidenced in the present work may affect the structure of the microalgal

populations in the vicinity of oyster beds. Our study suggests that the preferential rejection of large-size cells in pseudofaeces could favour the development and/or the maintenance of sub-populations of large-size *Haslea*, given that *H. ostrearia* cells rejected in pseudofaeces have a revival capacity that is not altered by pre-ingestive processing (Barillé and Cognie 2000). Furthermore, as many pennate diatoms, sexually competent cells of *H. ostrearia* can carry out auxosporulation to compensate for the size reduction associated to vegetative divisions, only when cell size decreases to 65-70 μm , which represents 50% of the maximum cell length of the species (Neuville and Daste 1979; Davidovich et al. 2009; Mouget et al 2009). Therefore, this study shows that *C. gigas* preferentially ingests sexually mature cells and rejects immature ones, which could modify *H. ostrearia* cell size distribution and population dynamics in oyster ponds. The importance of this predation pressure on the life cycle of *H. ostrearia* in oyster ponds remains to be assessed, however, especially considering the importance of marennine in the greening process, but also regarding its many biological activities (e.g., antibacterial) that could be exploited in aquaculture.

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3 CHAPTER 3: GREENING OF BIVALVE BY *HASLEA OSTREARIA*

SUMMARY

The second article, entitled “Integrative study on greening Pacific oyster *Crassostrea gigas* by marennine produced by *Haslea ostrearia*”, was co-authored by myself and by Ikha Safitri, Bruno Cognie, Priscilla Decottignies, Romain Gastineau, Michèle Morançais, Eko Windarto, Réjean Tremblay and Jean-Luc Mouget. This article will be submitted in Journal of Aquaculture. As the first author, my contribution to this article was the execution of experiments, laboratory and data analysis as well as writing for the manuscript preparation. Jean-Luc Mouget, Bruno Cognie, Priscilla Decottignies and Michèle Morançais provided the original idea and assisted in the method development. All of the co-authors participated in this work. Several results obtained from this work was presented as oral presentation in International Conference of Physiomar 2014 in La Serena, Chile, 3 to 6 November 2014.

Integrative study on the greening of Pacific oyster *Crassostrea gigas* by marennine produced by *Haslea ostrearia*

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Abstract

The oyster greening phenomenon due to the fixation of marennine, the blue-green pigment produced by *Haslea ostrearia*, gives added value to the French oyster industry. Additionally, *in vitro* studies have shown that the pigment displays antibacterial activities, which could be advantageous in the field of aquaculture. Nevertheless, the mechanism of greening in oyster pallial organs, as well as its consequence on oyster's feeding behavior, remains poorly documented. The present study was thus performed to evaluate (1) the greening mechanism in the gills of oyster *Crassostrea gigas* (2) marennine effects on the

clearance rate of oyster (3) oyster recovery from marennine exposure. Firstly, the greening experiment was conducted to understand the mechanism of marennine fixation on oyster pallial organs. Concomitantly, another feeding experiment was conducted where clearance rate (CR) was measured on control and marennine exposed oysters. Both groups of oysters were fed simultaneously by a culture of *Skeletonema costatum*, which is commonly used in the oyster refining process. We demonstrated that the extracellular form of marennine significantly contributed to the greening compared to the intracellular form. Fixation of marennine is likely to be visible in the mucocyte of the gills. Furthermore, marennine significantly decreased CR by 51% with respect to control ($p < 0.05$) as a consequence of its fixation on oyster gills. However, the CR of oyster recovers to normal state after 7 days of exposure, suggesting that oyster is able to depurate the effect of marennine. However, on a longer-term (weeks), influence of marennine on the growth performance of the bivalves should be further studied, for any possible use of marennine as nutraceutical in aquaculture.

Keywords: *Crassostrea gigas*, *Haslea ostrearia*, marennine, clearance rate

3.1 Introduction

Oyster greening in the South-West of France is a seasonal phenomenon that occurs mainly in spring and autumn in oyster ponds. It is due to the proliferation of the diatom *Haslea ostrearia*, which can outcompete other microalgae that co-exist in the ponds. Indeed, *H. ostrearia* becomes recurrently dominant, possibly through the mechanism of allelopathy, by releasing its blue water-soluble pigment, marennine (Moreau 1970; Neuville and Daste 1978; Turpin et al. 1999; Pouvreau et al. 2007). Previous studies have shown that marennine concentration in oyster pond seawater can reach 3.4 mg L^{-1} (Turpin et al. 2001). During the greening, marennine released into the ponds is filtered by the oysters and fixed on the gills, giving them a specific green color. The oyster greening is considered to be important as this phenomenon contributes economically to the French

oyster industry. Moreover, potential exploitation of marennine as a natural anti pathogen agent draws attention since the extraction and purification methods were developed, and antimicrobial activities demonstrated *in vitro* (Pouvreau et al. 2006; Gastineau et al. 2012). However, to achieve the maximum benefit of the application of marennine in conchyliculture, it is necessary to determine the effects of this pigment on the response traits of oyster.

The direct role of marennine in oyster greening has been previously demonstrated in the laboratory, by using either the supernatant (hereafter named as ‘crude extract’) of *H. ostrearia* culture (Carazzi 1897; Neuville and Daste 1972; Ranson 1927) or the purified form (Pouvreau 2006). In oyster ponds, however, it is not known whether the pigment transfer to gills is only caused either by the simple filtration of seawater and the extracellular form of marennine (EMn) contained in *H. ostrearia* supernatant, or if it is closely related to the ingestion by the oyster of *H. ostrearia* cells containing Intracellular form of marennine (IMn). Moreover, despite that the oyster *C. gigas* is able to ingest *H. ostrearia* (Barillé et al. 1994; Piveteau 1999) and both purified forms of marennine, EMn and IMn, are able to color oyster gills (Pouvreau 2006), information on the cellular mechanisms involved in the greening of oyster gill’s tissue remains unclear, as well as possible consequences of the greening on oyster physiological state is not yet documented.

Among all physiological indicators in bivalves, the clearance rate (CR) is considered as a reliable physiological parameter and its fluctuation describes the impacts of chemical stress, including microalgae exudates, on the organism at the whole body level (Bricelj et al. 2001; Ishida et al. 2004; Echevarria et al. 2012). Moreover, CR reflects the activity of filtering surfaces, particularly gills (Jorgensen 1990). Therefore, CR in suspension feeder can be used as an indicator of short- and long-term effect of chemical substances.

The aim of the present study was to give better insight on the greening phenomenon of oysters and determine the mechanisms of interaction between marennine and oyster gills. Several questions were thus addressed in this study regarding the greening process and its consequences. First, does the greening in presence of *H. ostrearia* occur due to the IMn or EMn? Secondly, does the greening occur actively through the feeding activity, or

passively by retention at the gill level? Thirdly, is there possible loss of color or depuration ('degreening') performed by the oyster with time? Fourthly, where and how does the marennine fix on the gills of oyster? Finally, does marennine fixed on gills modify oyster behaviour (e.g., at the CR level)?

3.2 Materials and methods

3.2.1 Culture of microalgae and maintenance of oysters

Juvenile and adult oysters with different shell lengths (mean \pm SE, $n= 16$, 6.0 ± 1.2 cm and 10.1 ± 1.5 cm, respectively) were collected from Bourgneuf Bay ($46^{\circ} 59' 19''$ N/ $2^{\circ} 14' 14''$ W), West of France, in June 2013. Shell epibionts were removed manually, by placing animals in a sodium hypochlorite solution for 2 minutes in order to eliminate shell borers such as *Polydora* sp. The cleaned individuals were then rinsed several times and were maintained in aquaria filled with oxygenated filtered seawater for 96 h prior to the experiments.

The microalgae strains used in this experiment were *H. ostrearia* (NCC-148.7) and *S. costatum* (NCC-53) obtained from NNC (Nantes Culture Collection, Université de Nantes). These two species were grown under non-axenic conditions in sterilized conical flasks, containing a medium of artificial seawater (ASW) (Mouget *et al.*, 2009) at $16^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with an irradiance of $100 \mu\text{mol photon m}^{-2}\text{s}^{-1}$ (Li-Cor quantum meter), and in a photoperiod light/dark of 14h / 10h (Philips TLD 36 watt/965 Fluorescent tubes).

3.2.2 Experimental design

3.2.2.1 Greening experiment

The greening experiment was conducted by placing the juvenile and adult oysters in an experimental chamber containing 1 L of filtered seawater (Instant Ocean®: NaCl, Na₂SO₄, KCl, NaHCO₃) with temperature and salinity of 16°C and 35 psu, respectively.

Greening was conducted in four different treatments as described in Fig. 3-1 (A, B, C and D). Culture of *H. ostrearia* maintained in an ASW medium during 3 weeks (cell densities between 30,000 and 120,000 cell mL⁻¹) was used for all treatments (B, C and D) except for control where *S. costatum* with the same range of cell concentration were given. As for treatment B (Fig. 3-1B), oysters were maintained in the experimental chamber containing cells of *H. ostrearia* and their culture supernatant or crude extract of EMn (final concentration: 5 mg L⁻¹). On the other hand, in the treatment C (Fig. 3-1C), the medium only contained the crude extract of EMn, without any presence of cells. This was obtained by filtering the crude extract through a GF/ filter F (Millipore). The last treatment (Fig. 3-1D) represents the medium containing *H. ostrearia* cells only. This was prepared extemporaneously by gentle centrifugation of *H. ostrearia* culture in order to eliminate the supernatant, which contained the extracellular form of marennine. After having discarded the supernatant, *H. ostrearia* cells (pellet) were resuspended in a fresh medium, and placed into the experimental chambers containing oyster for 6h, (short 'feeding period') to limit possible release of EMn by algae. The observation on the greening effect was determined every 24 hours since the oysters maintained at the experimental chamber during 96 hours and up to 12 weeks for treatment A, B and C. Whereas for the treatment D, the medium was re-newed daily after 6 hours during the 12 weeks of the experiment to determine the possible accumulative effect of intracellular marennine in the gills of oyster.

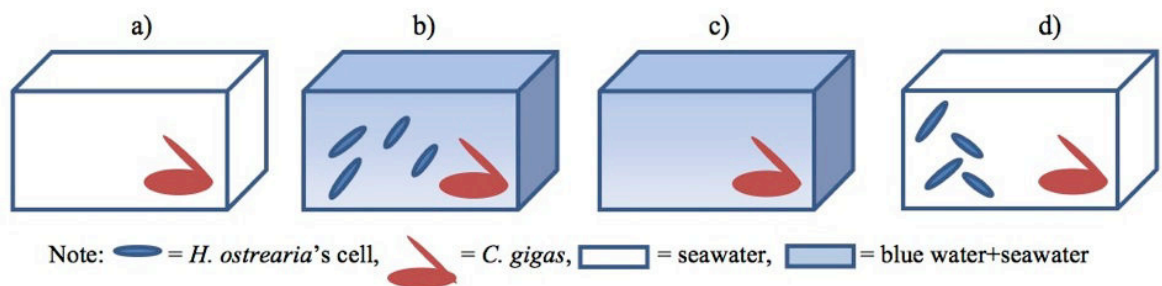


Figure 3-1. Scheme of greening experiment *in vitro*. Oysters were maintained in the chamber containing A) filtered artificial seawater (0.2 μm); B) culture of *Haslea ostrearia* with EMn; C) crude extract of EMn without *H. ostrearia* cells; D) filtered artificial seawater containing *H. ostrearia* cells only without any presence of EMn.

3.2.2.2 Feeding experiment

Experiment 1: short-term effect of marennine

Two groups of juvenile *C. gigas* (n=8, for each control and treatment group) were provided. One group was exposed to crude extract of EMn and the other group as control (here after named “green-oyster” and “normal-oyster”, respectively). Green-oysters were prepared by exposing them to EMn crude extract (with final concentration 5 mg L⁻¹) 96 h prior to the feeding experiment. CR was quantified using a flow through chamber system, which was composed of ten individual chambers, equipped with a flow meter and a dispatcher (Barillé et al. 2006). Two chambers were provided as sediment chambers that contained only oyster shell. Flows in the individual chambers were controlled using valves fitted at their entrance. In each chamber, two partitions were provided to limit the turbulence on biodeposit suspended matter. Both groups of oysters were then fed with cultures of *Skeletonema costatum* (300 L in scobalith) with a final concentration of 30,000 cell mL⁻¹. To simulate particulate inorganic matter (PIM), silt (kaolinite BS1, AGS) was added to the algal mixtures, because it is well ingested by oyster comparatively to other clays (Sornin et al. 1988). Sampling at the outflow of the individual chamber was conducted in 20 minute intervals during one hour after all oysters were sufficiently acclimated. Samples were then analyzed using a particle counter multisizer (Beckman Coulter Counter Z2, Mississauga, ON, Canada).

Experiment 2: long-term effect of marennine

To determine the possible mechanism of depuration ('degreening'), the two groups of oysters, as stated above, were re-placed into seawater medium and fed with the same diet. CR in both control and treatment groups was measured by using static chamber. Two chambers were used as sediment control chambers as stated above. Furthermore, experimental conditions and sampling were performed as previously described in experiment 1.

Physiological measurements

CR is defined as the volume of water cleared of suspended particles per unit of time (Macdonald and Ward 1994). In experiment I, by using flow through chamber system, we assumed that the particles were 100% retained on oyster gills. CR ($L h^{-1}$) was calculated as follows:

$$CR = F \times [(inflow - outflow) \times inflow^{-1}] \quad (1)$$

Where F is the flow rate of water through the chambers ($L h^{-1}$).

In experiment 2, CR was measured in a static system and was calculated as follows:

$$CR = (V/t) \ln (C_0/C_t) \quad (2)$$

Where V is the volume of water and t is the incubation time. C_0 and C_t are the initial and final particle concentrations in the closed chambers.

Calculated CR was then standardized to the individual's equivalent of 1 g dry weight (Bayne et al. 1987; Barillé et al. 1997). This standardization was done by applying the allometric coefficient ($b = 0.65$), determined by (Shpigel et al. 1992) for *C. gigas*, as follows:

$$Y_s = (1 / W_{ex})^b Y_{ex} \quad (3)$$

Where Y_s corresponds to the physiological rate for an animal of standard weight, Y_{ex} is the value of experiment and W_{ex} is the weight of oysters used in the experiment.

3.2.2.3 Histology sample preparation

Preliminary experiments to observe green gills using *in toto* staining and classic histological method were unsuccessful due to the leaching of marennine, thus unabling the determination of which part of gills marennine was fixed. In this study, the frozen section technique was used in order to observe the fixation of marennine on oyster gills. Briefly, fresh gills ($n=3$) were dissected from the oyster shell and immediately immersed in 3 to 30

kDa ultra-filtered EMn (e.g. final concentration, 500 mg L⁻¹) for 24 h until they turn green. Gills with marennine were then fixed in 10% formaldehyde for 10 days prior to the sectioning process. Marennine fixation was evaluated *in vitro* in cryostat section and sections (40 µm) were cut along the antero-posterior axis of the gills with a pre-frozen cryostat (MICROM International GmbH) at -28°C prior to observation under microscope with 100 magnifications (Keyence VHX-2000E).

3.2.2.4 Marennine quantification

The concentration of extracellular marennine (EMn) was calculated by filtering the crude extract with Millipore filter (0.22 µm) prior to the measurement with a spectrophotometer. Afterwards, the absorbance of crude extract was measured by UV-visible spectrophotometry (PerkinElmer Lambda 25), and the concentration was determined as described in Robert et al. (2002). The concentration of EMn $[C]$ (mg L⁻¹) was calculated according to the following formula:

$$[C] = \frac{A_{\lambda_{max}}}{\epsilon_{\lambda_{max}} \times l}$$

Where $A_{\lambda_{max}}$ is the absorbance at the peak wavelength in red region, $\epsilon_{\lambda_{max}}$ is the specific extinction coefficient at the peak wavelength, and l is the cuvette path length.

Because no method to quantify marennine fixed on the gills has been developed yet, two methods of estimation were used, one qualitative and one quantitative. In the former, the amount of marennine fixed in oyster gills was expressed as a greening intensity with the scoring method (Fig. 3-2). Briefly, the scoring method was applied by establishing the greening scale in by using DigitalColor Meter application in Mac. The scale was made based on the red green blue (RGB) ratio obtained by applying the maximum aperture on the area of interest from the captured image in control and treatments, which will give the numerical value. The control scale (0) was represented by the color of gills of the oysters in

control, whereas the maximum scale (10) was represented by the maximum of green coloration by marennine. In the latter, frozen gills were defrosted on ice, blotted on absorbent paper and the wet mass determined. Marennine in the gills was extracted using urea ($\text{CO}(\text{NH}_2)_2$) 8M after grounding with an Ultraturax tissue tearor (BIOSPEC, Inc) at maximum speed in a 15 mL Falcon tube. Ground tissue with urea was then centrifuged at 7800 rpm for 10 min and the absorbance of the supernatant measured by UV-visible spectrophotometry (UV-6300 PC double beam, VWR). The marennine concentration was measured using the same formula as described above (Eq. 4). Furthermore, marennine concentration fixed in the gills was obtained by removing the absorbance of gill tissue alone from the control by using a calibration curve.

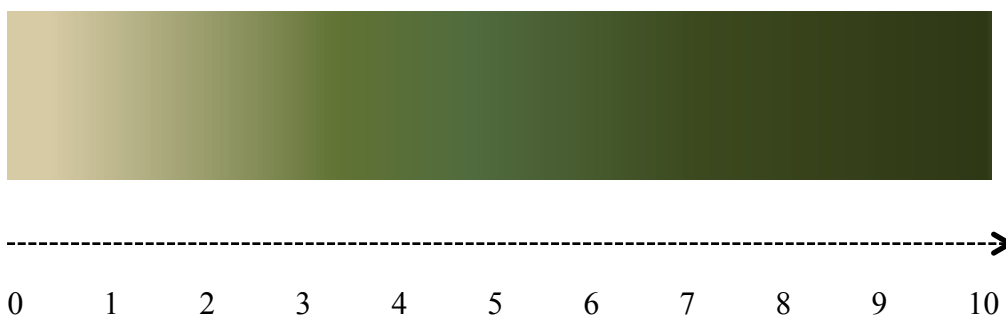


Figure 3-2. Scale of greening intensity used to estimate marennine in oyster gills qualitatively.

3.2.3 Statistical analyses

All data was analyzed using JMP Pro 11 statistical software (©SAS Institut Inc.). Normality and homogeneity tests were used prior to statistical analyses by Shapiro-Wilk and Levene's test, respectively. Scores obtained in the greening experiment were analyzed by using one-way ANOVAs. In contrast, two-way ANOVAs were performed to determine differences in CR between different groups of treatment (normal- vs green-oyster) and also group of time (week). Where differences were detected, Tukey-HSD multiple comparison tests were used to determine which means was significantly different.

3.3 Results

3.3.1 Greening experiment

Qualitative measurements showed that the greening occurred in most of the treatments except the control where the *H. ostrearia* and the crude extract of EMn was absent (Fig. 3-3A). Strong greening intensity in *C. gigas*'s gills was visually observed when the oysters were exposed to *H. ostrearia* concomitantly with marennine and marennine's crude extract only (Fig. 3-3B&C). In contrast, weak coloration appeared on the oysters fed only by the cells of *H. ostrearia* without any presence of marennine's crude extract (Fig. 3-3D). Statistical analysis showed that greening intensity appeared to be significant between treatment and control (ANOVA: $F_{3, 37} = 13.67$, $p < 0.0001$). Tukey-HSD post-hoc analysis showed that greening intensity in the treatment B and C were significantly different compared to the control ($p < 0.0001$ and $p < 0.0005$, respectively). In contrast, no significant difference in greening intensity was observed between D and control ($p > 0.05$). However, greening in oysters fed with *H. ostrearia* cells were only approximately four times lower compared to those are exposed to crude extract of marennine and the combination between the cells and crude extract ($p = 0.0026$ and $p = 0.0004$, respectively). Last, it has been observed that after the exposure to *H. ostrearia* supernatant and the greening, oyster gills kept the green color for at least 3 months.

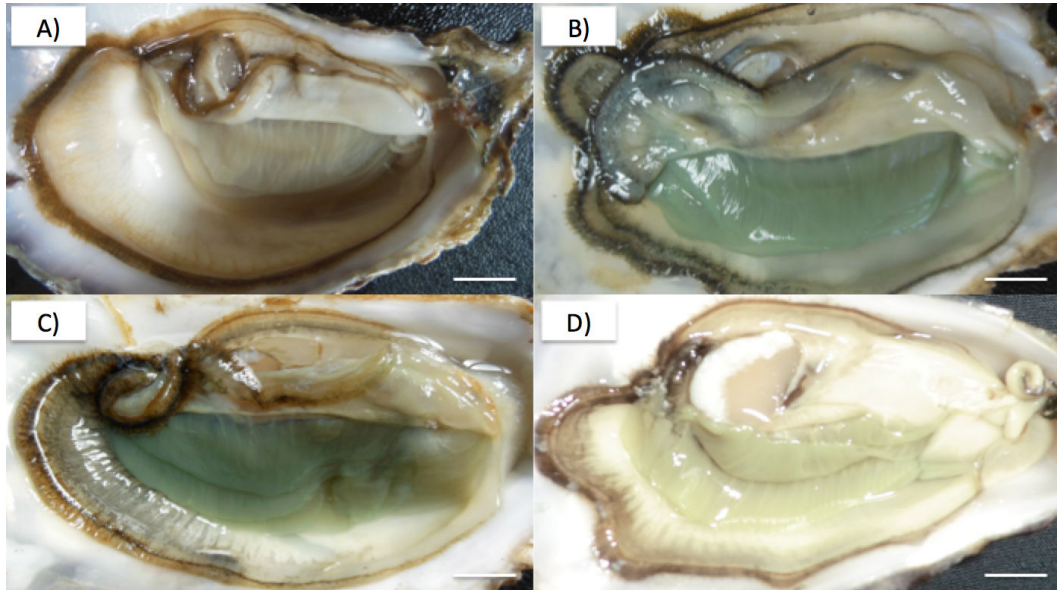


Figure 3-3. Effect of oyster treatments on the coloration of gills. A) Oyster fed with *S. costatum* as control; B) oyster exposed to *H. ostrearia* and EMn containing supernatant; C) oyster exposed to EMn containing supernatant; and D) oyster exposed to *H. ostrearia*'s cell only. Scale bar correspond to 1 cm.

We observed that the greening in all treatments was time exposure dependent. This was confirmed by the significant relationship between greening intensity and time of exposure (Fig. 3-4A). A strong relationship was observed between the greening intensity for all treatments (B, C and D) and the time exposure ($r = 0.89, 0.90$ and 0.85 , respectively). In addition, linear regression showed that a significant relationship was obtained between the EMn concentration obtained by qualitative method and those are obtained by urea-extraction method (Fig. 3-4B).

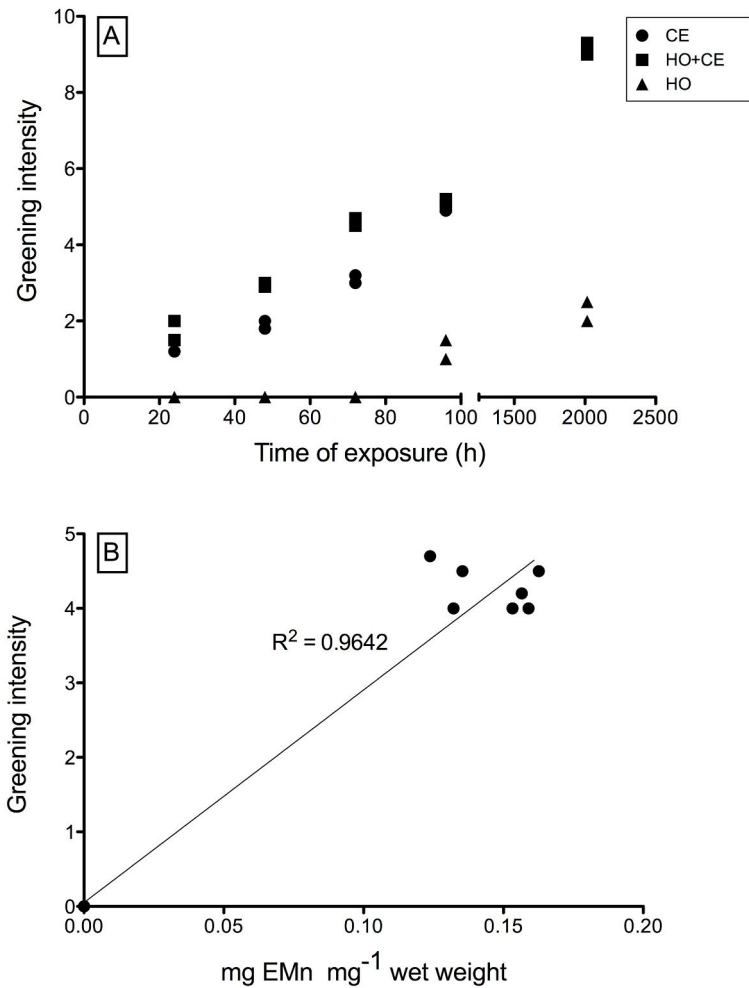


Figure 3-4. Greening intensity in function of (A) time exposure and concentration of EMn obtained by urea-extraction method (B).

3.3.2 Feeding experiment

In the present study, the body mass (g DW) ranged from 0.58 to 1.03 for normal-oyster (mean: 0.81 ± 0.19 , $n = 14$), and from 0.62 to 1.35 for green-oyster (mean: 0.92 ± 0.31 , $n = 14$). Additionally, no significant difference in oyster weight was observed between normal- and green-oysters ($F_{1,14} = 2.55$, $p = 0.132$).

In terms of physiological rates, CR of oysters with green gills was significantly reduced by 51% compared to the control after a short exposure to marennine (start of the experiment, day 0) (Fig. 3-5; $F_{1,30} = 7.45$, $p = 0.015$). However, on a longer term, this initial reduction by marennine had leveled off at day 7, CR of green-gill oysters reaching rates similar to those observed in control (Fig. 5; $F_{1,30} = 3.43$, $p = 0.173$).

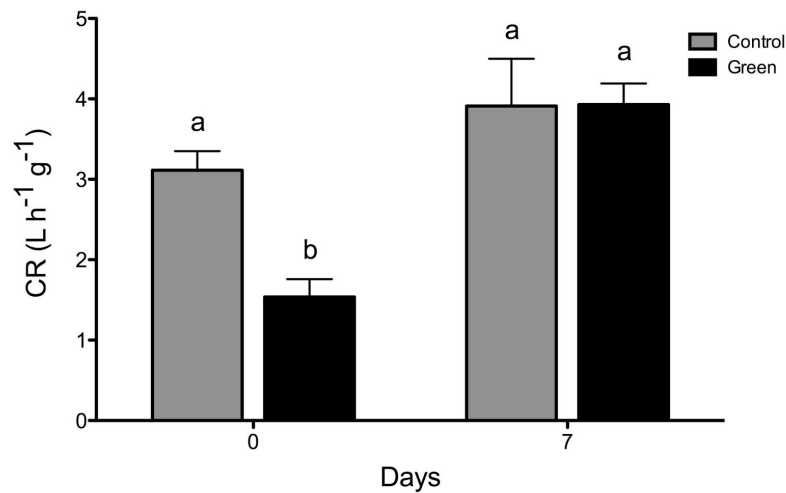


Figure 3-5. CR of oysters exposed to marennine (green) compared to control. Values are mean \pm standard error ($n = 8$).

3.3.3 Histological experiment

Histological analysis showed that marennine was fixed in oyster gills regardless of passive and active filtration by oyster. Histological cut by cryostat microtome demonstrated that EMn penetrated not only outside but also inside of the gills' ordinary filament (Fig. 3-6A, B). However, marennine appeared likely to be preferentially fixed at the mucocytes of ordinary filaments since higher greening intensity was observed in this region (Fig. 3-6B, indicated by arrow).

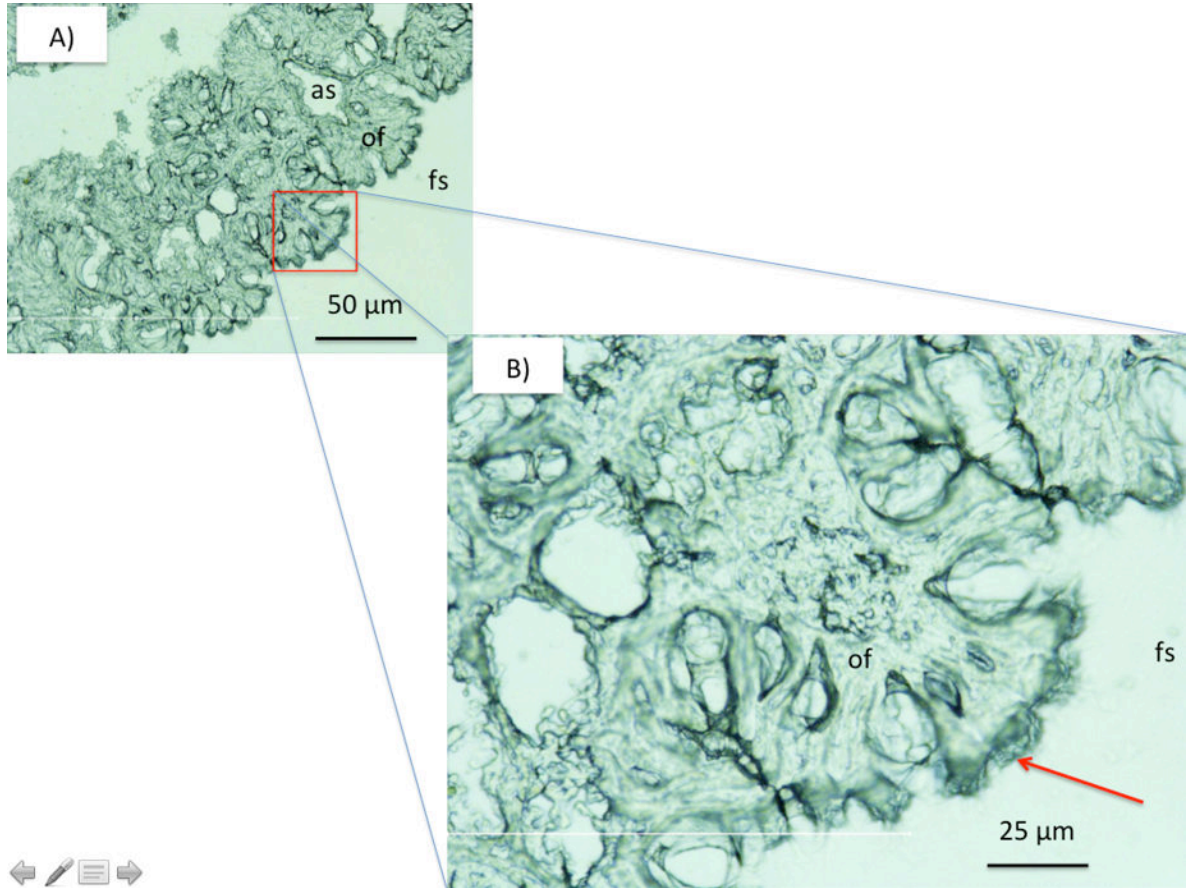


Figure 3-6. Histological observation on longitudinal section of oyster *C. gigas* gill that previously immersed in highly concentrated semi-purified EMn (A and B). A) Marennine penetrating the oyster gills, red rectangle indicates magnified region with higher greening intensity. B) Magnified gills region with marennine fixation on the mucocytes of the gill's ordinary filament. fs = frontal surface; as = abfrontal surface; of = ordinary filament.

3.4 Discussion

This study addressed several points regarding the interactions between marennine and gills of oysters, in particular concerning the form of marennine that is preferentially fixed in gills, which is likely the extracellular one (EMn). This phenomenon changes with time of exposure, and as a consequence, can result in an increase of color intensity. Moreover, the capacity of EMn to fix to, and to color oyster gills appeared stronger and faster as compared to the IMn through the ingestion of *H. ostrearia* cells, and an

hypothetical internal transfer to gills. Indeed, a lower greening intensity was caused by IMn through ingestion of cells, and it appeared much slower and requires approximately 2.16×10^7 cells of *H. ostrearia* during 3 months of experiment. Our results are in agreement with Pouvreau (2006) who observed the stronger ability of EMn in coloring oyster gills even though the pigments used in these experiments were the purified forms of EMn and IMn, with shorter period of experiment. Considering low ability of IMn in coloring oyster gills, we hypothesize that EMn mainly undergoes the greening in the natural environment. Although experiments were designed to specifically prevent or limit contact between gills and algal supernatant containing EMn, involvement of EMn cannot be fully dismissed. By reducing the 'feeding period', thus the time of possible exposure to EMn, however, our result leads to hypothesis that the pigment transfer mainly occurs by filtration of seawater containing EMn by the oyster.

For this study, a method for estimating the quantity of marennine fixed on the gills needed to be developed, and two methods were used, one qualitative and the other quantitative. A significant relationship between the two methods was observed, but the extraction protocol of marennine from oyster gills allowed recuperating approximately 10% of marennine given into the medium, suggesting a mechanism of strong binding of marennine on gills, and a quantitative method still to be improved. The long-term persistence of the green color on gills is likely a consequence of the strong attachment of marennine in gill tissue. However, it is likely that a lower concentration of marennine on the gills can be considered logical since marennine could also be fixed in other organs apart from gills (e.g. labial palps and mantle).

Apart from the greening itself, marennine can have some impact on physiological traits in bivalves, for instance the CR, but its mechanism of action is poorly documented, in contrast with other algal molecules or toxins, for instance, for Paralytic Shellfish Toxin (PST)-like produced by toxic dinoflagellates. Indeed, such decrease in CR is also observed with this type of toxin (Hégaret et al. 2007; Mafra et al. 2009; Navarro and Contreras 2010; Manfrin et al. 2012; Jauffrais et al. 2012). Marennine significantly reduced the CR of oyster approximately by half of CR in the control group. However, this reduction of CR on the short-term by marennine was followed by a recovery at day 7 after the exposure,

indicating that the organisms acclimate to, or 'depurate' marennine within this period of time. Indeed, mechanism of depuration in bivalves has been documented and it is likely that the rate of depuration depends on the type of chemical or biotoxin and also the species (Corrêa et al. 2007, 2012; Love et al. 2010; Navarro and Contreras 2010; Luna-Acosta et al. 2011; Medhioub et al. 2012; Phuvasate et al. 2012). For instance, Navarro and Contreras (2010) observed that the depuration in *Mytilus chilensis* exposed to the toxic dinoflagellate *Alexandrium catenella* occurred within 3 days after the exposition. On the other hand, recovery from the toxic *Alexandrium ostenfeldii* in *C. gigas* was complete within 7 days after exposure (Medhioub et al. 2012).

Histological study to better understand the greening mechanism in oyster is also little documented. In the present study, we used the frozen section technique and demonstrated with *in toto* method that marennine is fixed in oyster gill by penetration throughout the gill filaments. Furthermore, *in toto* method used in histological analysis suggests that pigment fixation can occur passively without any filtration activity by the bivalve. Our results were in agreement with earlier histological study of the greening in *Ostrea edulis* and *O. angulata*, which demonstrated that marennine was mainly fixed in pallial organs of these two bivalves (Carazzi 1897). Furthermore, it seems that marennine is fixed more intensively on the mucocytes of the gills. In the heterorhabdic gills of pseudolamellibranchs such as *C. gigas* and *C. virginica*, mucocytes can be divided into two types, the acid mucopolysaccharide (AMPS) and the neutral mucopolysaccharide (NMPS) secretion type (Beninger and Dufour 1996; Dufour and Beninger 2001). Due to the leaching when using the classic histological method, it was not possible to identify in which type of mucocyte marennine was mainly fixed.

In the present study, it is likely that EMn perturbs the feeding behavior of oyster *C. gigas* in addition to coloring the gills. Recently, purified EMn has been shown to affect CR and scope for growth in eastern oyster *C. virginica* and blue mussel *M. edulis* (Prasetya et al. 2015, submitted) suggesting that marennine can be considered as a disturbing bioactive compounds in oysters. Nevertheless, in public health perspective, marennine can be considered as harmless substance since the green oyster has been consumed for centuries and no deleterious effect was reported (Gastineau et al. 2012). In addition, detrimental

effects of marennine could be largely compensated for by the numerous beneficial factors especially as the natural anti pathogens, and also by the higher market value of green oysters.

3.5 Conclusion

The present study demonstrates that the greening of oysters occurred through the pigment transfer, which associated with the solubility of marennine in the medium. The marennine that is released by *H. ostrearia* can be fixed in oyster's gills either actively by filtration activity or passively by direct contact with the gill. The extracellular form has stronger ability in coloring oyster gill. In addition, marennine-pallial organ interaction is a complex process and histological observation demonstrated that the mucocyte of the gills appeared to be the main site of fixation. This pigment fixation on the gills of oyster is likely to be persistent. As physiological consequence, marennine fixation on the gills significantly reduces the clearance rate of oyster. However, the oyster *C. gigas* is able to compensate for the effect of marennine within 7 days after exposure. It is noteworthy to test a longer and higher exposure of marennine to determine whether effect increase in time and type of pigment and whether oyster can still recover thereafter. Last but not least, the efficiency of marennine as a natural antipathogen agent merits further investigation to achieve the maximum benefit particularly in aquaculture perspective.

3.6 Acknowledgements

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4 CHAPTER 4: CONSEQUENCES OF GREENING BY MARENINE ON THE INTEGRATIVE RESPONSE OF BIVALVE

SUMMARY

The third article, entitled “Effect of marenine produced by the blue diatom *Haslea ostrearia* on behavioral, physiological and biochemical traits of *Mytilus edulis* and *Crassostrea virginica*”, was co-authored by myself and by Luc Comeau, Romain Gastineau, Priscilla Decottignies, Bruno Cognie, Michèle Morançais, François Turcotte, Jean-Luc Mouget and Réjean Tremblay. This article was submitted in Journal of Aquaculture in September 2015. As the first author, my contribution to this article was the execution of experiments, laboratory and data analysis as well as writing for the manuscript preparation. Jean-Luc Mouget, Réjean Tremblay, and Bruno Cognie provided the original idea and assisted in the method development. All of the co-authors participated in this work. Results of this work was presented as oral presentation in both International Conference of Physiomar 2014 in La Serena, Chile, 3 to 6 November 2014, and the International Elsevier Conference of Aquaculture 2015: Cutting edge science in aquaculture, in Montpellier, France, 23-26 August 2015.

Effect of marennine produced by the blue diatom *Haslea ostrearia* on behavioral, physiological and biochemical traits of *Mytilus edulis* and *Crassostrea virginica*

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Abstract

Haslea ostrearia is a marine diatom that synthesizes and releases marennine, a water-soluble blue-green pigment responsible for the greening of the gills and labial palps of bivalves. The present study evaluated the effect of different marennine concentrations (0, 0.5, 1.0 and 2.0 mg L⁻¹) on the behavior (valve opening), physiology (clearance rates, oxygen consumption, assimilation efficiency and scope for growth) and biochemistry (fatty

acid composition of neutral and polar lipids) of two commercially important bivalves, the blue mussel *Mytilus edulis* and the eastern oyster *Crassostrea virginica*. Under short-term (<1 day) exposure, the concentration of marennine found on the gills of both species was positively correlated to the concentration of dissolved marennine in the water medium. However, a behavioral response was detected at the higher marennine concentration (2.0 mg L⁻¹) and both species displayed curtailed valve opening compared to control groups. Under longer-term (8 weeks) exposure, marennine (at 2 mg L⁻¹) significantly decreased scope for growth by 58% and 85% (ANOVA; $F_{3,31} = 3.39$, $p = 0.034$ and $F_{3,31} = 3.08$, $p = 0.044$) for *M. edulis* and *C. virginica* respectively. The greening process had an effect on total fatty acids contained in the digestive gland of mussels only, suggesting that marennine interferes with the accumulation of energy reserves in this bivalve. In the polar lipids of the gills, greening increased the concentration of unsaturated fatty acids in the gills of *C. virginica* but not *M. edulis*, suggesting for oyster a possible regulatory mechanism counteracting marennine effect.

Keywords: *Crassostrea virginica*, fatty acid composition, *Haslea ostrearia*, marennine, *Mytilus edulis*, scope for growth, valve activity

4.1 Introduction

Molluscan bivalves acquire food and oxygen by filtering water across their gills. Water movement inside the shell is ensured by gill ciliary motion, triggered by an active mechanism responding to the presence of a dissolved chemical or particulate matter in water. The most visible behavior associated to filtration or ventilation is the opening of the valves, whereas the closing of valves may signal satiation (Morton, 1973; Bayne, 1998; Cranford, 2001) or protection of soft tissues (Ait Ayad et al., 2011; Ait Fdil et al., 2006; Gainey and Shumway, 1988; Hégaret et al., 2007; Katticaran and Salih, 1992). For example, previous work has shown that toxic microalgae producing the paralytic shellfish toxin (PST) can alter bivalve behavior, physiology, and cellular activity (Bricelj et al., 2005). Toxic dinoflagellates in particular can result in valve closure and lowered filtration rate, oxygen consumption, absorption efficiency and Scope for Growth (SFG) (Bardouil et

al., 1993; Bricelj et al., 1996; Hégaret et al., 2011,2007; Navarro and Contreras, 2010). However, little attention has been given to the effects of non-toxic organic compounds on bivalves. Moreover, the few studies on this topic focus exclusively on non-PST compounds produced by dinoflagellates and the effect that these compounds have on bivalve hemocytes (Ford et al., 2008; Hégaret and Wikfors, 2005).

The aim of the present study was to assess whether a non-PST compound can affect behavioral, physiological and biochemical traits of bivalves. Specifically, the study focuses on the effects of marennine, a water-soluble blue pigment that is excreted by the non-toxic pennate diatom *Haslea ostrearia* (Gaillon) Simonsen. Marennine binds preferentially to the gills and labial palps of bivalves (Robert, 1975) and is responsible for the greening of Pacific oysters *Crassostrea gigas*. This natural phenomenon, which occurs erratically in oyster ponds on the French Atlantic Coast, increases the economic value of cultured oysters due to the organoleptic modification in green oysters. Moreover, marennine has antibacterial and antiviral properties and therefore acts as a natural protective agent against pathogens (Gastineau et al., 2014, 2012a, 2012b; Pouvreau et al., 2008, 2007). Interestingly, several species from the same genus *Haslea* produce “marennine-like” pigments in other parts of the world (Gastineau et al., 2014, 2012a). Hence, there are potential worldwide applications of marennine in shellfish aquaculture.

With respect to response variables, our study focused on valve movement, scope for growth (SFG) and the buildup of energy reserves. SFG represents the energy absorbed during feeding subtracted by the energy lost through respiration and excretion (Gilek et al., 1992; Widdows and Johnson, 1988). SFG is sensitive to environmental stressor such as salinity, temperature and toxic substances (Navarro and Contreras, 2010; Pernet et al., 2008, 2007; Widdows and Johnson, 1988; Widdows et al., 2002). Regarding energy, ectotherm animals such as bivalves have the capacity to remodel their lipid membranes to maintain fluidity in response to temperature or environmental changes (Hulbert and Else, 1999; Hulbert, 2007; Pernet et al., 2007; Parent et al., 2008; Parrish, 2013). Recent works (Parent et al., 2008; Parrish, 2013; Pernet et al., 2008, 2007) suggest that the fatty acid composition of neutral (NL) and polar (PL) lipids is indicative of acclimation. In particular NL represent energetic reserves that support metabolism and growth of organisms, while

PL constitute membrane structure where its fluidity may change due to the enlargement or reduction in unsaturated fatty acid content (Bergé and Barnathan, 2005; Fokina et al., 2015, 2014). Responses to marennine were investigated using two commercially important bivalves, the eastern oyster *Crassostrea virginica* and the blue mussel *Mytilus edulis*. First, we tested the hypothesis that dissolved extracellular marennine affects the valve opening of bivalves. We then examined whether greened oysters had different physiological (clearance rates, oxygen consumption, assimilation efficiency and scope for growth) and biochemical characteristics (tissues fatty acids composition). We predicted that responses would be marennine dose-dependent.

4.2 Materials and methods

4.2.1 Animals and diet preparation

Juveniles (aquaculture spat) of mussels (*M. edulis*) and oysters (*C. virginica*) were obtained from shellfish aquaculture leases in Prince Edwards Island, Canada (46°25.963 N; 62°39.914 W). Water temperature at the time of collection (end of August 2014) was 16°C and salinity was 29 ppt. Animals were immediately transported to the Station Aquicole de Pointe-aux-Pères (Institut des Sciences de la Mer, Rimouski, Canada). Upon arrival, 128 individuals of both species (mean shell length = 28.8 ± 0.7 mm for mussels and 25.9 ± 0.9 mm for oysters) were numbered with bee tags and acclimated to laboratory conditions for 30 days prior to starting the experiment. The two species were equally distributed in two 300 L maintenance tanks with light aeration, salinity of 29 ppt, temperature of 16°C and natural photoperiod. Animals were continuously fed with *Pavlova lutheri*, *Tisochrysis lutea* and *Chaetoceros muelleri* at 1:1:1 equivalent volume and a daily ration of 40 cells µL. Algae were sampled two times during the experiment for fatty acid analysis (Table 4-1).

Table 4-1. Fatty acid composition of microalgal species used in the diet for mussels and oysters. Values are mean \pm standard error.

Variable	<i>Chaetoceros gracilis</i> (CHGRA)	<i>Tisochrysis lutea</i> (TISO)	<i>Pavlova lutheri</i> (PLUTH)
Fatty acid composition (mol %)			
14:0	0.00 \pm 0.00	15.74 \pm 0.96	9.93 \pm 0.48
15:0	0.82 \pm 0.07	0.58 \pm 0.02	0.45 \pm 0.01
16:0	29.37 \pm 1.31	18.63 \pm 0.32	19.87 \pm 0.40
17:0	0.25 \pm 0.02	0.59 \pm 0.05	0.44 \pm 0.02
18:0	2.02 \pm 0.02	2.86 \pm 0.18	2.71 \pm 0.10
20:0	0.14 \pm 0.00	0.87 \pm 0.17	0.59 \pm 0.04
21:0	0.00 \pm 0.00	0.28 \pm 0.27	0.28 \pm 0.27
22:0	0.21 \pm 0.01	1.60 \pm 0.38	0.96 \pm 0.07
24:0	0.17 \pm 0.07	1.26 \pm 0.28	0.49 \pm 0.48
Σ SFA	33.11 \pm 1.42	42.40 \pm 0.81	35.71 \pm 0.12
14:1	0.07 \pm 0.00	0.54 \pm 0.09	0.00 \pm 0.00
16:1	29.81 \pm 0.35	7.19 \pm 0.89	20.21 \pm 0.40
17:1	0.00 \pm 0.00	0.81 \pm 0.05	0.00 \pm 0.00
18:1 n-9c	2.69 \pm 0.27	12.60 \pm 0.06	2.31 \pm 0.16
18:1 n-9t	2.40 \pm 0.28	0.00 \pm 0.00	0.00 \pm 0.00
18:1 n-7	0.03 \pm 0.00	3.08 \pm 0.04	2.94 \pm 0.03
20:1	0.22 \pm 0.01	0.82 \pm 0.14	0.64 \pm 0.02
22:1 n-9	0.08 \pm 0.00	0.76 \pm 0.13	0.51 \pm 0.03
22:1 n-11	0.02 \pm 0.00	0.77 \pm 0.14	0.53 \pm 0.04
24:1	0.15 \pm 0.00	0.55 \pm 0.10	0.43 \pm 0.02
Σ MUFA	35.48 \pm 0.19	27.14 \pm 0.33	27.56 \pm 0.41
18:3 n-3	0.53 \pm 0.05	3.93 \pm 0.21	1.14 \pm 0.01
18:3 n-6	2.72 \pm 0.15	1.57 \pm 0.09	0.55 \pm 0.50
18:4 n-3	3.01 \pm 0.32	0.00 \pm 0.00	0.00 \pm 0.00
18:2 n-6c	2.50 \pm 0.09	2.47 \pm 2.40	1.16 \pm 0.04
18:2 n-6t	0.48 \pm 0.05	1.04 \pm 0.34	0.61 \pm 0.05
20:4 n-6	5.84 \pm 0.07	0.93 \pm 0.09	0.90 \pm 0.02
20:5 n-3	9.67 \pm 0.30	4.51 \pm 0.41	20.36 \pm 0.48
20:3 n-6	0.67 \pm 0.14	0.00 \pm 0.00	0.00 \pm 0.00
20:3 n-3	0.11 \pm 0.01	0.80 \pm 0.16	0.00 \pm 0.00
20:2	0.13 \pm 0.02	0.71 \pm 0.15	0.60 \pm 0.03
22:6 n-3	4.34 \pm 0.31	12.77 \pm 1.53	9.92 \pm 0.05
22:2	0.15 \pm 0.01	0.00 \pm 0.00	0.00 \pm 0.00
22:5 n-3	1.27 \pm 0.01	1.72 \pm 0.03	1.49 \pm 0.02
Σ PUFA	31.41 \pm 1.23	30.46 \pm 1.15	36.72 \pm 0.28
Total fatty acids ($\mu\text{g g}^{-1}$)	185.11 \pm 15.70	36.56 \pm 10.75	34.40 \pm 5.42

4.2.2 Marennine extraction and purification

H. ostrearia was produced in 100 L photobioreactors as described in Gastineau et al. (2014) and extracellular marennine was extracted and purified as described in Pouvreau et al. (2006). Briefly, 1 µm-filtered culture supernatant of *H. ostrearia* was passed through a two-step ultrafiltration process (30 and 3 kDa) with cartridges fitted with regenerated cellulose spiral membranes (Prep/Scale Spiral Wound TFF-6 0.54 m² Emd Millipore). At the end of filtration, the retentate was collected and the pigment was then separated by anion-exchange chromatography on a 100 mL column of DEAE sepharose fast flow media using an ÄKTAFPLC system and a XK50 column (GE Lifesciences). Finally, the EMn collected was then dialyzed, freeze-dried and stored in the dark at -20°C until use.

4.2.3 Behavioral response to marennine

Valve opening was closely monitored during a short-term (17.5h) exposure to marennine. The experiment simultaneously tested four marennine concentrations (0, 0.5, 1.0 and 2.0 mg L⁻¹) and included 8 individuals per species per concentration. The holding chambers were cylindrical forms of jars containing 1 L of filtered seawater corresponding to different concentrations of marennine. Valve measurement was started following the transfer of animals into the jars. Valve opening was measured using a valvometry system described in (Comeau et al., 2012). A coated Hall element sensor (HW-300a, Asahi Kasei, Japan) was attached to one of the valve at the maximum distance from the hinge. A small magnet (4.8 mm diameter x 0.8 mm height) was then attached to the other valve, right below the Hall sensor. The magnet weight was 0.1 g, while the Hall element was 0.5 g. The magnetic field (flux density) between the sensor and magnet was a function of the gap between the two valves. The magnetic field in the form of output voltage (µV) was acquired by strain recording devices (DC 104R, Tokyo Sokki Kenkyujo Co., Japan). Voltage measurements were recorded once every minute. At the end of the experiment, voltage values were converted into absolute valve openings by applying conversion algorithms specific to each sensor assembly. Precisely, the adductor muscle was severed,

and small calibration wedges were manoeuvred between the two valves at the point farthest from the hinge. Wedge height was 1–6 mm. The relationship between voltage and valve opening in mm were non-linear and strong. To control for the influence of animal size, valve opening data were converted from millimetres to angles (θ in degrees) using the following equation (Wilson et al., 2005):

$$\theta = 2 \arcsin (0.5 W/ SH)$$

Where W is the valve opening (mm) and SH (mm) is the mussel or oyster shell height. Additionally, the proportion of time the valves of the animals were open in the study population was calculated on an hour-per-hour basis and was expressed as valve open (%):

$$\text{Valve open (\%)} = (n_{\text{valve opening}} / n_{\text{total recording time}}) \times 100$$

Where $n_{\text{valve opening}}$ is the total number of individuals with valves open during a 1-h period of measurement and $n_{\text{total recording time}}$ is the total number of individuals. A value of 100% means that the valves of all individuals were open during the 1-h period of measurement and 0% means that no individuals had their valves open during this time. Results were compiled separately for two consecutive 6-h phases, an acclimation phase (0–6 h) and a post-acclimation (6–12 h) phase.

At the end of the behavioral response experiment, individuals were measured for shell height, weighed and dissected. Gills were sampled and preserved at -80°C until biochemical analyses.

4.2.4 Physiological and biochemical response to marennine

Eight individuals from each species were held in water containing either 0 (control), 0.5, 1.0 or 2.0 mg of marennine per liter of water (i.e., 8 individuals \times 4 marennine

concentrations = 32 individuals per species). Following the 16-h treatment period, all individuals were transferred into maintenance tanks for 8 weeks, after which time physiological measurements were realized (see 2.4.1). Individuals were ultimately sacrificed and the total wet mass of tissue was estimated. The digestive gland and gills were isolated. Gills were divided into two sections, one for the measurement of marennine concentration (see 2.4.2) and the other for the lipid analysis (see 2.4.3). Tissues were preserved at -80°C until biochemical analyses.

4.2.4.1 Physiological measurements

Physiological measurements were conducted over 5 days on individuals randomly sampled from the maintenance tanks. Ten chambers filled with 1 μm -filtered and UV treated seawater were used simultaneously, which allowed measuring eight animals and two controls (empty shell). Animals that remained closed in the chamber were excluded from physiological analysis. Physiological rates were converted to mass-specific rates equivalent to animals of 1-g dry mass using weight exponents (Pernet et al. 2007).

Clearance rate (CR), defined as the volume of water cleared of suspended particles per unit time, was measured using a static system (Pernet et al., 2008, 2007). Animals were placed in 1.3 L metabolic chambers for 1 h before measurements started, then provided with *Tisochrysis lutea* at an initial concentration of 40 cells μL^{-1} . Mixing was promoted by fine bubble aeration around the wall of the metabolic chamber to minimize the resuspension of feces. Food particles were counted every 15 min during 60 min of measurement using an electronic particle counter (Beckman Coulter Counter Z2, Mississauga, ON, Canada). The instrument was fitted with a 100- μm aperture tube and was set to measure particles in the size range of 5–19 μm which are known to be efficiently retained by mussels and oysters (Ward and Shumway, 2004). The greatest difference between two consecutive measurements was used to calculate CR as described in Gilek et al. (1992). CR (L h^{-1}) was then used in conjunction with the algal biomass (mg mL^{-1}) to estimate the amount of ingested energy, assuming that the energy content of the diet was 23.5 J mg^{-1} (Widdows and Johnson, 1988).

Oxygen consumption was determined by individually placing the same individuals above in 0.85 L metabolic chambers. The water within these chambers was continually mixed using magnetic stirrers and animals were acclimated for 60 min prior to measurements. The rate of oxygen uptake was determined by sealing the metabolic chambers and measuring the decrease in dissolved oxygen using a YSI (5331) polarographic electrode coupled to a YSI micro-oxymeter (Yellow Springs, OH, USA). The output signal was monitored continuously on a chart recorder until there was a 20% decrease in oxygen saturation. Respiration was then expressed as $\text{ml O}_2 \text{ g}^{-1} \text{ tissue wet weight h}^{-1}$ and then converted into energy equivalents using the conversion factor $1 \text{ ml O}_2 = 20.33 \text{ J}$ (Widdows and Johnson, 1988).

Following oxygen uptake measurements, all animals were transported individually into 1 L buckets that were placed into the maintenance tank and continuously fed with *Tisochrysis lutea* at 40 cells μL . After 24 h, feces from each individual were collected using a pipette. Assimilation, defined as the product of ingested energy and absorption efficiency (Widdows and Johnson, 1988), was estimated from the Conover ratio (Conover, 1966). Thus, food and faecal samples were filtered onto pre-combusted pre-weighed 25 mm GF/F filters, rinsed with isotonic ammonium formate (3.2%), dried at 70°C for 24 h, cooled to room temperature in a desiccator, and then re-weighed. Afterwards, filters were combusted for 4 h at 450°C, cooled to room temperature in a desiccator, and finally re-weighed. This procedure provided estimates of the organic and inorganic fractions contained in the food and faeces.

Scope for growth (SFG), or the energy available for growth, provides a quantitative assessment of the energy status of the bivalves as well as insight into individual physiological parameters that affect changes in growth rate (Widdows and Johnson, 1988). SFG was estimated by subtracting the energy lost through respiration and excretion from the energy obtained by food assimilation (Widdows and Johnson 1988).

4.2.4.2 Estimation of marennine concentration in the gills

Frozen gills were defrosted on ice, blotted on absorbent paper and the wet mass determined. Marennine in the gills was extracted using urea ($\text{CO}(\text{NH}_2)_2$) 8M after grounding with an Ultraturax tissue tearor (BIOSPEC, Inc) at maximum speed in a 15 mL Falcon tube. Ground tissue with urea was then centrifuged at 7800 rpm for 10 min and the absorbance of the supernatant measured by UV-visible spectrophotometry (UV-6300 PC double beam, VWR). The concentration of marennine, C (mg L^{-1}), was calculated according to the following formula:

$$[C] = \frac{A_{\lambda_{\max}}}{\epsilon_{\lambda_{\max}} \times l}$$

Where $A_{\lambda_{\max}}$ is the maximum absorbance at the peak of wavelength in the red region (674 nm), $\epsilon_{\lambda_{\max}}$ is the specific extinction coefficient at the maximum wavelength, and l is the cuvette path length (Robert et al. 2002). Marennine concentration fixed in the gills was obtained by removing the absorbance of gill tissue from the control using a calibration curve.

4.2.4.3 Lipid analysis

Lipid analysis was performed on 100 mg wet mass of gill or digestive gland from mussels and oysters that were exposed to 0 and 2 mg L^{-1} of marennine and also on triplicate samples of 5 mL of each microalgae species culture used to feed the animals. Microalgae were filtered on pre-combusted 25 mm GF/F filters and second triplicates were sampled for dry mass determination. All lipid samples were stored in lipid-free amber glass vials with TeflonTM-lined caps under nitrogen in 1 mL dichloromethane at -80°C until lipid analyses. Lipids were extracted according to Folch et al. (1957). For bivalve samples, extracts were separated into neutral lipids (including triglycerides and sterols) and polar lipids (including mainly phospholipids) on silica gel hydrated with 6% water (Marty et al., 1992; Pernet et al., 2008, 2007). No neutral and polar fraction separation were applied for microalgae used as bivalve diet. Only the neutral fraction of digestive gland and the polar

fraction of gills were analyzed. The internal standard, 19:0 was added and fatty acid methyl esters (FAME) were prepared using 2% H₂SO₄ in methanol (Lepage and Roy, 1984). The neutral fraction was purified on an activated silica gel with 1 mL of hexane:ethyl acetate (v/v) to eliminate free sterols. FA composition was analyzed in the full scan mode (ionic range: 50–650 m/z) on a Polaris Q ion trap coupled to a multichannel gas chromatograph Trace GC ultra (Thermo Scientific) equipped with an autosampler model Triplus, a PTV injector and a mass detector model ITQ900 (Thermo Scientific). The separation was performed with an Omegawax 250 (Supelco) capillary column with high purity helium as a carrier gas. Data were treated using Xcalibur v.2.1 software (Thermo Scientific). FAME were identified and quantified using known standards (Supelco 37 Component FAME Mix and menhaden oil; Supelco), and were further confirmed by mass spectrometry (Xcalibur v.2.1 software).

4.2.5 Statistical analyses

Analyses of variance (ANOVA) were realized using JMP Pro 11. Normality and homoscedasticity were tested by Kolmogorov-Smirnov and Levene test, respectively. One-way ANOVAs were performed to determine differences in valve opening and physiological rates between different groups of marennine exposition. Where differences were detected, Tukey-HSD multiple comparison tests were used to determine which means were significantly different.

Multivariate analyses on total FA composition were conducted using permutational multivariate analysis of variance (PERMANOVA) using PRIMER version 6.1.15 with PERMANOVA+ (Anderson et al., 2005) based on Bray-Curtis dissimilarities with two sources of variation: marennine concentration and species of bivalves. Homogeneity was evaluated using the permutation analysis of multivariate dispersion (PERMDISP) routine. When a PERMANOVA was significant, pair-wise multiple comparison tests were used. Variations in percentage of FA composition were visualized using non-metric multidimensional scaling (nMDS) ordination based on dissimilarities between samples

after standardization of data. Similarity of percentage (SIMPER) analysis was performed on untransformed data to identify FAs that contributed the most dissimilarity between treatments. Afterwards, these FA were further analyzed by t-test for the two treatment groups. Similar statistical treatments were also applied to compare sum of saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), poly-unsaturated fatty acids (PUFAs), essential fatty acids (EFAs) and the unsaturation index (UI) in the polar lipids.

4.3 Results

4.3.1 Behavioural responses to marennine

The valve gaping activity of control individuals were carefully inspected to gauge acclimation in holding chambers. Acclimation was arbitrary set at 6 hours based on the frequency/amplitude of micro-closures, i.e., shell closures of a relatively short duration. For instance, Fig. 4-1A shows the micro-closures displayed by a control mussel. Micro-closures were frequent (~ 15 times h^{-1}) within the first 2.5 hrs but then gradually decreased after 4.5 hrs (< 10 times h^{-1}); in the post-acclimation phase (Fig. 4-1B), micro-closures were not as frequent (< 5 times h^{-1}). Similarly, in oysters, micro-closure amplitude generally declined sharply after 2.5 hrs and remained stable thereafter (Fig. 4-1C, D).

It was found that mussels initially responded to marennine (Fig. 4-2A, C; $F_{3,31} = 4.68$, $p = 0.010$). Specifically, during the acclimation phase, the highest marennine concentration (2.0 mg L^{-1}) curtailed significantly absolute valve gaping by 44% on average (Tukey: $p = 0.011$). Similarly, during the acclimation phase, the same marennine concentration sometimes resulted in complete valve closures. The proportion of the population with opened valves fell significantly from 100% (control) to 95% (marennine 2.0 mg L^{-1}) (Tukey: $p = 0.028$). These effects of marennine on mussel behavior were no longer present in the post-acclimation phase ($p > 0.3$) (Fig. 4-2E, G).

In oysters, marennine had a delayed effect on behavior. During the acclimation phase no significant effect of marennine was observed, both in terms of valve opening (Fig. 4-2B, $F_{3,31} = 2.37$, $p = 0.089$) and percent of the population with opened valves (Fig. 4-

2D, $F_{3,31} = 1.29$, $p = 0.296$). However, significant differences were detected during post-acclimation (Fig. 4-2F, $F_{3,31} = 4.87$, $p = 0.009$ and Fig. 4-2H, $F_{3,31} = 3.14$, $p = 0.039$). Marennine at higher concentration (2 mg L^{-1}) reduced 62% of valve gaping (Tukey-HSD, $p = 0.006$). Furthermore, the proportion of the population with open valves also fell significantly from 100% (control) to 41% (marennine 2.0 mg L^{-1}) (Tukey: $p = 0.002$).

As expected a positive correlation was found between the concentration of marennine in the water and the concentration of marennine measured in the gill tissues of mussels ($\rho = 0.71$, $p = 0.0001$) and oysters ($\rho = 0.44$, $p = 0.025$) (Fig. 4-3A). Surprisingly valve opening in both mussels and oysters was negatively correlated with marennine concentration in the gill ($\rho = -0.57$, $p = 0.001$ and $\rho = -0.43$, $p = 0.021$, respectively) (Fig. 4-3B).

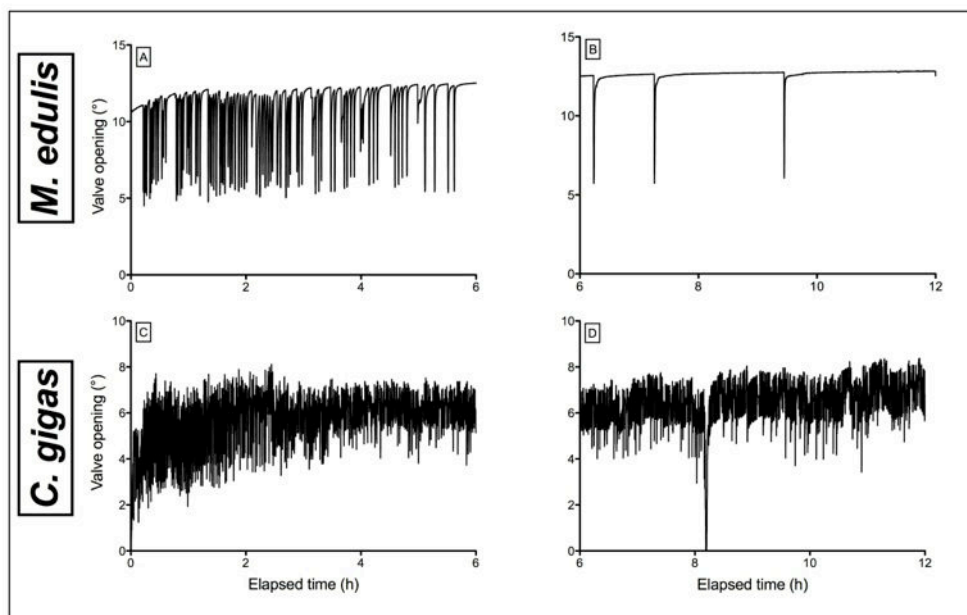


Figure 4-1. Valve movements recorded for *M. edulis* (A,B) and *C. virginica* (C,D) in acclimation (A,C) and post-acclimation phase (B,D) under control condition. Shell valve movement data obtained from a single animal as an example of a typical set of results.

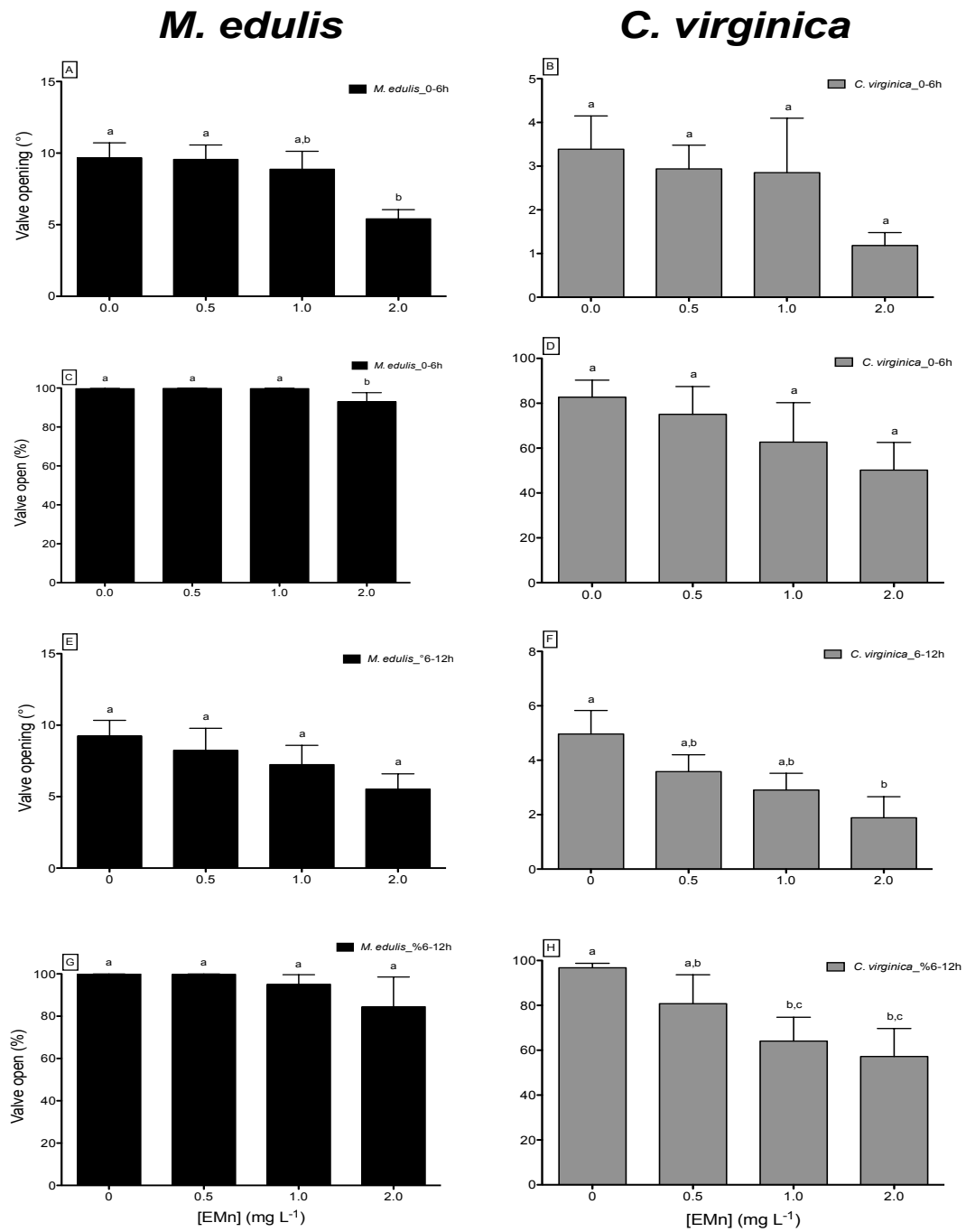


Figure 4-2. Mean of valve gaping (°) and percentage of time valve open (%) of *M. edulis* (A,C,E,G) and *C. virginica* (B,D,F,H) in different time frame of experiment and under different concentration of marennine exposure. Values are mean ± standard error. Error bars represent the 95% confidence interval of the mean. Error bars with different lower case letters are significantly different.

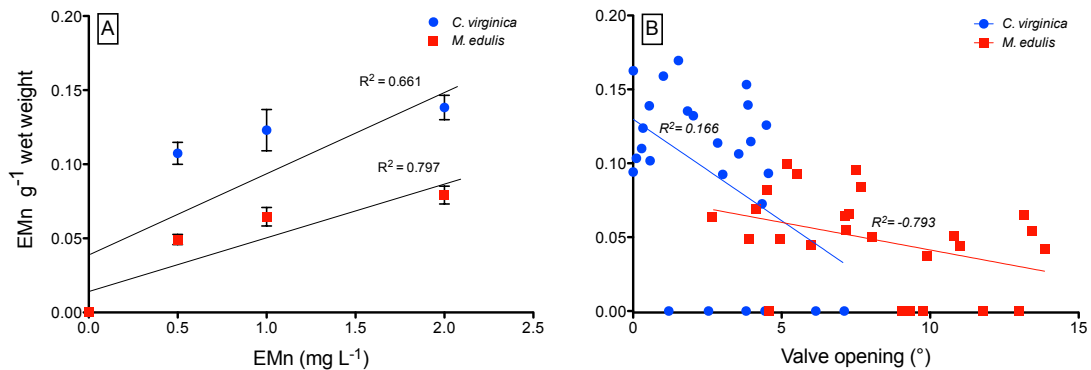


Figure 4-3. Pooled marennine concentration that fixed on the gills of animals as function of marennine concentration on the medium and valve gaping. Coefficient Spearman correlation for mussel and oyster for A were $\rho = 0.71$ and $\rho = 0.44$, respectively. Whereas for B were $\rho = -0.57$ and $\rho = -0.43$ for mussel and oyster, respectively.

4.3.2 Physiological responses to marennine

In oysters oxygen consumption was significantly affected by marennine ($F_{3, 31} = 2.42$, $p = 0.029$). Oysters exposed to 2 mg L^{-1} displayed 31.8% lower oxygen uptake comparatively to those in the control group (Tukey; $p = 0.023$) (Fig. 4-4B). In mussels however no significant effect of marennine on oxygen consumption was observed ($p > 0.05$).

Marennine significantly affected CR in mussels and oysters (Fig. 4-4C, D; $F_{3, 31} = 3.22$, $p = 0.041$; $F_{3, 31} = 1.22$, $p = 0.029$, respectively). Posthoc analysis demonstrated that marennine at 2 mg L^{-1} lowered CR of mussels by 56% on average compared to the control group (Fig. 4-4C, Tukey; $p = 0.026$). On the other hand, oyster with 2 mg L^{-1} of marennine cleared less particles per unit of time compared to the control group (Fig. 4-4D, Tukey; $p = 0.031$).

Marennine had no detectable effect on food absorption rates (Fig. 4-4E,F). Nevertheless, it seems that marennine negatively impacted energy available for growth or the SFG. Marennine significantly affects mussel and oyster's SFG (Fig. 4-4G, H; $F_{3, 31} = 3.39$, $p = 0.034$ and $F_{3, 31} = 3.08$, $p = 0.044$, respectively). Mussels and oysters exposed to 2

mg L⁻¹ of marennine had 58 and 85% lower SFG compared to the control group (Tukey; $p = 0.023$ and $p = 0.044$). These outcomes were presumably related to energy intake, given positive correlations between SFG and CR (Pearson correlation; $r = 0.997$ and $r = 0.988$ for mussel and oyster, respectively). A negative correlation was observed between the SFG and the concentration of EMn that was fixed on the gills, but it was only significant in oysters ($r = -0.629$).

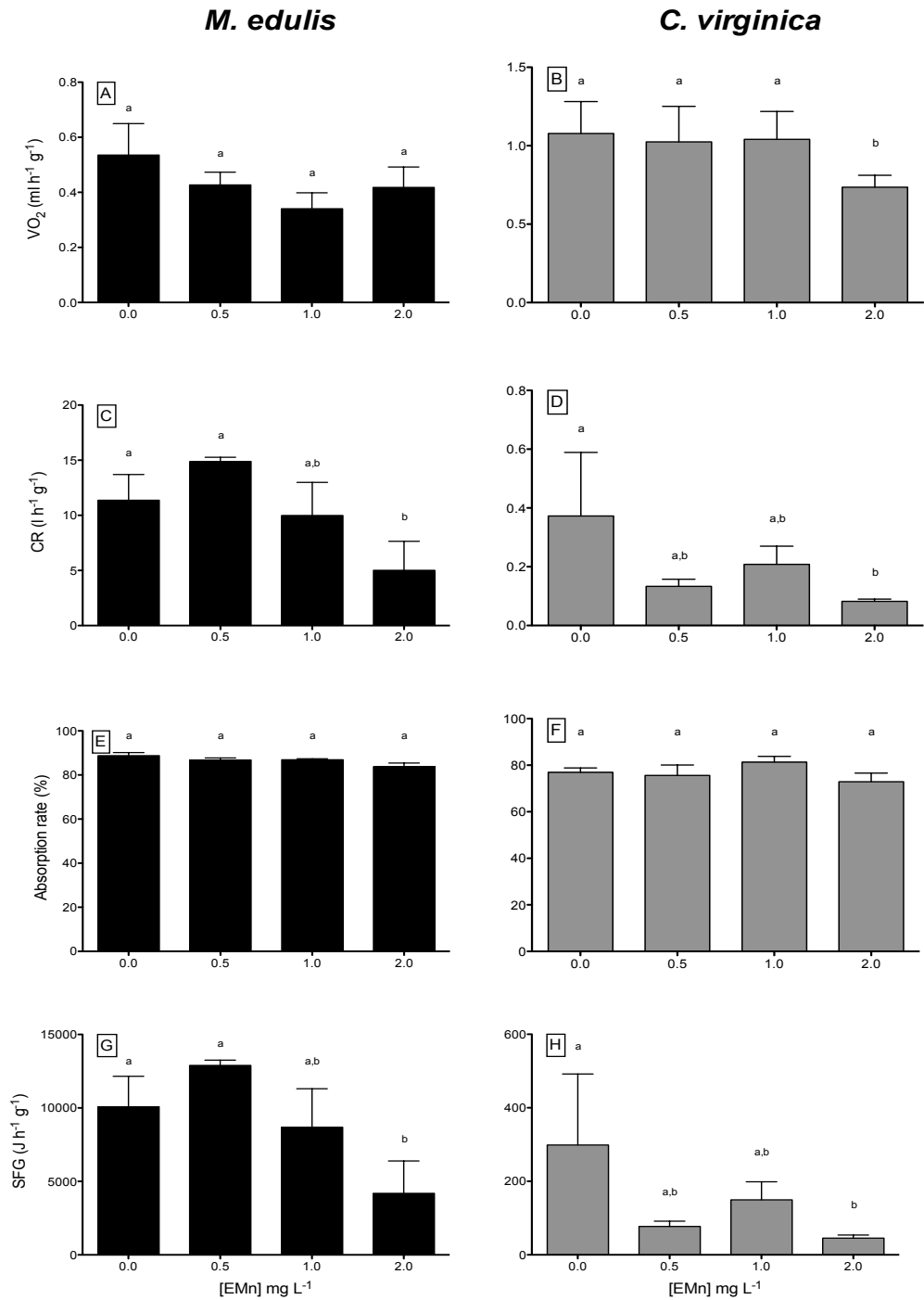


Figure 4-4. Physiological rates of *M. edulis* (A,C,E,G) and *C. virginica* (B,D,F,H) exposed to different concentration of marennine exposure. Values are mean \pm standard error. Error bars represent the 95 % confidence interval of the mean. Error bars with different lower case letters are significantly different.

4.3.3 Biochemical responses to marennine

4.3.3.1 Fatty acids composition of digestive gland neutral lipids

FAs in neutral lipids (NL) in the digestive glands were measured to evaluate the effect of marennine on energetic reserves accumulation. After 8 weeks of exposure to marennine, it was found that the FA composition of the NL in the digestive glands of both species differed significantly between marennine exposed and control groups (Table 4-2, $P_{(perm)} = 0.031$ and $P_{(perm)} = 0.009$ for mussel and oyster, respectively). SIMPER analysis showed that the 18:3 n-6 and 20:5 n-3 contents explained most of the FA composition differences in both species. An effect of marennine on total FAs concentration accumulated in the digestive glands was detected in mussels only. Specifically, total FAs concentrations in NL were significantly lower in mussels exposed to marennine compared to a control group (t-test; $p = 0.014$). Decreasing total FAs concentration in NL of digestive glands were correlated to decreasing CR in mussels exposed to marennine ($r = 0.54$, $p = 0.046$). Moreover, the decrease in CR seemed to be observed for all microalgae species in the diet, as no difference in fatty acid profiles was observed in the NL of digestive glands between the marennine exposed and the control group of mussels. However, there was no significant difference ($p > 0.05$) in total FAs in the NL of oyster's digestive glands even though the CR was significantly affected by the marennine treatment.

Table 4-2. Characteristics of the fatty acids of the neutral lipids (NL, energetic reserves) of digestive glands (DG) and the polar lipids (PL, membrane composition) of gills of mussels *Mytilus edulis* and *Crassostrea virginica* in both control (0) and after 8 weeks (8) exposure to marennine. Values are mean \pm standard error.

Variable	Mussel				Oyster			
	DG (NL)		Gills (PL)		DG (NL)		Gills (PL)	
	0	8	0	8	0	8	0	8
12:0	0.59 \pm 0.5	0.50 \pm 0.4	0.00 \pm 0.0	0.25 \pm 0.2	1.22 \pm 1.1	0.00 \pm 0.0	1.70 \pm 1.2	0.61 \pm 0.4
14:0	7.60 \pm 1.3	6.49 \pm 2.3	3.02 \pm 0.9	2.95 \pm 0.4	13.0 \pm 1.0	8.93 \pm 0.7	7.36 \pm 0.8	3.09 \pm 0.2
15:0	0.66 \pm 0.2	0.00 \pm 0.0	0.48 \pm 0.2	0.33 \pm 0.2	0.00 \pm 0.0	0.74 \pm 0.4	0.00 \pm 0.0	0.24 \pm 0.2
16:0	10.4 \pm 1.5	5.93 \pm 0.6	3.85 \pm 1.0	3.85 \pm 0.4	10.13 \pm 0.9	12.43 \pm 1.2	4.38 \pm 0.6	3.94 \pm 0.2
17:0	1.05 \pm 0.3	0.35 \pm 0.3	1.83 \pm 0.4	1.51 \pm 0.1	0.82 \pm 0.5	2.55 \pm 0.5	3.44 \pm 0.3	1.73 \pm 0.1
18:0	1.75 \pm 0.1	1.77 \pm 0.4	2.03 \pm 0.4	2.66 \pm 0.1	1.86 \pm 0.2	2.06 \pm 0.1	3.61 \pm 0.4	2.81 \pm 0.1
20:0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0
21:0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0
22:0	0.34 \pm 0.1	2.83 \pm 1.4	0.13 \pm 0.0	0.15 \pm 0.0	0.66 \pm 0.2	0.30 \pm 0.1	0.06 \pm 0.0	0.07 \pm 0.0
23:0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0
24:0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0
ΣSFA	22.44\pm1.8	17.9\pm2.5	11.34\pm0.6	11.70\pm0.5	27.73\pm0.4	27.01\pm0.4	20.56\pm0.4	12.49\pm0.1
14:1	0.14 \pm 0.10	0.00 \pm 0.0	0.28 \pm 0.2	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.55 \pm 0.4	0.27 \pm 0.2
15:1	0.20 \pm 0.1	0.00 \pm 0.0	0.77 \pm 0.6	2.51 \pm 1.2	0.00 \pm 0.0	0.00 \pm 0.0	1.61 \pm 1.1	0.64 \pm 0.5
16:1	13.11 \pm 1.1	9.11 \pm 2.6	4.81 \pm 0.8	4.42 \pm 0.3	11.39 \pm 0.7	8.63 \pm 0.5	4.18 \pm 1.1	4.36 \pm 0.1
17:1	3.59 \pm 1.1	6.46 \pm 1.9	31.83 \pm 2.3	30.55 \pm 1.5	6.00 \pm 1.5	5.25 \pm 1.1	22.03 \pm 0.6	24.22 \pm 1.9
18:1 n-9	4.83 \pm 1.0	1.77 \pm 0.6	1.23 \pm 0.3	0.75 \pm 0.2	6.38 \pm 0.7	9.04 \pm 0.7	0.50 \pm 0.2	1.24 \pm 0.1
20:1 n-9	4.23 \pm 0.7	2.84 \pm 1.8	7.99 \pm 1.5	9.94 \pm 0.4	0.00 \pm 0.0	2.08 \pm 0.9	6.66 \pm 0.2	4.37 \pm 0.1
22:1 n-9	0.00 \pm 0.0	0.92 \pm 0.8	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	1.22 \pm 0.8	0.00 \pm 0.0	0.21 \pm 0.2
24:1 n-9	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0
ΣMUFA	26.09\pm1.6	21.1\pm1.7	46.9\pm1.3	48.2\pm1.3	23.76\pm0.5	26.22\pm0.5	35.52\pm0.9	35.31\pm0.8
18:2 n-6	5.16 \pm 1.2	3.11 \pm 1.8	2.97 \pm 1.2	5.01 \pm 0.5	1.26 \pm 1.1	2.48 \pm 1.2	0.00 \pm 0.0	3.35 \pm 0.9
18:3 n-3	1.75 \pm 0.7	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.32 \pm 0.3	0.00 \pm 0.0	0.00 \pm 0.0
18:3 n-6	6.88 \pm 2.9	22.4 \pm 8.6	8.11 \pm 3.1	4.95 \pm 0.5	16.04 \pm 3.1	7.38 \pm 1.3	14.05 \pm 2.0	5.47 \pm 0.7
18:4 n-3	3.73 \pm 0.6	1.58 \pm 0.9	3.72 \pm 0.7	3.25 \pm 0.3	2.97 \pm 1.4	3.91 \pm 0.9	0.89 \pm 0.6	2.99 \pm 0.1
20:2	0.79 \pm 0.3	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0
20:3 n-3	0.51 \pm 0.5	0.00 \pm 0.0	2.52 \pm 1.7	8.70 \pm 1.8	0.00 \pm 0.0	0.00 \pm 0.0	3.33 \pm 2.3	11.91 \pm 0.4
20:3 n-6	0.30 \pm 0.2	0.00 \pm 0.0	0.88 \pm 0.4	1.16 \pm 0.4	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0	0.00 \pm 0.0
20:4 n-6	2.75 \pm 0.7	6.59 \pm 1.8	0.00 \pm 0.0	0.00 \pm 0.0	3.36 \pm 1.1	2.95 \pm 0.9	0.00 \pm 0.0	2.77 \pm 1.8
20:5 n-3	15.04 \pm 1.9	14.2 \pm 3.0	7.97 \pm 1.3	7.39 \pm 0.4	12.79 \pm 2.0	13.77 \pm 0.4	11.41 \pm 0.4	9.59 \pm 0.3
22:6 n-3	14.57 \pm 1.3	12.5 \pm 2.5	14.97 \pm 1.9	9.68 \pm 0.5	12.08 \pm 1.9	15.33 \pm 1.0	14.24 \pm 0.8	15.42 \pm 0.7
22:2	0.00 \pm 0.0	0.57 \pm 0.4	0.62 \pm 0.4	0.00 \pm 0.0	0.00 \pm 0.0	0.63 \pm 0.6	0.00 \pm 0.0	0.30 \pm 0.2
ΣPUFA	51.47\pm2.1	61.0\pm3.7	41.75\pm0.6	40.14\pm0.4	48.50\pm0.7	46.77\pm0.6	43.93\pm0.9	51.80\pm0.6
ΣEFA	29.6\pm3.2	27.3\pm5.9	23.56\pm3.7	17.07\pm0.9	24.87\pm4.2	29.73\pm2.1	25.65\pm1.2	25.31\pm1.3
Unsaturation index	254.8\pm7.0	273.7\pm15	233.1\pm10	210.6\pm4.5	236.2\pm13	242.6\pm5.4	233.7\pm6.3	258.42\pm5.9

4.3.3.2 Fatty acids composition of gill polar lipids

The unsaturation index, which is the number of double bonds per 100 molecules of FAs, was compared the FA composition in the lipid polar fraction (PL) of gills to determine the potential effect of marennine exposure. An increase of the unsaturation index in the marennine exposed group was observed but only in oyster's gills (two sample t-test; $p = 0.017$) (Fig. 4-5B). Pairwise correlation was performed using groups of FAs as explanatory variables and the unsaturation index as the response variable. We observed that the unsaturation index of PL was variably correlated with PUFA in oyster's and mussel's gills ($r = 0.936$, $p < 0.001$ and $r = 0.355$, $p = 0.003$, respectively). In polar lipids of oyster's gills, both the 18:2 n-6, 20:3 n-3 and 22:6 n-3 were positively correlated ($r = 0.598$, 0.674 and 0.576 , respectively) with the unsaturation index, whereas the 18:3 n-6 was negatively correlated ($r = -0.622$) with the same index.

A significant difference in FAs composition of PL was observed between control and the marennine exposed group in both mussel's and oyster's gills ($p = 0.003$ and $p = 0.005$, for mussel and oyster, respectively). SIMPER analysis showed that the difference between control and marennine exposed groups could be explained by five FAs, such as the 18:2 n-6, 18:3 n-6, 20:3 n-3, 20:5 n-3 and 22:6 n-3 (Table 4-2, Fig. 4-6A,B). In oysters gills treated to marennine, the percentage value of 18:2 n-6 and 20:3 n-3 in PL significantly increased over controls by 33.5 and 72% (t-test; $p = 0.011$ and $p = 0.040$, respectively); in contrast, the percentage of 18:3 n-6 declined by 61.1% (t-test; $p < 0.001$). In mussel's gills, a similar tendency was observed for the 22:6 n-3. The percentage value of this particular FA in marennine-treated mussels gills declined by 35.3% in the PL compared to the control group (t-test; $p = 0.017$).

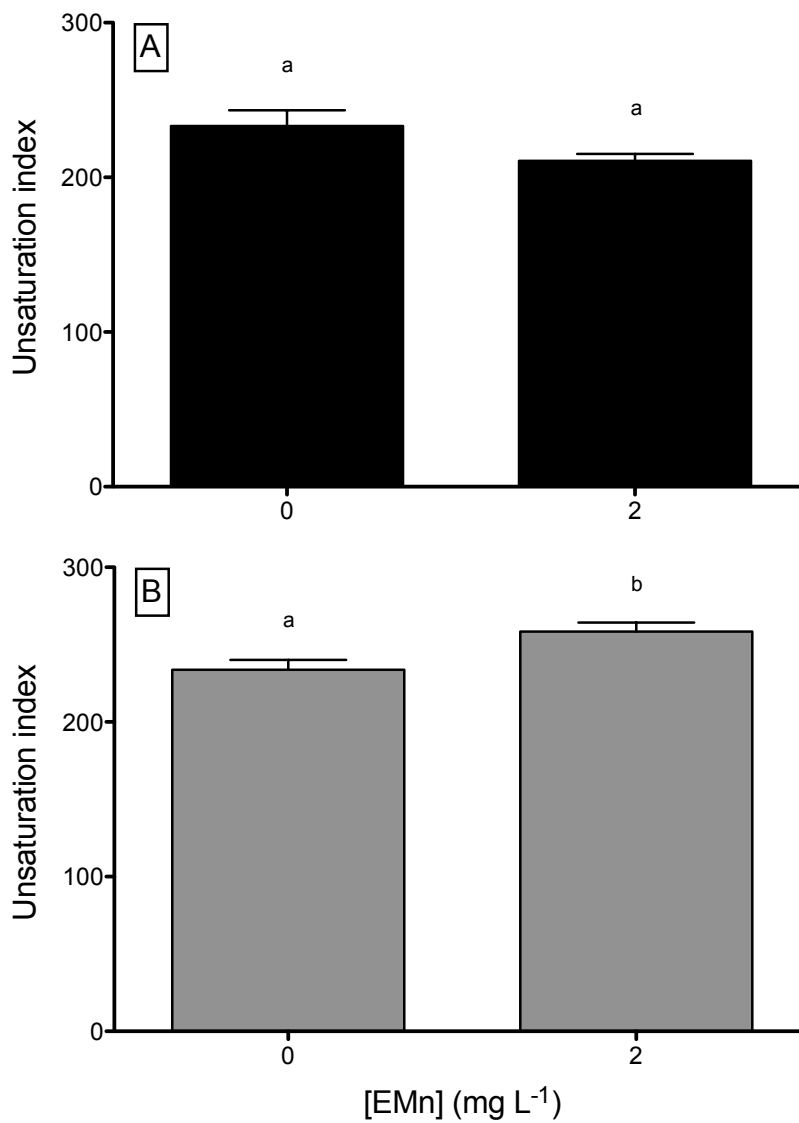


Figure 4-5. Unsaturation index of polar lipids in *M. edulis* (A) and *C. virginica* (B) exposed to different concentration of marennine exposure. Values are mean \pm standard error. Error bars represent the 95 % confidence interval of the mean. Error bars with different lower case letters are significantly different.

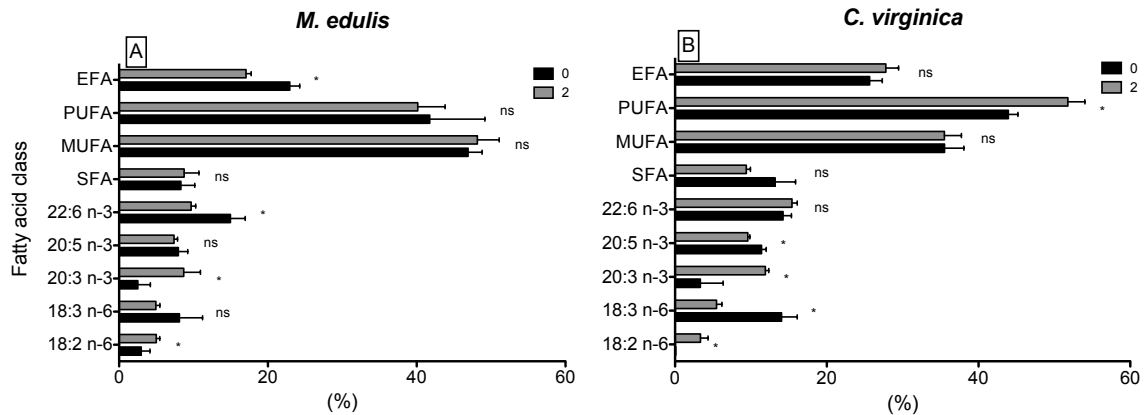


Figure 4-6. Variations in the proportions of selected fatty acid classes in polar lipid (gills) fraction of *M. edulis* (A) and *C. virginica* (B) with different exposition to marennine (solid bars indicate control group while shaded bars indicate EMn-exposed group). Values are mean \pm standard error. Error bars represent the 95 % confidence interval of the mean (ns: not significant; *: $p < 0.05$).

4.4 Discussion

The aim of this study was to determine the effects of excreted and purified marennine on two commercially important bivalve species, *M. edulis* and *C. virginica*. To the best of our knowledge, no other study has yet reported how a non-PST bioactive compound such marennine can influence bivalve behaviour, physiological rate, and biochemical state of cell membranes. In our study, it was found that the two bivalve species generally were impacted by this compound and exhibited similar responses to marennine, which is summarized and discussed below.

4.4.1 Behavioural responses to marennine

Marennine (2.0 mg L^{-1}) impacted the behaviour of both *M. edulis* and *C. virginica*. The most striking effect was related to absolute valve opening, measured in degree angles using a high frequency valvometry system. Marennine lowered valve opening by 44 - 62%. In *M. edulis* this effect was immediate but transient, lasting < 6 hours, whereas in *C.*

virginica the effect was delayed by several hours. It was also found that marennine marginally but significantly lowered the proportion of the population with opened valves, reducing this proportion from 100% (control) to 95% (*M. edulis*) and 41% (*C. virginica*) (marennine 2.0 mg L⁻¹). The underlying reason(s) for the bivalves closing their valves, sometimes completely, in response to marennine is unclear. However, the earlier effect of marennine on valve opening of *M. edulis* can be explained by their higher CR compared to *C. virginica*. It is noteworthy that valve opening was negatively correlated with the concentration of marennine on the gill, suggesting that the behaviour response is somehow related to the progressive accumulation of marennine in the feeding apparatus. Overall it seems marennine causes moderate behavioural effects when compared to bioactive compounds related to harmful algae. For instance, Hégaret et al. (2007) reported that *M. edulis* remains open while *C. virginica* closed their shell for most of the time when exposed to harmful algal. In other studies valvometry was applied to measure the sensitivity of bivalves to the presence of chemical substances such as cadmium or the PSP biotoxin (Nagai et al., 2006; Tran et al., 2003). Marennine on the other hand is completely distinct from metal compounds or the PSP toxin since the consumption of green oysters has never been associated to specific human disorders. Moreover, recent findings suggest that marennine could be applied broadly in aquaculture, since it has antibacterial properties against detrimental pathogens (Gastineau et al., 2014, 2012b). In summary, our results showed that marennine has significant but moderate effects on the valve opening of *M. edulis* and *C. virginica*.

4.4.2 Physiological responses to marennine

The few studies concerning bivalves and natural toxic substances report various effects on CR, pseudofaeces production, and scope for growth (Basti et al., 2013; Hégaret et al., 2007; Jauffrais et al., 2012; Navarro and Contreras, 2010). This previous work is mostly related to PST produced by dinoflagellates. Physiological responses to natural bioactive compounds such as marennine have never been reported. Firstly, our study

demonstrated that marennine fixes to the gills for a long period of time (*ca.* 8 weeks in this study). Secondly, our study indicated that marennine has a significant effect on some of the measured physiological rates. Specifically, marennine lowered SFG by 58% (*M. edulis*) and 85% (*C. virginica*). This impact on SFG was mainly due to curtailed oxygen consumption and CR in marennine-exposed groups. Thus it seems that marennine affects the mechanism of oxygen uptake on gills of bivalves. The parallel impact on CR was particularly striking, with marennine (2 mg L⁻¹) reducing CR by 56% (*M. edulis*) and 78% (*C. virginica*). Such inhibition of oxygen uptake, filtration activity and SFG has been previously observed in studies involving toxic algae (Bricelj and Shumway, 1998; and Li et al., 2002; Navarro and Contreras, 2010). For instance, Li et al. (2002) observed lower SFG in the manila clam *Ruditapes phillipinarum* and the green-lipped mussel *Perna viridis* that were exposed to *Alexandrium fundyense*. We conclude that marennine, a non-PST bioactive compound, has similar inhibitory effects on the growth of *M. edulis* and *C. virginica*.

4.4.3 Biochemical responses to marennine

We provide the first evidence that brief marennine exposures (16 h) affects physiological and biochemical traits of bivalves for an extended period of time (8 weeks). Over such time bivalves are known to allocate part of their energy retrieved from either feeding or energetic reserves. Here we report an augmented depletion of energetic reserves in the digestive gland, which was evidenced by a decrease (-67.58%) in total FAs of NL contained in the digestive glands of *M. edulis* exposed to marennine (2 mg L⁻¹). A similar trend was found in *C. virginica* but with less magnitude (8%) and without significant value. The recorded drop in total FAs was presumably related to corresponding reductions in CR (food intake) observed in the marennine-exposed animals. This interpretation is corroborated by a strong correlation between CR and SFG in *M. edulis* ($r^2 = 0.997$). Decrease in total FAs in NL is related to SFAs, PUFAs and SFA/PUFA ratio. Overall it seems marennine induces stress and depletes energetic reserves accumulated in the

digestive gland of bivalves. A similar outcome was previously demonstrated in oyster larvae (*C. gigas* and *C. virginica*) subjected to bacterial infection (Genard et al., 2013, 2011).

We also found that marennine alters the unsaturation index of membrane phospholipids in the gills of both species. *M. edulis* and *C. virginica* changed the FA composition of their gills' membrane (polar fraction), affecting probably their membrane fluidity, in response to marennine exposition. This structural modification, which in oysters significantly increased the unsaturation index of gill membranes, may have related to the observed reduction in oxygen consumption. In mussel and oyster a relationship between unsaturation index of gills' PL and metabolic rate have been already demonstrated (Pernet et al. 2007). A closer examination revealed that the augmented unsaturation index was mainly due to an increase of several PUFAs in the gills PL. Membrane alteration in bivalves as a response to environmental stress has been previously documented (Genard et al., 2013; Parent et al., 2008; Pernet et al., 2008, 2007; Rochetta et al., 2014). The different amounts of PUFAs found in animals exposed to marennine and control animals may reflect differences in their capacity for selective incorporation of dietary PUFAs into membrane lipids. For instance, long-chain PUFAs are essential for sustaining optimal growth in bivalves (De Moreno et al., 1976). Moreover, while animal cells are capable of synthesizing *de novo* SFAs and MUFAs, the limited or absence of biosynthesis of major highly PUFAs is unlikely due to deficient activity of specific enzymes, like elongases and desaturases (Glencross, 2009). The FAs composition of lipids contained in bivalve tissues generally reflect the combined influences of the dietary regime and selective incorporation or elimination of FAs (Delaunay et al., 1993). In our study, however, it is improbable that the reported differences in PUFAs reflect the dietary regime since all experimental animals were maintained under the same conditions throughout the experiment. Thus, the differences in FA composition are likely due to the impact of marennine on the gills.

4.4.4 Marennine and greening consequences in bivalves: advantageous or detrimental

Despite its unknown chemical nature, marennine-colored oysters have been consumed for centuries and no specific human disorders have been reported. More recently, studies have suggested that marennine and greened-oyster could be considered as healthy food (Gastineau et al., 2014; Pouvreau et al., 2008). From a shellfish aquaculture perspective, supplementing the diets with *H. ostrearia*, which produces marennine, could be advantageous due to the ease of digestibility of the microalgae (Barillé et al., 1994; Cognie et al., 2001) and perhaps more importantly to its properties as a natural antibiotic or bacteriostatic agent (Gastineau et al., 2014, 2012b). For instance, Gastineau et al. (2012b) reported that a purified form of marennine inhibited the growth of bacterial pathogens that contribute to the mass mortality of oyster in Europe such as *Vibrio aestuarianus* and *Vibrio splendidus*. Unpurified marennine was found to improve the survival rate of scallop (*Placopecten magellanicus*) and *M. edulis* larvae by more than 60% and 90%, respectively (Turcotte et al. 2015, unpublished data). The same investigators noted that crude extract of marennine inhibited the growth of *V. splendidus* by as much as 20 to 30% in terms of total number of bacteria. By contrast, our work shows that marennine has detrimental effects on bivalves themselves, namely by hampering their feeding process and reducing their SFG. Overall it seems that such detrimental effects would be largely compensated by the numerous beneficial factors listed above, and also by the high market value of greened-oysters.

4.5 Conclusion

This study provides the first detailed assessment of how bivalves respond to marennine, a natural pigment produced by *H. ostrearia*. Specifically, the impact of purified extracellular marennine on juvenile *M. edulis* and *C. virginica* was characterized from behavioral, physiological and biochemical standpoints. It was found that marennine (2.0 mg L⁻¹) reduces valve opening, oxygen consumption, CR and SFG. Marennine also

changes the unsaturation index in membrane phospholipids. These results enhance our understanding of marennine and particularly the consequence of greening bivalves. From an aquaculture perspective, the efficiency of marennine as a natural antipathogen agent merits further investigation.

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5 CHAPTER 5: ALLELOPATHIC EFFECT OF *HASLEA OSTREARIA* ON OTHER MICROALGAE RELEVANT TO AQUACULTURE

SUMMARY

The fourth article, entitled “Allelopathy can affect co-culturing *Haslea ostrearia* with other microalgae relevant to aquaculture”, was co-authored by myself and by Ita Widowati, Ikha Safitri, Bruno Cognie, Priscilla Decottignies, Romain Gastineau, Michèle Morançais, Eko Windarto, Réjean Tremblay, and Jean-Luc Mouget. This article was submitted in the Journal of Applied Phycology in May 2015. As the first author, my contribution to this article was the execution of experiments, laboratory and data analysis as well as writing for the manuscript preparation. Jean-Luc Mouget, Bruno Cognie, and Michèle Morançais provided the original idea and assisted in the method development. All of the co-authors participated in this work. Result of this work was presented as poster presentation in the International Elsevier conference of Aquaculture 2015: Cutting edge science in aquaculture, in Montpellier, France, 23-26 August 2015.

Allelopathy can affect co-culturing *Haslea ostrearia* with other microalgae relevant to aquaculture

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Abstract

Haslea ostrearia is a marine diatom known to produce marennine, a water-soluble blue-green pigment responsible for the greening of oysters in ponds along the French Atlantic coast. This phenomenon occurs seasonally when *H. ostrearia* blooms in oyster ponds and it increases the economic value of cultured oysters. From an ecological perspective, *H.*

ostrearia blooms are accompanied by a decrease in the abundance of other microalgae, suggesting that this diatom produces allelochemicals. Recent studies showed that purified marennine has other biological activities, antioxidant, antibacterial and antiviral which could be used in aquaculture to promote this pigment as a natural antipathogen agent. One important issue regarding the possible use of *H. ostrearia* in aquaculture as a mixed algal diet, however, is the importance of marennine allelopathy. In this study, we investigated the allelopathic effect of *H. ostrearia* on the growth of five microalgal species relevant to aquaculture: *Chaetoceros calcitrans*, *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Tetraselmis suecica* and *Tisochrysis lutea*. Allelopathic tests were realized in realistic conditions by co-culturing these microalgae with *H. ostrearia* in batch and in semi-continuous mode, based on initial biovolume ratios. Our findings showed that inhibition of the growth of microalgae due to the presence of *H. ostrearia* and marennine was species-dependent. *S. costatum*, *C. calcitrans* and *T. lutea* were significantly more sensitive, whereas *T. suecica* and *P. tricornutum* appeared to be more resistant. Growth irradiance significantly influenced the allelopathic effect against the sensitive species *S. costatum*, *H. ostrearia* production of marennine increasing with irradiance. This study confirmed that marennine released into the culture medium possibly acts as an allelochemical compound, thus explaining the dominance of *H. ostrearia* and the loss of sensitive algae in oyster ponds, but also that some species are insensitive, which allows co-culturing and use in a mixed algal diet in aquaculture.

Keywords: allelopathy, aquaculture, *Haslea ostrearia*, marennine, microalgae

5.1 Introduction

Autotrophic micro-organisms are subjected to important competition phenomenon for resources, such as light and nutrients, which can superimpose with chemical interactions with toxins or allelopathic compounds (*e.g.*, Legrand et al. 2003; Leflaive and Ten-Hage 2007). In general, allelopathy is defined as a toxicological interaction between

an ‘emitter’ and its direct competitors or predators, the ‘target organisms’ (Leflaive and Ten-Hage 2007). The emitter organism produces and releases metabolites that cause a variety of negative effects, for instance growth inhibition, cell lysis, loss of motility and even death of the target organisms (Arzul et al. 1999; Inderjit and Duke 2003; Tillman et al. 2007; Tang and Gobler 2011). In an aquatic ecological context, allelopathic interactions play important roles in species successions and occurrence of blooms (Keating 1977; Takano et al. 2003). Like many phytoplankters, diatoms produce allelochemical and toxigenic compounds (e.g., Sharp et al. 1979; Yamasaki et al. 2007; Ianora and Miralto 2010), among which polyunsaturated fatty acids (PUFAs) (Jüttner 2001), and polyunsaturated aldehydes (PUAs) (Adolph et al. 2003; Jüttner 2005; Ribalet et al. 2007, 2009). These compounds can have negative effects on bacteria (Desbois et al. 2008, 2009), phytoplankton competitors from different taxonomic groups, including diatoms (Sharp et al. 1979; Casotti et al. 2005; Yamasaki et al. 2007), and also grazers (Jüttner 2001, 2005; Pohnert et al. 2002).

The pennate diatom *Haslea ostrearia* can co-occur with other phytoplankton in oyster fattening ponds along the West coast of France. This diatom has the particular feature of synthesizing and excreting the water-soluble blue pigment marennine, responsible for the greening of oysters, an erratic and hardly controlled phenomenon, which gives added value to the bivalve *Crassostrea gigas* in French oyster industry. Previous works hypothesized that marennine accumulation in cells was correlated with an unfavorable environment, such as nutrient deficiency (Neuville and Daste 1978; Robert 1983). This could be, however, the transient result of cell division slow down, as it was further observed that light was an important factor for both growth and marennine production (Mouget et al. 1999, 2004, 2005; Rech et al. 2008). The presence of *H. ostrearia* and marennine in oyster ponds may have an impact on co-occurring phytoplankton. Indeed, during the greening process in oyster ponds, a bloom of *H. ostrearia* is observed, concomitant with a significant decrease in phytoplankton, particularly *Skeletonema costatum* and *Nitzschia closterium* populations, and some authors suggested that the dominance of *H. ostrearia* could occur by allelopathic interactions (Moreau 1970; Neuville and Daste 1978; Turpin 1999). This was confirmed by Pouvreau et

al. (2007), who showed that the purified form of marennine could act as an allelopathic compound, affecting algal growth and development of some diatom species, not only directly by contact, but also indirectly through a shading effect in the water column (absorption in the red part of the spectrum). Thus, the persistency as well as the erratic dominance of *H. ostrearia* in oyster ponds could be explained by possible allelopathic interactions with co-occurring microalgae.

Apart from allelopathic property, it has been demonstrated that purified marennine presents other biological activities, for instance antioxidant (Pouvreau et al. 2008), antibacterial, antiviral and antiproliferative (Gastineau et al. 2012c). Some of these biological activities could be of great importance in the field of aquaculture. For instance, *in vitro* study showed that purified forms of marennine significantly inhibited the development of *Vibrio splendidus* and *Vibrio aestuarianus*, which are likely important pathogens contributing to summer mass mortality of oysters in European region and worldwide (Gastineau et al. 2012c, 2014). Both antibacterial and antiviral activities observed *in vitro*, could lead to potential development of *H. ostrearia* and marennine with maximum benefit to the oyster industry, and more widely to sustainable aquaculture. Indeed, conventional methods for controlling microbial pathogens in aquaculture by use of chemical disinfectants and antimicrobial drugs have led to antibiotic-resistant bacterial strains that may cause significant decrease in animal production (Alderman and Hastings 1998; Cabello 2006). Therefore, in recent aquaculture development, utilization of natural antibiotics becomes progressively favored as a feasible method in management practices for disease prevention in bivalve hatcheries (Van den Bogaard and Stobberingh 2000; De et al. 2014). Hence, *H. ostrearia* and marennine could reveal good candidates for use as natural protector in shellfish larviculture, a very susceptible stage to pathogenic bacteria and viruses in mass mortality events (Paillard et al. 2004).

The present study focuses on the assessment of the allelopathic effect of *H. ostrearia* in realistic conditions of microalgal co-cultures, to identify which algal species relevant for aquaculture are either sensitive or resistant to *H. ostrearia* and its supernatant containing marennine. The hypothesized source of allelopathy, marennine in solution in the growth medium, was measured throughout the body of *H. ostrearia* cultures and filtered

culture supernatants. Growth kinetics and allelopathic tests were conducted on several microalgae species commonly used in aquaculture as feed of *C. gigas*, or in hatcheries, and possible diet combinations are discussed. To circumvent any bias on allelopathic effect resulting from possible competition or nutrient and light limitations, cultures were maintained at low cell density using a semi-continuous mode.

5.2 Materials and methods

5.2.1 Culture conditions

Six marine microalgal strains relevant for aquaculture were used in this work. *Haslea ostrearia* (NCC-148.7), *Skeletonema costatum* (NCC-53), *Pheodactylum tricorutum* (NCC-18), were obtained from the Nantes Culture Collection (NCC), *Tetraselmis suecica*, *Chaetoceros calcitrans*, and *Tisochrysis lutea*, were provided by IFREMER-Nantes. All species were cultured under non-axenic conditions in sterilized 500 mL Erlenmeyer flasks, containing 250 mL of artificial seawater medium (Mouget et al. 2009) at $16^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Cultures were grown at irradiance of $100 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ provided by Philips TLD 36 watt/965 fluorescent tubes or a high-intensity discharge lamp (Osram HQI T, 400 W). Irradiance was measured with a Li-Cor LI-189 quantum meter and a 2π Li-Cor Q21284 quantum sensor, in a 14h / 10h light/dark cycle.

5.2.2 Experimental set up

All series of experiments encompassed the measurement of the cellular volume of the different species of microalgae. The rationale was to consider possible allelopathic effects in function of both cell volume ratios and cell concentrations. For each target species, allelopathic effects were inferred from difference in growth rates and kinetics

between monospecific cultures (controls) and co-cultures with *H. ostrearia*, and from marennine concentration in the culture medium.

5.2.2.1 Biovolume measurement of microalgae

The aim of this measurement was to estimate the cell volume of each microalgal species, and to start allelopathic experiments (co-cultures) using a same volume of biomass (see Table 5-1). The purpose of using a same volume of biomass was to remove bias resulting from differences in biomass initial value (inoculums) when species were co-cultured (*i.e.*, same initial biovolume, but different initial cell concentration). Calculations of surface area and biovolume of the different species of microalgae were performed according to the method of Hillebrand et al. (1999). The ratios between the biovolumes of *H. ostrearia* and those of other microalgae tested are presented in Table 1. Biovolume measurement was conducted when cells were in the exponential phase of growth. A light microscope (Zeiss Axiostar Plus) with magnification of 400-1000 x depending on the species, was directly connected to a camera (AxioCam iCc1) and computer. ImageJ software was used to measure the length, width and height of microalgal cells.

Table 5-1. Mean cell growth rate, cell biovolume and cell surface area during the exponential growth phase of several microalgae in semi-continuously cultivated mono-specific culture. Cell biovolume for each species was calculated based on geometric shape for cell biovolume in Hillebrand et al. (1999).

Species	Exponential growth rate (day⁻¹)	Cell biovolume (μm³)	Cell surface area (μm²)	Shape model of biovolume used	Biovolume ratio
<i>H. ostrearia</i>	0.51 ± 0.03	758 ± 11	765 ± 7	Prism on elliptic base	1
<i>T. suecica</i>	0.44 ± 0.02	382 ± 17	155 ± 6	Prolate spheroid	2
<i>P. tricorutum</i>	0.41 ± 0.01	80 ± 2	117 ± 2	Half-elliptic prism	9
<i>T. lutea</i>	0.75 ± 0.04	58 ± 5	64 ± 4	Prolate spheroid	13
<i>S. costatum</i>	0.29 ± 0.02	29 ± 0	81 ± 1	Cylinder + 2 half spheres	27
<i>C. calcitrans</i>	0.22 ± 0.01	17 ± 1	40 ± 1	Half-elliptic prism	44

Note: values are means ± Standard Error. Mean growth rates are the average of the rates in exponential phase in each dilution cycles (n = 4) at each replicate of species (n = 3). Cell biovolume for each species was calculated at the exponential phase (n = 40).

5.2.2.2 Allelopathic test of *Haslea ostrearia* on microalgae tested

Algal cultures were conducted in batch to identify the beginning and the range of the exponential phase for all species tested and in semi-continuous modes to maintain the culture in exponential growth at low cell density. The semi-continuous mode of culture minimizes the competition for nutrients and light, which possibly occurred when cells are maintained in batch culture. For each species, cells were acclimated to their growth conditions for at least one week, and maintained in exponential growth phase by dilution with fresh medium every 4 days. Approximately 75% of culture total volume at the 4th day was discarded, and fresh medium was added to the remaining 25% to complete to the initial volume (250 mL). The cell concentration and the cell biovolume for each microalga were determined every 2 days in batch mode, every 4 days in semi-continuous mode, during 12 to 16 days (at least 3 consecutive dilution cycles). On each sampling day, samples were gently stirred in the Erlenmeyer prior to cell counting to avoid aggregates. Furthermore,

cell growth was monitored by measuring cell densities with Nageotte or Neubauer counting chambers. Growth rate was calculated as μ (day^{-1}) using eq. 1:

$$\mu = \frac{\ln N_2 - \ln N_1}{d_2 - d_1} \quad (1)$$

where N_1 and N_2 represent cell density at the start and the end of each growth period, and d_1 and d_2 are the time of measurement.

Tests for the allelopathic effect were performed in sterile Erlenmeyers containing *H. ostrearia* (final cell density, 5×10^3 cells mL^{-1}), and *T. lutea*, *T. suecica*, *C. calcitrans*, *S. costatum* or *P. tricornutum* (final cell density varying according to the species, to reach the same total biovolume as *H. ostrearia*) (see biovolume ratio, Table 1). To estimate the effect of allelopathy of *H. ostrearia* and supernatant containing marennine, the percent inhibition (I%) was calculated as percent difference in growth rate of the treatment relative to control (eq. 2):

$$\text{Percent inhibition (I\%)} = \frac{\mu_c - \mu_t}{\mu_c} \times 100 \quad (2)$$

Where μ_c and μ_t represent the growth rate controls (mono-specific culture) and treatments (co-culture) at the exponential phase, respectively.

5.2.2.3 Effect of light intensity on marennine production and allelopathic activity

This series of experiments were run using one sensitive microalgal species, *S. costatum*, evidenced as previously described. Co-cultures of microalgae (*H. ostrearia* and the target species) were prepared according to the biovolume ratio method, and grown at different irradiances: 20, 100 and 500 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ representing low (limiting), medium and high (saturating) light, respectively. For each co-culture, marennine

concentration was determined as described below. For each algal species, cell density, growth rate and percent inhibition were determined as described above.

5.2.2.4 Estimation of marennine concentration

The concentration of extracellular marennine (EMn) present in each sample was calculated at the end of the growth period (at $t = 12^{\text{th}}$ day and at each dilution cycle or 4^{th} day, for batch and semi-continuous culture, respectively). Co-culture supernatants were filtered on Millipore filter ($0.22 \mu\text{m}$) prior to the measurement. Afterwards, the absorbance of the supernatant was measured by UV-visible spectrophotometry (PerkinElmer Lambda 25), and the concentration was determined as described in Robert et al. (2002). The concentration (C) of EMn (g L^{-1}) was calculated according to the following formula (eq. 3):

$$[C] = \frac{A_{\lambda_{\text{max}}}}{\epsilon_{\lambda_{\text{max}}} \times l} \quad (3)$$

Where $A_{\lambda_{\text{max}}}$ is the absorbance at the peak wavelength in red region (674 nm), $\epsilon_{\lambda_{\text{max}}}$ is the specific extinction coefficient at the peak wavelength, and l is the cuvette path length.

5.2.2.5 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 one-way analyses of variance (ANOVA) followed by Tukey-HSD posthoc multicomparison test. In addition, analysis of co-variance (ANCOVA) was performed to test the effect of different light levels and marennine concentrations on allelopathic activity of *H. ostrearia* on the most sensitive target microalgal species.

5.3 Results

5.3.1 Biovolume and growth kinetics of *Haslea ostrearia* and microalgae tested

Microalgal biovolume allows estimating the relative importance of co-cultured algae in a global biomass. For a single cell, the variation in cell biovolume corresponds to the different shape and size (length, width, and depth) (Table 5-1). Based on the calculation of the length of transapical axis of the microalgae tested, they can be classified into two classes, *P. tricornutum* and *T. suecica* representing the large class (mean transapical axis: $22.3 \pm 0.3 \mu\text{m}$ and $12.6 \pm 0.2 \mu\text{m}$, respectively), whereas *S. costatum* ($4.4 \pm 0.1 \mu\text{m}$), *C. calcitrans* ($4.5 \pm 0.1 \mu\text{m}$), and *T. lutea* ($5.1 \pm 0.1 \mu\text{m}$) as the small class. The determination of cell biovolumes, using the standard geometric shapes by Hildebrand et al. (1999), showed that *H. ostrearia* had the highest average cell biovolume followed by *T. suecica*, *P. tricornutum*, *T. lutea*, *S. costatum*, and *C. calcitrans* (Table 5-1). For the allelopathy tests with co-cultures in batch and in semi-continuous mode, experiments were run taking into account the cell biovolume ratios, to set a same initial total biovolume for each species.

During growth kinetics in batch mode, *P. tricornutum* and *T. lutea* showed the highest cell concentration ($702 \cdot 10^4 \text{ cell mL}^{-1}$ and $650 \cdot 10^4 \text{ cell mL}^{-1}$, respectively) by the end of the growth phase ($t = 12$), whereas *H. ostrearia* had the lowest value ($13 \cdot 10^4 \text{ cell mL}^{-1}$, Fig. 5-1A). Variation was also observed in terms of total biovolume (μL) (ANOVA: $F_{5, 18} = 1821.1$, $p < 0.0001$). Biovolume of *P. tricornutum* was significantly higher than other species (Tukey HSD: $p < 0.0001$) whereas *S. costatum* had the lowest. As to *H. ostrearia*, it showed the lowest cell concentration, but a significantly higher total biovolume than the two other diatoms, *S. costatum* and *C. calcitrans* (Tukey HSD: $p < 0.0001$ and $p = 0.0004$, respectively) (Fig. 5-1B).

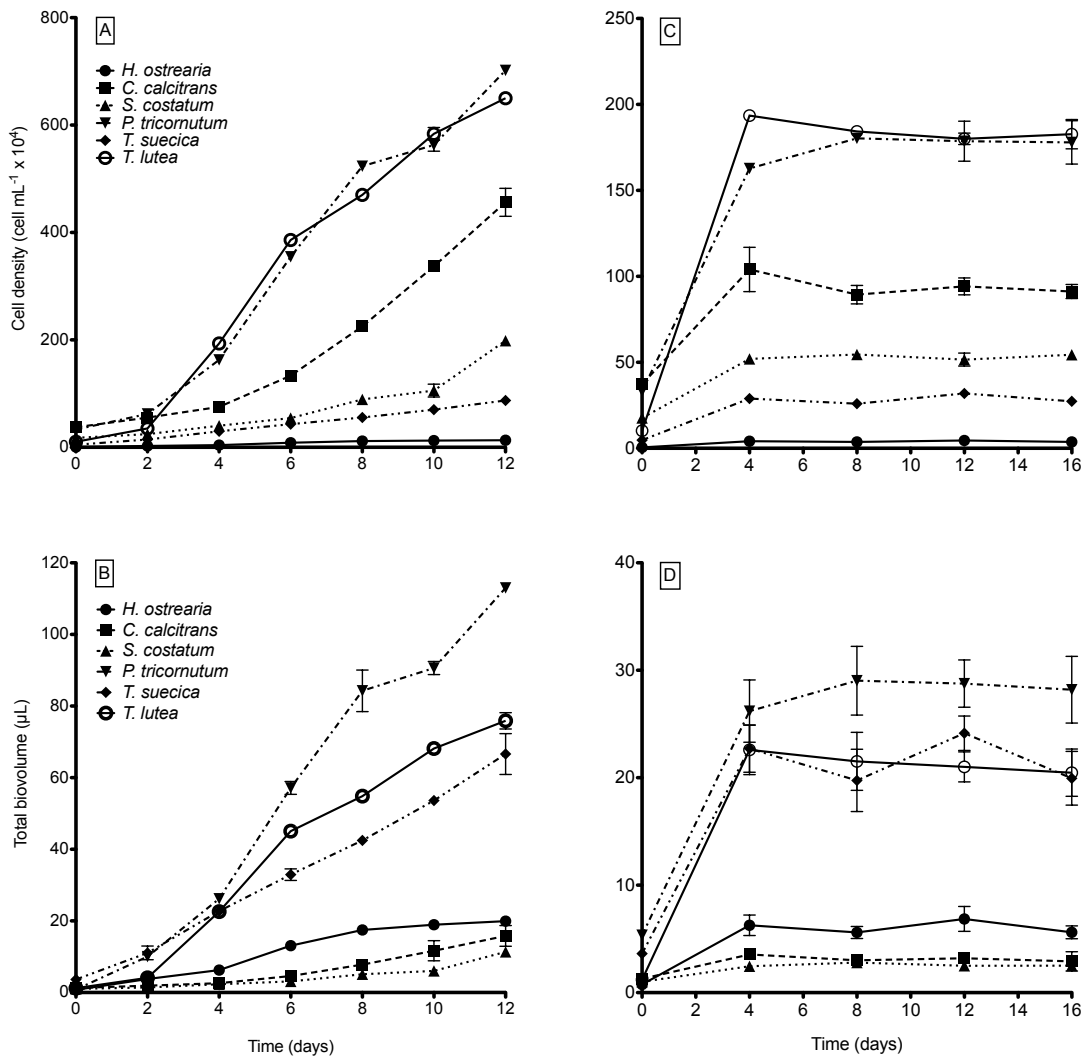


Figure 5-1. Growth kinetics of different species of microalgae cultivated in batch (A, B) and semi-continuous (C, D) mode, expressed as cell concentration (A, C) (10^4 cell mL⁻¹), and total biovolume (B,D) (μ L). Values are mean \pm standard error (n = 3).

Semi-continuous cultures were conducted subsequently to batch cultures by dilution of algal suspensions every 4 days to maintain cells in exponential growth phase at low cell density. Each microalga cultivated in a semi-continuous system showed a stabilization of growth, with only slight differences in cell concentration on the 4th day for each dilution (Fig. 5-1C). Variation occurred in terms of total biovolume (ANOVA: $F_{5,54} = 132.8$, $p < 0.001$), where *P. tricorutum* has significantly higher total biovolume than other

microalgae (Tukey HSD: $p < 0.0001$) (Fig. 5-1D). Furthermore, *T. lutea* showed the highest average growth rate whereas *C. calcitrans* exhibited the lowest value (0.75 d^{-1} and 0.22 d^{-1} , respectively) (ANOVA: $F_{5,54} = 500.1$, $p < 0.001$). For each species, no significant difference was observed in terms of cell density, total biovolume and growth rate in all cycles of dilution ($p > 0.05$) illustrating that the semi-continuous mode was stable (Figs. 5-1C, D).

5.3.2 Growth inhibition in co-culture of *Haslea ostrearia* with different microalgae

At the beginning of allelopathic co-culture experiments, the initial cell concentration of *H. ostrearia* in each flask was $5000 \text{ cell mL}^{-1}$, while the cell number of target species was different according to cell biovolume ratios. Allelopathic test kinetics showed variations in cell concentration (not shown) and in total biovolume of the target species, in response to the presence of *H. ostrearia* when they were co-cultured (Figs. 5-2A and B, batch and semi-continuous mode of culture, respectively). For algae co-cultured in a semi-continuous mode, pooled mean from the three consecutive cycles of dilution showed that *H. ostrearia* significantly hampered the growth rate of all species tested as compared to the respective controls (ANOVA: $F_{15,54} = 366.1$, $p < 0.001$). The decrease in growth rate, expressed as the percent inhibition (I%), was the highest for *C. calcitrans* ($79.4 \pm 5.5\%$) and *S. costatum* ($74.2 \pm 2.5\%$) then followed by *T. lutea* ($52.1 \pm 1.8\%$), and the lowest for *T. suecica* ($37.2 \pm 3.6\%$) and *P. tricorutum* ($29.5 \pm 1.9\%$) ($p < 0.05$) (Fig. 5-3A). Pearson correlation showed that the size of microalgae influenced the allelopathy interaction in co-culture. Percent inhibition of target species tested was negatively correlated with their cell biovolume and also their cell surface area ($r = -0.727$ and -0.754 , respectively) (Fig. 5-4A, B). The percent inhibition was positively correlated with the cell surface area to volume ratio ($r = 0.776$) (Fig. 5-4C).

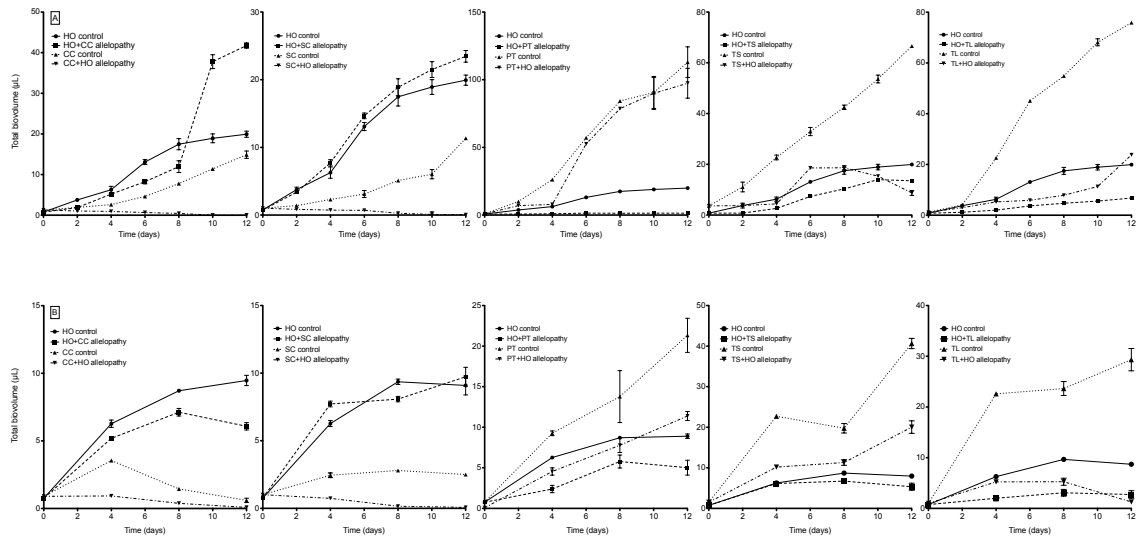


Figure 5-2. Growth kinetics of different microalgae co-cultured in allelopathic tests in batch (A) and semi-continuous (B) mode. Values are means \pm standard error ($n = 3$) of total biovolumes (μL).

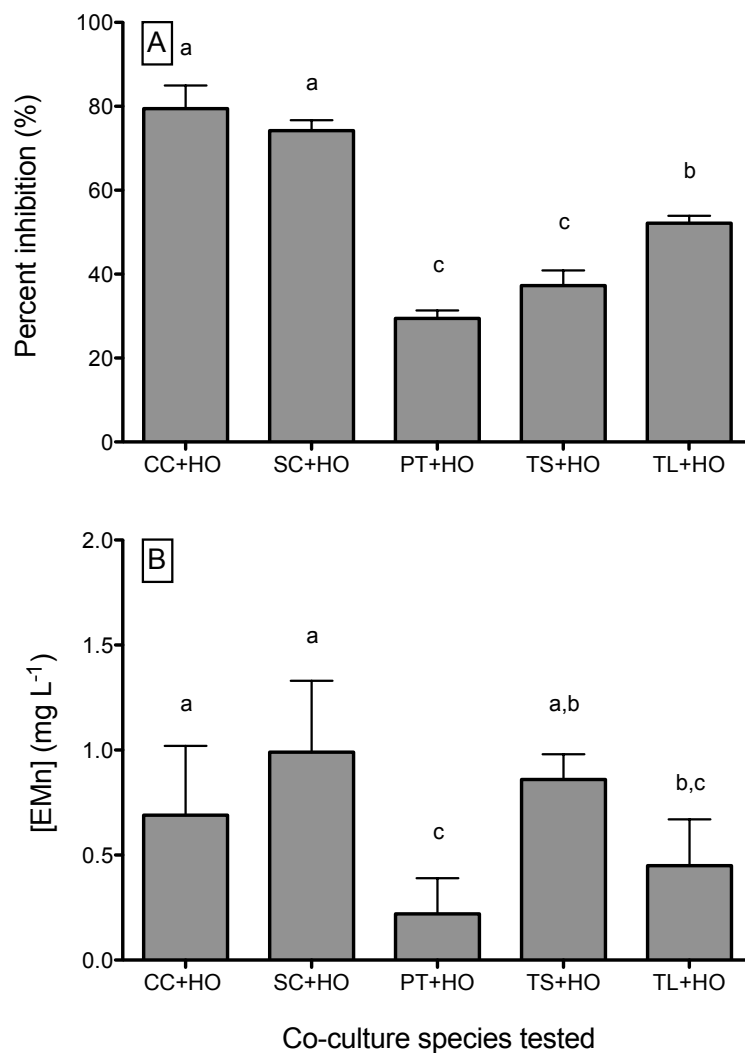


Figure 5-3. Percent inhibition (I%) of target algae (A) and concentration of extracellular marennine (EMn, mg L⁻¹) (B) in co-cultures with *Haslea ostrearia*. Values are means ± standard error (n = 3) obtained from three consecutive cycles of dilutions. Different letters indicate significant differences. CC = *Chaetoceros calcitrans*; SC = *Skeletonema costatum*; PT = *Phaeodactylum tricornutum*; TS = *Tetraselmis suecica*; TL = *Tisochrysis lutea*; HO = *Haslea ostrearia*.

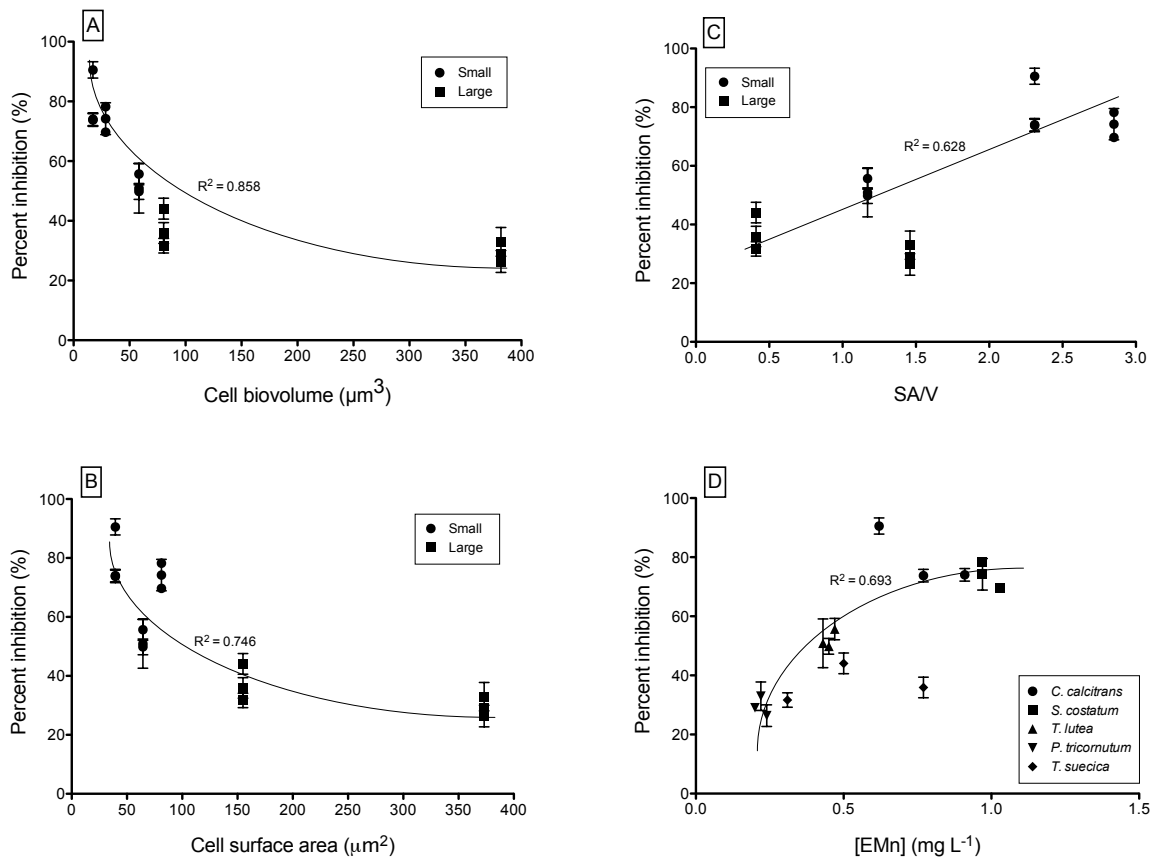


Figure 5-4. Percent inhibition ($I\%$) of microalgal target species tested in co-culture with *Haslea ostrearia* as a function of their respective cell biovolume (A), cell surface area (B), surface to volume ratio (C), and of marennine concentration measured at the end of the cycles of dilution (D). Data points are means \pm standard error ($n = 3$). Pearson correlation showed strong negative correlation between ($I\%$) and cell biovolume ($r = -0.731$), total surface area ($r = -0.778$), yet positive correlation was observed between percent inhibition and the total surface volume ratio ($r = 0.774$) and marennine concentration in co-culture of microalgal target species tested ($r = 0.775$).

5.3.3 Concentration of extracellular marennine (EMn) in co-culture supernatants

During growth, the blue diatom *H. ostrearia* synthesizes and releases to the culture medium the pigment marennine, which has been shown responsible for the inhibitory effect of growth of target species when they were co-cultured (Pouvreau et al. 2007). In the

present work, *a posteriori* quantification of EMn concentration in the supernatants was conducted every 4 days, at the end of each dilution cycle yet during the exponential growth phase. For all co-cultures, the EMn concentration was not significantly different between all three cycles of dilution ($p > 0.05$). The highest EMn concentration was observed in the co-culture of *S. costatum*, *C. calcitrans*, or *T. suecica* + *H. ostrearia* (maximum value of $1.04 \pm 0.25 \text{ mg L}^{-1}$, mean \pm SE, $n=3$) and the lowest in the co-culture of *P. tricornutum* or *T. lueta* + *H. ostrearia* (maximum value of $0.49 \pm 0.10 \text{ mg L}^{-1}$, mean \pm SE, $n=3$) (Fig. 5-3B).

Significant relationship was observed between concentration of EMn and the percent inhibition (I%) in target species tested. Pearson correlation showed that I% was positively correlated with the amount of EMn released into the medium ($r = 0.775$) (Fig. 5-4D). In contrast, negative relationship was observed between EMn concentration and the growth rate of microalgae tested in co-culture ($r = -0.750$), suggesting that a higher decrease in growth rate of the target microalgae resulted from a higher EMn released in the culture (data not shown).

A complementary experiment was conducted with the sensitive species *S. costatum* to test the hypothesis that growth inhibition was not the direct consequence of the presence of *H. ostrearia* cells in the co-culture. Actively growing *S. costatum* was subcultured at low density (initial cell concentration, $37 \times 10^4 \text{ cell mL}^{-1}$) with a mixture of fresh medium and *H. ostrearia* culture supernatant, in proportions (100/0, 70/30 and 30/70 v/v), which corresponded to 0.0, 3.6 and 8.4 mg L^{-1} of marennine, respectively. In comparison with the control (no marennine, 100% fresh medium), the growth of *S. costatum* decreased significantly when exposed to marennine-containing supernatant, percent inhibition I% increasing with time, and proportion of supernatant or marennine concentration (Fig. 5-5).

5.3.4 Effect of irradiance level on allelopathic activity of *Haslea ostrearia*

In this series of experiments, *S. costatum* was again chosen as the vulnerable species to determine whether irradiance level may influence the allelopathic activity of *H.*

ostrearia. For monospecific batch and semi-continuous cultures, the growth rate of *S. costatum* increased significantly with irradiance ($p < 0.05$) (Table 5-2). As for *H. ostrearia*, both in semi-continuous mode and in batch mode, however, the growth rate of *H. ostrearia* at low irradiance was significantly lower compared to medium and high irradiance, but no significant difference was observed between the last two levels of irradiance ($p > 0.05$).

Table 5-2. Mean cell growth rate (d^{-1}) during the exponential growth phase of mono- and co-cultured (allelopathy) microalgae (Mo. and Al., respectively), in batch and semi-continuous mode, under different irradiances.

Parameters	Type of culture	Species	Irradiance ($\mu\text{mol photon m}^{-2} \text{s}^{-1}$)		
			20	100	500
Growth rate (day^{-1})	Mo. Batch	Ho	0.21 ± 0.03	0.53 ± 0.06	0.59 ± 0.05
		Sc	0.14 ± 0.20	0.21 ± 0.04	0.40 ± 0.01
	Mo. Semi-continuous	Ho	0.36 ± 0.00	0.52 ± 0.01	0.57 ± 0.03
		Sc	0.16 ± 0.00	0.23 ± 0.01	0.41 ± 0.01
	Al. Batch	Ho	0.20 ± 0.03	0.55 ± 0.01	0.58 ± 0.01
		Sc	-0.03 ± 0.00	-0.08 ± 0.01	-0.11 ± 0.01
	Al. Semi-continuous	Ho	0.37 ± 0.01	0.51 ± 0.03	0.57 ± 0.02
		Sc	-0.07 ± 0.03	-0.16 ± 0.06	-0.15 ± 0.02
[EMn] (mg L^{-1})	Al. Batch	-	5.15 ± 0.53	8.43 ± 1.68	11.03 ± 0.33
	Al. Semi-continuous	-	0.57 ± 0.03	0.99 ± 0.20	1.43 ± 0.40
Percent Inhibition (I%)	Al. Batch	Sc	60.87 ± 0.72	87.66 ± 0.41	89.57 ± 0.26
	Al. Semi-continuous	Sc	61.99 ± 0.73	74.22 ± 2.48	82.10 ± 4.19

Note: EMn concentration (mg L^{-1}) and percent inhibition (I%) were measured at the end of allelopathy experiment and at each cycle of dilution in batch and semi-continuous culture, respectively. Values are means \pm standard error ($n = 3$) obtained from three consecutive cycles of dilutions.

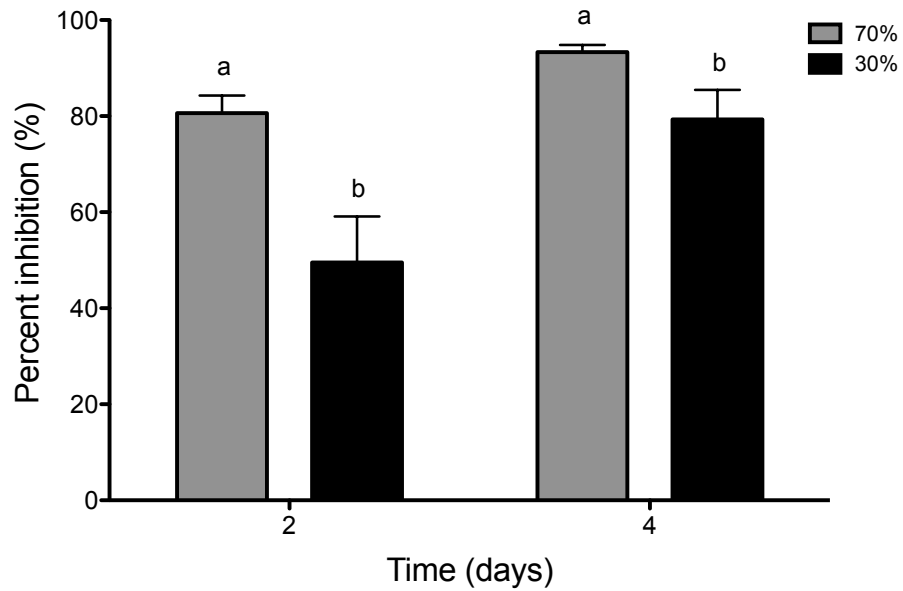


Figure 5-5. Percent inhibition of *Skeletonema costatum* grown for 4 days at low density (initial cell concentration, 37×10^4 cell mL^{-1}) in a mixture of fresh medium and *H. ostrearia* culture supernatant (100/0, 70/30 and 30/70 v/v, which corresponded to 0.0, 3.6 and 8.4 mg L^{-1} of marennine, respectively). Values are means \pm standard error (n = 3).

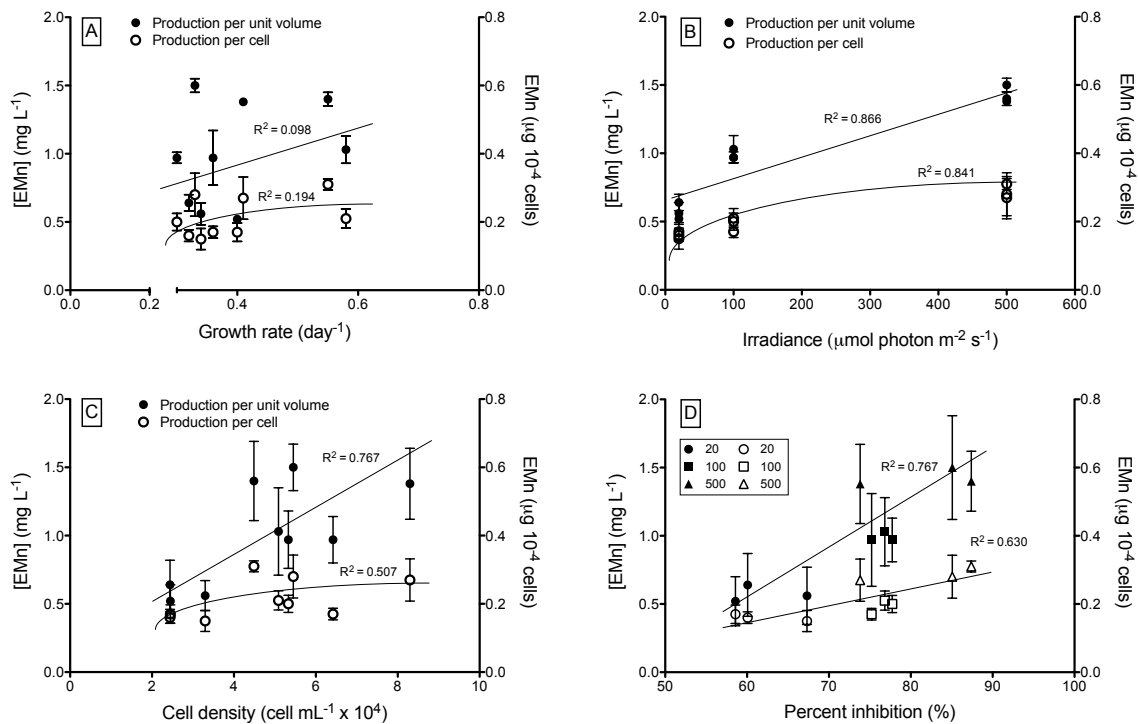


Figure 5-6. Marennine concentration (mg L^{-1} or $\mu\text{g } 10^{-4}$ cells) in co-cultures of *Skeletonema costatum* with *Haslea ostrearia* at different irradiances as a function of *H. ostrearia* growth rate (A), irradiance level (B), *H. ostrearia* cell density (C), and percent inhibition I% (D). Filled symbols indicate marennine production per unit volume, empty symbols indicate marennine production per cell. Data points are means \pm Standard Error ($n = 3$). Pearson correlation for A, B, C and D in marennine production per unit volume are $r = 0.314, 0.931, 0.724$ and 0.876 , respectively. Pearson's correlation coefficient values for A, B, C and D in marennine production per cell are $r = 0.440, 0.959, 0.507$ and 0.794 , respectively.

Following the acclimation in batch mode, co-cultures of *H. ostrearia* and *S. costatum* were performed in semi-continuous mode at limiting or saturating irradiance, to observe manifestation of the allelopathic effect, in terms of growth rate and percent inhibition. Pooled mean from three consecutive cycles of dilution in co-culture showed that significant difference was observed in cell concentration (not shown) and growth rate of *S. costatum* at all irradiance levels comparatively to the respective controls ($p < 0.05$) (Table

5-2). The decrease in *S. costatum* cell concentration and growth rate at all irradiances was concomitant with an increase in I%. At low irradiance I% was significantly lower than at medium and high irradiance ($p = 0.012$ and 0.011 , respectively). Furthermore, I% increased with EMn concentration (Table 5-2). Pooled data from all cycles of dilution showed that EMn concentration at medium and high irradiance levels in co-culture was significantly higher than at low irradiance ($p < 0.05$). It appeared that EMn concentration in the medium was not correlated significantly with *H. ostrearia* growth rate ($r = 0.314$, Fig. 5-6A) but with irradiance ($r = 0.931$), cell density ($r = 0.724$) and percent inhibition ($r = 0.876$) (Figs 5-6B, C and D, respectively). Furthermore, a good relation was observed between EMn production per day and EMn production per cell in function of the irradiance level, in both batch and semi-continuous co-cultures (Figs 5-7A and B, respectively).

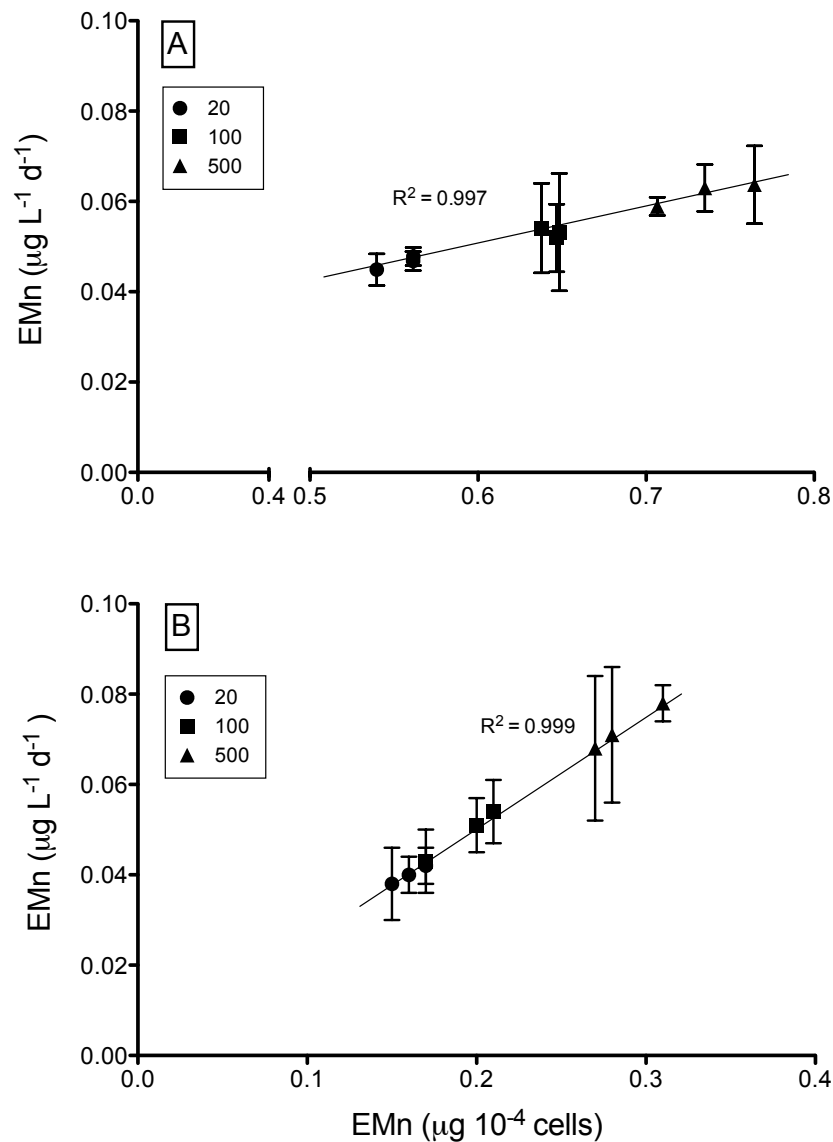


Figure 5-7. Relationship between production of marennine per day and per cell for monospecific cultures of *Haslea ostrearia* grown at different irradiances in batch (A) and semi-continuous mode (B). Data points are means \pm standard error ($n = 3$). Pearson's correlation coefficient values between production of marennine per day or per cell and irradiance are $r = 0.915$ and 0.875 in batch culture, $r = 0.959$ and 0.956 in semi-continuous culture, respectively.

5.4 Discussion

Green oysters (known as ‘vertes de claires’ in France) contribute to the economy and the standing local oyster production in Marennes-Oléron and Bourgneuf Bay. The importance of *H. ostrearia* and marennine as greening agent in oyster-fattening ponds has long been recognized (Gaillon 1820; Lankester 1886). It has also been demonstrated that *H. ostrearia* as a mono-specific algal diet can sustain the growth of the oyster *C. gigas* for 8 weeks (Piveteau 1999; Cognie 2001), and that it is well digested with almost 90% of digestibility (Barillé et al. 1994). Moreover, recent findings on marennine biological activities have awoken interest as a possible natural antibiotic compound for aquaculture (Gastineau et al. 2012c, 2014), but also demonstrated it has allelopathic activity on some diatoms usually encountered in oyster ponds (Pouvreau et al. 2007). Thus for potential industrial applications in aquaculture or larviculture, it has become important to determine the possible allelopathic pressure of *H. ostrearia* cultures on other microalgae in realistic conditions. For this reason, in the present work, *H. ostrearia* cultures or raw supernatants were tested, in contrast to purified pigments in Pouvreau et al. (2007). As a consequence, any inhibitory effect observed in co-culturing the blue *Haslea* with aquaculture relevant algae could be interpreted as a true allelopathic effect, either due to marennine or another compound released in the supernatant, or to a competition between phytoplankton species, or cell-to-cell interactions (effets of contact). However, competition for light and nutrients was highly minimized, as co-cultures were grown in a semi-continuous mode and maintained in exponential phase at low cell density. Moreover, effects of contact interactions should have been rare, if not unlikely, *H. ostrearia* behaving like a benthic species, whereas most of the target microalgae are rather pelagic, and *Haslea* culture supernatants (not containing cells) have been shown to exert allelopathic pressure.

5.4.1 Allelopathic effect of *Haslea ostrearia* on the growth of co-cultured algae

Allelopathic effect of *H. ostrearia* on the growth of other microalgal species has already been demonstrated in the laboratory, both using pigmented supernatants of *H. ostrearia* cultures (crude extracts, Neuville and Daste 1978; Robert and Turpin 1993) and purified marennine solutions (Pouvreau et al. 2007). Moreover, the growth of *H. ostrearia* can itself be inhibited by marennine, at concentrations ranging *ca.* 30-50 mg L⁻¹, depending on the form used, either purified pigment (Pouvreau et al. 2007) or crude extract (Robert and Turpin 1993), respectively. Crude extracts thus correspond to marennine produced and excreted by *H. ostrearia*, and accumulated in the growth medium. In the present study, the allelopathic effect of *H. ostrearia* presumably caused by the release of marennine was investigated by co-culturing this species with other microalgae commonly used in aquaculture as bivalve feeds, thus by testing the allelopathic effect of *H. ostrearia* culture supernatants. Our *in vivo* experiments showed that for a same initial biovolume of *H. ostrearia* and under stable environmental conditions, without light or nutrient limitation (cells maintained in exponential growth at low cell concentration, using a semi-continuous mode of culture), the diatom *H. ostrearia* triggered a reduction in cell concentration of some target species, and that the magnitude of this impact was species-dependent. For instance, *C. calcitrans* and *S. costatum* were significantly inhibited by *H. ostrearia* and marennine. Their growth rate was negatively affected when cultured in presence of *H. ostrearia* cells and marennine concentration ranging from 0.7 to 1 mg L⁻¹, with percent inhibition (I%) of more than 70%. Thus, these two microalgae can be considered as highly susceptible species, a result in accordance with Pouvreau et al. (2007), who observed that *S. costatum* was inhibited by marennine concentration less than 2 mg L⁻¹ (purified pigment). Although less susceptible, the haptophyte *T. lutea* is also a species, which growth was slowed down in the presence of *H. ostrearia*. Interestingly, a lower relative growth was also observed for *H. ostrearia* when co-cultured with *T. lutea*, suggesting that there is an inhibition caused by a allelopathic substance released by *T. lutea*, as previously observed, for instance using *C. gracilis*, *C. muelleri*, and *Nitzschia closterium* (Sun et al. 2012).

In contrast, *P. tricornutum* and *T. suecica* were less affected, as confirmed by an increase in cell concentrations and total biovolume despite the presence of *H. ostrearia* and marennine. Among target species tested, *P. tricornutum* and *T. suecica* exhibited certain tolerance, displaying an increase of cell concentrations and total biovolume despite allelopathy exposure, although their cell numbers in the treatment were still lower than those in controls. Moreover, these two microalgae outcompete *H. ostrearia* not only in terms of cell concentration but also of the total biovolume. The present work, however, cannot allow concluding about the intrinsic insensitiveness of *P. tricornutum* and *T. suecica*, as experiments were not designed to study specifically their response to change in marennine concentration. Indeed, previous studies demonstrated that biotic factors such as changes in cell concentration or dilution rates, can modulate allelopathic effects between species (Sharp et al. 1979; Tillmann et al. 2007, 2008; Lyczkowski and Karp-Boss 2014).

Susceptibility or insensitiveness of the target species could be related to their cell biovolume, as the largest species *P. tricornutum* and *T. suecica* were less sensitive to marennine than the smallest ones (Fig. 5-4A). Additionally, our study revealed that the susceptibility of target species to allelopathy was also influenced by the cell surface area as it is negatively correlated to percent inhibition (I%) (Fig. 5-4B). Marennine allelopathic effect is linearly correlated to surface area-volume (SA/V) ratio as I% in target species increased with SA/V ratio (Fig. 5-4C), which could reflect interactions at the cell membrane level. Indeed, our results are in coherence with the previous work by Lyczkowski and Karp-Boss (2014) who observed the negative relationship between cell size and allelochemical impact of *Alexandrium fundyense* on *Thalassiosira* cf. *gravidia*. These authors suggested that the toxicity of allelochemicals was biovolume- or biosurface-dependent, meaning that large cells would need to absorb a greater amount of allelochemical compound to be as inhibited as smaller species, which could present higher flux of allelochemicals inside the cells. In other words, small cells would tend to accumulate more allelochemicals or toxins per unit volume due to a higher flux rate (Ribalet et al. 2007). Thus, these mechanisms could explain why, in our study, microalgae that have a smaller cell biovolume were significantly more vulnerable to marennine

although the initial total biovolume for both microalgae species at the beginning of their growth kinetics in co-culture were the same.

The alleopathic effect could also be influenced by the biomass of target species, which is also related to their growth rate. In our study, we observed that the percent inhibition I% due to allelochemical released was negatively correlated with the growth rate of target species ($r = -0.890$). This result is in coherence with previous works, where the growth rate of target species had significant role in susceptibility to allelochemical. For instance, Tillmann (2003) observed that the lytic activity of *Prymnesium parvum* decreased when increasing the cell concentration and growth rate of target organisms.

If a lower susceptibility to alleopathic compounds in large cells could be related to a lower surface-volume ratio, in some species, resistance against allelochemical compounds could also be related to the structure and properties of cell wall, which could represent an impermeable barrier. For instance, the resistance of *P. tricornutum* to allelochemicals has been reported where this microalga can outcompete *Thalassiosira pseudonana* (Sharp et al. 1979). Additionally, Vasconcelos and Leal (2008) also demonstrated that the alleopathic exudates could promote the growth of *P. tricornutum*. Ribalet et al. (2007) hypothesize that membrane characteristics and cell wall properties may have a role in cell vulnerability to allelochemical compounds. This would be a possible explanation why among large cells, *P. tricornutum* was less sensitive to marennine than *T. lutea* since the diatom has a more highly structured cell wall as compared to the haptophyte *T. lutea* (Johansen 1991; Bartual et al. 2008; Tesson et al. 2009).

5.4.2 Marennine alleopathic effect on vulnerable species is function of irradiance

This series of experiments was based on the hypothesis that *H. ostrearia* cultured at different irradiances could generate different alleopathic effects, depending on marennine production and accumulation at cell apices (intracellular marennine, IMn), and excretion in the growth medium (EMn). Our study showed that *H. ostrearia* growth rate increased with irradiance, a result in concordance with a previous work by Mouget et al. (1999), where *H.*

ostrearia specific growth rate was high and almost constant from 100 to more than 750 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. These authors also observed that at high irradiance, *H. ostrearia* cells displayed a reduction in size of the chloroplasts and an increase of marennine accumulation at the apices (IMn), a result confirmed by Rech et al. (2008), which showed that IMn increased with irradiance. In the present study, we observed that *H. ostrearia* produces and releases more marennine (EMn) at high irradiances (100 and 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), as compared to low irradiance (20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Furthermore, a good relation was observed between growth irradiance and EMn production per day and EMn per cell, which might influence allelopathic susceptibility of the target species. Indeed, this demonstrated that change in irradiance level can modulate marennine concentration in the medium, and significantly influence *H. ostrearia* allelopathic pressure on sensitive co-cultured microalgae.

Marennine allelopathic effect varied with irradiance and thus with concentration, *S. costatum* co-cultured with *H. ostrearia* being significantly more inhibited at high irradiance, as compared to co-culture at low irradiance. Thus, light plays a significant role in allelochemical production by *H. ostrearia*, which consequently inhibited the growth of *S. costatum* despite non-limiting nutrient and light conditions. This result illustrates that light is possibly an important abiotic factor on allelopathy, not only because of possible photo-oxidation and degradation of allelochemicals, as hypothesized by Granéli and Hansen (2006), but also because of its direct influence on photosynthesis (*e.g.*, Figueredo et al. 2007), growth and relative cell concentrations of emitter and target species, and the production of allelochemical compound (this study).

5.4.3 Chemical warfare between algae and ecological importance of marennine

When greening occurs in oyster ponds, *H. ostrearia* blooms and produces huge amount of marennine, a phenomenon that is accompanied by the decrease in abundance of other microalgal species (Robert 1983; Turpin 1999). Turpin (1999) observed that concentration of marennine in oyster ponds during a bloom of *H. ostrearia* might reach 5

mg L⁻¹. This concentration is considerably high to inhibit the growth of *S. costatum* and other sensitive species (Pouvreau et al. 2007; this study), thus participating in the decline of their abundance (Robert 1983; Turpin 1999). Consequently, marennine released in oyster ponds is likely able to participate in the dominance of *H. ostrearia*, thus changing the community structure of microalgae through allelochemical mediation in such closed ecosystems.

Greening of marine invertebrates possibly due to blooms of *H. ostrearia* does not occur in oyster ponds in West of France only. It has been observed spontaneously elsewhere, in Great Britain (Sprat 1667), Denmark (Petersen 1916), United States (Mitchell and Barney 1918), Canada (Medcof 1945), and Australia (Gastineau et al. 2014). Moreover, recent findings revealed that other species of pennate diatoms from the genus *Haslea* that produce a blue pigment have been reported from different places in the world, for instance *H. karadagensis* in the Black Sea (Gastineau et al. 2012a), *H. provincialis* from the Mediterranean Sea (Gastineau et al. 2015), *H. silbo* sp. inedit from the Canary Islands, (Gastineau et al. 2014). Thus different species of blue *Haslea* produce “marennine-like” pigments, which can interact with bivalves (Gastineau et al. 2012b) in the same way as purified marennine or *Haslea* culture supernatants, and the resulting greening of marine invertebrates elsewhere in the world could probably be linked to as many allelopathic phenomena and competition between phytoplankton.

5.4.4 Co-culture of *Haslea ostrearia* with aquaculture relevant microalgae: why and how?

In aquaculture, massive use of conventional antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the microbial communities and favors the natural emergence of bacterial resistance (Alderman and Hastings 1998; Van den Bogaard and Stobberingh 2000; Cabello 2006). For biohazard control and health management consideration, the utilization of *H. ostrearia* as oyster feed could be advantageous since marennine produced during algal growth could act as natural

antibiotics or bacteriostatic agent. Indeed, confirming previous studies on the biological properties observed using *H. ostrearia* supernatants (e.g., Carbonnelle et al. 1999; Bergé et al. 1999), Gastineau et al. (2012b, 2012c, 2014) showed that purified marennine inhibited the growth of bacterial pathogens such as *V. aestuarianus* and *V. splendidus*. For larviculture, considering that the cell size of *H. ostrearia* is overly large for larvae, supernatant containing extracellular marennine could be added to a diet consisting in resilient species of microalgae, in order to benefit from marennine protecting properties. In contrast, for adult stages *H. ostrearia* could be provided, either as the sole source of feed, or alongside robust species for an optimization of the feeding. Hence, aside being a “greening agent,” *H. ostrearia* could act as a nutraceutical, a feed with several benefits regarding animal health.

As a feed source, *H. ostrearia* has been used as a mono-specific diet to sustain the growth of *C. gigas* for weeks (Piveteau 1999; Cognie 2001), with almost 90% of digestibility (Barillé et al. 1994). Although the specific nutritional value of *H. ostrearia* for aquaculture is not yet documented like other diatoms, it has been shown that this diatom is a good source of eicosapentaenoic acid (EPA, 20:5n-3), with a relative proportion of total fatty acids of 12.2% (Dunstan et al. 1994), and 14.5% (Groth-Nard 1994). These authors showed that other main fatty acids in *H. ostrearia* are 16:0 (20.4 and 21.3%, respectively), 16:1 (28.6 and 29.9), and 16:3 (10.6 and 15.1%). The diatom *H. ostrearia* could thus be used as an unique source of feeding for animals, depending on their growth phase. However, several studies conducted over the last decades on the alternative feeding of oysters, especially on their larval stage in which they are considerably vulnerable to pathogens, emphasized the idea that a mixed diet composed of several microalgal species gave better results than a monospecific diet (Guedes and Malcata 2012; Becker 2004). An explanation is that microalgae vary significantly in their nutritional value (Enright et al. 1986; Brown et al. 1997). For instance, the haptophyte *T. lutea* is a rich source of docosahexaenoic acid (DHA, 22:6n-3), which represents 8-10% of total fatty acids, while diatoms are a good source of eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6) (Volkman et al. 1989; Dunstan et al. 1994). However, utilization of *T. lutea* as monospecific diet seems not sufficient to optimize growth and survival, particularly for

bivalves' larvae (Da Costa et al. 2015; Marshall et al. 2010; Pernet and Tremblay 2004; Pernet et al. 2005). In our study, the allelopathic tests showed that *T. suecica* was one of the most tolerant species to marennine. However, we demonstrated also that diatoms rich in EPA and AA are susceptible to marennine and others sources of these essential fatty acids will need to be identified. One possibility could be a mixed diet composed of *H. ostrearia* and *T. suecica*, but such suggestion needs to be validated by nutritional study.

5.5 Conclusion

The present study shows that *H. ostrearia* can influence growth of microalgae species relevant for aquaculture possibly through allelopathic interactions in co-culture system. The magnitude of this allelopathic-like effect is species-dependent, *S. costatum*, *C. calcitrans* and *T. lutea* revealed as vulnerable species, whereas *P. tricornutum* and *T. suecica* were more resistant. The study also distinctly showed that the supernatants produced in realistic conditions of co-cultures of *H. ostrearia* with another species acted as an allelochemical mixture with marennine concentrations lower than *ca.* 1 mg L⁻¹. These values are consistent with those observed in oyster ponds where oysters are fattened and greened, which could explain the domination of *H. ostrearia* in these ponds. Regarding a putative exploitation in aquaculture of marennine antibacterial properties, a mixture of *H. ostrearia* and insensitive species such as *P. tricornutum* and *T. suecica* is recommended to circumvent allelopathic interactions between phytoplankton used as feed for animals.

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6 GENERAL CONCLUSION

Among global aquaculture production, molluscs are the second fastest growing sector with approximately 11.5% of world aquaculture's total revenue in 2012 (FAO 2014). However, this widespread growth of aquaculture co-occurred with the intensive use of a wide range of chemicals like antimicrobial drugs (Cabello 2006b). The use of antimicrobial drugs (AMDs) has been considered a common strategy to avoid disease outbreak, which later may cause economic losses. Despite the fact that efficiency of AMDs in eliminating pathogens has been evidenced (Defoirdt et al. 2007), their utilization has been restricted because of their potential side-effects on the health of fish, terrestrial animals, human beings and aquatic environments in general (Alderman and Hastings 1998; Miranda and Zemelman 2002; Cabello 2006a; Maas et al. 2007; Cabello et al. 2013). Additionally, in the new era of sustainable aquaculture, the usage of chemical product either in food product or in antibiotics should be limited or even be avoided. Thus, use of probiotics and natural antibiotics has been considered as a prospective solution in supporting sustainable aquaculture practices (Lee et al. 2009; Citarasu 2010; Sasidharan et al. 2010; Gastineau et al. 2012b, c; Fleurence et al. 2012; Mohapatra et al. 2013; De et al. 2014; García-Bueno et al. 2014; Reverter et al. 2014).

The interest of using marine organisms as potential natural prophylactics in aquaculture has increased during the past few years. Microalgae have been considered as prospective resources due to their bioactive compounds, which can inhibit pathogens in fish and shellfish aquaculture (Desbois et al. 2008, 2009; Vo et al. 2011; Molina-Cárdenas et al. 2014). Among microalgae, *H. ostrearia* is a pennate diatom able to synthesize and excrete water-soluble blue pigment, marennine, which could potentially be used in aquaculture as a natural antipathogen (Gastineau et al. 2012c, 2014c). *In vitro* study revealed that both the intracellular- (IMn) and extracellular-form (EMn) of marennine are able to inhibit the growth of *V. aestuarianus*, *V. splendidus* and the proliferation of herpesvirus OsHV-1 (Gastineau et al. 2012c, 2014d), the oyster pathogen causing the major casualties in France and worldwide. Another advantage of this type of microalga is the biodiversity and spreading of *H. ostrearia*-like diatoms. Indeed, recent studies have

revealed that several species from the genus *Haslea* produce ‘marennine-like’ pigment and that they exist worldwide (Fig. I-4). Preliminary studies unveiled the similar biological activities displayed by the pigment of one of these species (Gastineau et al. 2012b). Therefore, studies on the blue diatoms from the genus *Haslea* are likely to be developed to obtain the maximum benefit for local shellfish industry specifically and to aquaculture in general. Nevertheless, several investigations need to be conducted prior to the development of the blue *Haslea* species and marennine-like pigments in shellfish culture.

In the second chapter, it has been clearly demonstrated that the size of *H. ostrearia*, regardless of its biochemical properties, plays a crucial role in the process of pre-ingestive selection. Indeed, *in vitro* and *in vivo* sampling revealed that *H. ostrearia* cells with the largest sizes were preferentially rejected as pseudofaeces due to their impossibility to enter the principal filaments. As a consequence, the preferential rejection of large-size cells could change the structure of microalgal populations in the oyster ponds. Rejected large-size cells in pseudofaeces could also favour the development and/or the maintenance of their sub-populations, specifically with their revival capacity after the preingestive process (Barillé and Cognie 2000). Furthermore, this study also revealed that the oyster preferentially ingests sexually mature cells over the immature ones, which could modify *H. ostrearia* cell size distribution and population dynamics in the oyster ponds. Indeed, this selective feeding process showed that only small-size and mature cells might be processed to the next feeding stage. Therefore, the importance of this predation pressure on the life cycle of *H. ostrearia* in oyster ponds remains to be assessed, especially considering the importance of marennine in the greening process, but also regarding its many biological activities (*e.g.*, antibacterial) that could be exploited in aquaculture.

To better understand the greening process in oyster, in chapter 3, it has been demonstrated that the greening of oysters occurred through pigment fixation, which results from the solubility of marennine in the medium. The EMn released from *H. ostrearia*, regardless its purity, can be fixed in oyster’s gills either by active (through mechanism of filtration) or passive (direct contact with oyster gill) mechanisms. Experiments confirmed the previous study by Pouvreau (2006) that the EMn is the main form of the pigment contributing to the greening process. Histological observation showed that the EMn

appeared to be fixed on the mucocytes of the gills and this fixation persisted for months. Possible ecophysiological impacts of EMn fixation on the gills of juvenile oysters have also been assessed. It has been showed that after short-term exposure, EMn fixation on the gills affects oyster feeding behavior, such as the clearance rate (CR). However, the CR of *C. gigas* recovered at a similar state with the control in less that 7 days after exposure to marennine, suggesting that the oysters are able to depurate or compensate for the adverse effect of marennine. Regarding toxicity of a compound, depuration mechanism has already been evidenced in bivalves and this process appeared to be species-specific and depending on the type of biotoxin or chemical substance as well as time of depuration (Smith et al. 2001; Boisson et al. 2003; Corrêa et al. 2007, 2012; Love et al. 2010; Navarro and Contreras 2010; Luna-Acosta et al. 2011; Phuvasate et al. 2012).

As stated in a previous chapter, in order to obtain the optimum benefit from marennine pigment, a holistic approach needs to be conducted. Following the preliminary results obtained on *C. gigas*, similar investigations were conducted on other bivalve species. Therefore, the aim of the study in chapter 4 is to determine the consequences of the greening on fonctionnal traits of other bivalve species than oyster *C. gigas*. Thus, in chapter 4 we demonstrated for the very first time the impact of purified EMn on juvenile *M. edulis* and *C. virginica*'s behavioral, physiological and biochemical characteristics. EMn fixed on the gills affected valve activity, with a stronger impact on oysters compared to mussels. After long-term exposure, SFG of both animals treated with EMn was significantly lower comparatively to control. Deterioration of SFG was mainly caused by the lower CR in EMn-exposed oysters and mussels. In addition, biochemical analysis revealed that EMn might increase unsaturation index on the gills membrane by changing PUFA concentration and FAs' composition, but only in oysters. For mussels, a decrease in total FAs in lipid neutral fraction as an energetic reserve indicator was observed after EMn fixation. These results suggest that the persistency of EMn fixation on bivalve's gills could also interfere with their biological performances to different extents. Moreover, these results will also improve our understanding about the impact of marennine and the greening consequences on the physiology of juvenile bivalves, and could possibly be extended to other bivalve species at different stages. Additionally, it should be kept in mind

that the concentration with the most significant effect on the parameters was 2 mg L⁻¹ and that at lower concentrations, effects are unlikely to be observed. Thus, EMn with approximately 1 mg L⁻¹ or lower concentrations could be used to take the benefit from the antibacterial properties without any detrimental effect on oyster or mussel performance. Indeed, EMn at this concentration has been shown to inhibit the growth of *V. aestuarianus* (Gastineau et al. 2012c).

Finally, to have an insight on the suitability of *H. ostrearia* and marennine in aquaculture despite some formerly described allelopathic properties on other micro algae (Pouvreau et al. 2007), experiments of co-cultivation were conducted. In the last chapter, studies were designed to identify which algal species known for their relevance in aquaculture were either sensitive or resistant to *H. ostrearia* and its supernatant containing marennine. Here, it has been demonstrated that *H. ostrearia* influences the growth of other species through allelopathic interactions in co-culture system. The magnitude of this allelopathic effect is species-specific, *S. costatum*, *C. calcitrans* and *T. lutea* revealed to be vulnerable species, whereas *P. tricornutum* and *T. suecica* were more resistant. Additionally, it has also been showed that supernatants containing marennine could act as an allelochemical mixture, possibly explaining the domination of *H. ostrearia* in oyster ponds. A suitable diet to be proposed to aquaculturists could therefore be a mixture of *H. ostrearia* and the insensitive species mentioned above, in order to benefit both from the antipathogenic effects of marennine and the nutritional properties of the other species.

A holistic point of view of all these works is provided in Figure 6-1. In regards to the results obtained during this thesis, several considerations could be made on the suitability of *H. ostrearia* and marennine in supporting bivalve's culture. However, it shall be kept in mind that the cell sizes considerably impact the digestibility of *H. ostrearia* by oyster specifically or by other species of bivalves. Therefore, a further study in which stage of development of bivalve, *H. ostrearia* and marennine will be used should be conducted. Additionally, even though at a certain concentration (for example 2 mg L⁻¹) EMn affects behavioural, physiological and biochemical performance, utilization of this pigment as a natural antibacterial agent seems possible to achieve bivalve sustainable culture, thus avoiding conventional methods for controlling microbial pathogens by use of disinfectants

and antimicrobial drugs, which have led to antibiotic-resistant bacterial strains and global health problems.

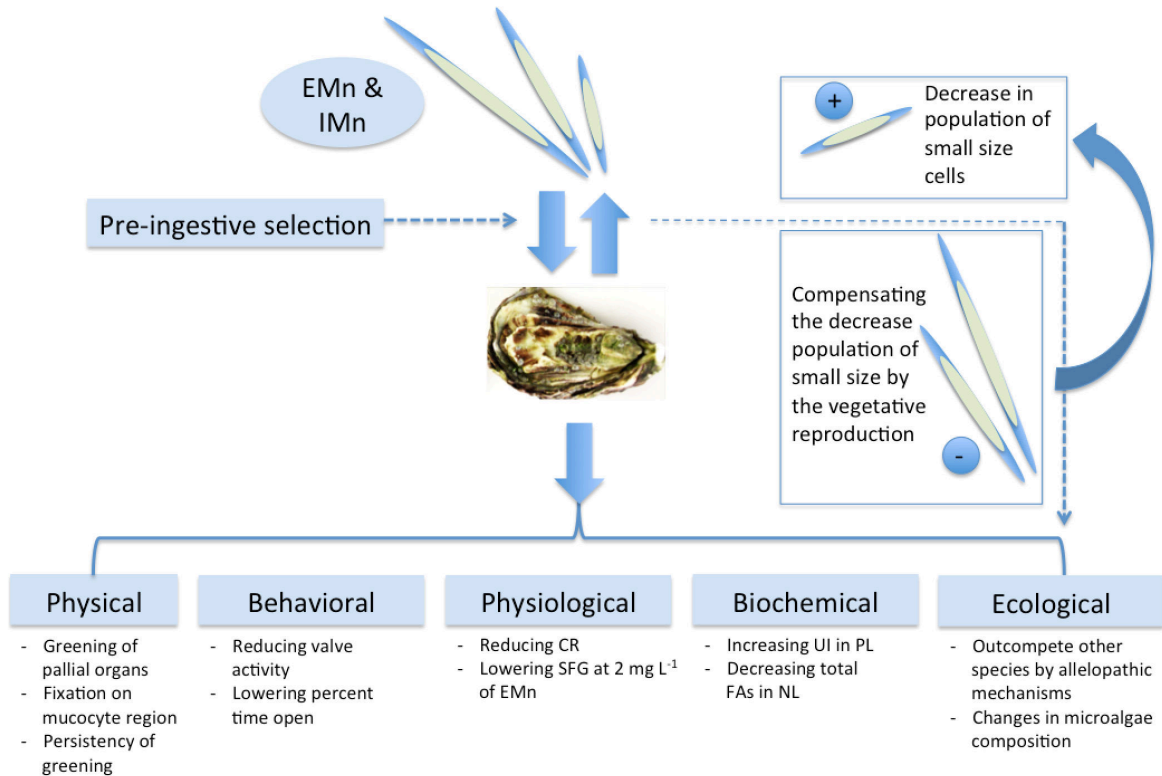


Figure 6-1. Scheme of holistic approach in utilization of *H. ostrearia* and marennine in bivalve's diets and their consequences on different aspect of studies. +) preferential selection on smaller *Haslea ostrearia* cell size; -) preferential rejection on larger size of *H. ostrearia*.

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Review

Marennine-like pigments: once in a blue moon diatoms

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Abstract: In diatoms, the main photosynthetic pigments are chlorophylls a and c, fucoxanthin, diadinoxanthin and diatoxanthin. The marine pennate diatom *Haslea ostrearia* has long been known for producing, in addition to these generic pigments, a water-soluble blue pigment, marennine. This pigment is responsible for the greening of oysters in western France, and presents different biological activities: allelopathic, antioxidant, antibacterial, antiviral, antiproliferative. A method to extract and purify marennine has been developed, but its chemical structure could hitherto not be resolved. For decades, *H. ostrearia* was the only organism known to produce marennine, and was considered worldwide in distribution. Our knowledge about *H. ostrearia*-like diatom biodiversity recently increased with the discovery of several new species of blue diatoms, the recently described *H. karadagensis*, and *H. silbo* sp. inedit. and *H. provincialis* sp. inedit., to be characterized soon. These blue diatoms produce different marennine-like pigments, which belong to the same

chemical family and present similar biological activities. Aside from being a potential source of natural blue pigments, *H. ostrearia*-like diatoms thus present a added-value potential that deserves to be exploited.

Keywords: aquaculture; biological activities; cosmetics and food industry; *Haslea ostrearia*-like diatoms; marennine-like blue pigments

1. Introduction

Seas and oceans cover more than 70% of the surface of the Earth, water mainly transmits and scatters blue wavelengths, and absorbs in the red part of the solar light spectrum, which makes astronauts say our planet is blue. *In* or *under* the sea, however, the blue color is scarcely distributed, as it can be observed only in a few organisms like the blue jellyfish *Cyanea lamarckii*, the blue coral *Heliopora coerulea*, the blue sea star *Linckia laevigata*, the giant clam *Tridacna maxima*, the mantle of which is commonly bright blue, and some species of surgeonfish from the genus *Acanthurus*, all being sorts of emblematic species. Micro-organisms appear better providers of blue color, as blue pigments have been evidenced long ago among bacteria, but first in terrestrial species. For instance in Proteobacteria, *Pseudomonas aeruginosa* produces pyocyanin [1, 2], a blue pigment that possesses antibiotic activities [3], and *Pantoea agglomerans* has recently been shown to produce a novel 'deep blue' pigment [4]. The study of marine bacteria started and expanded later, but a few species are known today to synthesize blue pigments [5]. For example, glaukothalin is produced by different species from the genus *Rheinheimera* [6, 7], and indigoidine is encountered in a strain of the marine bacteria genus *Phaeobacter*, in which it plays a role in the colonization of surfaces [8]. It is worth noting that indigoidine is also encountered in the terrestrial plant pathogen *Erwinia chrysanthemi* [9], in which this blue pigment seems to be partly responsible for its pathogenicity [10].

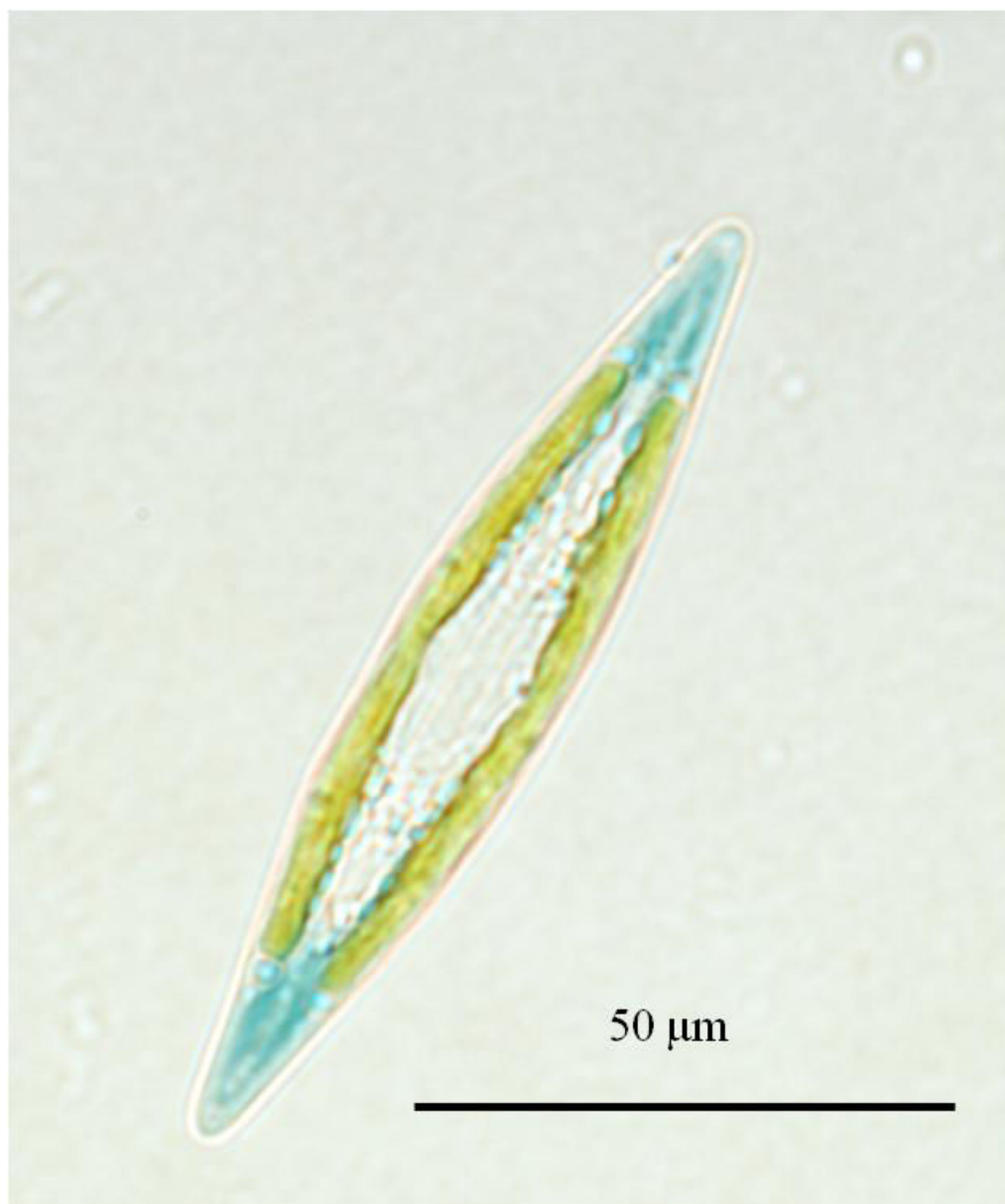
Regarding photosynthetic organisms, blue pigments can be observed in prokaryote as well as in eukaryote species. Formerly known as 'blue algae', cyanobacteria have specific accessory protein-pigment complexes, the phycobiliproteins, some of them, phycocyanin and allophycocyanin having a blue color, because they absorb orange and/or red light [11]. Phycobiliproteins were discovered in the 19th century, phycocyanin being first described in a strain of *Oscillaria* sp. [12], allophycocyanin in the red macroalga *Porphyra vulgaris* [13]. The partial protein nature of these two molecules was suggested by Mölisch [14], in the line of his work on phycoerythrine [15]. Kylin [16] further demonstrated that each

molecule was a complex of a chromophore [phycobilin] covalently bound to a protein. Phycocyanin and allophycocyanin are not restricted to cyanobacteria, as they have been evidenced in two groups of photosynthetic eukaryotes, Rhodophyta and Glaucophyta. Members of another group, the Cryptophyta, contain only one of these two blue pigments, phycocyanin [e.g., 17]. Until very recently, only two other photosynthetic eukaryotes, both members of the Heterokontophyta, were known to produce blue pigments, the recently discovered *Aurearena cruciata* (Aurearenophyceae) in its senescent stage [18], and the long-known pennate diatom *Haslea ostrearia* (Bacillariophyceae), during its exponential phase of growth and ageing [19]. *H. ostrearia*, the ‘blue navicula’, produces the specific pigment marennine, responsible for the ‘greening’ of oysters.

An explanation why the color blue is relatively seldom in organisms and organic molecules could be related to the biophysical processes required to absorb light, red or yellow wavelengths carrying less energy than blue ones. If the color is not stemming from metal centers, it is usually the excitation of delocalized electrons from their molecular orbital ground state π to the excited state π^* which makes a molecule appear colored. One double bond alone is usually not sufficient as it absorbs light in the UV range. Only the conjugation of several double bonds broadens the energy range of the different π orbitals, reducing the energy gap between the highest π and the lowest π^* orbital. More than ten conjugated double bonds are necessary to reduce the energy gap to absorb red light [20], so that the molecule appears blue, and this is a rather seldom constellation. Similar considerations count for aromatic rings, where usually adjacent groups can reduce the absorption energy, but hardly into the red region.

In this review, we focus on the diatom *H. ostrearia*, its blue pigment marennine, and their many interactions with oysters. We present a historical perspective of our knowledge of marennine complemented by some new insights, regarding its chemical nature, characterization, and biological properties. We also present data about *H. karadagensis*, the second species of blue diatoms recently discovered in the Black Sea, and *H. provincialis* sp. inedit. and *H. silbo* sp. inedit., two newly blue diatom species discovered in the Mediterranean Sea and the Canary Islands, respectively [21]. At least one of the blue pigments produced by these new species (*H. karadagensis*) is different from marennine but all belong to the same chemical family. Finally, we address the unexpected biodiversity of blue diatoms, and the added-value potential of marennine-like pigments, in aquaculture, cosmetics, food and health industry.

Figure 1. *Haslea ostrearia* in optical microscopy.



2. The marennine-oyster connection: trademark of *Haslea ostrearia*

In the following, we will refer to blue pigments in diatoms as ‘marennine’ when produced by *H. ostrearia*, and as ‘marennine-like pigments’, when produced by another species of blue diatoms (Figure 1). Indeed, no other name can be provided in accordance with the IUPAC standards, as the chemical structures of these pigments are unresolved yet.

Marennine is the gallicized version of ‘marennin’, proposed by Lankester [22], in reference to Marennes-Oléron, a region of Western France where oysters have been farmed for centuries, especially renowned for producing ‘green oysters’. In the Marennes-Oléron Bay, as well as in Bourgneuf Bay, oysters are matured and fattened in old salt marshes, the so-called ‘claires’. In these shallow and nutrient-rich ponds, phytoplankton proliferates and may feed oysters whose size rapidly increases and organoleptic properties improve. The duration of the fattening period and the density of the oysters in the ponds are regulated according to specific standards for the bivalves to be commercialized as ‘fines de claires’. In these ponds, *H. ostrearia* may become dominant year after year, producing large amounts of marennine, which are released to the seawater. It adheres to oyster gills, a phenomenon that increases palatability and therefore market value of the bivalves (red label ‘fines de claires vertes’).

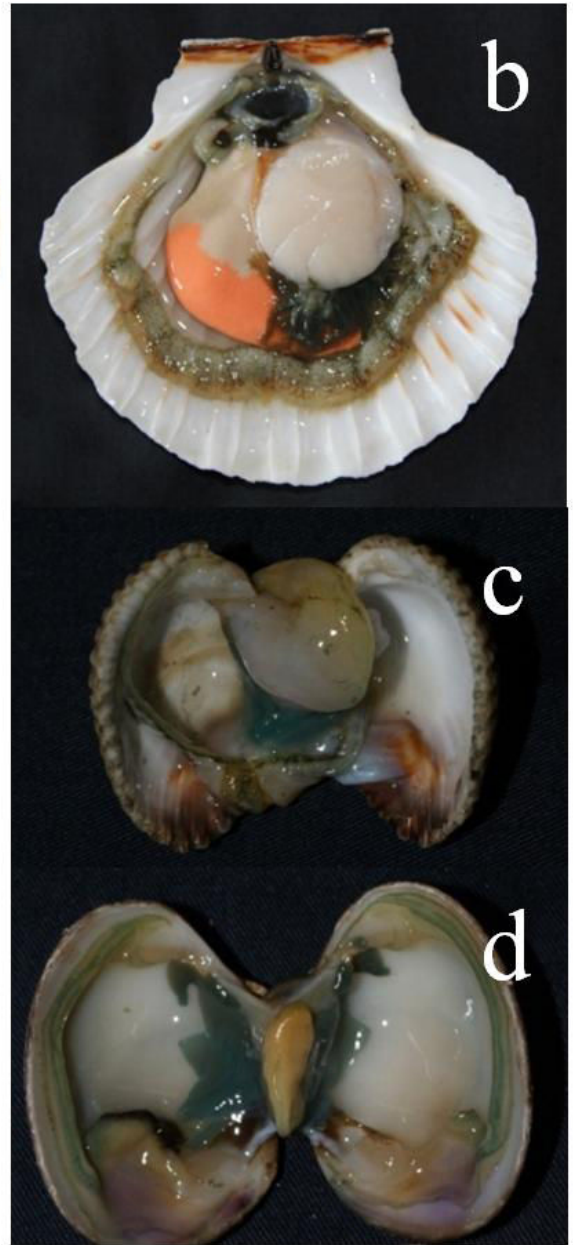
2.1. The greening of oysters, signature of the diatom Haslea ostrearia

As a biological model, green oysters provoked questioning and wondering for centuries. The earliest notification known is ‘The history of the generation and ordering green oysters, commonly called Colchester-oysters’, by Thomas Sprat, by the end of the 17th century [23]. Thomas Sprat was an Anglican bishop of Rochester, famous for his ‘History of the Royal Society of London’, in which he included this article on the green oysters of Colchester. He mentioned that oysters from the Tolesbury ponds could present a green color during summer, a phenomenon he suspected resulting from the actions of sun and earth, leading to a green coloration of pond bottom, before oyster gills turn green. The same environmental causal factors, along with the brackishness of waters, were addressed 150 years later [24]. Other hypotheses, not referring to environmental issues, ascribed the greening to a disease similar to obesity [25], or to ‘liver malfunction’ [26]. The role of grass, plants and mosses grown on the shores of the ponds and possibly ingested by oysters or some kind of Priestley's green matter was also hypothesized [27-29]. In line with these speculations, some authors suspected that the water of ponds, and consequently oysters, could be colored by pigments originating from green macroalgae [30]. Finally, some authors estimated that greening could result from the presence of specific metallic ions in pond sediments [31], or from unusual interactions with copper [32, 33], a metal long-known for inducing changes in color in oysters [34]. Indeed, such unusual color may represent a real turn-off, possibly reflecting the presence of pollutants as zinc or copper

[35-37]. In France, however, green oysters are gastronomically famous and more expensive than ordinary oysters. Moreover, from a historical perspective, green oysters have always been culinarily celebrated in this country [38-40], even being considered a dish fit for a king, at the very least one of the Sun King, Louis XIV's favorite meals [41].

The first experimental work on green oysters was reported by Benjamin Gaillon [42], an officer of French Customs during the Restauration and the early July Monarchy. Gaillon worked in the town of Dieppe [Normandy] and dedicated his free-time to life sciences, with a special interest in green oysters, common at that time in Dieppe area. Gaillon sampled green oysters, scrapped their shells and made microscopic examinations. He observed small motile organisms with 'green' color, not blue like *H. ostrearia* cells, possibly because of the limits of his microscope device. Gaillon hypothesized these motile organisms could be responsible for the greening of oysters, and considered they were animals, which he called *Vibrio ostrearius*, based on the classification of worms by Bruguière [43]. Controversially but not impartially, Goubeau de la Bilennerie [24] argued that the greening exclusively depended on environmental factors and oyster farmers' know-how, a phenomenon thus appearing more acceptable for oyster consumers. Indeed, a possible impact of Gaillon's theory on consumers and green oyster sales was of some concern for Goubeau de la Bilennerie, president of the court in Marennes [for a full study, see 44].

Figure 2. Greening effect of marennine on bivalves. [a] Pacific oyster; [b] Scallop; [c] Cockle; [d] Clam.



2.2. *Haslea ostrearia*, a very singular diatom

The topic of green oysters involved Gaillon in a second controversy, with Jean-Baptiste Bory de Saint-Vincent, botanist, explorer, and Dragons cavalry officer during the Napoleonic wars. Whereas Gaillon advocated for an animal nature of *V. ostrearius*, Bory de Saint Vincent classified it alongside with diatoms into his 'psychodiaire' reign, which contained all organisms whose position between animal and plant was unclear. Bory [45]

thus proposed the name *Navicula ostrearia*, which remained for 150 years. Taking profit from scanning electron microscopy facilities, Simonsen [46] transferred the 'blue navicula' from the genus *Navicula* to *Haslea*, a new genus he created for this purpose, based on specific morphological features of the frustule, and he used *H. ostrearia* as the type-species. By doing so, Simonsen acknowledged G.R. Hasle for her considerable work on phytoplankton in general, and on diatoms in particular.

Haslea ostrearia is a tychopelagic diatom [47] - an organism that can be benthic or epiphyte - but also planktonic [48]. *H. ostrearia* is euryhalin [49, 50], and can develop in high light environments [51]. Thus this diatom seems well adapted to oyster ponds, characterized by shallow and nutrient-rich water, where it mainly proliferates in Autumn and Spring, and can outcompete other microalgae [47, 52]. Marennine produced during *H. ostrearia* blooms is released into the seawater, and the ponds turn green. In such ponds, oysters can become green in a few days, by exhibiting light to dark green gills (Figure 2a). This phenomenon is not restricted to oyster ponds in Western France, as it can happen spontaneously elsewhere, in Great-Britain [23], Denmark [53], the United States [36, 54], Canada, Australia, etc. It is worth noting that naturally, the greening happens in peculiar environments, usually protected bays, with quiet waters and fresh water inlets. The greening is not limited to oysters, as it also occurs in other invertebrates, polychaetes, crabs, littorina, mussels [55], sea anemones [42], scallops, clams and cockles (Figure 2b-d). If greening invertebrates is not the blue diatoms' *raison d'être*, it is at least their signature.

3. Historical perspective on marennine studies

3.1 First hypotheses regarding the chemical nature of marennine

For more than hundred years, the presence of such intriguing and unusual blue pigment inside unicellular algae has been a fruitful matter for contradictive theories and inconclusive experimentation. The hypothesis of a metallic salt was early proposed and rapidly rejected [22, 26, 35, 36, 56]. Then there was the 'pigment connection', with hypotheses successively relating marennine to carotenoids [57], or to chlorophylls [32, 47, 55, 57, 59, 60, 61], possibly resulting from their degradation. Other authors linked marennine with cyanobacterial pigments, thus suggesting a protein nature for this water soluble molecule [54, 62, 63] or proposed it could be an anthocyanin, with respect to stress and pigment accumulation in cells [64]. Finally, recent advances related marennine to a mixture of different macromolecules [65], or to a polymer possibly of polyphenolic nature [66]. It has been shown that marennine exists in two slightly different forms, intracellular

and extracellular [67], and that for both forms the color changes with pH, from blue (acidic pH) to green (basic pH) [66].

3.2 Biosynthesis of marennine: when, where, why, and how it happens

Although the work of Pouvreau *et al.* [66] represents the most achieved investigation on marennine characterization so far, the exact structure of marennine is still unknown, and the description of its biosynthesis pathway a far-off goal. It is also unclear when marennine synthesis is triggered in the cell. There is a consensus on the stimulating effect of high light conditions and long photoperiods [49, 68-70] with just a few authors advocating the opposite theory [71]. An impact of the light quality, with a positive effect of blue wavelength, has been evidenced [19, 68, 70]. Some authors suggested that marennine production could be influenced by organic substances like glucosamines [57, 71], or result from salt-induced stress [32, 49, 58, 59], or nutrient deficiencies, *e.g.*, iron, vitamins [47, 49]. In the line of the ‘nutrient deficiency hypothesis’, it has also been suggested that marennine synthesis could result from, or be enhanced by the senescence of cells [72]. It is worth noting that this could be the result of an apparent and transient accumulation inside the cells, due to a reduced division rate, rather than an increase in production yield. Furthermore, it has been shown that *H. ostrearia* cultures in exponential growth phase release marennine continuously in the medium [19], but the mechanisms responsible for the release of marennine out of the cell, and the factors that control this release are not determined yet. However, according to Nassiri *et al.* [73], marennine is accumulated in vesicles at the apex of the cells and excreted by exocytosis. A sort of secretory vesicles containing marennine (a few μm in diameter) can be observed in contact with cells in active growth in Petri dishes [unpublished]. These vesicles attach to the cells during their erratic movements, then they detach from the cells, further float for a few minutes, before they finally collapse and release marennine in the medium.

There is no consensus on a possible relation between marennine production and photosynthetic pigment content, some authors having observed a negative correlation [32, 49, 60, 61, 71], others no correlation [19, 47, 74, 75]. A striking feature associated with marennine production and accumulation in the cell apices is the reduction in size of chloroplasts [32, 49, 55, 71, 76- 81], possibly related with a decrease in photosynthetic activity [74]. However, the plastid size change could also reflect the decrease of the cellular content of major photosynthetic pigments (chlorophylls *a* and *c*, fucoxanthin), when growth irradiance increases [51], a common photoacclimation response in phytoplankton. Moreover, the impact of this size modification on the organisation and stability of thylakoids remains uncertain, as observations were contradictory [61, 73].

Regarding the autecology of *H. ostrearia* in oyster ponds, a few authors hypothesized a correlation between the accumulation of marennine in the cells and a change in algal behaviour, switching from the planktonic to the benthic stage, together with changes in its metabolism [47, 57, 71], blue cells of *H. ostrearia* becoming able to assimilate reduced forms of nitrogen and carbon [52]. Moreover, the many biological activities displayed by marennine and marennine-like pigments [see below] could explain why these specific pigments represent a competitive advantage for the blue diatoms.

3.3 First attempts regarding production, extraction, and purification processes

A series of engineering- and process-oriented works were conducted at the end of the 90's dealing with bioprocessing issues of *H. ostrearia* production and marennine release and extraction with photobioreactors and membrane separations [82, 83]. Vandanjon *et al.* [84] proposed a method to produce a large amount of extracellular marennine (EMn) by simultaneous concentration and desalting of the pigment released into the culture medium (Blue Water) using a 3 kDa cut-off ultrafiltration membrane. The resulting permeate contained EMn and a mixture of several components in a large range of molecular weights, as only a 3 kDa cut-off membrane was used. Thus this experimental approach did not allow obtaining very pure pigment. On the other hand, the aim of this work was mainly to concentrate a large amount of marennine for developing potential industrial applications, which did not require absolutely pure product.

Then, in order to increase the recovery of the intracellular marennine (IMn), a continuous-flow-high-pressure disrupter was evaluated [85]. Cells were partly broken from 30 MPa, but a pressure of 100 MPa (1 cycle) was required to obtain optimal pigment release. The latter was directly linked to the physical cell breakage dependent upon the applied pressure and the number of disintegration cycles. Granulometric analyses by laser diffraction (0.04–2000 μm) revealed a size reduction of cell fragments when increasing these two operating parameters.

In view of optimizing both production rates in photobioreactors and extraction yields of marennine, Vandanjon *et al.* [84] studied the effects of shear stress on *H. ostrearia* cells due to circulation in pumps and valves of the production or harvesting systems. For the pumps, it was shown that shear stress was dependent on the type of pump, but that mechanical shear could have different effects even if the pumps and the number or frequency of loops were the same. In throttling valves, the aim was to correlate the effect of shear to a parameter related to the inner geometry of the valve and to operating conditions. An overall parameter was then evaluated, *i.e.*, the pressure drop coefficient K_v which integrates both the type of valve and its opening degree. As a consequence, the modeling of

the shear effects was conceivable, basic descriptive data used so far (type of pump, geometry or opening degree of the valve, etc.) being completed and partially substituted by quantitative parameters (rotating velocity, capacity, or internal leakage for the pumps, K_v coefficient for the valves).

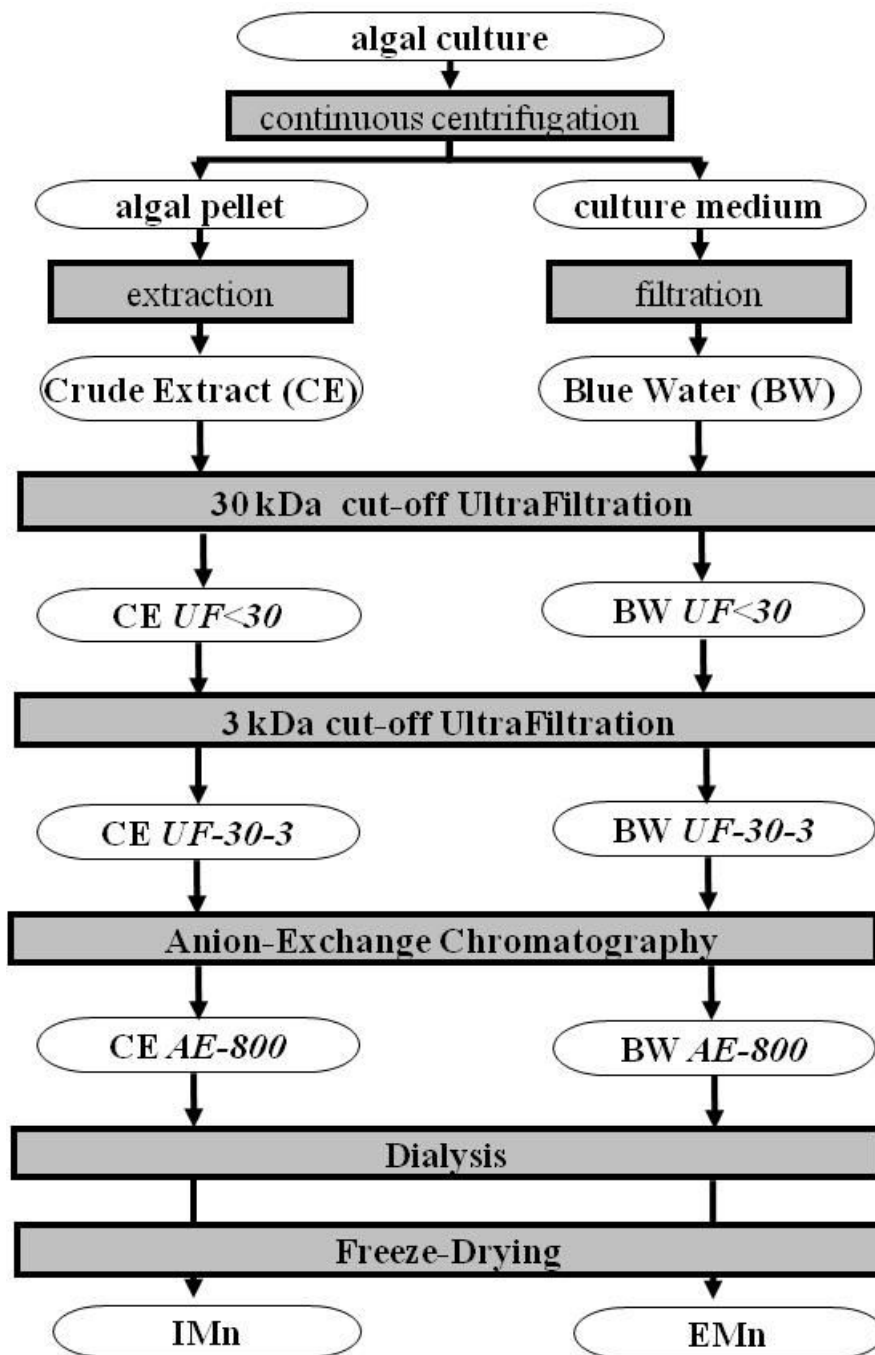
A new photobioreactor coupled with an ultrafiltration system [immersed membranes] was investigated for the continuous culture of *H. ostrearia* in order to improve marennine production and recovery [86]. The system, with a simple design, was particularly interesting, because energetic costs were minimized, and the cells were not submitted to any shear stress due to pumping or circulation. The photobioreactor was of cylindrical type, a membrane module was placed at the bottom of the reactor and the hydrostatic pressure was used as driving force both for the permeation and periodical backflushing steps. The production of biomass and marennine was stable for a three-week period, with marennine concentration three times higher than in a conventional batch photobioreactor.

A last study dedicated to bioprocessing aimed to compare the pigment productivity obtained with two types of photobioreactors [87]. In the first process, cells were free and recycled in a photobioreactor combined with a membrane ultrafiltration equipment (external loop). In the second system, cells were entrapped in a tubular agar gel layer in a photobioreactor of original design. The influence of nitrate concentration and renewal rate was examined. Experiments, conducted on long term periods (up to 40 days) without any external contamination revealed that marennine productivities of more than $5\text{--}7 \text{ mg } 10^9 \text{ cell}^{-1} \text{ d}^{-1}$ could be reached with both bioreactors. The advantages and drawbacks of each process design were also discussed.

Marennine is insoluble in all organic solvents, and first attempts to extract it from algal biomass were made using various aqueous solvents, *e.g.*, pure distilled or tap water, or bicarbonate or phosphate buffers. Regarding purification, Robert *et al.* [19] published a purification procedure in which intracellular marennine (IMn) was extracted using organic solvent and water mixtures and further solubilized in $500 \text{ mmol L}^{-1} \text{ K}_2\text{SO}_4$ at 80°C . This method obviously increased the possibility of obtaining denaturated pigment and was not convenient to produce large amounts of IMn. More recently, Pouvreau *et al.* [67] developed a new method for the extraction and purification of both forms of marennine (Figure 3). EMn and IMn were selectively extracted from Blue Water (blue-coloured culture medium) and algal pellet, respectively, and were then purified by a three-step semi-preparative procedure using two ultrafiltration steps and one anion-exchange chromatography step. This method was easily applicable to a large production system. Finally, after dialysis, UV-Visible-photodiode array analysis showed that EMn and IMn

were not contaminated, suggesting that these compounds reached the degree of purity required for further biochemical investigations.

Figure 3. Purification process of marennine and marennine-like pigments.

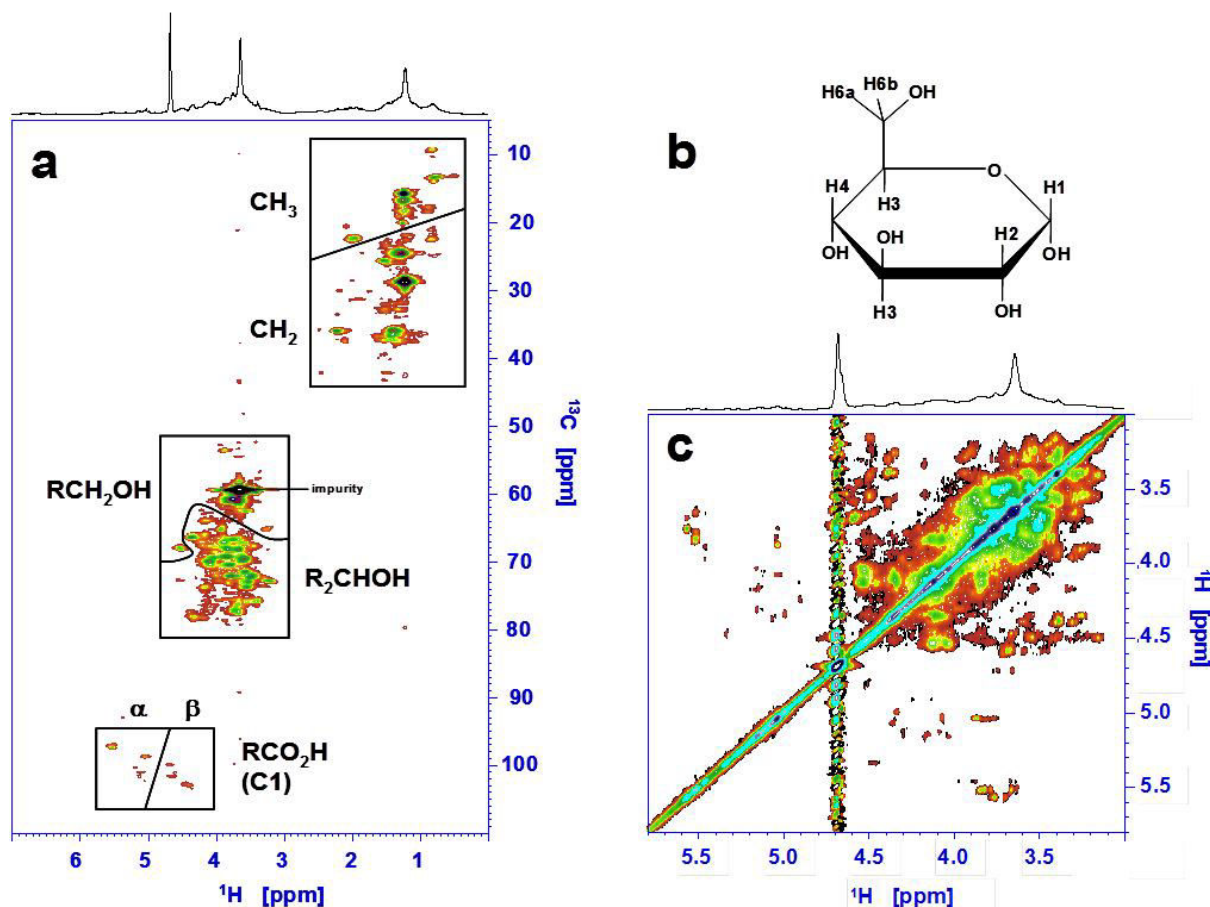


3.4 Marennine structure: what's new?

In the line of the preliminary characterization achieved by Pouvreau *et al.* [66], a series of experiments are currently conducted to increase our knowledge about the chemical structure of marennine, mainly using nuclear magnetic resonance (NMR) techniques. All current NMR experiments have been carried out on a Bruker Avance 400 MHz spectrometer equipped with a 5 mm BBFO⁺ probehead. Samples of EMn, purified as described in Pouvreau *et al.* [66] were dissolved in 0.5 mL of 25 mM phosphate buffer in D₂O with 40 mM NaN₃ at pD 6.6 (corresponding to pH 7.0) to a concentration of 2.4 mM. A ¹H-¹³C HSQC was acquired with 64 repetitions and 800 increments in the indirect dimension, applying the echo-antiecho scheme, until a maximal *t*₁ of 24.8 ms. A ¹H TOCSY with 90 ms DIPSI-2 mixing was acquired using 16 repetitions and 800 increments in the indirect dimension until a maximal *t*₁ of 106 ms.

The standard one-dimensional ¹H spectrum of EMn shows signal groups without any resolved individual signals (top of Figure 4a). Marennine is, thus, a macromolecule with a relatively complex structure. ¹H diffusion spectra [diff] [“DOSY”] reveal a diffusion coefficient of about 10⁻¹⁰ m²/s for both EMn and IMn, consistent with the mass in the 10 kDa range determined by mass spectrometry [66]. The high signal at 3.58 ppm is identified as impurity.

Figure 4. [2DNMR]. [a] ¹H-¹³C HSQC of the extracellular form of marennine (EMn) in deuterated phosphate buffer. On top the corresponding 1D ¹H spectrum. The lines separate areas of carbons with – among others – two hydrogen atoms and with one or three hydrogens, as determined by an additional edited HSQC with lower resolution and signal-to-noise ratio. **[b]** Generic pyranose. **[c]** Ring region of a ¹H-¹H TOCSY of EMn with 90 ms mixing time. The strong ¹H signal at 3.65 ppm stems from an impurity.



A [partial] resolution of signals can only be achieved by two-dimensional [2D] NMR. The correlation signals in a ^1H - ^{13}C HSQC spectrum identify chemical groups by a combination of the hydrogen and carbon chemical shifts. An edited HSQC allows, in addition, determining the number of hydrogen atoms attached to a carbon by the sign of the signal. The sensitivity is however lower. We have acquired an HSQC (Figure 4a) and an edited HSQC of EMn (not shown). Three groups of signals can be seen in the HSQC spectrum, the most striking being in the chemical shift ranges of 60 – 75 ppm in the ^{13}C dimension and 3.5 – 4.5 ppm [^1H]. This chemical shift distribution matches – among others – the ring carbons (C2 – C5 in the case of a pyranose) and hydrogens of saccharides. This is supported by a small group of signals in the relatively unusual chemical shift region of 95 – 105 ppm [^{13}C] and 4.3–5.8 ppm [^1H] characteristic of anomeric carbons [C1] and aldose hydrogens. According to the edited HSQC, these signals belong to a carbon bound to one hydrogen atom. Only some signals with relatively small carbon shifts correspond to carbons bound to two hydrogens, corresponding to a CH_2OH group [C6; also C1 in some

furanoses]. According to the chemical shifts of both hydrogen and carbon atoms, the anomeric signals can further be divided into a group attributable to carbohydrates in the α [97 – 102 ppm] \times [4.9 – 5.7 ppm] and the β form [usually 103 – 106 ppm, here lower] \times [4.3 – 4.8 ppm]. The interpretation of the HSQC spectrum is confirmed by ^1H - ^1H correlation experiments. Figure 4b displays the ring region of the TOCSY spectrum and shows spectral overlap typical for polysaccharides in the non-anomeric ring proton region (Figure 4c). Cross-peaks of H2 to α -anomeric hydrogen signals H1 are weak even after relatively long mixing time (90 ms), because the equatorial-axial J -coupling is weak [88].

The fact that marennine consists to a large extent of glycosidic elements suggests a connection to the main energy storage compound of diatoms, chrysolaminarin, in particular under consideration of the large amount of marennine produced by *H. ostrearia*. The spectra are however more complex than that of chrysolaminarin [89]. Moreover, there is a large number of signals in the aliphatic region at about [10 – 40 ppm] \times [1.0 – 2.5 ppm]. The CH_3 and CH_2 areas are separated by their chemical shifts and the edited version of the HSQC. A particular high $^{13}\text{CH}_2$ signal has a chemical shift of 28 ppm; it is well visible already in the 1D ^1H spectrum at 1.22 ppm. Diffusion experiments [DOSY] confirm that this signal comes from the macromolecule and not from an impurity. It could indicate the presence of longer CH_2 chains. Such a unit would however conflict with the general hydrophilic character of marennine. Steroids are an aglycone substance group that could explain the general dispersion of aliphatic signals.

In a previous study using primarily biochemical tests on various potential substance groups [66], a glycosidic digestion test on sugars had been negative. It is however possible that the other chemical groups compromised the detection by this method. On the other hand, it was concluded that marennine was a substance with polyphenolic or similar rings, but signals from the aromatic region in the HSQC spectrum are too weak to detect any correlation under the present experimental conditions (see also section 5). The Folin-Ciocalteu and the Prussian Blue tests on polyphenols might have been positive due to these few groups, while NMR identifies the scaffold of the macromolecule. This also counts for the chromophore. Whatever its nature is, polyphenolic or a different type, it is well possible that it constitutes only a small part of the molecule, which does not become apparent on the first impression from the principal NMR signals of this molecule. Pouvreau *et al.* [66] also report the elemental composition of marennine, where, remarkably, oxygen constitutes about 50% of the molecular mass. This result, since then confirmed using different samples of purified marennine [unpublished data], is consistent with a polyphenol but also with a glycoside.

4. Mass production of *Halsea ostrearia*: there is many a slip from cup to lip

Fundamental research regarding the characterization of marennine-like pigments can be sustained by algal production at the laboratory scale, but mass production of *H. ostrearia* is a prerequisite before achieving any added value at the industrial scale. Therefore different attempts were made to grow this peculiar diatom. According to the literature, *H. ostrearia* has been cultivated mostly at the laboratory scale (< 100 L), but also at a semi-pilot scale (up to 10 m³) [90], or using photobioreactors (< 7 L) [86]. At a larger scale, *H. ostrearia* was grown industrially for several years under indoor controlled conditions to produce marennine, mainly for the intensive greening of oysters [knowledge transfer from U. de Nantes to SOPROMA, Bouin, France]; however this production unit ceased its activity about 7 years ago for economic reasons. In fact, the only structures for the mass production of *H. ostrearia* that have ever been fully operational are oyster ponds. Unfortunately, blooms of *H. ostrearia* in these ponds [and as a consequence the greening of oysters] remain erratic and non-controllable. So far, production units of *H. ostrearia* are still to be developed, taking into account the specificities of benthic microalgae, that use tank surface to grow and generally tend to form biofilm, which makes them difficult to grow in conventional culture systems designed for suspended microalgae. Since the technology to produce massive algal biofilms is not readily available, a simple photobioreactor could be designed in order to maximise *H. ostrearia* productivity and, mostly, excretion of marennine. Some relevant traits of *H. ostrearia* autecology must be considered to design the culture system: growth is characterized by a settlement on the marine floor, where maximal biomass (as far as 350 000 cells L⁻¹) is obtained in conditions of shallow waters (as in the oyster ponds) and high light intensity [47]. Marennine is then released in the water, with concentration ranging between 2 to 5 mg L⁻¹ [90].

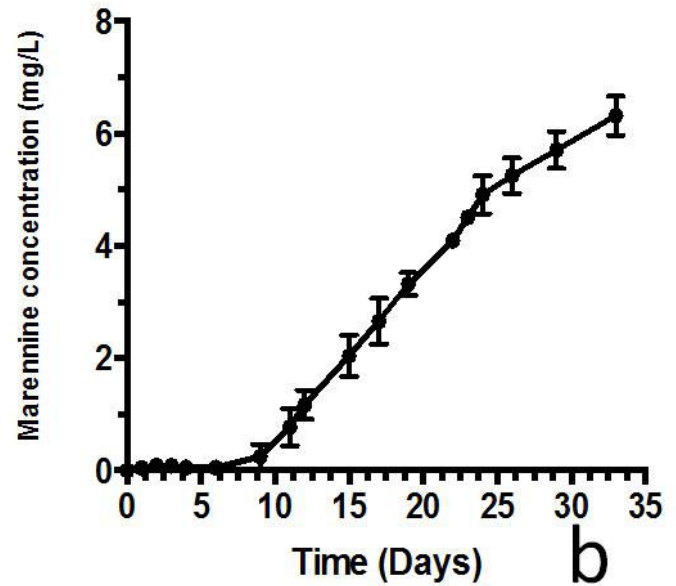
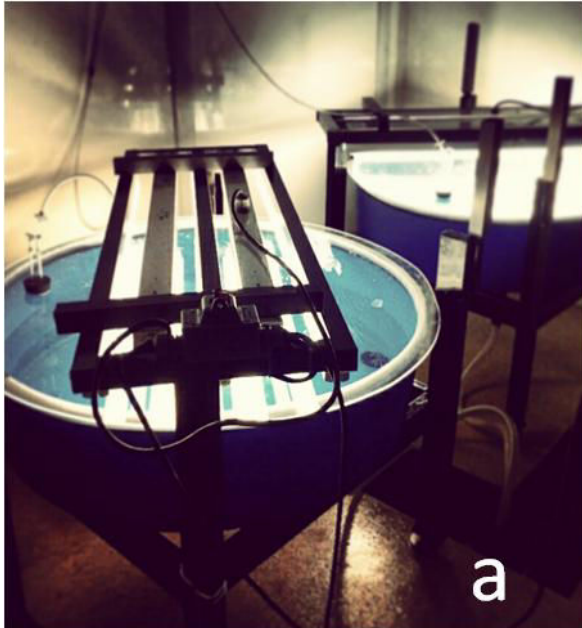
With this goal but also these constraints in mind, a photobioreactor (PBR) dedicated to the production of *H. ostrearia* and marennine has been devised. This simple PBR was designed in order to obtain large quantities of marennine to further study its biological activities and chemical structure. Also, it was designed in a simple manner so that it could be easily set-up by end-users, in laboratories as well as in hatcheries. Flat bottom polyethylene circular tanks of 200 L were used (diameter: 122 cm), covered by acrylic glass with light supplied from above by T5-5000K high-output fluorescent tubes (General Electric) at about 25 cm from the cover. A drain was installed at the lowest point of the tank, allowing water sampling and harvesting. Pre-filtered air (0.22 µm) was supplied to the dead space inside the tank through the cover to keep a positive pressure inside the tank, preventing contamination from the outside and purging air heated by the proximity of the

light source. The proximity of the air source relative to the water surface also generated a slight agitation at the surface, thus ensuring a slow but constant water movement in the tank and providing nutrient renewal to the cells.

The study was conducted at the Université du Québec à Rimouski (UQAR) Station Aquicole at Pointe-au-Père (Québec, Canada), with the use of two PBRs. Algal production was carried out using the NCC-136 strain of *H. ostrearia* isolated from Bourgneuf Bay [France] and provided by the Nantes Culture Collection (NCC). Cells were grown in a semi-continuous mode in sterilized seawater enriched with F/2 [91] and 30 mg L⁻¹ silicates. Culture was initiated in 500 mL Erlenmeyer flasks filled with 200 mL seawater, and then transferred to 2.8 L Erlenmeyer flasks filled with 2 L seawater. Growth irradiance was 125 μmol photons m⁻² s⁻¹ and room temperature was maintained at 20°C. Two of these 2 L flasks were then used to inoculate one PBR with approximately 2000 cells mL⁻¹. To minimize light attenuation by the water column (<10 cm), tanks were half filled with 100 L filtered (1 μm) natural seawater (salinity 28) supplemented with commercial nutrients (f/2 and silicate from Fritz) and ultrafiltered at 50 kDa (Romicon, KOCH Membrane, Wilmington, Massachusetts, USA). The irradiance level was 180 μmol photons m⁻² s⁻¹ (PAR Radiometer, Macam, Q201), measured under the light source at the bottom of the empty tank. Light intensity decreased in a linear way to reach 100 μmol photons m⁻² s⁻¹ at the border of the PBR (Figure 5a). Room temperature was kept at 16°C by an air-conditioner, for a maximal water temperature of 19.5°C. Both rooms were kept in a 14/10h light/dark cycle. Two cultures were run in separate PBRs, under the same conditions. Marennine concentration was determined on the cell-free culture water (syringe-filtered on 0.22 μm) by optical absorption according to the Beer-Lambert law. Optical density (OD) was measured at 677 nm in a 10 cm cell by means of a Cary 100 Bio UV-Visible spectrophotometer (Agilent Technologies, Mississauga, Ontario, Canada), using the specific extinction coefficient for EMn following Pouvreau *et al.* [92]. Optimal algal biomasses in the tanks, based on cells and biofilm appearance, were obtained after 12 days of culture growth, like in pre-culture realized in smaller volumes (confirmed with Nageotte counting chambers). Marennine release into the medium started after 10 days of growth (Figure 5b), which corresponds to the end of the exponential phase. Marennine production was relatively constant between day 10 and day 33, with a production rate of about 0.3 mg L⁻¹ d⁻¹. Maximal extracellular concentration obtained was about 6.2 mg L⁻¹ in both PBRs.

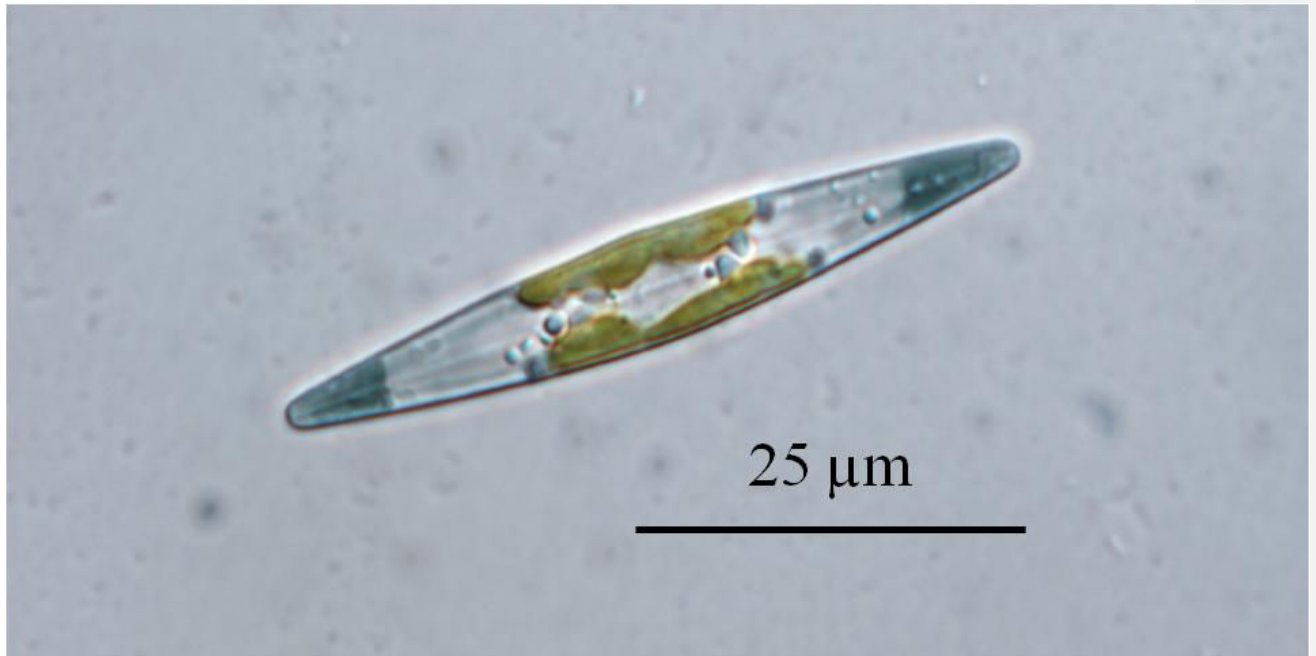
Figure 5. [a] Photography of the two PBR's used in this experiment. [b] Marennine concentration (mg L⁻¹) in the PBR, measured

spectrophotometrically on the culture medium. Values are means of concentrations obtained in each PBR (n=2).



Different culture methods were tried in the past by different authors, with estimated marennine productivities ranging from 3 to 100 mg L⁻¹ [86, 87, 90, 93-96]. However, according to these authors, these values were obtained using a cytophotometric method and a calibration curve established with unpurified marennine, and not referring to a published extinction coefficient [63], which is more an estimation of marennine production in a culture rather than a true quantification of the pigment [66]. In the literature, there is no quantification of marennine production in natural environments or in a culture that uses the molar extinction coefficient determined on purified marennine by Pouvreau *et al.* [66]. Therefore, a direct comparison of our results with other results in the literature is difficult.

Figure 6. *Haslea karadagensis* in optical microscopy.

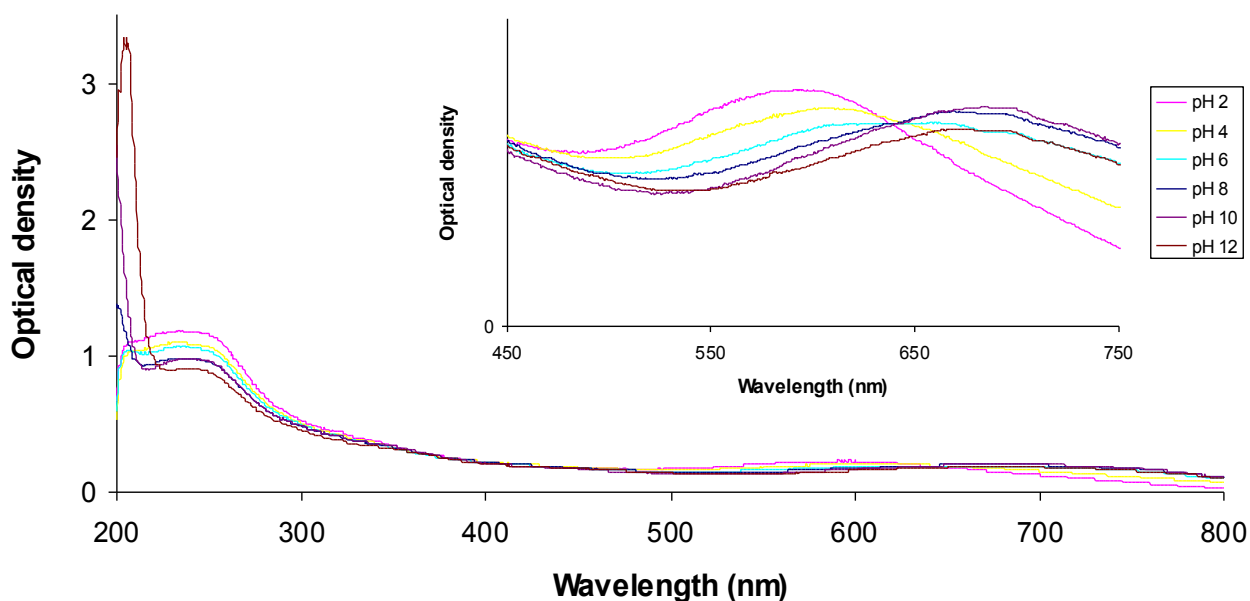


5. Marennine-like pigments and *Haslea ostrearia*-like diatoms: expanding families

Over the past two centuries, diatoms with blue tips, or marine invertebrates with greened gills were reported from almost all seas and oceans, which made *Navicula* or *Haslea ostrearia* considered as unique and cosmopolitan species [97, 98], and marennine a specific curiosity. Indeed, all blue diatoms observed were ascribed to *N.* or *H. ostrearia*, with the possible exception of a sample collected in Honduras, identified as *N. fusiformis* var. *ostrearia* by Grunow [99]. The biodiversity of blue diatoms has recently been unravelled, with the collection of phytoplankton samples in different countries and continents, and their examination using various and complementary methods, *i.e.*, biometry of natural populations, morphometry of the frustules, molecular markers, reproductive compatibility, UV-visible spectrophotometry. The first novelty came from the shores of the Black Sea in the Crimea peninsula (Ukraine), with a second species of blue diatom characterized and named *Haslea karadagensis* [97]. Despite a global similarity with *H. ostrearia* when observed in light microscopy, stria density of the frustule, *rbcL* and ITS markers, and the inability to interbreed [97, 100], constitute as many evidences that the two species are different. Another major difference that allows distinguishing between the two species concerns their pigments, as blue apices in *H. karadagensis* appear darker than in *H. ostrearia* (Figure 6). Both pigments exhibit a comparable and reversible bathochromic shift when pH increases, but different λ_{\max} (Figures 7 and 8). Furthermore, UV-visible

spectrophotometry shows that the pigment produced by *H. karadagensis* presents two isobestic points when pH varies from 2 to 12 (Figures 7 and 8), in comparison with only one in marennine [66]. Regarding biological properties, both pigments demonstrated a greening effect on bivalves, and as detailed below, antibacterial, antiviral and antifungal activities [98, 101].

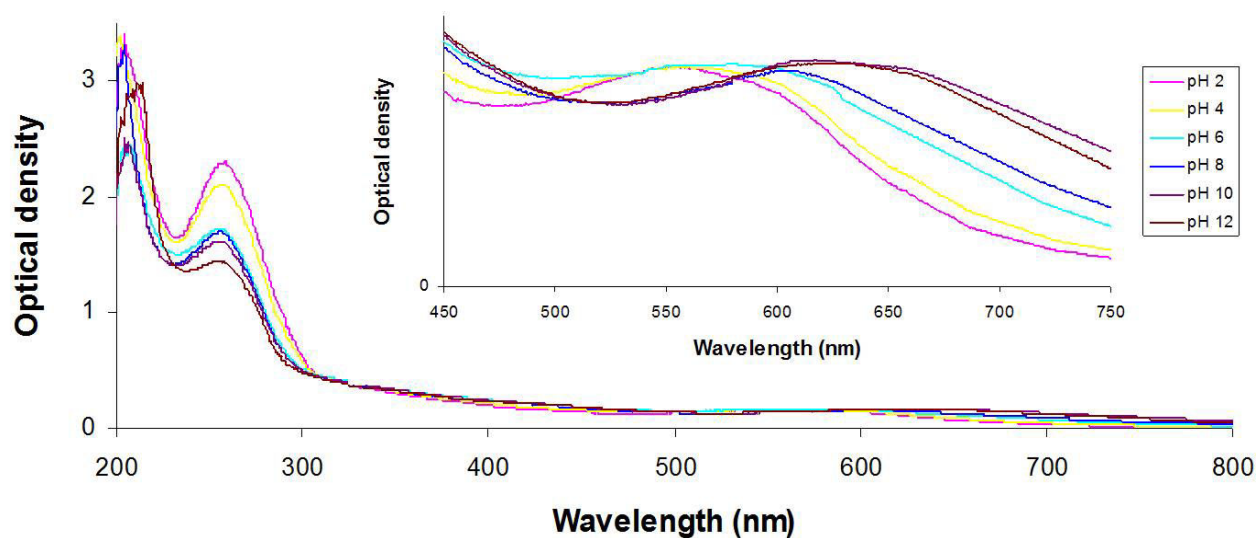
Figure 7. Absorption spectra of purified intracellular marennine from *H. ostrearia* depending on the pH (ranging from 2 to 12). Inset: position of the peak of absorption in the visible part of the spectrum.



More samples of blue diatoms were obtained from the Canary Islands (La Gomera), and the Mediterranean Sea (French coast, between Toulon and Nice). Investigated by the same complementary approaches, these samples proved to be two new species of blue diatoms. They were named *Haslea silbo* sp. inedit. and *Haslea provincialis* sp. inedit., respectively [21]. They are currently being characterized. Both species produce a blue pigment, which is highly similar if not identical to marennine, as evidenced by UV-visible spectrophotometry (not shown), and Raman spectrometry (Figure 9). Both techniques did not allow discriminating between intracellular and extracellular forms of these pigments, in contrast to marennine. However, these two techniques could be less suited for going further in the study of these pigments. For instance, Raman spectroscopy showed that the pigment purified from a supernatant (extracellular form) of *H. provincialis* sp. inedit. displays a

spectral signature similar to IMn, but the NMR spectra appear to be more distinctive. Figure 10 [^1H NMR] compares the ^1H NMR spectra of the extracellular form of the pigment from *H. provincialis* sp. inedit. with EMn and IMn from *H. ostrearia*. All three spectra show a general similarity in the represented signal groups, which manifests that the molecules belong to the same substance class. There are, as already seen in the ^1H - ^{13}C correlation spectrum of EMn, essentially an important signal group at 3.4 to 5.4 ppm, assigned to sugar ring hydrogens, and the signals in the aliphatic region between 0.8 and 2.4 ppm. There are only weak signals in the aromatic region. However, there are differences in the details. Most strikingly, both extracellular forms contain a strong CH_2 signal at 1.22 ppm discussed in paragraph 3.4.1. Furthermore, the intracellular form of marennine appears to contain less anomeric protons in alpha conformation (4.9 – 5.7 ppm). These preliminary results appeal for a more thorough study of the marennine-like pigments produced by the different species of blue diatoms, which constitute an original family of natural blue pigments.

Figure 8. Absorption spectra of purified intracellular pigment from *H. karadagensis* depending on the pH (ranging from 2 to 12). Inset: position of the peak of absorption in the visible part of the spectrum.

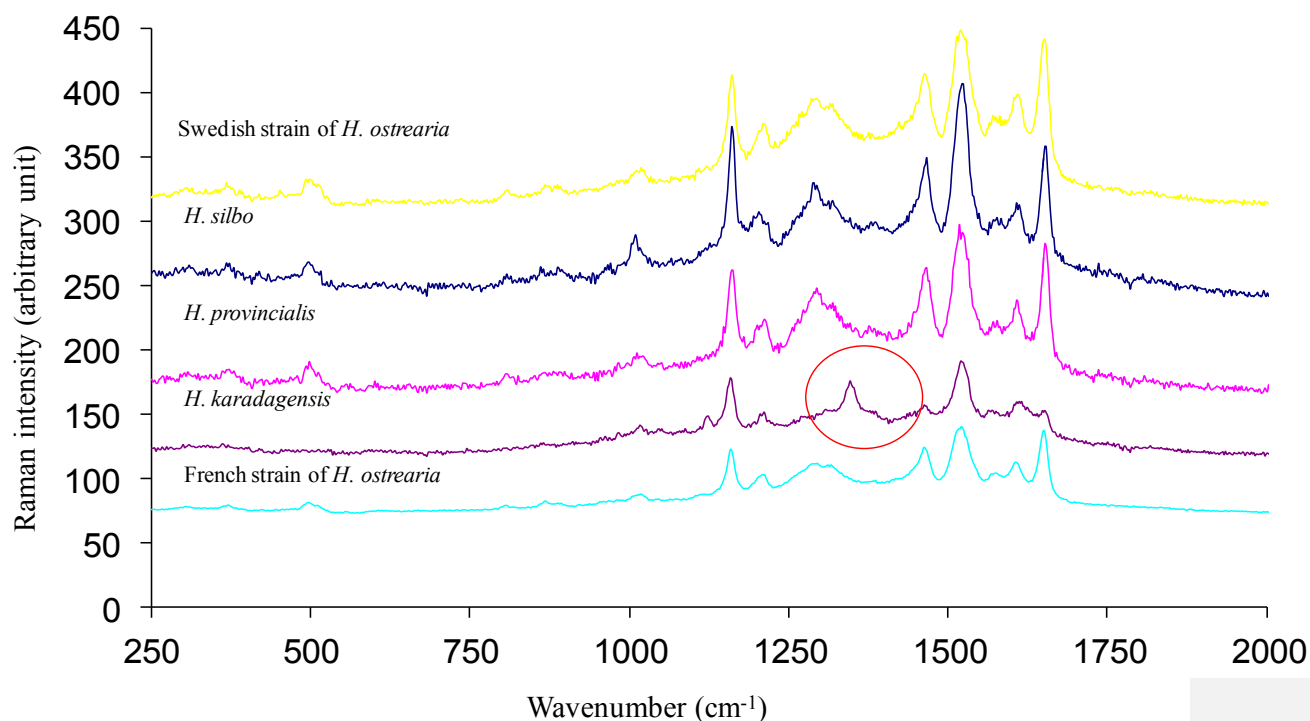


6. Biological functions of marennine-like pigments

During a bloom of *H. ostrearia* in an oyster pond, the quantity of marennine released into the environment should represent a high ‘cost of production’, which raises a question

about the advantages that the species may retrieve from this peculiar biosynthesis pathway. More generally, the ecological significance of marennine-like pigments remains to be considered, especially in regard of the amount of pigment produced during the diatom life cycle. As presented above, historically, a wealth of information is available on marennine, but concerning the diatom *H. ostrearia* itself, information about the biological function of this specific blue pigment is scarce and inconclusive. The longest known and most obvious biological effect of marennine is the greening of oysters. Experimentally, oysters placed in either a suspension of *H. ostrearia* or a green supernatant of a culture, turn green within a few hours [55, 72, 78, 98, 102, 103], but little is known about the mechanism. Some authors suggested there might be interaction between marennine and some proteins in the gills [36], especially in some specialized secreting cells [22, 36]. In oyster gills *in vivo*, it is possible that marennine binds and precipitates proteins like tannins, as observed *in vitro* [unpublished data]. A tannin-binding protein effect would be in agreement with the suggested polyphenolic nature of marennine proposed by Pouvreau *et al.* [66]. Regarding *H. ostrearia* itself, Schubert *et al.* [104] showed that marennine did not play any role in light capture and photochemical activity; however, it could indirectly influence photosynthesis by absorbing in the red part of the spectrum, with peaks at 672 and 677 nm for the intracellular and the extracellular forms of the purified pigment (neutral pH), respectively [66], and at 669 nm for raw supernatants of *H. ostrearia* cultures [19]. Marennine could thus be considered a photoprotective molecule at high irradiance levels [104], or a factor able to modify the light spectrum in the water column when accumulated in the medium [68, 92].

Figure 9. Raman spectra obtained *in vivo* on the blue pigments contained in the apices of different strains of blue diatoms. Noticeable differences can be seen between the pigment of *H. karadagensis* and the others in the 1240 cm^{-1} to 1420 cm^{-1} region.

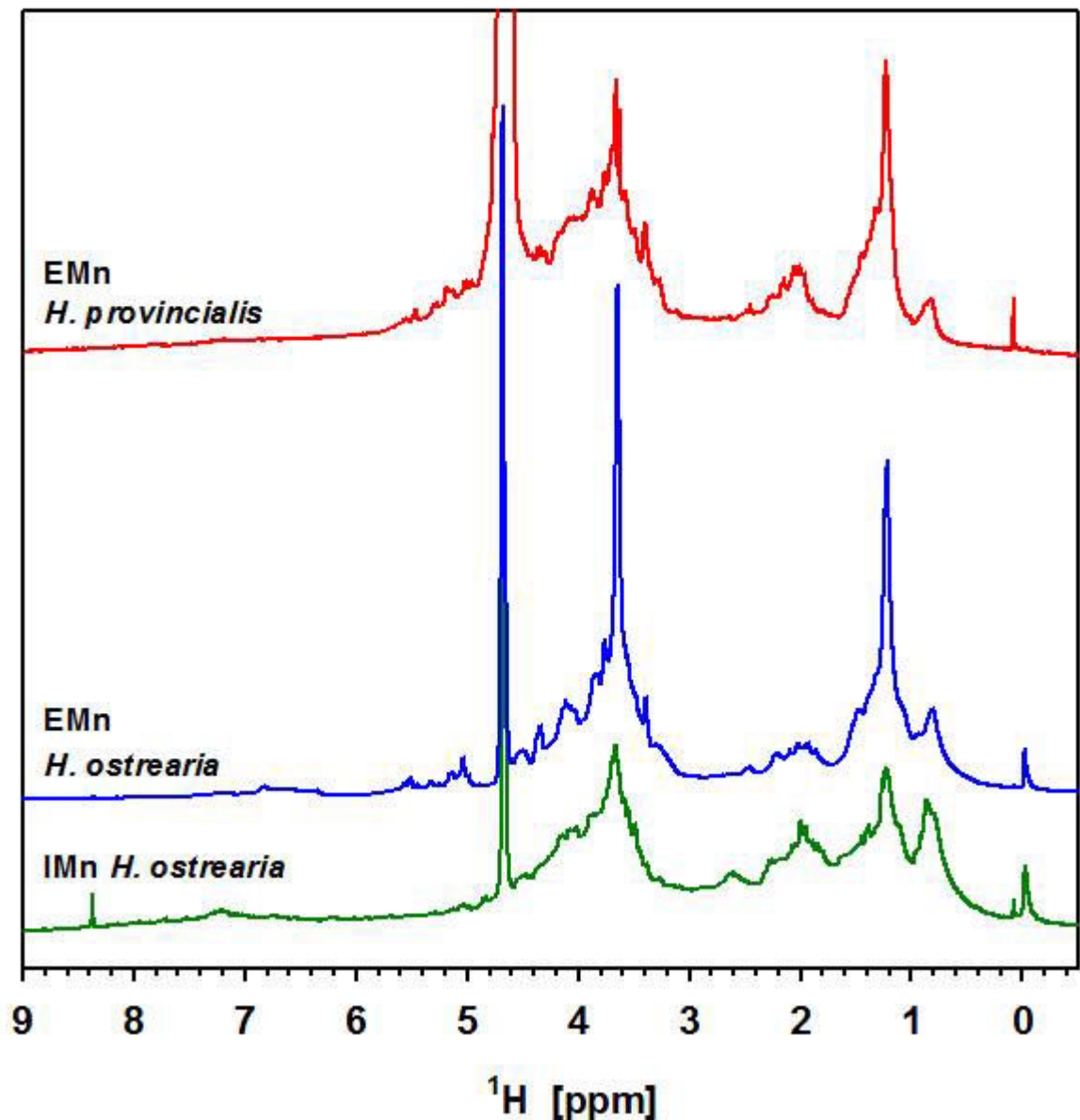


6.1 Marennine, weapon of chemical warfare

Aside this shading effect, it was shown that marennine has antioxidant activity [105], that it can afford some protection against metals such as copper [106], and act directly as an allelochemical by inhibiting the growth of some algal species encountered in oyster ponds and modifying inter-specific competition among phytoplankton [49, 92]. These results have been reinforced by co-cultivation experiments of *H. ostrearia* with other species, which underlined the sensitivity of centric species like *Skeletonema costatum*, *Chaetoceros calcitrans*, *C. gracilis*, (all species abundantly used in aquaculture), as well as the insensitivity of others like *Pavlova lutheri* (not shown). This could explain the occasional dominance of *H. ostrearia* in oyster ponds, concomitant with an almost elimination of other diatom species [47, 71, 90], thus revealing the importance of chemical ecology in marine phytoplankton and environments [107]. A few authors hypothesized that *H. ostrearia* could itself be affected by marennine, a sort of autotoxin associated with pathological processes [32, 49, 71]. However, it was further demonstrated that *H. ostrearia* was rather tolerant to marennine [92].

Figure 10. [1H NMR]. ¹H 1D NMR spectra of the intracellular form (IMn) of marennine from *Haslea ostrearia* (bottom, green), the extracellular form (EMn)

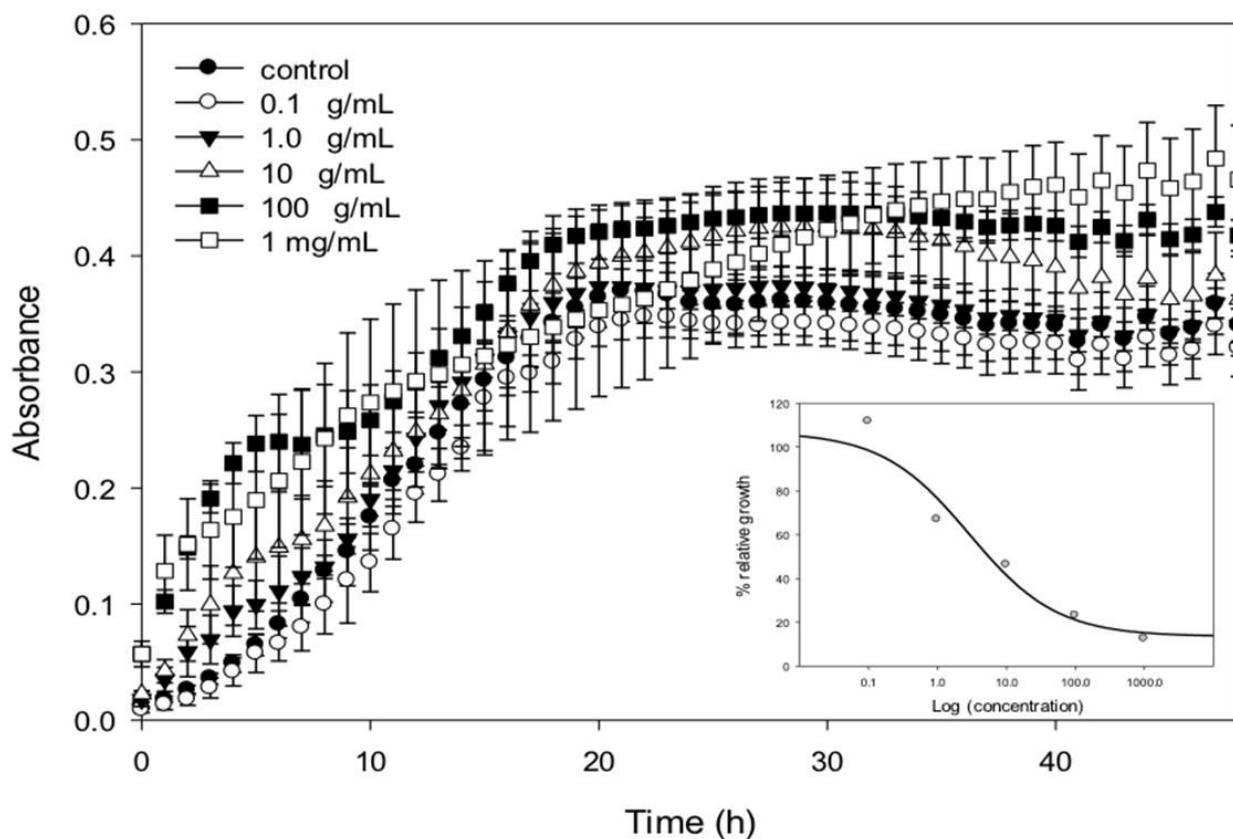
of marennine (middle, blue), and the extracellular form of the marennine-like pigment from *Haslea provincialis* sp inedit. (top, red), all dissolved in D₂O. The signal at 4.7 ppm stems from residual HDO, the signal at 3.64 ppm in the extracellular marennine partly stems from an impurity.



Last but not least, preliminary works conducted on *H. ostrearia* aqueous extracts, thus containing marennine, displayed antiviral, anticoagulant [108] or antiproliferative properties [109]. These activities have been recently confirmed using purified marennine,

which exhibited antibacteria, antiviral, and antiproliferative activities [101], or using the pigment synthesized by *H. karadagensis*, the pigment of which demonstrated antifungi, antibacteria, and antiviral activities [98]. Hence, aside the greening action, marennine and marennine-like pigments could be especially useful in prophylaxis in the context of oyster farming, due to their antibacterial and antiviral activities.

Figure 11. Growth of *Vibrio splendidus* after 3 h in contact with marennine. *V. splendidus* was grown in modified marine media overnight. Cells were washed and then incubated for 3 h in 0, 0.1, 1.0, 10, 100, or 1000 $\mu\text{g mL}^{-1}$ marennine. Cells were washed in sterile water and brought to an optical density of 0.5 before the 3 h incubation. Cells were then added to fresh marine media in a 96 well plate and growth kinetics were done for 48 h with measurements every 30 min. Inset: maximum growth rates expressed in function of the control (100%) with marennine concentrations.



6.2 Marennine-like pigments, magic potion for oysters?

In the last decades, it has been observed that the cultivated Pacific oyster *Crassostrea gigas* presents massive and recurrent summer mortalities, this being of great concern for oyster industry all over the world. In France, for instance, bacteria such as *Vibrio aestuarianus* [110], *V. splendidus* [111], and viruses belonging to the Malacoherpesviridae, like the ostreid herpesvirus OsHV-1, distantly related to other members of the Herpesviridae [112] are frequently associated to, if not responsible for these severe summer mortality events.

Marennine-like pigments displayed antibacterial activities against three marine bacteria, *V. aestuarianus*, *Pseudoalteromonas elyakowii*, *Polaribacter irgensii* [98, 101]. In a first attempt to explain the mechanism of action of the antibacterial activity exhibited by marennine-like pigments, Tardy-Laporte *et al.* [113] demonstrated, using ^2H solid-state NMR on intact *Escherichia coli*, that the extracellular form of the pigment produced by *H. provincialis* sp. inedit. perturbs the bacteria membranes, unlike the intracellular form. More specifically, their results suggest that the pigment released in the medium exerts its antibiotic action by interacting with the lipopolysaccharides on the bacterium's surface, thus rigidifying the outer membrane.

In the line of this membrane-mediated inhibition effect, a series of experiments were conducted with *V. splendidus* cells incubated for 3 h with different concentrations of EMn ($0.1 \mu\text{g mL}^{-1}$ to 1.0mg mL^{-1}), then washed and resuspended in fresh marine medium, for 48 h. The highest the marennine concentration, the highest the inhibition of the growth (Figure 11). The effective concentration reducing bacteria growth rate by 50%, EC_{50} , was $2.89 \mu\text{g mL}^{-1}$, a value in the range of many anti-bacterial marine compounds described so far [114].

Regarding antiviral activities, due to a lack of a suitable bivalve cell line for culturing oyster herpes virus, a heterologous model using fibroblastic Vero cells and human HSV-1 was chosen in some studies, to investigate the effect of antiviral activity [115]. It has been shown that marennine intracellular and extracellular forms exhibited antiviral activity against the HSV-1 herpes virus [101], with 50 % antiviral effective concentration (EC_{50}) values of 24.0 and $27.0 \mu\text{g mL}^{-1}$, respectively [for a multiplicity of infection (MOI) of $0.001 \text{ID}_{50}/\text{cells}$]. The blue grey pigment produced by *H. karadagensis* presents similar antiviral activity [98], with the extracellular form being more active than the intracellular form (EC_{50} of $23 \mu\text{g mL}^{-1}$ and $62 \mu\text{g mL}^{-1}$, respectively). Both intracellular forms of the pigments present a relative cytotoxicity against the fibroblastic Vero cells, not observed with their extracellular counterparts. This underlines the need for further experiments

regarding a possible toxicity, and the mode of action of marennine-like pigments. A sulfated polysaccharide, naviculan, was isolated from *Navicula directa*, a diatom collected from deep-sea water in Toyama Bay, Japan [116]. This compound was shown to inhibit HSV-1 and HSV-2 (half maximal inhibitory concentration, $IC_{50} = 7\text{--}14 \mu\text{g mL}^{-1}$) by interfering with the early stages of viral replication, most likely affecting viral adhesion and penetration into host cells. Very few other biologically active secondary metabolites have been reported from diatoms [117, 118], and except for a few studies [98, 101, 108], little is known about the antiviral activity of diatom extracts or compounds. The selection of antiviral marine compounds for aquaculture environments being a possible solution to control viral disease transmission [119], a possible valorisation of the biological activities of marennine-like pigments as probiotics would constitute a new and promising field of research.

The fact that marennine-like pigments seem to have the potential to inhibit growth of pathogenic marine bacteria and virus replication, is of special interest for oyster producers, as bivalves lack antibody-mediated humoral immunity, but possess an innate immune system. Experiments are in progress to test the hypothesis that marennine in solution or present on gills could protect oysters from these pathogens in realistic *in vivo* conditions.

7. Do marennine-like pigments present a real added-value potential?

At the industrial scale, marennine is solely exploited in aquaculture for the greening of oysters, thus as a natural blue-green colorant. The main source of natural blue colorants is indigo dye, extracted from different species of higher plants. For food market, however, there is a lack of available natural blue pigment alternatives compared to red, orange, and yellow natural colorants [120]. More simple to produce and less expensive to use, some of the synthetic blue colorants used in food chemistry have to face alleged or assessed reputations of harmfulness, some of them possibly encountering commercial restrictions or banishment soon (*e.g.* Patent Blue V E131). Presently, the use of marine blue dye in food industry is restricted to phycocyanin, mainly produced by cyanobacteria but also some Rhodophyta [121]. Phycocyanin is a food colorant, which is known in Japan under the name of ‘Lina-Blue’, and it is mainly used in ice-creams or drink preparations. In this context, marennine is also a natural blue pigment, which could complete the source of marine blue dyes available for the food industry, and it presents some merits but also a few flaws. People have consumed green oysters for centuries, without any disease or anaphylactic reactions recorded, which should suggest that this pigment is non-toxic, at least considering a standard food intake. Marennine is produced by a marine microalga, which augurs well for an environmental-friendly production system. It is water soluble, and

its extraction process does not require massive use of solvents. However, the structure of marennine is still unknown, and because marennine exhibits a wide range of biological activities, its possible cytotoxicity and its stability as a pigment have to be carefully tested before considering a possible use in cosmetology. Indeed, before turning to reality in Europe, and in France especially, this type of application will be submitted to a previous acceptance by the regulation authority. For example, the use of ‘pure marennine’ as a food colorant would indeed require nutritional and toxicity studies on the rat, and cellular tests to demonstrate the pure molecule has no mutagenic properties. These necessary steps represent a strong restriction before the use of marennine as food additive. An alternative way, already applied for the up-grading of phycocyanin and phycoerythrin, would be to promote the use of aqueous extracts enriched in pigment for their colorant properties. Generally, this application is authorized, at least for the phycocyanin extract, and appears to be realistic from an economic point of view regarding cosmetics, but this would remain a more crippling problem for any development towards food and health industry

Illustrating the added-value potential some microalgae in cosmetic industry, some companies such as LVMH group and Daniel Jouvance have invested in their own microalgal production units. Indeed, some microalgae (*e.g.*, *Chlorella*, *Odontella*, *Tetraselmis*, *Dunaliella*, *Emiliana*, *Noctiluca*) are established in the skin care market. Microalgae extracts are mainly found as skin care products, *e.g.*, anti-aging, emollient or moisturizing, and also sunscreen products. Preliminary experiments were conducted to study possible photo-protective and anti-inflammatory effects of marennine. The photo-protective potential was thus studied, to determine sun protection factor (SPF) and UVA protection factor (PF-UVA) of marennine, using an *in vitro* method. An O/W emulsion placebo was prepared in the laboratory as previously described [122]. Marennine was incorporated into the formulation components at 10% (w/w) in order to study the potential biological properties. Thirty milligrams of product exactly weighed were spread on polymethylmetacrylate (PMMA) plates over the whole surface (25 cm²) using a cot-coated finger (15 mg remain on the finger cot). SPF and PF-UVA of the creams were measured *in vitro*. Three plates were prepared for each product to be tested and 9 measures were performed on each plate. Transmission measurements between 320 and 400 nm were carried out using a spectrophotometer equipped with an integrating sphere (UV Transmittance Analyzer UV1000S, Labsphere, North Sutton, US). The calculations use the following equations for SPF and PF-UVA:

$$SPF = \frac{\sum_{290}^{400} E_s S_i d_i}{\sum_{290}^{400} E_s T_i d_i}$$

$$FP-UVA = \frac{\sum_{320} E_{\lambda} S_{\lambda} d_{\lambda}}{\sum_{320} E_{\lambda} S_{\lambda} T_{\lambda} d_{\lambda}}$$

where E_{λ} is CIE erythral spectral effectiveness, S_{λ} is solar spectral irradiance and T_{λ} is spectral transmittance of the sample [123, 124]. Emulsions containing 10% (w/w) marennine exhibited SPF (1.28 ± 0.05) and PF-UVA (1.24 ± 0.04) values (means \pm SD, $n=3$), which demonstrate that marennine could not be considered a molecule interesting enough in the domain of the topical photo-protection.

The determination of a possible anti-inflammatory effect of marennine incorporated into a cream formulated in the laboratory was carried out using a test with Phorbol-12-Myristate-13-Acetate (PMA). Introduction of mouse ear edema was based on the method of Carlson *et al.* with some modifications [125-127]. Firstly, the thickness of the mouse ears was measured using a model micrometer gauge (Oditest[®], Kroeplin, Schlüchtern, Germany). 10 mL of preparation with marennine or 0.1% (w/w) butyrate hydrocortisone-based lotion (Locoïd[®], Astellas Pharma – Levallois-Perret, France) were applied using a ripette genix electro dispenser (Fisher scientific, Illkirch, France), on the mice's right ears, twice at 5 min intervals. 10 mL of placebo emulsion were applied according to the same protocol, on the mice's left ears. Thirty minutes later, 10 mL of a hydro-alcoholic solution of Phorbol-12-Myristate-13-Acetate (250 mg/mL) were then applied on each ear, in order to cause an edema. After 3.5 h, the thickness of the ears was once again determined using the Oditest[®]. Five mice were used for each product tested. The cream containing 10% (w/w) marennine demonstrated a moderate anti-inflammatory effect, with an edema inhibition of 62.5%, as compared to 100% for the control butyrate d'hydrocortisone (0.1% w/w). These preliminary experiments show that marennine can hardly be considered as a potential UV filter, but it could be valorised in soothing creams. It might be interesting to explore potential applications in the management of atopic skin or rosacea, for example.

7. Conclusion

Aquaculture still represents the most immediate and 'natural' valorisation segment of marennine and marennine-like pigments, due to the greening of bivalves. Aside this colouring action, it is hypothesized that marennine-like pigments could act as natural prophylactic agents in hatchery and oyster farming, due to their antibacterial and antiviral activities, which were demonstrated *in vitro* at the laboratory scale. Together with a possible use as natural blue colorants for cosmetics, provided they are inexpensive to produce, stable in the formulations, and safe for use, the many biological activities

evidenced so far represent a new research avenue, and a great potential of valorisation for the marennine-like pigments.

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Conflicts of Interest

The authors declare no conflict of interest.

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