Valorisation des activités biologiques de la diatomée marine Haslea ostrearia

Charlotte Falaise

▶ **To cite this version:**

Charlotte Falaise. Valorisation des activités biologiques de la diatomée marine Haslea ostrearia. Microbiologie et Parasitologie. Le Mans Université, 2019. Français. ⟨NNT : 2019LEMA1011⟩. ⟨tel-02127553⟩

HAL Id: tel-02127553
https://theses.hal.science/tel-02127553v1

Submitted on 13 May 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

HAL Authorization

THESE DE DOCTORAT

LE MANS UNIVERSITE
COMUE UNIVERSITE BRETAGNE LOIRE

ECOLE DOCTORALE N° 598
Sciences de la Mer et du littoral
Spécialité : « *Biologie marine - Microbiologie* »

Par
Charlotte FALAISE

Valorisation des Activités Biologiques de la Diatomée Marine *Haslea ostrearia*

Thèse présentée et soutenue à Le Mans, le 5 avril 2019
Unité de recherche : Mer Molécules Santé (MMS) - EA2160, Le Mans Université
UFR Sciences et Techniques, Avenue Olivier Messiaen 72085 Le Mans Cedex 9
Thèse N° : 2019LEMA1011

Rapporteurs avant soutenance :

Lucie BEAULIEU, Professeure
Université Laval de Québec

Laurent PICOT, Maitre de conférences, HDR
La Rochelle Université

Composition du Jury :

Patrick CORMIER, Professeur
Sorbonne Université

Juliette FAUCHOT, Maîtresse de conférences
Caen Normandie Université

Présidente du jury :
Claire HELLIO, Professeure
Bretagne Occidentale Université

Marie-Agnès TRAVERS, Chargée de recherche, HDR
Ifremer SG2M-LGPMM, La Tremblade

Directeur de thèse
Jean-Luc MOUGET, Professeur
Le Mans Université

Co-directeur de thèse
Vincent LEIGNEL, Maître de conférences, HDR
Le Mans Université

“Always look on the bright side of life [whistles]”

Brian

Remerciements

Cette thèse a été réalisée au laboratoire Mer, Molécules, Santé de Le Mans Université et a bénéficié d'une allocation du Ministère de l'Enseignement Supérieur et de la Recherche (2015-2018). Ce projet a également bénéficié de financements du programme de recherche et d'innovation Horizon 2020 de l'Union européenne//au titre de la convention de subvention n° 734708 / GHANA / H2020-MSCA-RISE-2016.

Je voudrais d'abord remercier les membres du jury qui ont accepté d'examiner mon travail, merci aux rapporteurs Lucie Beaulieu et Laurent Picot, ainsi qu'aux examinateurs Patrick Cormier, Juliette Fauchot, Claire Hellio, Vincent Leignel et Marie-Agnès Travers.

Merci à mes encadrants de m'avoir donné l'opportunité de réaliser ce travail de doctorat et un merci tout particulier à mon directeur de thèse Jean-Luc Mouget. Merci pour tes conseils, tes encouragements pendant mes moments de doutes et ta disponibilité tout au long de mon doctorat. Merci d'avoir toujours pris le temps de répondre à mes questions et également d'avoir été aussi réactif pour me renvoyer toutes tes corrections (et particulièrement en cette fin de thèse !). Ces trois années ont été une belle aventure, que j'ai pu enrichir de séjours dans différents laboratoires en France et à l'étranger grâce à ton réseau et au programme européen. Donc un grand merci pour ton encadrement, j'ai été très contente que tu diriges ma thèse, de découvrir l'univers de la marennine et de voir la vie en bleu !

Merci aux membres de mon CSI, Tristan Renault et Sébastien Lefebvre, pour leurs regards extérieurs et leurs conseils sur mon travail de doctorat.

Merci à tous mes collègues du Laboratoire MMS du Mans, j'ai eu beaucoup de plaisir à travailler à vos côtés, à discuter autour d'un café et à partager les pauses repas. Merci Brigitte, Justine et Marie pour nos séances de bavardages et de rigolades, nos pique-niques au soleil et parties de Mölkky, mais également pour votre aide et vos conseils tout au long de ma thèse. Merci Nathalie et Aurore d'être toujours disponibles et à l'écoute et en particulier pour votre soutien au début de mon doctorat. Merci à ma co-bureau Myriam pour toutes nos belles conversations, pour les brassages de meubles et pour les chocolats

sur-emballés ; tu as vraiment apporté la touche d'énergie positive dont j'avais besoin en cette fin de thèse ! Merci Françoise et Gérard pour les pauses café animées et les discussions variées. Merci à vous tous, Benoit, Annick, et merci aussi Marie pour ton aide dans la préparation des TP, Valérie pour ton éternelle bonne humeur et pour avoir géré tout un tas de dossiers au cours de ces années. Bref, un grand merci à tous d'avoir rendu la vie au travail si agréable !

Merci également à tous ceux qui ne sont aujourd'hui plus au labo mais avec qui ça a été un réel plaisir de travailler, merci Sophie, Alexandre et Kajani. Merci Romain pour tout ce que tu m'as transmis et pour avoir veillé même à distance que ma thèse se passait bien. Bien sûr une pensée spéciale pour les « anciens » thésards, merci pour le soutien moral et l'entraide, merci Stéphane ma co-bureau de début de thèse, merci Hélène, Fiddy, Stan, Bing et Wided. La relève est maintenant assurée : merci Matteo, Orlane, Mariem et Julie !

Merci à Intan et Hamza, j'ai fait mes premiers pas d'encadrante de stage avec vous et j'espère vous avoir transmis au moins autant que ce que vous m'avez apporté.

Merci à toute la team G*H*aNA et marennine, à Pamela, Emmanuelle, Boris et bien sûr merci à toi Lila, pour nos nombreux fous rires même dans nos moments de galère à essayer de trouver un sens à nos résultats, merci pour ta délicieuse cuisine (qui me manque !) et pour ta joie de vivre .

Merci Patrick pour ton accueil à Roscoff, ça a été un réel plaisir de travailler avec toi. J'ai beaucoup apprécié mes séjours à la station biologique, tant pour la sympathie et l'accueil chaleureux de toute l'équipe TCCD que pour le cadre de travail et les magnifiques couchers de soleil qu'offre la ville !

Merci aux équipes du LGPMM de la station de la Tremblade. Merci Cyrille pour ton accueil, tes conseils et pour tous nos échanges intéressants, merci à Marie-Agnès, Joël et à tous les chercheurs et doctorants pour leur aide et avoir rendu ces séjours à La Tremblade si agréables.

Merci aux équipes de l'ISMER, pour leur accueil et leur aide lors de mes trois séjours rimouskois ! Merci beaucoup Réjean de m'avoir permis de travailler dans ton labo, merci pour ta disponibilité, ton optimisme et de m'avoir offert la possibilité de participer à différents congrès. Merci Nathalie, Jordan, Sarah-Béatrice et François pour votre aide à la station aquicole. Merci Karine et Kim de m'avoir donné accès au labo de bactériologie et pour tous vos conseils. Merci Bertrand et Renée pour le temps que vous m'avez accordé et pour votre aide en biologie moléculaire. Merci Efflam et Jean-Nicolas pour votre soutien au cours de mes expérimentations. Enfin merci à tous ceux qui ont contribué à rendre ces séjours au Canada aussi enrichissants sur les plans professionnels que personnels, merci notamment à Constance pour nos balades dans la neige ou au bord du Saint Laurent, à Inès pour nos sessions de travail tardives et nos discussions autour d'une poutine, et bien sûr un grand merci à mes supers colocataires Marion et Florian pour tous ces moments partagés !

Je tiens également à remercier tous mes proches qui m'ont soutenue et encouragée au cours de mon doctorat.

Un merci tout particulier à ma famille, « pour l'amour, le partage… la générosité !!! ». Merci Kathleen et Leslie d'être toujours là pour moi, je ne pourrais pas imaginer avoir des sœurs plus géniales, vous êtes des piliers dans ma vie et je sais qu'on pourra toujours compter les unes sur les autres. Merci maman pour tout ce que tu fais pour moi, d'être toujours aussi positive et confiante, de t'assurer que je vais bien (que je bois du thym-miel-citron en hiver !) et pour toute la jovialité et la force que tu me transmets. A big thank you to my little Nany, thank you for always being there for me and for all your love and your support. Merci papa d'avoir été aussi présent pendant mon doctorat, de m'avoir soutenu, de t'être intéressé à mes travaux et de m'avoir même insufflé des pistes de réflexion ! Merci à Mathieu, à Aurélien et bien sûr à Robinson qui grandit tellement vite et qui égaye nos vies avec toute son énergie et sa bonne humeur. Un grand merci à vous tous, la famille proche et plus lointaine, c'est un vrai bonheur de se sentir si bien entourée !

Merci bien sûr à tous mes ami(e)s aux côtés de qui je peux recharger les batteries, me confier et rigoler. Merci à Elodie, Manon, Melou, Coco, Damien, Thomas et Marion, même si

on ne se voit pas forcément souvent je sais que je peux compter sur vous et ça fait toujours un bien immense de vous retrouver ! Merci aussi à toute la team de Poitiers, pour nos joyeux moments de détentes et de festivités !

Un énorme MERCI à mes colocs Marie-Laure, Elodie, Léonie, Carole (et Loulou !). Quel plaisir de rentrer le soir à la maison et de papoter autour d'une « infu » et des cookies (ou d'une bière et des Munster Munch selon l'heure). Merci pour tous ces chouettes moments partagés dans « notre maison du bonheur », nos discussions parfois philosophiques et profondes et parfois beaucoup moins, nos larmes de rire ou de tracas, nos activités artistiques, nos playlists musicales qui tournent en boucle, ~~nos séances de sport~~, nos soirées soupe-télé ou apéros ensoleillés. Merci d'avoir été là pour moi, à l'écoute, attentionnées et d'avoir rendu ces trois années si riches et si belles !

And last but not least, merci à toi David, pour tout. Tu as toujours su me motiver, trouver les mots pour m'encourager, me tirer vers le haut et me faire relativiser quand je commençais à dramatiser sur ma vie de thésarde (parce-que non, ce n'est pas si grave si les bactéries n'ont pas poussé ou que les moules ne veulent pas pondre). Merci de t'être autant impliqué et intéressé à mon travail ; je ne suis pas sûre de pouvoir parler des moteurs CFM et LEAP aussi bien que tu parles de microalgues ou de réponse hormétique ! Merci de ta patience et de ton soutien. J'ai une chance folle de t'avoir auprès de moi, et maintenant l'aventure du doctorat terminée j'ai hâte d'en entamer une toute nouvelle avec toi.

SOMMAIRE

REMERCIEMENTS……………………………………………………………………………II

SOMMAIRE………………………………………………………………………………VII

LISTE DES FIGURES……………………………………………………………………XI

LISTE DES TABLEAUX…………………………………………………………………XVII

LISTE DES FIGURES ET TABLEAUX SUPPLEMENTAIRES………………………………..XVII

LISTE DES PRODUCTIONS SCIENTIFIQUES…………………………………………...XVIII

CHAPITRE I. INTRODUCTION GÉNÉRALE.. 1

1 Les diatomées 3
1.1 Ecologie des diatomées.. 3
1.2 Caractères généraux des diatomées... 4
1.3 Classification des diatomées 6
1.4 Cycle de vie et reproduction des diatomées... 7
1.5 Utilisation et valorisation des diatomées.. 10
2 **Les diatomées du genre *Haslea*.. 11**
2.1 Caractéristiques générales du genre *Haslea*.. 11
2.2 La diatomée bleue *Haslea ostrearia*.. 12
2.2.1 Caractéristiques morphologiques 12
2.2.2 Écologie 13
2.2.3 Physiologie... 13
2.2.4 Reproduction... 14
2.2.5 Culture.. 14
2.2.6 Distribution géographique 16
2.3 *Haslea ostrearia*, unique en son genre ? La diversité insoupçonnée des *Haslea* bleues. 17
3 Les pigments bleus d'*Haslea*... 21
3.1 La marennine et le verdissement des huîtres.. 21
3.1.1 Les huîtres « fines de claires vertes » 21
3.1.2 La fixation de la marennine sur les branchies 22
3.1.3 La consommation des huîtres vertes, une spécialité française.................... 23
3.2 La marennine et les pigments de type marennine.................................. 24
3.2.1 Production et excrétion de la marennine par Haslea.................................... *24*
3.2.2 Caractérisations préliminaires du pigment.. 25
3.2.3 Techniques de purification du pigment... 26
3.2.4 Un nouveau procédé pour obtenir des solutions enrichies en pigment bleu 27
4 Activités biologiques et valorisations potentielles des *Haslea* bleues et de leurs pigments 30
4.1 Les biotechnologies bleues 30
4.1.1 Le secteur de l'agroalimentaire.. 30

4.1.2 Les secteurs de la cosmétique et de la pharmacologie *32*
4.2 L'aquaculture 33
4.2.1 Les microalgues comme probiotiques *33*
4.2.2 Les Haslea *bleues pour limiter les infections microbiennes ?* *34*
4.3 Perspectives et limites actuelles 35
5 Contexte et objectifs du sujet de recherche 36

CHAPITRE II. MICROALGAL COMPOUNDS AGAINST PATHOGENS: EMPHASIZE ON DISEASES IN AQUACULTURE 39

1 Introduction 43
2 Antibacterial activity from microalgae 46
2.1 Antibacterial activity from microalgae against human pathogenic bacteria 46
2.1.1 Toward improving extraction techniques *49*
2.1.2 Diversity of antibacterial compounds extracted from microalgae *50*
2.2 Use of microalgae against pathogenic bacteria in aquaculture 52
2.2.1 Benefits of the "green-water" technique against bacterial diseases in aquaculture *54*
2.2.2 Microalgae to improve the live-food quality *56*
2.2.3 In vitro efficiency of microalgal compounds against marine bacteria *56*
2.2.4 Interactions between microalgae and marine bacteria *59*
3 3. Antifungal activity from microalgae 61
3.1 Antifungal activity from microalgae against human pathogens 61
3.2 Potential use of microalgae against fungal diseases in aquaculture 64
4 Antiviral activity from microalgae 66
4.1 Antiviral activity from microalgae against human pathogenic viruses 66
4.2 Potential use of microalgae against viruses in aquaculture 70
5 Conclusion 73

CHAPITRE III. EFFECTS OF MARENNINE SOLUTIONS ON THE GROWTH OF MARINE *VIBRIO* 75

CHAPITRE III.Partie 1. Complex relationships between the blue pigment marennine and marie bacteria of the genus *Vibrio* **77**

1 Introduction 80
2 Materials and Methods 82
2.1 *Vibrio* strains 82
2.2 *Vibrio* exposure to Blue Water (BW) solutions 82
2.2.1 Preparation of bacterial inocula *83*
2.2.2 Blue Water (BW) production *83*
2.2.3 Antibacterial essay *84*
2.3 Growth curve analyzes and statistics 85

3 Results **86**
3.1 Different patterns of *Vibrio* growth curves evidenced by the screening experiment 86
3.2 Experiment with BW concentration range 89
4 Discussion **92**
5 Conclusion **95**

CHAPITRE III. Partie 2. Co-culture of *Haslea ostrearia* with an oyster virulent *Vibrio* strain **101**

1 Introduction **104**
2 Results and discussion **106**
3 Conclusion **110**
4 Materials and Methods **111**

CHAPITRE IV. NEW INSIGHTS INTO THE EFFECTS OF MARENNINE SOLUTIONS ON MARINE ORGANISMS **113**

CHAPITRE IV. Partie 1. Harmful or harmless: biological effects of marennine on marine organisms **115**

1 Introduction **118**
2 Materials and Methods **121**
2.1 Preparation of the purified extracellular marennine (EMn), Blue Water (BW) and Concentrated Supernatant (CS) 122
2.2 Exposure of the mussel *Mytilus edulis* larvae to BW 123
2.3 Exposure of the barnacle *Chthamalus bisinuatus* larvae to BW 124
2.4 Exposure of the winter flounder *Pseudopleuronectes americanus* larvae to BW 125
2.5 Exposure of the sea urchins *Sphaerechinus granularis* and *Paracentrotus lividus* to EMn and CS 126
2.6 Exposure of *Vibrio* species to BW and CS 128
2.7 Exposure of fish gill cell lines to EMn 129
2.8 Exposure of the oyster *Crassostrea gigas* haemocytes to EMn 130
2.9 Statistics 131
3 Results **132**
3.1 Differences in the solutions containing the extracellular marennine 132
3.2 Effects of BW on *Mytilus edulis* larvae 132
3.3 Effect of BW on the barnacle *Chthamalus bisinuatus* larvae 135
3.4 Effect of BW on the winter flounder *Pseudopleuronectes americanus* larvae 135
3.5 Effects of CS and purified EMn on *Sphaerechinus granularis* and *Paracentrotus lividus* 136
3.6 Antibacterial effects of BW and CS on *Vibrio* species 139
3.7 Effects of EMn on fish gill cell line RTgill-W1 140

3.8 Effects of EMn on *Crassostrea gigas* haemocytes ... 141
4 Discussion ... 142
4.1 Limitations of the study ... 143
4.2 The unpredictable “Jekyll and Hyde”, good and bad nature of marennine ... 145
5 Conclusion ... 148

CHAPITRE IV. Partie 2. Preliminary results on marennine effects on the mussel *Mytilus edulis* larvae ... **149**

1 Introduction ... 152
2 Results and discussion ... 154
2.1 *Mytilus edulis* larvae challenged with *Vibrio* ... 154
2.2 *Mytilus edulis* exposure to different BW concentrations ... 155
2.2.1 Effect of BW on Mytilus edulis *larval mortality ... 155*
2.2.2 Molecular response of Mytilus edulis *larvae under BW exposure ... 156*
3 Conclusion ... 160
4 Materials and methods ... 161
4.1 Experimental design ... 161
4.1.1 Mytilus edulis *rearing procedure and exposure to BW ... 161*
4.1.2 Mytilus edulis *challenged with* Vibrio *species ... 161*
4.2 Sample collection ... 162
4.3 Studied genes and RT-qPCR ... 163
4.4 Statistical analysis ... 164

CHAPITRE V. DISCUSSION GÉNÉRALE ET PERSPECTIVES ... 165

1 Contexte des travaux de recherche ... 167
2 Bilan des effets biologiques de la marennine ... 169
3 Valorisation de la marennine en aquaculture : limites actuelles et perspectives de recherche ... 173

RÉFÉRENCES BIBLIOGRAPHIQUES ... 179

ANNEXES ... 213

Annexe 1. Falaise et al. (2016), *Marine Drugs* [texte complet]
Annexe 2. Falaise et al. (2019), *Aquatic Toxicology* [texte complet]
Annexe 3. Falaise et al. (2019), *Marine Drugs* [texte complet]
Annexe 4. Prasetiya et al. (sous presse), *Plant Ecology & Evolution* [texte complet]
Annexe 5. Chapitres de Livre : Falaise, Dewi et al. (2018) ; Gastineau et al. (2018)
Annexe 6. Essais préliminaires

LISTE DES FIGURES

Figure 1.1. Diversité de la structure des frustules siliceux des diatomées nettoyés à l'acide et observés en microscopie électronique à balayage (MEB). (A,F) Diatomées centriques radiales, (B,C) diatomées pennées et (D,E) diatomées centriques polaires. Les frustules des diatomées sont constitués de deux valves : l'épivalve et l'hypovalve liées par un ensemble de bandes cingulaires (le cingulum). Barres d'échelles : 10 µm. Cette figure est adaptée de photographies provenant de Kociolek et al. (2015a, 2015b) pour (A,B,C) et de Losic et al. (2009) pour (D,E,F)…………………………………………………………………………...**5**

Figure 1.2. Schéma décrivant le cycle de vie monogénétique diplophasique des diatomées. Durant la multiplication végétative, les cellules filles synthétisent une nouvelle valve à l'intérieur de la valve fournit par la cellule mère. Cela entraîne une diminution progressive de la taille cellulaire de la population. Les cellules pouvant initier le processus de reproduction sexuée (auxosporulation) sont appelées gamétocystes. Chez les diatomées centriques, l'auxosporulation se produit entre deux cellules d'une même lignée sexuelle et la méiose donne deux types de gamètes : un macrogamète (oogonie) et des microgamètes uniflagellés (spermatocytes). Chez les diatomées pennées, l'auxosporulation se produit lorsque deux souches de types sexuels différents (types + et -) sont en contact étroit. La conjugaison des gamètes haploïdes produit un zygote qui se développera en auxospore. Une future cellule initiale aux parois siliceuses se forme dans l'auxospore et sera libérée dans le milieu……...**9**

Figure 1.3. Photographies de la diatomée pennée *Haslea ostrearia*. (A) Observations en microscopie permettant de distinguer deux chloroplastes pariétaux et la présence de vésicules contenant un pigment bleu (la marennine). Barre d'échelle 5 µm. (B-E) Photographies du frustule siliceux en microscopie électroniques à balayage (MEB) avec observations des apex de la cellule (B,C) et du centre de la cellule (D,E) en vue externe (B,D) et interne (C,E). Barres d'échelle 1 µm. Photographie optique communiquée par A. Alverson et photographies MEB d'après Gastineau et al. (2014a)……………………..……**12**

Figure 1.4. (A) Photographie d'une cellule de grande taille de *Haslea ostrearia* prise quelques jours après son isolement dans une claire ostréicole de la région de Bourgneuf (à gauche), et d'une cellule après un an de culture en laboratoire (à droite) présentant une petite taille et une forme atrophiée. (B) Courbe de croissance d'une culture de 200 mL en batch de *H. ostrearia*. Après une croissance exponentielle, la culture entre en phase de déclin sous 15 jours et nécessite un nouveau repiquage………………………………………**15**

Figure 1.5. Répartition mondiale de *Haslea ostrearia* d'après la littérature. La présence de *H. ostrearia* a été déduite de l'observation de diatomées avec des apex bleus ou de la présence d'huîtres aux branchies vertes ; d'après Gastineau et al. (2014a)…………………**16**

Figure 1.6. (A) Comparaisons de données morphométriques des frustules de plusieurs espèces de *Haslea* bleues. (B) Arbre phylogénétique de différentes espèces de *Haslea* basé sur le gène chloroplastique rbcL (les valeurs des bootstraps du maximum de vraisemblance (en noir) et du maximum de parcimonie (en bleu) ont été obtenues avec 1000 réplicats). Photographies au microscope électronique à balayage (MEB) de la fin du raphé central de plusieurs espèces d'*Haslea* référencées dans l'arbre phylogénétique. Une fine barre parallèle au raphé central (flèche blanche) peut être observée chez certaines espèces de diatomées bleues telles que *H. nusantara* et *H. silbo*. Cette caractéristique morphologique est commune aux diatomées non bleues telles que *H. pseudostrearia*. Cette fine barre n'est pas observée parmi les espèces de diatomées bleues génétiquement plus lointaines telles que *H. ostrearia*, *H. karadagensis* et *H. provincialis*. Barres d'échelle = 1 µm. Figures adaptées de Gastineau et al. (2014a) et Falaise et al. (2016a)……………………………..**18**

Figure 1.7. Efflorescences de *Haslea bleues* observées (A) en mer Mediterranée (Corse, France), (B,C) dans l'océan Atlantique (Caroline du Nord, États-Unis) et (D) en mer Adriatique (Dalmatie, Croatie). Photographies communiquées par D. Sirjacobs (A), N. Lindquist (B,C) et Y. Hardivillier (D)…………………………………………..……………**20**

Figure 1.8. (A) Affinage des huîtres dans des bassins d'eau de mer peu profonds appelés « claires » dans la région de Marennes-Oléron. (B) Huître « fine de claire » et huître « fine de claire verte » (label Rouge français) dont les branchies ont été verdies par la présence de *Haslea ostrearia* et de son pigment (marennine) dans les claires lors de l'affinage…………**22**

Figure 1.9. (A) Technique de purification de la marennine intracellulaire (IMn) et extracellulaire (EMn) de *Haslea ostrearia* proposée par Pouvreau et al. (2006b). La *Blue Water* (BW), un surnageant de culture concentré en marennine, est obtenue par ultrafiltration de la EMn. (B) Spectres UV-Visible caractéristiques des fractions de IMn et EMn purifiées, adapté de Pouvreau et al. (2006b)……………………………………………**27**

Figure 1.10. Nouveau procédé de concentration de la marennine et des pigments de « type marennine » des espèces de *Haslea* bleues (n° de brevet 1872316). Procédé développé en collaboration entre le laboratoire Mer, Molécule, Santé (MMS) et l'Institut des Molécules et Matériaux du Mans (I3M) de le Mans Université……………………………………………...**29**

Figure 2.1. Growth inhibition of *Vibrio splendidus* by purified marennine, the blue pigment produced by *Haslea ostrearia*. (A) *V. splendidus* was grown over night at 25°C, cells were then washed with sterile water 2 times and adjusted to an OD_{600}=0.5. Cells were added to wells with marennine in the following concentrations: 0, 0.1, 1, 10, 100 and 1000 µg mL^{-1}. Kinetics were run at OD_{600} for 48 h, with measurements taken every 30 min (n=3). (B) Relative values were graphed in order to account for the absorbance differences due to the

pigment. The effective concentration reducing bacteria growth rate by 50 %, EC50, was estimated at 19.14 µg mL^{-1} (Standard error: 6.736; Pasetto et al., unpublished)...............**57**

Figure 2.2. Relative growth inhibition of three *Vibrio* species: *V. aestuarianus*, *V. coralliilyticus*, *V. tubiashii*, after a 48 h exposition to purified extracellular marennine, produced by the diatom *Haslea ostrearia*. Each strain was grown over night in a Mueller-Hinton Broth medium at 22°C and their concentration was then adjusted to an OD_{600}=0.1. Bacterial cultures were exposed for 48 h to marennine at a concentration of 100 µg mL^{-1} before OD measurement. The relative growth inhibition was assessed in comparison with the growth of the control, not exposed to marennine. A difference of sensitivity between the two *V. aestuarianus* and *V. coralliilyticus* strains was observed. Results are means ± SD, for two separate experiments conducted using triplicates (ANOVA statistical test, *p*-value 0.01 and 7.10^{-4} respectively; Falaise et al., unpublished)...**58**

Figure 3.1. Growth kinetics of four *Vibrio* strains (#) exposed to 0, 10 or 25 µg mL^{-1} of Blue Water (BW) over a 24 h period with growth characteristic features observed for the 30 *Vibrio* strains tested in the screening experiments. BW exposure inhibited the growth of (A) *Vibrio sp.* #36, (B) *Vibrio chagasii* #11 and (C) *Vibrio harveyi* #21 but stimulated the growth of (D) *Vibrio fortis* #9. Vibrios presented a diauxic growth characterized by two distinct exponential growth phases with a diauxic lag phase in between. Results are means ± SE (n=3)..........**86**

Figure 3.2. (A) Relative maximum population size (%) of *Vibrio* strains exposed over a 24 h period to concentrations of 10 and 25 µg mL^{-1} of Blue Water (BW). (B) Graphical illustrations of the dose-response results presented in (A). (C) Relative maximum population size (%) after the first growth phase (see insert) for some of the *Vibrio* strains presenting a diauxic growth curve and exposed to 10 and 25 mg mL^{-1} of BW. (D) Graphical illustrations of the screening experiment results presented in (C). Significant growth inhibitions are presented in black, growth stimulations in red and no observed effects in green. Asterisks (*) indicate a statistical difference with the control and letters (A.B) significant differences between the two BW concentrations tested. Values are means ± SE (n=3)..**88**

Figure 3.3. Growth kinetics and dose-response curves of *Vibrio* strains exposed to a concentration range (µg mL^{-1}) of Blue Water (BW) over a 24 h period presenting: (A,B) a linear response, (C) a "U shape" response or (D) a hormetic response. Growth of *V. harveyi* #23 was analyzed in two independent phases, with phase 1 from 0 to 9 h and phase 2 from 9 to 24 h. Values in the growth curves are means ± SD (n=3)......................................**91**

Figure 3.4. Observation of *Haslea ostrearia* cells under light microscopy (Gx400). (A) *H. ostrearia* cell under low light treatment (LL; 80 µmol m^{-2} s^{-1}). Chloroplasts (chl) occupy a large part of the cell and cytoplasmic vesicles containing marennine (v) are very reduced. (B) *H. ostrearia* under high light treatment (HL; 580 µmol m^{-2} s^{-1}). Chloroplasts (chl) are reduced,

many cytoplasmic vesicles containing marennine (v) are observable, with those at the ends of the cell particularly developed. Scale bars 10 µm……………………………………….**105**

Figure 3.5. Growth kinetic of *Vibrio aestuarianus* (strain 12/016) over a 24 h period under Blue Water (BW) exposure (µg mL^{-1}). BW had no effect on *V. aestuarianus* growth with 6.8 ± 2.9 % of relative inhibition at the higher concentration tested (*p*-value 0.4). Results are means ± SE (n=3)…………………………………………………………………………………**106**

Figure 3.6. *Haslea ostrearia* cultures under different light treatments: high light (HL) and low light (LL) and co-cultured with *Vibrio aestuarianus* (HLV and LLV). (A) *H. ostrearia* concentration (cell mL^{-1}) in a semi-continuous culture mode and sub-cultured every 3 days (d 6, d 9 and d 12). (B) Extracellular marennine concentration (µg mL^{-1}) in the culture media of the four different treatments. (C) Correlation between *H. ostrearia* concentration (cell mL^{-1}) and marennine concentration (µg mL^{-1}). (D) Marennine production per *H. ostrearia* cell (µg $cell^{-1}$). Results in (A) and (B) are means ± SE (n=3) and graphics in (C) and (D) gather results from d 12 and d 15 (n=6)…………………………………………………………………**107**

Figure 3.7. (A) Bacterial concentration (CFU mL^{-1}) in *H. ostrearia* cultures under different light treatments: High Light (HL) or Low Light (LL) and in co-culture with *Vibrio aestuarianus* (HLV, LLV). The total bacterial concentration (Total) was calculated after the associated bacteria (AB) concentration and the *V. aestuarianus* (Vibrio) concentration. (B) Number of bacteria per *H. ostrearia* cell (bacteria $cell^{-1}$) in the four different treatments. Results are means ± SE and gather data from d 12 and d 15 (n=6)……………………………………**108**

Figure 4.1. Underwater pictures of natural blue *Haslea* blooms forming biofilms observed (A) in macro-algae *Padina sp*. in the Mediterranean Sea, Corsica (France) and (B) on sediments in the Beaufort Strait, North Carolina (United States)…………………………………………**118**

Figure 4.2. Exposure of the mussel *Mytilus edulis* embryos to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 48 h period. (A) Percentage of embryos that reached the D-larval stage (grey bars) and percentage of mortality (black bars). (B) Length of the larvae that reached the D stage (µm). Values are means ± SE (n=3). Error bars with different lower case letters are significantly different………………………………………..………**133**

Figure 4.3. Exposure of the mussel *Mytilus edulis* D-larvae to different concentrations (µg mL^{-1}) of Blue Water (BW). (A) Percentage of swimming D-larvae exposed during 24 h (light grey bars) and 72 h (dark grey bars) to BW. Values are means ± SE (n=6). Error bars with different lower case letters are significantly different. (B) Observation of D-larvae of the control condition (left picture) and exposed to 5 µg mL^{-1} of BW (right picture) after 24 h under light invert microscopy. D-larvae exposed to 5 µg mL^{-1} of BW were totally motionless with marennine agglutinated on the velum (arrow). Scale bars: 100µm…………………………..**134**

Figure 4.4. Mussel *Mytilus edulis* veliger larvae exposed to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 5 d period. (A) Percentage of veliger larvae mortality and (B) veliger larvae mean length after BW exposure. Values are means ± SE (n=3). Error bars with different lower case letters are significantly different..**134**

Figure 4.5. Percentage of mortality of the barnacle *Chthamalus bisinuatus* larvae exposed to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 9 d period. Results are means ± SE (n=4)……………………………………………………………………………………..**135**

Figure 4.6. Winter flounder *Pseudopleuronectes americanus* larvae exposed to different concentration (µg mL^{-1}) of Blue Water (BW) over a 14 d period. (A) Mean lengths (mm) of *P. americanus* larvae. (B) Bacterial load (bacteria mL^{-1}) in larval tanks and (C) larval energy reserves: percentages of triglyceride (TAG), sterol (ST), acetone mobile polar lipids (AMPL), and phospholipids (PL) and TAG/ST ratio. Results are means ± SE (n=3)…………………**136**

Figure 4.7. Sea urchin *Sphaerechinus granularis* eggs exposed to different concentrations (µg mL^{-1}) of *Haslea ostrearia* Concentrated Supernatant (CS) before fertilization. (A) Fertilization rate and (B) observation under phase contrast (left panels) and fluorescence (right panels) microscopy (Gx400) of *S. granularis* eggs of the control condition (upper raw) and exposed to CS at 50 µg mL^{-1} (lower raw) 2 h post exposure to CS. Eggs were fixed in a DNA fluorescent stain (Hoechst fixative) to observe cell nuclei of the control dividing egg and spermatozoa agglutinated around the unfertilized egg exposed to 50 µg mL^{-1} of CS. Values in (A) are means ± SE (n=3). Error bars with different lower case letters are significantly different. Scale bars in (B): 25 µm…………………………………………………………………**137**

Figure 4.8. Exposure of newly fertilized sea urchin *Sphaerechinus granularis* eggs to different concentrations (µg mL^{-1}) of concentrated supernatant (CS) of *Haslea ostrearia* culture. (A) Kinetic of first cleavage of *S. granularis* eggs exposed to the CS 10 min post fertilization. (B) Observation of *S. granularis* embryos under phase contrast rosmiccopy 8 h post exposure to CS (Gx200) and (C) 48 h (top raw) and 72 h (lower raw) post exposure to the CS (Gx400). Embryos of the “unwashed” conditions were still incubated in the CS while embryos of the “washed” condition were rinsed 6 h post exposure to the CS. The kinetics of first cleavage presented in (A) is representative of 3 replicate experiments. Views in (B) and (C) are representative of the observed stages, scale bars: 50 µm…………………………...**139**

Figure 4.9. Relative growth inhibition of the bacteria *Vibrio chagasii*, *Vibrio crassostreae* and *Vibrio sp.* exposed to 10 µg mL^{-1} of Blue Water (BW) and 10 µg mL^{-1} of Concentrated Supernatant (CS) over a 24 h period. Values are means ± SE (n=3)………………………..**140**

Figure 4.10. Percentage of viable cells of the fish gill cell line Rtgill-W1 exposed to different concentrations (µg mL^{-1}) of purified Extracellular Marennine (EMn). Values are means ± SE (n=4)………………………………………………………………………………………………**140**

Figure 4.11. Percentage of viable oyster *Crassostrea gigas* hemocytes exposed to different concentrations (µg mL^{-1}) of purified Extracellular Marennine (EMn). Values are means and SE (n=6)……………………………………………………………………………………..……**141**

Figure 4.12. Percentage of mortality of *Mytilus edulis* veliger larvae over a 3 d exposure period to Blue Water (BW) at 0.5 µg mL^{-1} and challenged with *Vibrio splendidus* or *Vibrio aestuarianus.* Results are means ± SE (n=3)…………………..………………………………**154**

Figure 4.13. Percentage of relative mortality of *Mytilus edulis* veliger, pediveliger and metamorphosed larvae over a 3 d exposure period to different Blue Water (BW) concentrations (µg mL^{-1}) and of veliger larvae challenged with *Vibrio splendidus* (insert). Results are means ± SE (n=3). Error bars with different lower case letters in are significantly different and asterisks indicate significant difference with the control (p-value < 0.05)………………………………………………………………………….…………………**156**

Figure 4.14. Relative expression of (A) HSP70, (B) SOD, (C) CTPB and (D) ASB genes in *Mytilus edulis* veliger, pediveliger and metamorphosed larvae exposed over a 3 d exposure period to different concentrations (µg mL^{-1}) of Blue Water (BW). Relative gene expression data were estimated by comparison with the corresponding level from larvae of the Control treatment at each developmental stage. Results are means ± SE (n=3)…………………....**158**

Figure 5.1. Exemple d'utilisation de la marennine pour lutter contre une infection bactérienne dans un bassin d'élevage de bivalves. (A) Pour une application de la marennine en aquaculture les doses administrées devront être maîtrisées, comme par exemple en délivrant le pigment sous forme de granulés afin d'éviter tout effet nocif sur les animaux élevés et notamment (B) sur les jeunes stades de développement particulièrement sensibles. La marennine pourrait interférer sur la croissance des bactéries : (C) rôle direct sur les membranes bactériennes ; (D) rôle indirect en formant des complexes avec des nutriments du milieu ; (E) rôle indirect en perturbant la communication (quorum sensing) des bactéries. (F) La marennine pourrait diminuer le pouvoir infectieux des bactéries et (G) exercer un effet prophylactique sur les animaux élevés. (H) Le pigment possédant des activités allélopathiques, les microalgues utilisées pour nourrir les animaux dans les bassins devront être choisies en fonction de leur sensibilité à la marennine…...…………………….**173**

LISTE DES TABLEAUX

Table 2.1. Antibacterial activity from microalgae against human pathogenic bacteria..........**47**

Table 2.2. Antibacterial activity from microalgae against diseases in aquaculture………......**53**

Table 2.3. Antifungal activity from microalgae..**62**

Table 2.4. Antiviral activity from microalgae...**68**

Table 4.1. Summary of the marennine based solutions and marine organisms used in the present study..**121**

Table 4.2. Summary of the marennine based solutions effects on the marine organisms tested...**142**

Table 4.3. Details of genes and primers used in the study..**164**

LISTE DES FIGURES ET TABLEAUX SUPPLEMENTAIRES

Supplementary Figure 3.1. Growth curves of 30 *Vibrio* strains (#) from 10 different species exposed over a 24 h period to 0, 10 or 25 µg mL^{-1} of Blue Water (BW), the concentrated supernatant of *Haslea ostrearia* containing the extracellular marennine. Results are means ± SE (n=3)……………………………………………………………………………………………**96**

Supplementary Figure 3.2. Growth kinetics and dose-response curves of *Vibrio* strains exposed to a concentration range (µg mL^{-1}) of Blue Water (BW) over a 24 h period. Values are means ± SD (n=3). ………………………………………………………………………….**98**

Supplementary Table 3.1. Preferential distribution, virulence to oysters and Blue Water (BW) effect over a 24 h period and over the first phase of growth of the 30 *Vibrio* strains tested...**99**

LISTE DES PRODUCTIONS SCIENTIFIQUES

- ## Articles Scientifiques

Prasetiya, F.S., Gastineau, R., Poulin, M., Lemieux, C., Turmel, M., Syakti, A.D., Hardivillier, Y., Widowati, I., Risjani, Y., Iskandar, I., Subroto, T., Falaise, C., Mouget, J.-L., Leignel, V., **sous press**. *Haslea nusantara*, a new blue diatom from the Java Sea, Indonesia: Morphology, biometry and molecular characterization. *Plant Ecology and Evolution*.

Falaise C., James A., Travers M-A., Zanella M., Badawi M., Mouget J-L., **2019**. Complex relationships between the blue pigment marennine and marine bacteria of the genus *Vibrio*, *Marine Drugs* 17(3), 160-172.

Falaise, C., Cormier, P., Tremblay, R., Audet, C., Deschênes, J.-S., Turcotte, F., François, C., Seger, A., Hallegraeff, G., Lindquist, N., Sirjacobs, D., Gobert, S., Lejeune, P., Demoulin, V., Mouget, J.-L., **2019**. Harmful or harmless: Biological effects of marennine on marine organisms. *Aquatic Toxicology* 209, 13–25.

Falaise, C., François, C., Travers, M.-A., Morga, B., Haure, J., Tremblay, R., Turcotte, F., Pasetto, P., Gastineau, R., Hardivillier, Y., Leignel, V., Mouget, J.-L., **2016**. Antimicrobial compounds from eukaryotic microalgae against human pathogens and diseases in aquaculture. *Marine Drugs* 14, 159-186.

- ## Chapitres de Livre

Gastineau, R., Prasetiya, F.S., Falaise, C., Cognie, B., Decottignies, P., Morancais, M., Méléder, V., Davidovich, N.A., Turcotte, F., Tremblay, R., Pasetto, P., Dittmer, J., Bardeau, J.-F., Pouvreau, J.-B., Mouget, J.-L., **2018**. Chapter 16. Marennine-Like Pigments: Blue Diatom of Green Oyster Cult?, in: *Blue Biotechnology, Production and Use of Marine Molecules*. Stéphane La Barre and Stephen S. Bates (Eds.). Wiley-VCH, Weinheim, Germany.

Falaise, C., Dewi, I.C., Hellio, C., Bourgougnon, N., Mouget, J.-L., **2018**. Chapter 12. Anticancer, antiviral, antibacterial and antifungal properties in microalgae, in: *Microalgae in Health and Disease Prevention*. Levine I. and Fleurence J. (Eds.). Elsevier Science, Amsterdam, Netherlands.

- **Dépôt de Brevet Français**

Falaise C., Zebiri L., Jacquette B., Pasetto P., Mouget J-L., Pigment bleu hydrosoluble de type marennine – *Haslea*. **Numéro : 1872316**, dépôt : décembre **2018**.

- **Communications Orales**

Falaise C., James A., Tremblay R., Cormier P., Mouget J-L., Harmful or harmless: effects of marennine on marine invertebrates and bacteria from the genus *Vibrio*. *67th Annual Meeting of the British Phycological Society (BPS)*, 7th-10th January **2019**, SAMS-Oban, Scotland.

Mouget J-L., Falaise C., Perkins R., Pasetto P., Witkowski A., Gastineau R., The genus *Haslea*: dress up something old as something new. *67th Annual Meeting of the British Phycological Society (BPS)*, 7th-10th January **2019**, SAMS-Oban, Scotland.

Falaise C., Tremblay R., Cormier P., François C., James A., Seger A., Hayashi H., Hallegraeff G., Mouget J.L., Can the blue diatom *Haslea ostrearia* with strong biological activity be considered as a harmful alga? *International Conference on Harmful Algae (ICHA)*, 21st-26th October **2018**, Nantes, France.

Zebiri L., Falaise C., Yusuf M., Dittmer J., Pasetto P., Mouget J-L., Dr Marennine and Mr Blue pigment: a diatom polymeric compound fit for food or for feed? *Biopolymers*, 29th November-1st December **2017**, Nantes, France.

Prasetiya F., Mouget JL., Decottignies P., Morançais M., Comeau L., Turcotte F., Gastineau R., Iskandar, Subroto T., Falaise C., Tremblay R., & Cognie B., Greening by the blue diatom *Haslea ostrearia*: consequences on bivalve feeding behaviour, physiological and biochemichal state. *Molluscan Forum*, 22th November **2017**, London, England.

Falaise C., Cormier P., Mouget J.L., Leignel V., Hardivillier Y., Lemarchand K., Tremblay R., Effects of marennine, the blue pigment synthesized by the diatom *Haslea ostrearia*, on early developmental stages of the mussel *Mytilus edulis* and the sea urchin Sphaerechinus *granularis*. *Physiomar*, 18th-22th September **2017**, Cambridge, England.

Dewi I.C., Falaise C., François C., Travers M.-A., Morga B., Haure J., Widowati I., Yanuhar U., Hardivillier Y., Leignel V., Mouget J.-L., Antimicrobial compounds from microalgae: potential application of the diatom *Haslea ostrearia* in aquaculture. *International Society of Applied Phycology (ISAP)*, 18th-23th June **2017**, Nantes, France.

- **Communications Écrites (Posters)**

Falaise C., Leignel V., Hardivillier Y., Mouget J-L., Marennine : Docteur Jekyll ou Mister Hyde ? Expose ta thèse, 17 Novembre **2017**, Le Mans, France.

Falaise C., Zebiri L., François C., Travers M.A., Dittmer J., Pasetto P., Mouget J.L., Marennine, the still mysterious pigment with antibacterial activities from *Haslea ostrearia*. *ANTIMIC*, 21st-23th June **2017**, Quebec city, Canada.

Falaise C., Gastineau R., Poulin M., Leignel V., Hardivillier Y., Widowati, I., Syakti, A.D., Lemieux, C., Turmel, M., Mouget, J.L, *Haslea nusantara sp.* nov., a novel species of blue diatom from Indonesia. *24th International Diatom Symposium*, 21st-26th August **2016**, Quebec city, Canada.

CHAPITRE I.

INTRODUCTION GÉNÉRALE

1 LES DIATOMEES

Les diatomées sont des algues microscopiques caractérisées par la présence d'une paroi cellulaire en silice, nommée le frustule. Elles sont communément appelées « microalgues brunes » en raison de la couleur brunâtre donnée par la fucoxanthine, un pigment qui masque la couleur verte des pigments chlorophylliens des cellules (Round et al., 1990). Les diatomées forment un groupe de microalgues très diversifié dont le nombre est estimé potentiellement à 200 000 espèces et dont moins de 10 % d'entre elles seraient décrites (Guiry et al. 2012 ; Mann et Vanormelingen, 2013 ; Medlin, 2018).

1.1 Ecologie des diatomées

Les diatomées sont majoritairement unicellulaires mais peuvent parfois former des colonies (Hoagland et al., 1982). Elles peuvent être soit planctoniques et se développer dans la colonne d'eau, soit benthiques et croître sur des substrats rocheux (*e.g.* épilithiques), sédimentaires (*e.g.* épipéliques ou épisammiques) ou sur d'autres végétaux (*e.g.* épiphytes). Les diatomées sont ubiquistes et sont retrouvées dans presque tout type de milieu : des eaux douces aux marais salants, des mers tropicales aux glaces polaires (Smol et Stoermer, 2010). On retrouve également des diatomées en association avec d'autres organismes vivants, par exemple dans le plumage d'oiseaux marins (Croll et Holmes, 1982), sur la peau de cétacés (Bennett, 1920) ou sur la carapace de tortues (Wetzel et al., 2010). Elles peuvent également être endosymbiotes de foraminifères (Lee, 2011), parasites d'éponges (Bavestrello et al., 2000) ou bien hôtes de cyanobactéries (Stewart et al., 1983).

Principaux constituants du phytoplancton et à la base des réseaux trophiques marins, les diatomées sont responsables d'environ 45 % de la production primaire des océans en ne représentant pourtant que 1 % de la biomasse terrestre photosynthétique (Benoiston et al., 2017 ; Field et al., 1998). Le microphytoplancton (principalement constitué de diatomées) pourrait même contribuer jusqu'à 70 % de la production primaire dans les

systèmes de remontées d'eau (*upwelling*) côtiers et 50 % dans les régions tempérées et subpolaires durant les saisons printanières et estivales (Uitz et al., 2010). Les diatomées représentent des maillons essentiels des cycles de la silice (Tréguer et De La Rocha, 2013) et du carbone océaniques, en contribuant notamment à la production de carbone organique dans les zones euphotiques profondes par transport à travers les réseaux trophiques (Tréguer et al., 2018).

1.2 Caractères généraux des diatomées

"*Few objects are more beautiful than the minute siliceous cases of diatoms: were they only created to be admired under microscope?*" (Darwin, 1859).

Le mot diatomée provient du grec *diatomos* qui signifie « deux parts», en référence à l'exosquelette de la microalgue constitué de deux valves siliceuses distinctes : l'épivalve et l'hypovalve. L'hypovalve (partie inférieure) a une taille sensiblement inférieure et s'insère à l'épivalve (partie supérieure). Les deux valves sont liées l'une à l'autre par des ceintures connectives qui forment le cingulum (Figure 1.1). La taille des diatomées varie généralement de 2 à 500 µm mais peut pour de rares espèces (*e.g. Ethmodiscus rex*, *Thalassiothrix antarctica*) atteindre une taille macroscopique de 2 à 5 mm (Sarthou et al., 2005 ; Smetacek, 2000).

Selon la forme de leur frustule, les diatomées peuvent être catégorisées en trois groupes morphologiques : les diatomées pennées de forme allongée et à symétrie bilatérale, les centriques radiales qui présentent des valves circulaires à symétrie radiale, et les centriques polaires dont les valves n'ont généralement pas une forme circulaire et présentent une symétrie multipolaire (Figure 1.1).

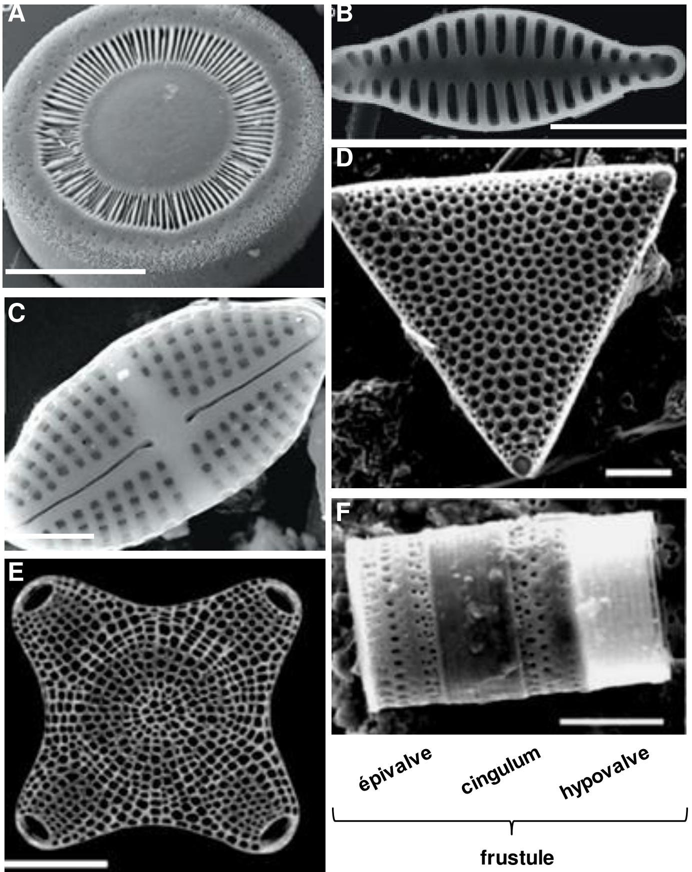


Figure 1.1. Diversité de la structure des frustules siliceux des diatomées nettoyés à l'acide et observés en microscopie électronique à balayage (MEB). **(A,F)** Diatomées centriques radiales, **(B,C)** diatomées pennées et **(D,E)** diatomées centriques polaires. Les frustules des diatomées sont constitués de deux valves : l'épivalve et l'hypovalve liées par un ensemble de bandes cingulaires (le cingulum). Barres d'échelles : 10 µm. Cette figure est adaptée de photographies provenant de Kociolek et al. (2015a, 2015b) pour (A,B,C) et de Losic et al. (2009) pour (D,E,F).

Les frustules des diatomées présentent une grande variété de formes. Ils sont percés de pores et ornementés d'aréoles ou encore de stries qui constituent des motifs propres à chaque espèce. Ces caractères morphologiques, visibles en microscopie électronique à balayage (MEB), sont en effet utilisés pour identifier les diatomées au niveau spécifique. Les pores ou les conduits présents sur le frustule permettent à la cellule de communiquer et d'échanger avec le milieu extérieur en captant notamment des

éléments dissous dans l'eau et en sécrétant des composés cellulaires dans le milieu extérieur. C'est le cas du raphé (*i.e.* fente dans la paroi de la valve mettant en contact la cellule et le milieu extérieur) chez les diatomées pennées ou du *fultoportula* et du *rimoportula* (*i.e.* structures tubulaires par lesquelles s'effectuent la sécrétion de substances vers le milieu extérieur) chez les diatomées centriques (Round et al., 1990 ; Ruck et Theriot, 2011). Bien que la fonction pour la cellule de cet exosquelette siliceux soit encore mal connue, il est suggéré que le frustule protégerait les diatomées de potentiels prédateurs (Hamm et al., 2003) ou constituerait une barrière physique limitant les infections virales ou fongiques (Hanic et al., 2009). Il est également démontré que la silice biogène (SiO_2) qui constitue les frustules protège les cellules des dommages induits par les rayonnements ultraviolets (Ingalls et al., 2010). Le frustule pourrait également permettre le positionnement des diatomées dans la colonne d'eau en contenant la pression de turgescence engendrée par le protoplaste (Raven et Waite, 2004).

1.3 Classification des diatomées

Les diatomées auraient émergé d'un ancêtre commun il y a plus de 250 millions d'années (Medlin, 2011). Il a été suggéré que l'origine des diatomées résulterait d'endosymbioses multiples entre des cellules hétérotrophes et des ancêtres autotrophiques de microalgues vertes puis de microalgues rouges (Armbrust, 2009 ; Benoiston et al., 2017). Cependant, l'origine endosymbiotique avec un ancêtre des microalgues vertes est aujourd'hui encore soumise à débat (Deschamps et Moreira, 2012 ; Kociolek et Hamsher, 2017).

La classification des diatomées est elle aussi en constante évolution, au gré de l'identification de nouvelles espèces et des progrès technologiques et scientifiques. Avant les années 90, la classification des diatomées était principalement basée sur des critères morphologiques par l'observation des frustules en MEB selon une morpho-taxonomie établie par Round et al. (1990). Les diatomées étaient alors catégorisées en tant que division (Bacillariophyta) regroupant trois classes : les pennées raphides, avec la

présence d'un raphé sur au moins l'une des valves (Bacillariophyceae), les pennées araphides, sans raphé (Fragilariophyceae) et les diatomées centrées (Coscinodiscophyceae). Cette classification basée sur la morphologie a été sujette à modification (Hoek et al., 1995) jusqu'à être remise en cause au début des années 2000 avec l'émergence des techniques de phylogénies moléculaires (Kooistra et al., 2003). Une nouvelle classification des diatomées, basée sur des analyses moléculaires, a donc récemment été établie par Medlin (2016) en classant les diatomées dans la lignée des Héterokontophytes, division des Bacillarophyta :

- Sous-division des Coscinodiscophytina
    - Classe des Coscinodiscophyceae (centrées radiales)
- Sous-division des Bacillariophytina
    - Classe des Mediophyceae (centrées polaires)
    - Classe des Bacillariophyceae (pennées)
        - Sous-classe des Urneidophycidae (pennées araphides)
        - Sous-classe des Fragilariophycidae (pennées araphides)
        - Sous-classe des Bacillariophycidae (pennées raphides)

1.4 Cycle de vie et reproduction des diatomées

Les diatomées sont des cellules diploïdes qui se multiplient par divisions végétatives. Lors de la mitose, les valves du frustule de la cellule mère deviendront les épivalves des deux cellules filles qui synthétiseront chacune l'hypovalve manquante, de plus petite taille (Figure 1.2). De ce fait, les phases de multiplications asexuées entrainent une diminution progressive de la taille cellulaire moyenne de la population ; jusqu'à atteindre une taille minimale critique au-delà de laquelle les plus petites cellules ne sont plus viables. Afin de restaurer des cellules de grandes tailles, les diatomées effectuent périodiquement une phase de reproduction sexuée, appelée auxosporulation. La cellule doit atteindre une gamme de taille favorable, spécifique à chaque espèce, pour que la reproduction sexuée

se produise (Kaczmarska et al., 2013). Ces cellules, dont la taille optimale est atteinte pour initier la méiose et la production de gamètes sont les gamétocystes.

Les diatomées centrées et pennées font appel à deux processus distincts lors de la reproduction sexuée :

Les diatomées centrées sont homothalliques, c'est-à-dire qu'il n'existe pas de lignées de types sexuels différents. Au moment de la reproduction sexuée, les diatomées centrées se différencient en cellule mâle ou en cellule femelle et produisent, suite à la méiose, deux types de gamètes : les spermatocytes (*e.g.* petits gamètes flagellés mâles) et les oogonies (*e.g.* gamètes femelles non mobiles de grande taille). La conjugaison des gamètes entraîne la formation d'un zygote diploïde (Figure 1.2).

Les diatomées pennées sont majoritairement hétérothalliques, c'est à dire que deux types de gamètes sont produits par deux cellules de types sexuels différents. Au moment de l'auxosporulation, les gamétocystes de types sexuels différents (type + et type -) s'apparient deux à deux, ce qui amorcera le processus de la méiose. Dans certains cas (anisogamie), les gamétocystes « + » produisent des gamètes actifs (mobiles) et les gamétocystes « - » des gamètes passifs (immobiles). Après ouverture des valves des gamétocytes, les gamètes « + » se déplacent pour fusionner avec les gamètes « - », restés associés au frustule du gamétocyste « - ». La conjugaison des gamètes entraîne la formation de deux zygotes diploïdes (Figure 1.2). Dans d'autres cas, chaque gamétocyste produit un gamète mobile et un autre immobile (isogamie).

Chez les diatomées centrées comme pennées le zygote diploïde s'allonge et se développe en auxospore dans laquelle une cellule initiale de grande taille se forme. La cellule initiale est ensuite libérée dans le milieu et formera à son tour une nouvelle lignée par multiplications végétatives (Figure 1.2).

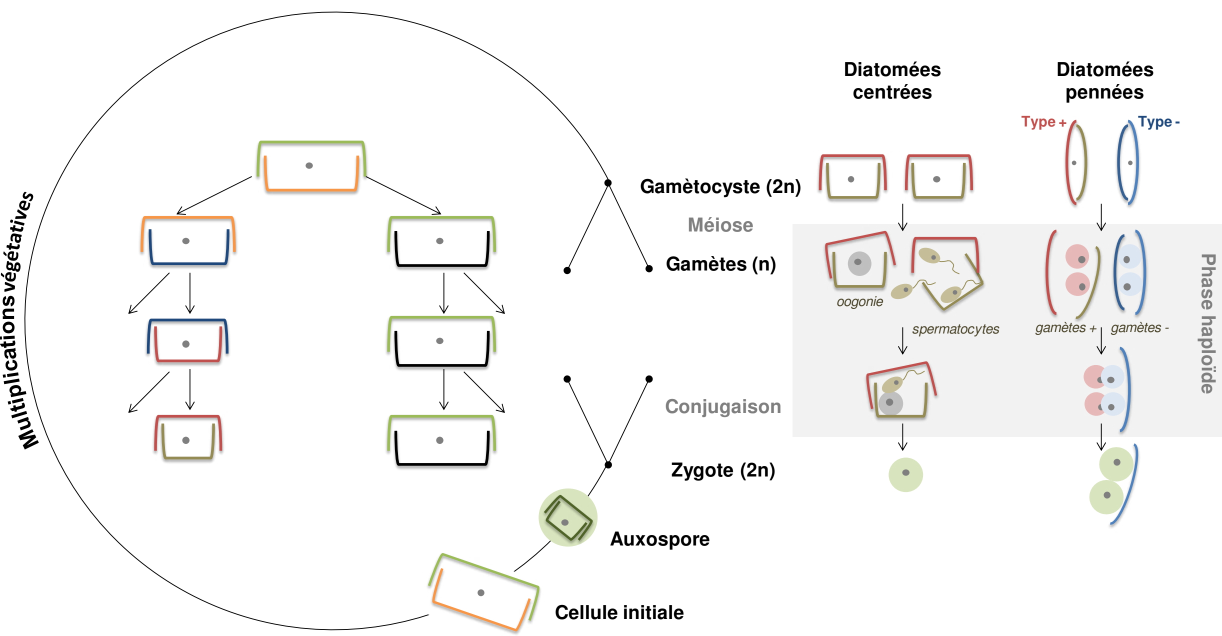


Figure 1.2. Schéma décrivant le cycle de vie monogénétique diplophasique des diatomées. Durant la multiplication végétative, les cellules filles synthétisent une nouvelle valve à l'intérieur de la valve fournit par la cellule mère. Cela entraîne une diminution progressive de la taille cellulaire de la population. Les cellules pouvant initier le processus de reproduction sexuée (auxosporulation) sont appelées gamétocystes. Chez les diatomées centriques, l'auxosporulation se produit entre deux cellules d'une même lignée sexuelle et la méiose donne deux types de gamètes : un macrogamète (oogonie) et des microgamètes uniflagellés (spermatocytes). Chez les diatomées pennées, l'auxosporulation se produit lorsque deux souches de types sexuels différents (types + et -) sont en contact étroit. La conjugaison des gamètes haploïdes produit un zygote qui se développera en auxospore. Une future cellule initiale aux parois siliceuses se forme dans l'auxospore et sera libérée dans le milieu. Schéma C. Falaise©.

1.5 Utilisation et valorisation des diatomées

Divers secteurs s'intéressent à l'utilisation et à la valorisation des diatomées. Les structures à potentiel de valorisation sont soit la cellule vivante entière, soit des métabolites produits par la diatomée ou encore le frustule siliceux.

La principale utilisation des diatomées est dans le secteur de l'aquaculture, notamment pour les écloseries de bivalves. Elles peuvent être délivrées dans les bassins d'élevage comme aliment « fourrage » ou intégrées dans la composition de compléments alimentaires (Bellou et al., 2016 ; Muller-Feuga, 2000). Les diatomées sont en effet réputées pour leur contenu lipidique riche en acides gras polyinsaturés, essentiels à la croissance des larves (Brown et al., 1997 ; Guedes et Malcata, 2012). Les diatomées benthiques sont également utilisées comme indicateur de la qualité biologique de l'eau. L'indice biologique des diatomées (IBD) a été normalisé par l'AFNOR pour estimer la qualité des cours d'eau de France métropolitaine (Norme AFNOR (NF T90-354), 2007, 2016). En paléontologie, la diatomite (*e.g.* roche formée par les frustules des diatomées fossiles) peut être étudiée pour la reconstitution de paléo-environnements (Mackay et al., 2003 ; Smol et Stoermer, 2010). Cette roche est également connue pour être utilisée, en association avec de la nitroglycérine, dans la fabrication de la dynamite. L'utilisation des frustules siliceux intéresse également le secteur des nanotechnologies pour des applications dans des composés électroniques, optiques ou encore dans la fabrication de batteries (La Barre et Bates, 2018). Certains composés produits par les diatomées peuvent également trouver une application en pharmacologie. C'est notamment le cas de l'acide domoïque produit par certaines diatomées du genre *Pseudo-nitzschia* et dont des applications sont envisagées en neuropharmacologie. En effet, ce composé, ingéré suite à la consommation de fruits de mer contaminés, provoque des pertes de mémoire parfois permanentes et pourrait représenter un modèle pour l'étude des maladies de d'Alzheimer ou de Parkinson (Lefebvre et Robertson, 2010 ; Trainer et al., 2012).

2 LES DIATOMEES DU GENRE *HASLEA*

2.1 Caractéristiques générales du genre *Haslea*

Les diatomées du genre *Haslea* appartiennent à la famille des *Naviculaceae*. Anciennement classées au sein des genres *Navicula, Amphipleura* ou encore *Pleurosigma*, elles ont été inclues dans un nouveau genre par Simonsen (1974) suite à l'observation approfondies des frustules en MEB. La dénomination de ce nouveau genre nommé *Haslea* fût donnée en l'honneur de la biologiste Norvégienne Grethe Rytther Hasle (1920-2013).

Morphologiquement, les diatomées du genre *Haslea* sont caractérisées par leur forme pennée en navette, et possèdent deux chloroplastes pariétaux. Les observations en MEB du frustule permettent de distinguer un raphé droit avec une terminaison peu accentuée sur chacune des deux valves. La partie externe du frustule est composée de stries siliceuses longitudinales parallèles au raphé tandis que la partie interne est creusée de rangées d'aréoles quadrilatères lui donnant un aspect gaufré (Figure 1.3). Ces deux parties sont reliées entre elles par des piliers.

Près d'une trentaine d'espèces de diatomées du genre *Haslea* ont été décrites (AlgaeBase, 2018). Ces différentes espèces peuvent présenter des variations sur les plans morphologiques, écologiques ou physiologiques (Gastineau et al., 2014a). Par exemple, l'espèce *Haslea wawrikae* est une diatomée planctonique, contrairement à la majorité des espèces de ce genre qui présente un mode de vie benthique (Simonsen, 1974). L'espèce *Haslea gigantea* est quant à elle catégorisée comme constituant du mésoplancton en raison de sa grande taille pouvant atteindre 500 µm, en opposition aux espèces du genre ne mesurant majoritairement qu'une centaine de micromètres (Simonsen, 1974). L'espèce *Haslea ostrearia* présente la particularité de synthétiser et de libérer dans son milieu un pigment bleu hydrosoluble, la marennine (Simonsen, 1974).

2.2 La diatomée bleue *Haslea ostrearia*

2.2.1 Caractéristiques morphologiques

Les dimensions de *Haslea ostrearia* en milieu naturel varient de 60 à 120 µm de longueur et 6 à 12 µm de largeur (Robert, 1973, 1978). En laboratoire, la longueur maximale d'une cellule initiale après reproduction sexuée est de 138 à 140 µm, et la taille minimale en deçà de laquelle une cellule ne peut plus se diviser est de 17 µm (Davidovich et al., 2009 ; Neuville et Daste, 1978). En microscopie optique deux chloroplastes pariétaux sont observables ainsi que des vésicules bleues, essentiellement concentrées aux apex de la cellule, contenant la marennine (Figure 1.3).

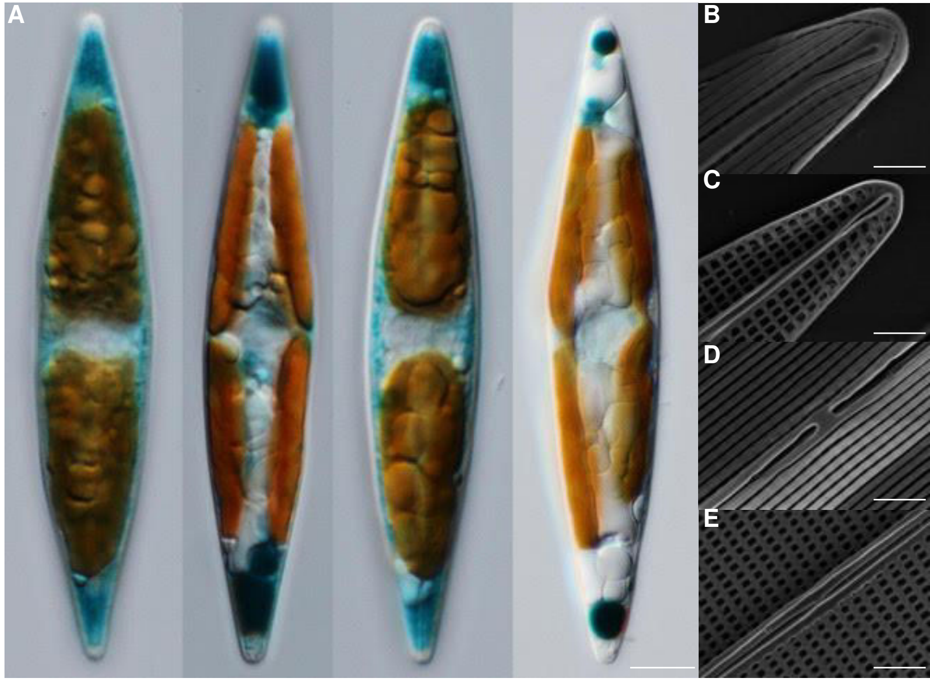


Figure 1.3. Photographies de la diatomée pennée *Haslea ostrearia*. **(A)** Observations en microscopie permettant de distinguer deux chloroplastes pariétaux et la présence de vésicules contenant un pigment bleu (la marennine). Barre d'échelle 5 µm. **(B-E)** Photographies du frustule siliceux en microscopie électroniques à balayage (MEB) avec observations des apex de la cellule (B,C) et du centre de la cellule (D,E) en vue externe (B,D) et interne (C,E). Barres d'échelle 1 µm. Photographie optique communiquée par A. Alverson et photographies MEB d'après Gastineau et al. (2014a).

2.2.2 Écologie

Haslea ostrearia est une diatomée benthique qui peut former des biofilms sur des surfaces sédimenteuses ou sur des macroalgues, ou d'autres supports immergés. Elle peut être occasionnellement rencontrée en milieu pélagique lors de la remise en suspension des sédiments ou par brassage des courants (Robert, 1983). *Haslea ostrearia* est une diatomée euryhaline qui peut s'acclimater à des salinités allant de 1,5 à 4,0 % (Wraige et al., 1998). Elle supporte également des gammes d'intensités lumineuses variant de 20 à 750 µmol m^{-1} s^{-1} (Mouget et al., 1999) et possède des mécanismes de défense contre le stress UV (Rech et al., 2005).

2.2.3 Physiologie

Haslea ostrearia est photosynthétique et photoautotrophe, bien qu'elle puisse présenter une photohétérotrophie ou photomixotrophie pour certaines substances azotées et carbonées (Neuville et Daste, 1978 ; Robert et al., 1982). Comme toutes les diatomées, *H. ostrearia* possède deux voies d'assimilation du carbone minéral : la voie de la ribulose-bisphosphate-carboxylase (RuBPC) associée au cycle de Calvin-Benson, et la voie de la phosphoénolpyruvatecarboxykinase (PEPCK ; Tremblin et Robert, 2001). Une voie alternative de β-carboxylation associée à une activité anhydrase carbonique dépendante de l'intensité lumineuse a également été démontrée (Rech et al., 2008 ; Tremblin et Robert, 2001). La composition biochimique d'*H. ostrearia* reste stable au cours du vieillissement des cultures, avec une teneur en glucides et en protéines moyennant 50 mg / 10^{-9} cellules et 200 mg / 10^{-9} cellules respectivement (Robert, 1983). En revanche, la teneur en lipides varie entre 60 et 200 mg / 10^{-9} cellules en fonction de la phase de croissance de la culture et les proportions en acides gras, lipides neutres et glycolipides sont semblables à celles d'autres diatomées (Robert, 1983). *Haslea ostrearia* synthétise des acides gras polyinsaturés ω-3 (Mimouni et al., 2003) et possède une voie de synthèse d'hydrocarbures insaturés (*i.e.* alcanes) polyramifiés tels que des isoprénoïdes, dont certains à 25 carbones ont été nommés haslènes en référence au genre *Haslea* (Allard et al., 2001 ; Wraige et al., 1998). Les pigments retrouvés chez *H. ostrearia* sont les chlorophylles *a* et *c*, la diatoxanthine, la

diadinoxanthine, la fucoxanthine et le pigment bleu marennine. Dans une culture en mode discontinu (batch), l'organisation des thylakoïdes et le comportement photosynthétique de *H. ostrearia* varient au cours de l'accumulation de marennine dans la cellule (Nassiri et al., 1998).

2.2.4 Reproduction

La reproduction sexuée chez *H. ostrearia* présente la particularité d'être hétérothallique, se produisant entre cellules compatibles de types sexuels différents (Davidovich et al., 2009), mais aussi homothallique, pouvant se produire entre deux cellules de même type sexuel (Neuville et Daste, 1978). Ces deux types de reproductions sexuées sont préférentiellement déclenchés sous faible éclairement (< 50 photons m^{-2} s^{-1}) et lors de courtes photopériodes (entre 6 et 10 h d'éclairement journalier), correspondant à des conditions hivernales (Davidovich et al., 2009 ; Neuville et Daste, 1978). La qualité de la lumière joue également un rôle dans l'induction de la reproduction sexuée (Mouget et al., 2009). La taille maximale des gamétocystes est estimée à 68 µm, soit environ la moitié de la taille de la cellule initiale (Davidovich et al., 2009).

2.2.5 Culture

Différents milieux de culture sont utilisés en laboratoire pour cultiver *H. ostrearia*. En général, des solutions d'enrichissement telles que celles proposées par Provasoli et al. (1957) ou par Robert (1983) sont ajoutées à de l'eau de mer naturelle filtrée ou à de l'eau de mer artificielle (Harrison et al., 1980). Un milieu de culture a également été développé spécifiquement pour *H. ostrearia,* nommé *diatom artificial medium* (Gagneux-Moreaux et al., 2007).

Sur le long terme, *Haslea ostrearia*, comme beaucoup de diatomées pennées, est difficile à maintenir en culture en laboratoire en raison de la réduction de la taille des cellules au cours de la multiplication végétative. Les mitoses successives conduisent au développement de petites cellules tordues, de forme tératogène, dont l'état physiologique est diminué (Falasco et al., 2009). De ce fait, une souche de *H. ostrearia* est difficilement maintenue en laboratoire au delà de 2-3 ans en culture en batch : la taille et la forme des

cellules devenant critiques (Figure 1.4). Lorsque la culture en batch est appliquée en laboratoire pour le maintien des souches, il est donc nécessaire d'effectuer des repiquages très réguliers (< 20 jours) pour éviter que les cultures n'entrent en phase de déclin (Figure 1.4). Pour maintenir les souches dans le plus long terme, une technique d'immobilisation des cellules dans de l'alginate à 4°C et sous faible éclairement a été proposée (Gaudin et al., 2006). En revanche, les techniques de cryopréservation ne sont pas recommandées pour *H. ostrearia* car elles peuvent induire plus de 90 % de mort cellulaire (Tanniou et al., 2012).

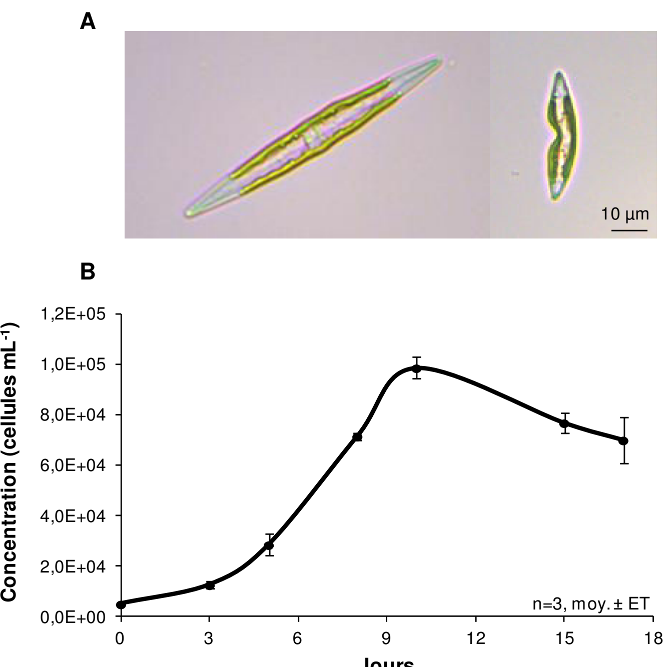


Figure 1.4. (A) Photographie d'une cellule de grande taille de *Haslea ostrearia* prise quelques jours après son isolement dans une claire ostréicole de la région de Bourgneuf (à gauche), et d'une cellule après un an de culture en laboratoire (à droite) présentant une petite taille et une forme atrophiée. **(B)** Courbe de croissance d'une culture de 200 mL en batch de *H. ostrearia*. Après une croissance exponentielle, la culture entre en phase de déclin sous 15 jours et nécessite un nouveau repiquage.

Haslea ostrearia est jusqu'à présent essentiellement cultivée à l'échelle du laboratoire (< 100 L), mais des cultures en photobioréacteurs (PBR, < 7 L), en semi-pilote (10 m^3) ou en bassins (200 L) ont également été menées (Gastineau et al., 2014b ; Rossignol et al., 2000 ; Turpin, 1999). Les unités de production à grande échelle ont encore besoin d'être optimisées car les PBR, généralement développés pour cultiver les

microalgues en suspension, ne conviennent pas pour *H. ostrearia* qui sédimente et forme des biofilms.

2.2.6 Distribution géographique

Haslea ostrearia est une diatomée cosmopolite qui peut être retrouvée sur les côtes Atlantiques françaises, et particulièrement dans les eaux saumâtres des claires à huîtres. La présence de *la* diatomée bleue a aussi été décrite en de nombreux endroits du globe tels que la mer Adriatique (Molisch, 1903), la mer Méditerranée (Sauvageau, 1906), l'océan Indien (Simonsen, 1974), la mer Baltique (Snoeijs et Kasperoviciene, 1996), les côtes japonaises (Ranson, 1937) ou encore les côtes de l'est des États-Unis (Mitchell et Barney, 1918) et de la Tasmanie (Hallegraeff et al., 2010). Ces données, parfois anciennes, sont cependant généralement basées sur des observations en microscopie optique ou sur l'occurrence de cellules aux extrémités bleues, sans que les caractéristiques morphométriques des cellules n'aient été étudiées (Figure 1.5).

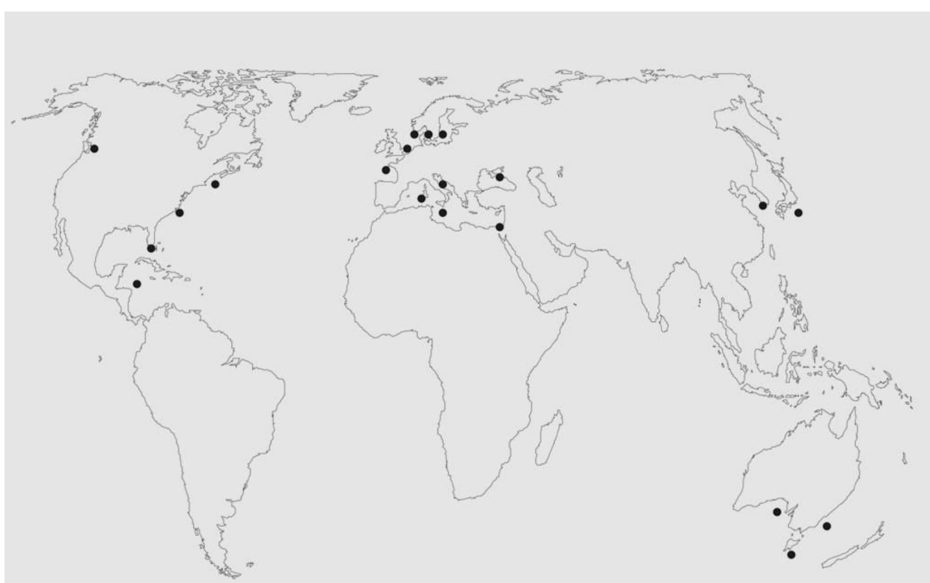
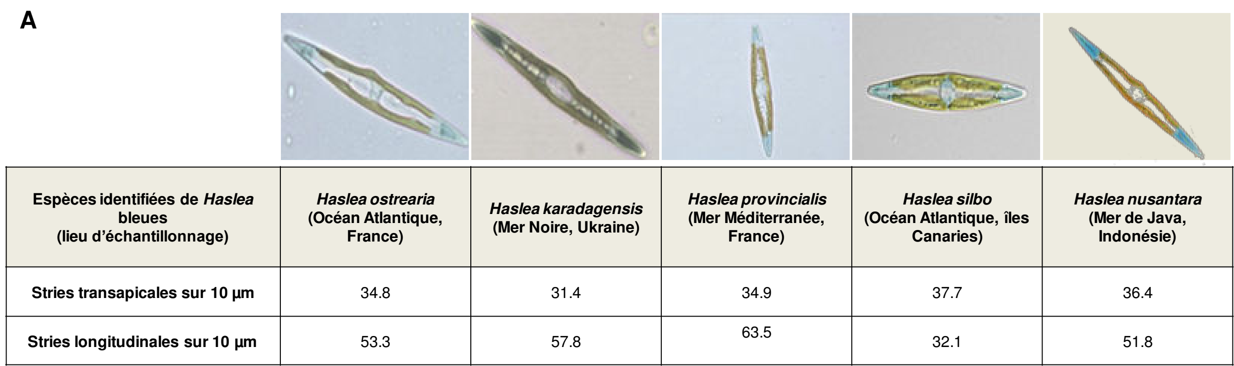


Figure 1.5. Répartition mondiale de *Haslea ostrearia* d'après la littérature. La présence de *H. ostrearia* a été déduite de l'observation de diatomées avec des apex bleus ou de la présence d'huîtres aux branchies vertes ; d'après Gastineau et al. (2014a).

2.3 *Haslea ostrearia*, unique en son genre ? La diversité insoupçonnée des *Haslea* bleues.

Haslea ostrearia a longtemps été considérée comme la seule diatomée du genre *Haslea* à synthétiser un pigment bleu. Cependant, de récentes campagnes d'échantillonnage de phytoplancton dans différents pays et continents, alliées à des analyses morphométriques des frustules en MEB ainsi qu'à l'utilisation de marqueurs moléculaires ont permis de révéler l'existence d'espèces différentes produisant également un pigment bleu hydrosoluble (Gastineau et al., 2014a). La première nouvelle espèce identifiée, *Haslea karadagensis*, a été échantillonnée en 2008 dans la mer Noire et décrite par Gastineau et al. (2012a). De nouvelles espèces ont ensuite été identifiées aux îles Canaries et en mer Méditerranée, nommées respectivement *Haslea silbo* (Gastineau, 2011) et *Haslea provincialis* (Gastineau et al., 2016). *Haslea silbo*, initialement échantillonnée sur l'île de la Gomera, a par la suite été identifiée en Caroline du Nord et dans la baie de Naples. Une autre espèce, encore partiellement identifiée, a également été retrouvée aux îles Canaries, sur l'île de Gran Canaria, et est pour l'instant nommée *Haslea acoran* (Gastineau, 2011). Ces différentes espèces sont sexuellement incompatibles et présentent des variations d'ornementations sur les frustules (Figure 1.6). Aussi, l'utilisation du marqueur moléculaire *cox1* (gène codant pour la première sous-unité de l'enzyme cytochrome oxydase) a démontré un pourcentage d'identité de paires de bases (pb) de seulement 90 % entre ces nouvelles espèces et *H. ostrearia*, tandis que deux sous-populations de *H. ostrearia* isolées sur les côtes françaises (baie de Bourgneuf) et australiennes (baie de Coffin) présentent quant à elles entre 98 et 100 % d'identité de pb (Gastineau et al., 2014a).

A



Espèces identifiées de *Haslea* bleues (lieu d'échantillonnage)	*Haslea ostrearia* (Océan Atlantique, France)	*Haslea karadagensis* (Mer Noire, Ukraine)	*Haslea provincialis* (Mer Méditerranée, France)	*Haslea silbo* (Océan Atlantique, îles Canaries)	*Haslea nusantara* (Mer de Java, Indonésie)
Stries transapicales sur 10 µm	34.8	31.4	34.9	37.7	36.4
Stries longitudinales sur 10 µm	53.3	57.8	63.5	32.1	51.8

B

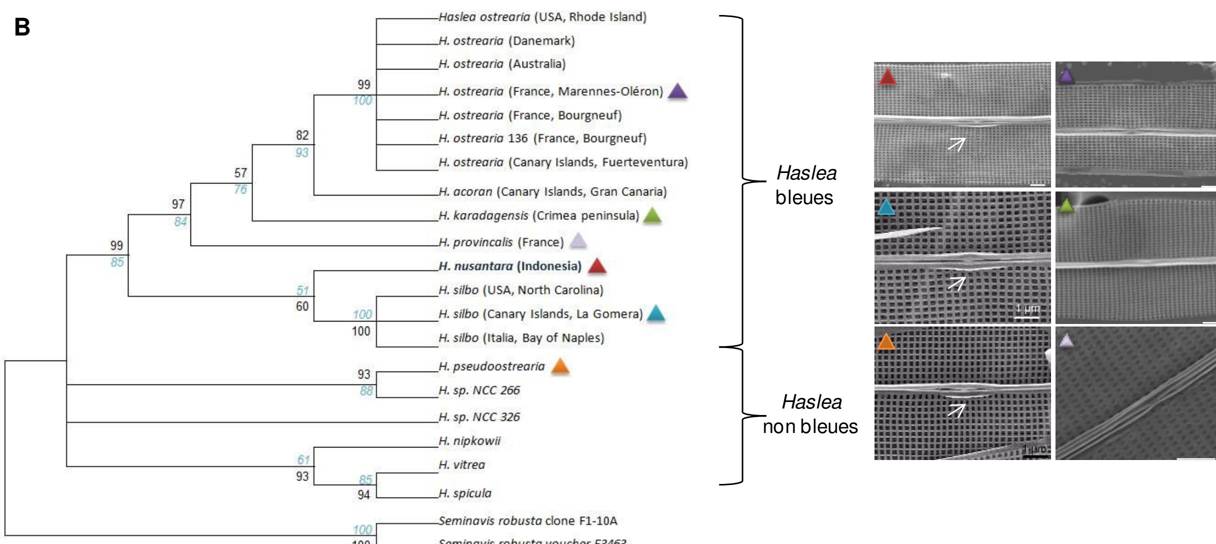


Figure 1.6. (A) Comparaisons de données morphométriques des frustules de plusieurs espèces de *Haslea* bleues. **(B)** Arbre phylogénétique de différentes espèces de *Haslea* basé sur le gène chloroplastique rbcL (les valeurs des bootstraps du maximum de vraisemblance (en noir) et du maximum de parcimonie (en bleu) ont été obtenues avec 1000 réplicats). Photographies au microscope électronique à balayage (MEB) de la fin du raphé central de plusieurs espèces d'*Haslea* référencées dans l'arbre phylogénétique. Une fine barre parallèle au raphé central (flèche blanche) peut être observée chez certaines espèces de diatomées bleues telles que *H. nusantara* et *H. silbo*. Cette caractéristique morphologique est commune aux diatomées non bleues telles que *H. pseudostrearia*. Cette fine barre n'est pas observée parmi les espèces de diatomées bleues génétiquement plus lointaines telles que *H. ostrearia*, *H. karadagensis* et *H. provincialis*. Barres d'échelle = 1 µm. Figures adaptées de Gastineau et al. (2014a) et Falaise et al. (2016a).

La nouvelle espèce de *Haslea* bleue la plus récemment identifiée est *Haslea nusantara*, isolée en mer de Java au printemps 2015, soit quelque mois avant le début de mon doctorat. Il parait ici opportun de préciser que mon sujet de recherche initial portait sur la « Caractérisation et valorisation de deux espèces de diatomées bleues du genre *Haslea* : étude comparative par approches physiologiques et moléculaires » (dirigé par Vincent Leignel, co-dirigé par Jean-Luc Mouget et co-encadré par Yann Hardivillier). Alors que *H. ostrearia*, disponible au laboratoire et dont les conditions pour le maintien des cultures sont connues, *H. nusantara* a quant à elle été difficilement maintenue en culture et a rapidement atteint une taille critique de dégénération. La souche d'*H. nusantara* a conséquemment été perdue en quelques mois et, malgré plusieurs campagnes d'échantillonnages menées en Indonésie fin 2016 et début 2017, aucune *Haslea* bleue n'a pu être de nouveau isolée. Mon travail de recherche, ne pouvant se porter sur la comparaison de ces deux espèces de diatomées, s'est donc centré sur *H. ostrearia* et il a été décidé, fin mars 2017, que le sujet de recherche s'articulerait autour de la « Valorisation des activités biologiques de la diatomée marine *Haslea ostrearia* » (dirigé par Jean-Luc Mouget et co-encadré par Vincent Leignel et Yann Hardivillier). Ce travail a nécessité au préalable de cultiver en masse *H. ostrearia* et de produire son pigment pour en étudier les activités. Néanmoins, la caractérisation de la nouvelle espèce de *Haslea* bleue indonésienne a été possible et a donné lieu à une publication dans le journal *Plant Ecology and Evolution* intitulée « *Haslea nusantara*, a new blue diatom from the Java Sea, Indonesia: Morphology, biometry and molecular characterization » (Prasetiya et al., in press).

Ainsi, bien que *H. ostrearia* ait longtemps été considérée comme la seule diatomée bleue de son genre, on dénombre aujourd'hui six autres espèces également capables de produire et de libérer un pigment bleu hydrosoluble et de colorer les branchies des bivalves (Gastineau et al., 2014a). Ces *Haslea* bleues sont retrouvées de part le monde et peuvent épisodiquement former de larges biofilms sur la surface des sédiments ou sur des macroalgues (Figure 1.7). Il est fort probable que de nouvelles campagnes d'échantillonnages permettent d'identifier davantage d'espèces de diatomées bleues qui viendront enrichir la diversité du genre *Haslea*.

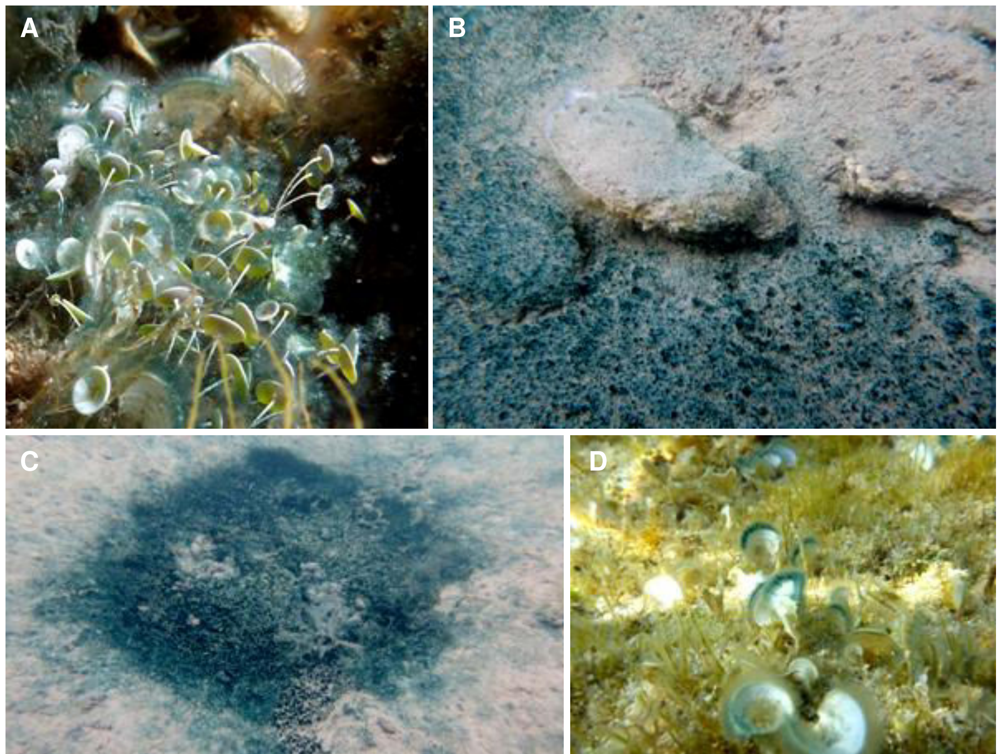


Figure 1.7. Efflorescences de *Haslea* bleues observées **(A)** en mer Mediterranée (Corse, France), **(B,C)** dans l'océan Atlantique (Caroline du Nord, États-Unis) et **(D)** en mer Adriatique (Dalmatie, Croatie). Photographies communiquées par D. Sirjacobs (A), N. Lindquist (B,C) et Y. Hardivillier (D).

3 LES PIGMENTS BLEUS D'*HASLEA*

3.1 La marennine et le verdissement des huîtres

3.1.1 Les huîtres « fines de claires vertes »

La marennine produite par *H. ostrearia* est connue pour induire le verdissement des branchies des huîtres dans les claires ostréicoles, des bassins d'eau de mer dans lesquels les huîtres sont mises en affinage (Figure 1.8). Dans ces bassins peu profonds et riches en nutriments, *H. ostrearia* peut proliférer et libérer de grandes quantités de marennine qui rendra l'eau des claires verte. Les huîtres obtiendront ainsi par filtration des branchies verdies en quelques jours seulement (Robert, 1983).

Ce phénomène de verdissement naturel présente un intérêt économique dans l'activité ostréicole des régions de Marennes-Oléron et de la baie de Bourgneuf dans l'ouest de la France, où l'affinage des huîtres en claires est pratiqué. Les huîtres verdies de Marennes-Oléron portent en effet un label rouge particulier « huîtres fines de claires vertes » et se vendent en moyenne 20 à 30 % plus cher que des huîtres affinées non verdies (Figure 1.8). Ce prix de vente plus élevé peut s'expliquer par la rareté du produit : les blooms de *H. ostrearia* dans les claires étant un phénomène erratique, mais également par une différence de saveur entre les huîtres vertes et non vertes. Cependant, l'amélioration des propriétés organoleptiques avancée par les amateurs d'huîtres ne proviendrait pas nécessairement du pigment lui-même, mais pourrait plutôt résulter de la modification de la communauté de phytoplancton des claires lors des blooms de *H. ostrearia*, modifiant ainsi l'alimentation des huîtres et potentiellement leur goût. En effet, la prolifération de *H. ostrearia* dans les claires génère un effet d'ombrage qui empêche la croissance d'autres microalgues (Pouvreau et al., 2007). De plus, il a été montré que la marennine possède des propriétés allélopathiques qui ralentissent, voire préviennent la croissance d'autres microalgues (Pouvreau et al., 2007 ; Prasetiya et al., 2016).

Figure 1.8. (A) Affinage des huîtres dans des bassins d'eau de mer peu profonds appelés « claires » dans la région de Marennes-Oléron. **(B)** Huître « fine de claire » et huître « fine de claire verte » (label Rouge français) dont les branchies ont été verdies par la présence de *Haslea ostrearia* et de son pigment (marennine) dans les claires lors de l'affinage.

3.1.2 La fixation de la marennine sur les branchies

Les mécanismes par lesquels la marennine se fixe aux branchies des huîtres sont encore aujourd'hui mal connus. Il a été montré que les huîtres gardent la signature de la marennine plusieurs mois sur leurs branchies même élevées en absence de *H. ostrearia* (Gastineau et al., 2014b). L'étude de coupes histologiques d'huîtres verdies montre que la marennine pénètre les plis des branchies et se fixe principalement dans les mucocytes ; des cellules produisant et excrétant du mucus (Gastineau et al., 2018 ; Semba Prasetiya, 2015).

Il est à noter que le verdissement des branchies induit par la marennine n'est pas exclusif aux huîtres et que d'autres organismes tels que la moule, le pétoncle, la coque ou la palourde présentent également un verdissement des branchies suite à une exposition au pigment (Gastineau et al., 2014b). Aussi, ce phénomène ne se produit pas uniquement dans les claires ostréicoles mais peut survenir en milieu naturel, au niveau de baies où la mer n'est pas trop agitée, ou dans des milieux totalement ouverts (voir Figure 1.7).

3.1.3 La consommation des huîtres vertes, une spécialité française

Les huîtres vertes sont à l'heure actuelle presque exclusivement consommées en France mais leur production et leur consommation commencent à se développer en Australie et aux États-Unis. Dans l'hexagone, les huîtres vertes sont considérées depuis plusieurs siècles comme un met de grande qualité et étaient d'ailleurs retrouvées à la table des rois de France (Grelon, 1978), alors qu'ailleurs la méconnaissance du phénomène à l'origine de ce verdissement et la couleur inhabituelle laissaient penser aux ostréiculteurs et aux consommateurs que ces huîtres étaient polluées et impropres à la consommation (Boyce et Herdman, 1896). D'ailleurs, les rapports du ministère du Commerce américain de 1916 indiquaient que les branchies vertes étaient un « problème », alertant qu'« aucune méthode de contrôle des branchies vertes n'a été déterminée » (US Report of the Secretary of Commerce and Reports of Bureaus, 1916). En effet, avant de pouvoir déterminer que le verdissement des branchies était causé par une microalgue, différentes hypothèses avaient été avancées pour tenter d'expliquer ce phénomène, décrit depuis le XVIIème siècle (Sprat, 1667). Ainsi, il a été suggéré que les branchies vertes étaient un signe de maladie des huîtres comme l'obésité ou le disfonctionnement de la glande digestive (Pasquier, 1818 ; Valenciennes, 1841). Il avait également été suggéré que le verdissement pouvait être dû à des causes environnementales telles qu'un effet du soleil, la présence d'eaux saumâtres ou de macroalgues vertes (Bosc et Lair, 1817 ; Sprat, 1667), ou résulterait d'interactions des huîtres avec des métaux tels que le cuivre (Boon, 1977).

Ce n'est qu'au début du XIXème siècle que Gaillon identifie la présence de microorganismes « verts » (appréciation incorrecte de la couleur due aux moyens d'observation limités de l'auteur) comme étant à l'origine du verdissement. Ces microorganismes sont d'abord considérés comme étant d'origine animale et sont nommés *Vibrio ostrearius* (Gaillon, 1820). L'appellation « Navicule bleue » ou *Navicula ostrearia* fût ensuite proposée par le botaniste Bory de Saint Vincent, qui suggéra une appartenance de ces microorganismes au règne végétal (Bory de Saint-Vincent, 1826). La microalgue bleue a ainsi été classée dans le genre *Navicula* pendant près de 150 ans puis, grâce aux progrès

de la microscopie, elle fût transférée dans le genre *Haslea,* crée spécialement pour cette espèce aux caractéristiques morphologiques bien particulières (Simonsen, 1974).

3.2 La marennine et les pigments de type marennine

La marennine est le pigment bleu produit par l'espèce *H. ostrearia*. Le nom du pigment, initialement *marennin*, fût proposé par (Lankester, 1886) en référence à la région de Marennes-Oléron. La marennine est un pigment hydrosoluble, insoluble dans les solvants organiques (Pouvreau et al., 2006a), et dont la couleur varie selon le pH, allant de violet à bleu dans des conditions acides et passant de vert clair à vert-gris dans des conditions basiques. Bien que *H. ostrearia* et son pigment aient fait l'objet de nombreuses études au cours des dernières décennies, la voie de biosynthèse du pigment n'est pas encore déterminée et sa structure chimique pas totalement élucidée.

Les pigments bleus produits par les autres espèces de *Haslea* bleues récemment identifiées sont nommés pigments « de type marennine », en l'absence d'identification de caractéristiques chimiques plus spécifiques. A l'heure actuelle, *H. ostrearia* et la marennine demeurent davantage étudiées que les autres *Haslea* bleues et leurs pigments « de type marennine ».

3.2.1 Production et excrétion de la marennine par *Haslea*

Bien que le processus soit encore mal identifié, il semblerait que la marennine soit produite *via* une voie de synthèse cytoplasmique (Nassiri et al., 1998). Des vésicules contenant la marennine se forment dans le cytoplasme avec une phase de concentration transitoire aux deux extrémités cellulaires. La marennine est ensuite libérée de la cellule par exocytose dans de petites vésicules qui éclatent au bout de quelques minutes permettant la libération de la marennine dans le milieu. Les conditions qui favorisent la production et la libération de marennine sont elles aussi incertaines. Des études ont suggéré une stimulation de production par des substances organiques comme les glucosamines (Ranson, 1937) ou

encore par un stress salin (Bachrach et Simonet, 1936 ; Neuville et Daste, 1978) ou des déficiences en fer ou vitamines (Neuville et Daste, 1978 ; Robert, 1983). La lumière est également avancée comme étant un facteur influençant la production du pigment. Des conditions de culture sous fort éclairement avec de longues photopériodes, ainsi que la qualité de la lumière avec les longueurs d'ondes dans le bleu semblent favoriser la production de marennine (Mouget et al., 2005 ; Tremblin et al., 2000). Sur des algues maintenues en phase de croissance exponentielle (pas de limitation en nutriments ni en lumière), plus le niveau d'éclairement est élevé, plus la production de marennine est importante (Prasetiya et al., 2016).

Le coût métabolique de la production de marennine implique nécessairement un intérêt pour la cellule et un rôle écologique, mais les fonctions biologiques du pigment restent incertaines. La marennine n'est pas directement impliquée dans la photosynthèse ou la captation lumineuse (Schubert et al., 1995). Des auteurs ont néanmoins suggéré une implication indirecte de la marennine dans la photosynthèse en modifiant quantitativement et qualitativement la lumière traversant la colonne d'eau (Mouget et al., 1999 ; Tremblin et al., 2000). Une fonction écologique peut également être avancée en raison des effets allélopathiques (Pouvreau et al., 2007 ; Prasetiya et al., 2016) et antimicrobiens (Gastineau et al., 2014b) exercés par la marennine et prémunirait *Haslea* d'un moyen de compétition envers d'autres microalgues ou bactéries, voire de potentiels prédateurs.

3.2.2 Caractérisations préliminaires du pigment

Aujourd'hui encore, la nature chimique de la marennine questionne. Différentes hypothèses se sont succédées pour tenter de caractériser ce pigment. Des auteurs ont ainsi relié la marennine à des caroténoïdes (Ranson, 1937), des produits de dégradation de la chlorophylle (Bachrach et Simonet, 1936 ; Geneves et al., 1976 ; Robert, 1983), ou encore des pigments de nature protéique tels que les phycobiliprotéines des cyanobactéries (Bocat, 1907). L'hypothèse la plus généralement admise est celle proposée par Pouvreau et al. (2006a) qui ont décrit la marennine comme un polymère complexe portant des groupements polyphénoliques. Une étude récente a également permis de caractériser le squelette

carboné de la molécule et d'identifier la présence de différents sucres (Gastineau et al., 2014b). La marennine existerait sous deux formes : intracellulaire (IMn) et extracellulaire (EMn) dont les poids moléculaires ont respectivement été estimés à 10,7 kDa et 9,9 kDa (Pouvreau et al., 2006a).

3.2.3 Techniques de purification du pigment

Une méthode de purification de la IMn et de la EMn a été développée par Pouvreau et al. (2006b). Elle comprend deux phases d'ultrafiltration, à 30 kDa et 3 kDa, ainsi qu'une phase de chromatographie par échange d'anions, une étape de dialyse puis de lyophilisation (Figure 1.9). Cette technique aboutit à l'obtention d'une poudre de marennine, partiellement purifiée, permettant notamment de réaliser des essais biochimiques afin de caractériser davantage le pigment. Des spectres « types » de la IMn et de la EMn en spectrophotométrie UV-Visible et Raman ont ainsi pu être établis (Pouvreau et al., 2006a). Le spectre UV-Visible de la marennine à pH 8 présente trois pics identifiables autour de 247, 322 et 675 nm (Figure 1.9). Les spectres UV-Visible et Raman des pigments de « type marennine » de *H. silbo*, *H. provincialis* et *H. nusantara* sont semblables à ceux de la marennine. Seul le pigment de type marennine produit par *H. karadagensis*, qui apparait plus foncé que la marennine en microscopie optique, présente des propriétés spectrales légèrement différentes (Gastineau et al., 2012a).

Néanmoins, le procédé de purification développé par Pouvreau et al. (2006b) n'est pas adapté pour le traitement de gros volumes de culture car il nécessite du matériel coûteux (*e.g.* colonnes de chromatographies à échanges d'ions) et ne présente qu'un faible rendement. Une telle technique de récupération de la marennine rend donc difficilement envisageable son utilisation pour des essais biologiques à large échelle ou pour la concentration du pigment en vue d'applications biotechnologiques et industrielles. La *Blue Water* (BW), une solution de marennine extracellulaire concentrée par ultrafiltration (Figure 1.9), peut être utilisée pour des essais biologiques. Elle peut en effet être produite plus rapidement et en plus grande quantité que la marennine purifiée, mais cette technique nécessite également d'effectuer des ultrafiltrations sur d'importants volumes de culture.

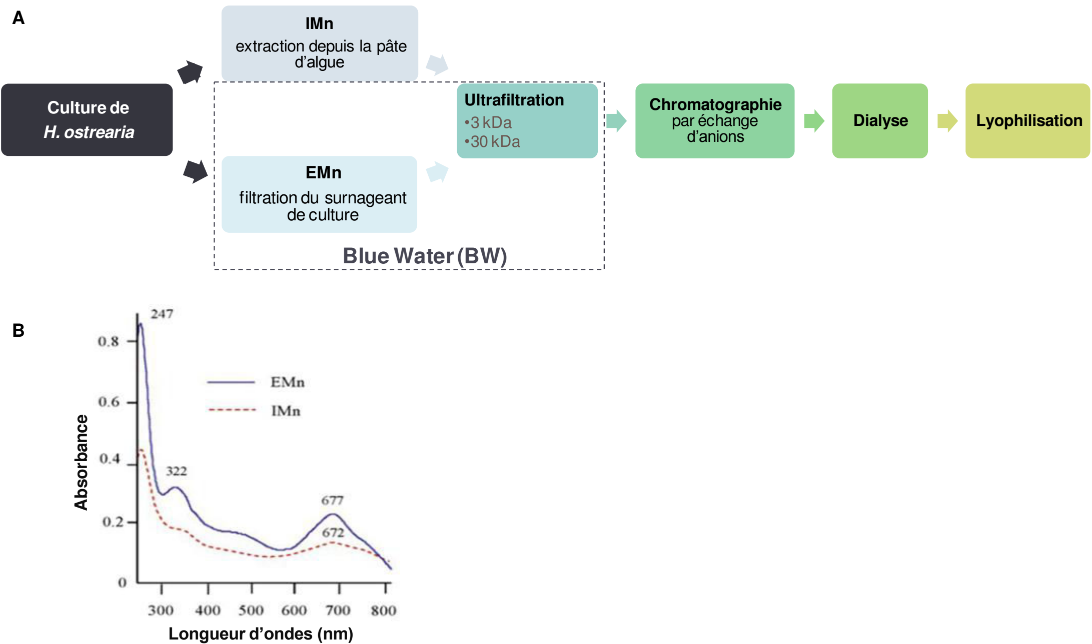


Figure 1.9. (A) Technique de purification de la marennine intracellulaire (IMn) et extracellulaire (EMn) de *Haslea ostrearia* proposée par Pouvreau et al. (2006b). La Blue Water (BW), un surnageant de culture concentré en marennine, est obtenue par ultrafiltration de la EMn. **(B)** Spectres UV-Visible caractéristiques des fractions de IMn et EMn purifiées, adapté de Pouvreau et al. (2006b).

3.2.4 Un nouveau procédé pour obtenir des solutions enrichies en pigment bleu

Il est ainsi rapidement devenu nécessaire de mettre au point de nouveaux procédés d'obtention de solutions aqueuses enrichies en pigment bleu plus simples et plus rapides que ceux utilisés à ce jour, en particulier à partir de grands volumes.

En collaboration avec des chercheurs de l'Institut des Molécules et Matériaux du Mans (I3M), nous avons développé une « Méthode de récupération et de concentration d'un pigment bleu hydrosoluble de type marennine produit par les diatomées bleues du genre *Haslea* » pour laquelle une déclaration d'invention et un brevet ont récemment été déposés (n° de brevet 1872316 ; Lila Zebiri (I3M), Charlotte Falaise (MMS), Boris Jacquette (I3M), Pamela Pasetto (I3M) et Jean-Luc Mouget (MMS)).

Cette méthode présente l'avantage d'être beaucoup plus rapide que celle développée par Pouvreau et al. (2006b), moins onéreuse et permet d'obtenir une solution de marennine concentrée avec un spectre UV-Visible semblable à la EMn. Cette technique peut s'appliquer à la marennine, mais également aux pigments de « type marennine » puisqu'elle a été testée avec des cultures de *H. provincialis*, *H. silbo* et *H. nusantara*. L'invention concerne un procédé d'obtention d'une solution aqueuse enrichie en pigment bleu comprenant diverses étapes : 1) la filtration d'un surnageant de culture de *Haslea* contenant la forme extracellulaire du pigment, 2) la précipitation du pigment bleu à l'hydroxyde de sodium, 3) la séparation de la phase précipitée de la phase aqueuse, 4) la solubilisation du pigment bleu compris dans la phase précipitée, 5) une dialyse et enfin 6) l'ultrafiltration de la solution concentrée (Figure 1.10).

Grâce à cette technique, l'utilisation d'une solution concentrée en pigment bleu pourrait être envisagée à l'échelle industrielle. Cependant, la structure chimique du pigment n'étant pas définie, l'application de la marennine ou des pigments de type marennine dans les secteurs de l'agroalimentaire ou de la pharmacologie ne peut pour l'instant pas être considérée. En revanche, ces solutions aqueuses enrichies en pigment bleu pourront être valorisées dans les secteurs de l'aquaculture ou de la cosmétologie, pour lesquels l'identification de la molécule ne présente pas un impératif pour l'autorisation de mise sur le marché.

Figure 1.10. Nouveau procédé de concentration de la marennine et des pigments de « type marennine » des espèces de *Haslea* bleues (n° de brevet 1872316). Procédé développé en collaboration entre le laboratoire Mer, Molécule, Santé (MMS) et l'Institut des Molécules et Matériaux du Mans (I3M) de le Mans Université.

4 ACTIVITES BIOLOGIQUES ET VALORISATIONS POTENTIELLES DES *HASLEA* BLEUES ET DE LEURS PIGMENTS

4.1 Les biotechnologies bleues

4.1.1 Le secteur de l'agroalimentaire

En plus d'applications en aquaculture, les pigments de type marennine peuvent avoir des débouchés sous-exploités mais néanmoins pertinents dans le domaine émergent des biotechnologies bleues. Pour le moment, le principal marché à considérer est « le pigment bleu naturel ». De nos jours, les colorants bleus les plus utilisés pour les aliments et les boissons sont le *Brillant Blue* (FD&C Blue n°1, E133), l'*Indigo Carmine* (FD&C Blue n°2, E132), approuvés dans l'Union européenne et aux États-Unis, ainsi que le bleu patenté V (E131) autorisé dans certains pays européens (Directive 94/36/CE ; Food and Drug Administration (FDA), 2016). Cependant, ces colorants bleus sont d'origine synthétique (Sharma et al., 2011). Étant donné que les colorants artificiels questionnent quant à leurs effets sur la santé et ont tendance à être évités par les consommateurs, la recherche d'homologues naturels ou de nouvelles molécules est actuellement un grand défi pour l'industrie alimentaire (Buchweitz, 2016 ; Frick, 2003).

Les composés de couleur bleue peuvent être retrouvés dans les plantes, les animaux, les champignons ou plus fréquemment chez les microbes, et appartiennent à différentes classes chimiques telles que les flavonoïdes ou les quinones (Newsome et al., 2014). Des pigments bleus tels que les phycocyanines ou allophycocyanines sont notamment retrouvés chez les cyanobactéries, d'ailleurs anciennement nommées les « algues bleues-vertes ».

Bien que présentes dans la nature, les teintes bleues sont très difficiles à reproduire dans les aliments. En effet les pigments sont, par définition, insolubles dans le milieu qui les contient (*e.g.* en suspension dans un liquide, en dispersion dans un solide), à la différence

des colorants qui sont des substances colorées solubles dans le milieu qu'elles colorent. Les termes de pigments et colorants font souvent l'objet de mésusages et sont génériquement utilisés pour décrire une « substance colorée » ou « donnant une couleur ». Les pigments d'origine organique ou minérale doivent donc être extraits et solubilisés par des méthodes telles que l'utilisation de solvant, la distillation ou la chromatographie pour pouvoir être utilisés en tant que colorants.

Le développement d'un nouveau colorant bleu doit répondre à plusieurs critères en termes d'intensité de couleur, de solubilité, de stabilité à la chaleur, à l'acidité, la lumière et l'oxygène, ainsi qu'en termes d'exigence de sécurité alimentaire. À ce jour, seuls quelques colorants bleus naturels sont utilisés dans l'industrie alimentaire. Le bleu spiruline, nom commercial de la phycocyanine, est un pigment bleu principalement produit par les cyanobactéries mais également par certaines algues eucaryotes, telles que les cryptophytes et les rhodophytes (Eriksen, 2008). Les extraits de spiruline sont utilisés depuis longtemps en Asie et ont récemment été approuvés par la FDA des États-Unis pour colorer les bonbons et les gommes à mâcher (FDA, 2016). Cependant, la phycocyanine ne peut pas remplacer l'utilisation du colorant synthétique *Brillant Blue* dans la plupart des aliments et boissons en raison de sa faible stabilité (Newsome et al., 2014). Le bleu Jagua, dérivé du fruit de *Genipa Americana* L., est également considéré comme une source prometteuse de pigment bleu naturel pour une application agroalimentaire en raison de sa bonne stabilité (Brauch et al., 2016). Son utilisation dans les aliments est déjà approuvée au Japon, mais des évaluations de sécurité supplémentaires sont nécessaires pour les marchés des États-Unis et de l'Union européenne (Wrolstad et Culver, 2012).

La marennine et les pigments de « type marennine » pourraient ainsi représenter des composés bleus intéressants pour l'industrie alimentaire, notamment en raison de leur solubilité dans l'eau et de l'intensité de leur couleur. Cependant, l'évaluation de la stabilité à long terme, de la toxicité potentielle ou des propriétés mutagènes de la marennine représente une étape importante pour son utilisation en tant qu'additif alimentaire. Néanmoins, la consommation humaine d'huîtres vertes pendant des siècles sans

enregistrement de maladies ni de réactions anaphylactiques laisse supposer la non-toxicité de ce pigment bleu dans un contexte de consommation alimentaire régulière.

4.1.2 Les secteurs de la cosmétique et de la pharmacologie

Outre de potentielles applications dans l'industrie alimentaire, une meilleure connaissance des propriétés de la marennine et des pigments de type marennine pourrait permettre leur utilisation dans les secteurs de la cosmétologie ou de la pharmacologie. Jusqu'à présent, très peu d'études ont été menées dans le domaine de la cosmétologie et celles-ci n'ont pas permis de mettre en évidence des activités cosmétologiques intéressantes. Les effets anti-inflammatoires de la marennine ont été évalués avec l'application d'une crème contenant 10 % (m/m) de marennine sur un œdème de l'oreille d'une souris. L'application de la crème a permis une inhibition de l'œdème de 62,5 %, un effet modéré comparativement à celui du dermocorticoïde contrôle utilisé pour l'étude (100 % d'inhibition, à 0,1 % m/m), supposant un faible potentiel de valorisation de la marennine dans des crèmes apaisantes (Gastineau et al., 2014b). Le potentiel photoprotecteur de la marennine a également été étudié *in vitro* pour évaluer les facteurs de protection contre le soleil et les UVA, mais la molécule n'a pas présenté de photoprotection topique intéressante (Gastineau et al., 2014b). Cependant, il a été prouvé que la marennine possède de fortes activités antioxydantes, supérieures même à certains antioxydants couramment utilisés dans les aliments, tels que l'apegénine flovonoïde (Pouvreau et al., 2008). L'utilisation de la marennine ou des pigments de « type marennine » en tant que substance antioxydante ou agent colorant naturel dans des produits cosmétiques représente donc une application potentielle intéressante ; la production de nouvelles crèmes à base de marennine a d'ailleurs débuté dans une *start up* canadienne.

En ce qui concerne les applications pharmaceutiques potentielles, la marennine a démontré plusieurs effets biologiques, notamment antiviraux, antiprolifératifs et antioxydants. Les extraits hydrosolubles et les formes EMn et IMn purifiées ont montré une activité contre le virus de l'herpès simplex de type 1 (HSV-1 ; Bergé et al., 1999 ; Gastineau et al., 2012b). Les deux formes extracellulaire et intracellulaire purifiées du pigment de type marennine extrait de *H. karadagensis* ont également démontré des activités anti-herpétiques en

inhibant l'infection et la destruction des cellules (Gastineau et al., 2012c). De plus, les propriétés antiprolifératives d'extraits bruts de marennine ou de la forme purifiée ont également été démontrées sur diverses lignées de cellules cancéreuses humaines telles que SKOV-3 (cancer de l'ovaire), SW116 (cancer du côlon) ou M113 (mélanome ; Carbonnelle et al., 1998 ; Gastineau et al., 2012b). Cependant, la marennine n'a montré aucun effet sur un large éventail de bactéries pathogènes terrestres importantes pour la sécurité alimentaire, telles que *Pseudomonas aeruginosa*, *Staphylococcus aureus* ou *Salmonella typhimurium* (Gastineau et al., 2012b).

4.2 L'aquaculture

4.2.1 Les microalgues comme probiotiques

La définition des probiotiques change avec l'apport de nouvelles connaissances dans les domaines d'applications concernés, tel que l'aquaculture (Newaj-Fyzul et al., 2014 ; Pandiyan et al., 2013). L'une des définitions la plus généralement acceptée est donnée par l'Organisation des Nations Unies pour l'alimentation et l'agriculture (*ONUAA*, ou plus couramment *FAO* pour Food and Agriculture Organization of the United Nations) et par l'Organisation mondiale de la santé (*OMS* ou *WHO* pour World Health Organization), qui considèrent les probiotiques comme des « microorganismes vivants qui, lorsqu'ils sont administrés en quantité suffisante ou consommés en quantité suffisante en tant qu'aliments, confèrent un avantage à la santé de l'hôte » (FAO/WHO, 2001).

Les microalgues ont longtemps été utilisées en aquaculture pour nourrir et favoriser la croissance de divers animaux aquatiques ou pour les processus d'affinage des stades adultes (Muller-Feuga, 2000). Elles jouent un rôle majeur dans le secteur de l'aquaculture, qui est le secteur de production alimentaire présentant le plus fort taux de croissance au niveau mondial, avec une croissance annuelle estimée à 6 % entre 2000 et 2016 (FAO, 2018a). L'addition régulière de phytoplancton dans des bassins d'élevage est appelée technique « d'eau verte » (Neori, 2010 ; Papandroulakis et al., 2001). Cette utilisation de

microalgues comme probiotiques présente de nombreux avantages pour les animaux d'élevage, tels que l'amélioration de la santé générale et une meilleure résistance aux maladies (Cahu et al., 1998 ; Van der Meeren et al., 2007), grâce à une meilleure nutrition (Marques et al., 2006) et à une amélioration de la qualité de l'eau du milieu de culture (Palmer et al., 2007). Plusieurs espèces de microalgues sont également connues pour leurs activités biologiques contre divers microbes marins, tels que des bactéries ou des champignons responsables de maladies en aquaculture (ces aspects seront développés dans le Chapitre II. de ce manuscrit).

4.2.2 Les *Haslea* bleues pour limiter les infections microbiennes ?

Les *Haslea* bleues ont également été étudiées pour leur utilisation potentielle en tant que probiotiques en aquaculture et pour une utilisation dans des systèmes en « eaux vertes ». La marennine purifiée extraite de *H. ostrearia* et le pigment purifié de *H. karadagensis* ont démontré une activité antibactérienne contre trois bactéries pathogènes d'intérêt en aquaculture : *Polaribacter irgensii*, *Pseudoalteromonas elyakowii* et *Vibrio aestuarianus* (Gastineau et al., 2012b, 2012c). La marennine purifiée présente également des activités antibactériennes contre d'autres espèces de *Vibrio*, telles que *V. anguillarum* et *V. tasmaniensis* (Pouvreau, 2006 ; Gastineau et al., 2014). De plus, il a été démontré que la marennine en solution dans l'eau de mer peut augmenter la survie et l'état physiologique des larves de moules bleues *Mytilus edulis* et de pétoncles *Placopecten magellanicus* exposées à une souche virulente de *V. splendidus* (Turcotte et al., 2016). *Haslea ostrearia* et son pigment bleu pourraient donc représenter un probiotique intéressant pour le contrôle des agents pathogènes dans les écloseries. En outre, la marennine purifiée extraite de *H. karadagensis* a démontré une activité antifongique *in vitro* contre *Corollospora maritima*, *Lulworthia sp.* et *Dendryphiella salina* ; des champignons souvent impliqués dans des phénomènes de *biofouling* (*i.e.* encrassement biologique) et qui peuvent s'accumuler sur les coquilles ou les cages d'huîtres (Gastineau et al., 2012c). Ainsi, compte tenu des activités biologiques de la marennine et des pigments de type marennine contre plusieurs agents pathogènes connus pour induire des maladies en aquaculture, l'utilisation des espèces

bleues du genre *Haslea* pourrait présenter de nombreux avantages. Cependant, d'autres études sont nécessaires pour 1) évaluer la concentration en marennine requise pour exercer une activité antimicrobienne sans induire de toxicité pour les animaux d'élevage, et 2) définir la faisabilité de la production de ces diatomées benthiques à une échelle industrielle. De plus, des informations sur le mode d'action de la marennine, et notamment contre les bactéries marines, sont également nécessaires. Jusqu'à présent, certains indices ont été apportés par Tardy-Laporte et al. (2013) qui ont démontré qu'un pigment de type marennine purifié, extrait de *H. provincialis*, induisait une perturbation de la membrane cellulaire d'*Escherichia coli*, mais le mécanisme d'action exact n'a pas encore été élucidé.

4.3 Perspectives et limites actuelles

La marennine et les pigments de « type marennine » sont des substances naturelles prometteuses pouvant être utilisées comme composés prophylactiques en aquaculture ou comme pigment bleu naturel dans les industries agroalimentaire, pharmaceutique ou cosmétique. Les principaux défis liés à la valorisation de ces pigments bleus sont de définir leurs structures chimiques, de déterminer leur absence de toxicité et d'améliorer les modes de culture des espèces de *Haslea* et les procédés d'extraction des composés d'intérêt, en particulier si la production de marennine à une échelle industrielle est envisagée.

5 Contexte et objectifs du sujet de recherche

Les travaux de ce doctorat s'inscrivent au sein d'un projet de recherche européen intitulé G*Ha*NA (The Genus *Haslea*, New marine resources for blue biotechnology and Aquaculture, H2020-MSCA-RISE-2016) et porté par Jean-Luc Mouget. Le projet G*Ha*NA s'articule autour de différents axes de recherche tels que l'étude de la physiologie des *Haslea* bleues, le criblage de produits à haute valeur ajoutée (*e.g.* silice, acides gras), le développement d'unités de production (PBR), la bioprospection pour la découverte et l'identification de nouvelles espèces ou encore la valorisation de *Haslea* et de leurs pigments bleus dans le secteur de l'aquaculture.

Mon projet de thèse se rattache ainsi à deux de ces axes de recherche : la bioprospection, avec des campagnes d'échantillonnages menées notamment sur les îles Canaries et l'isolement de souches d'*Haslea* bleues et, essentiellement, l'étude des activités biologiques de la marennine en vue d'une potentielle valorisation en aquaculture.

Comme précédemment évoqué dans l'introduction générale de ce manuscrit, la marennine a démontré *in vitro* un effet sur la prolifération de certaines bactéries marines et également un effet protecteur *in vivo*, dit prophylactique, à faible dose sur des larves de bivalves. De tels résultats ont donc encouragé la poursuite des recherches en vue de l'utilisation de solutions concentrées en marennine pour une application dans les bassins d'élevage, notamment ceux de bivalves. Cependant, avant d'envisager une telle application de la marennine, il est nécessaire d'identifier davantage les effets de ce pigment sur la croissance de bactéries d'intérêt en aquaculture, de s'assurer de son innocuité sur d'autres organismes marins ou de définir des doses seuils d'exposition pour prévenir de tout effet adverse sur la survie ou le développement des animaux exposés au pigment.

Ainsi, ce manuscrit se développe en quatre parties principales présentant **1)** un état de l'art des activités antimicrobiennes des microalgues, et particulièrement envers des microorganismes responsables d'infections dans le secteur de l'aquaculture (Chapitre II) ; **2)** l'étude des effets de la marennine sur la croissance de bactéries marines du genre *Vibrio* pouvant être impliquées dans des épisodes de mortalité notamment chez les bivalves

(Chapitre III) ; **3)** l'étude des effets de la marennine sur la survie et le développement d'organismes marins d'intérêt en aquaculture (Chapitre IV) ; et enfin **4)** une discussion générale soulevant des perspectives de recherche suite aux résultats obtenus dans ce travail de thèse (Chapitre V).

SYNOPSIS

- **Chapitre II.** *Microalgal compounds against pathogens: emphasize on diseases in aquaculture.*

Ce chapitre dresse un état de l'art de la découverte et de l'identification de composés extraits de microalgues avec des activités antimicrobiennes pour des valorisations dans les secteurs de la santé, mais également dans le secteur de l'aquaculture.

Dans cette revue de littérature, seuls les composés de microalgues *stricto sensu* sont présentés, les activités antimicrobiennes des composés extraits de cyanobactéries ont quant à elles été développées au sein d'un chapitre de livre intitulé « Anticancer, antiviral, antibacterial and antifungal properties in microalgae » et publié dans l'ouvrage *Microalgae in Health and Disease Prevention* (Falaise et al., 2018).

- **Chapitre III.** *Effects of marennine solutions on the growth of marine* Vibrio

Les bactéries du genre *Vibrio* sont les bactéries les plus fréquemment impliquées dans les épisodes de mortalités associés à des infections bactériennes chez les bivalves (Travers et al., 2015). Cependant, peu d'études ont été menées concernant les effets de la marennine sur la croissance de bactéries du genre *Vibrio*. De plus, les évaluations des activités anti-*Vibrio* précédemment réalisées se sont basées sur des méthodes *in vitro* de diffusion sur boite de Petri (Gastineau et al., 2012b ; Pouvreau, 2006), qui sont des techniques principalement qualitatives, qui ne permettent pas de rendre compte quantitativement des effets d'une substance sur la croissance bactérienne (Jorgensen et al., 2007).

Ce chapitre se divise en deux parties et vise à estimer les effets de solutions de marennine sur la croissance de bactéries du genre *Vibrio.* La **partie 1)** *« Complex*

relationships between the blue pigment marennine and marine bacteria of the genus Vibrio *»* présente une approche *in vitro* des effets de la marennine sous forme de *Blue Water* (BW) à différentes concentrations sur la croissance de trente souches de bactéries du genre *Vibrio.* La **partie 2)** *« Co-culture of* Haslea ostrearia *with an oyster virulent* Vibrio *strain »* présente une approche *in vivo* pour estimer les effets de la marennine libérée par *H. ostrearia* sur la croissance d'une souche de *Vibrio* dans un système de co-culture. Les parties 1) et 2) sont respectivement présentées sous forme d'article scientifique et de *Short communication*.

- **Chapitre IV.** *New insights into the effects of marennine solutions on marine organisms*

À la lumière des diverses activités biologiques exercées par la marennine telles que des actions antimicrobiennes ou antioxydantes, l'utilisation de ce pigment est présentée comme bénéfique dans un secteur tel que l'aquaculture (Gastineau et al., 2014b). Cependant, bien que quelques études récentes aient souligné l'occurrence d'effets adverses sur la survie ou le comportement de larves ou juvénile de bivalves suite à une exposition à la marennine (Prasetiya et al., 2017 ; Turcotte et al., 2016), les effets du pigment sur les organismes marins ont peu été explorés.

Ce chapitre se divise en deux parties et vise à identifier les effets de la marennine sur divers animaux marins, choisis selon l'intérêt qu'ils représentent comme organisme modèle pour des essais écotoxicologiques, ou pour leur intérêt en aquaculture. La **partie 1)** *« Harmful or harmless: biological effects of marennine on marine organisms »* présente les effets de différentes solutions de marennine (sous forme de marennine extracellulaire purifiée (EMn), de BW et de solution de marennine concentrée selon le nouveau procédé présenté dans la section 3.2.4) sur la survie et le développement d'organismes marins appartenant à divers phyla. La **partie 2)** *« Preliminary results on marennine effects on the mussel* Mytilus edulis *larvae »* propose une approche biomoléculaire pour évaluer les effets de la marennine sous forme de BW chez des larves de moules à différents stades de développement. Les parties 1) et 2) sont respectivement présentées sous forme d'article scientifique et de *Short communication*.

CHAPITRE II.

MICROALGAL COMPOUNDS AGAINST PATHOGENS: EMPHASIZE ON DISEASES IN AQUACULTURE

Chapitre II. Microalgal compounds against pathogens: emphasize on diseases in aquaculture.

Ce chapitre a bénéficié de l'expertise de Cyrille François[2], Joël Haure[2], Marie-Agnès Travers[2], Réjean Tremblay[3], François Turcotte[3], Pamela Pasetto[4], Romain Gastineau[1], Yann Hardivillier [1], Vincent Leignel [1] et Jean-Luc Mouget[1].

Cette revue de littérature, qui a été publiée dans le Journal *Marine Drugs* en septembre 2016 sous l'intitulé « Antimicrobial compounds from ekaryotic microalgae against human pathogens and diseases in aquaculture », contient également des résultats originaux. Ces résultats ont été obtenus suite à des expérimentations menées au Laboratoire de Génétique et de Pathologie des Mollusques Marins (IFREMER, La Tremblade) sous la supervision de Cyrille François et Marie-Agnès Travers en février et avril 2016.

Les résultats originaux contenus dans cet article ont été présentés sur des Posters lors du congrès *ANTIMIC* à Québec (Canada), les 21-23 juin 2017 et du congrès *Biopolymers* à Nantes (France), les 29 novembre-1er décembre 2017, ainsi que lors d'une présentation orale au congrès de l'*International Society of Applied Phycology* (ISAP) à Nantes (France), les 18-23 juin 2017.

Affiliations des différents contributeurs de ce travail :

[1]Laboratoire Mer Molécules Santé (EA 2160/FR CNRS 3473 IUML), Le Mans Université, France ; [2]Laboratoire de Génétique et de Pathologie des Mollusques Marins (RBE/SG2M/LGPMM/Ifremer), Station La Tremblade, France ; [3]Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, Canada ; [4]Institut des Molécules et Matériaux du Mans (UMR CNRS 6283), Le Mans Université, France.

Abstract

The search for novel compounds of marine origin has increased since the last decades for their application in various areas such as pharmaceutical, human or animal nutrition, cosmetics or bioenergy. In this context of blue technology development, microalgae are of particular interest due to their immense biodiversity and their simple growth needs. In this review, we discuss about the promising use of microalgae and microalgal compounds as sources of natural antibiotics against human pathogens but also their potential to limit microbial infections in aquaculture. An alternative to conventional chemical antibiotics is needed as the microbial resistance to these drugs is increasing in human and animals. Furthermore, using natural antibiotics for livestock could meet the consumer demand to avoid chemicals in food, would support a sustainable aquaculture and present the advantage of being environmentally friendly. Using natural and renewable microalgal compounds is still at its early days, but considering the improvement in culture and extraction process, the valuation of microalgae will surely extend in the future.

Keywords: biological activity; antimicrobial; antibacterial; antifungal; antiviral; *Haslea;* microalgae; sustainable aquaculture

1 INTRODUCTION

Microalgae are present in almost all ecosystems around the world. They evolved in extreme competitive environments, abundant in phytoplankton grazers and microbial pathogens such as bacteria, viruses and fungi. In order to survive, they had to develop tolerance or defense strategies. The variety of these mechanisms resulted in a high diversity of compounds synthesized from diverse metabolic pathways. It appears that many of these metabolites present very specific chemical structures that are not encountered among terrestrial organisms, and sometimes with a structural complexity very difficult , often impossible to produce by hemi-synthesis or complete synthesis (Borowitzka, 1995).

Since the last decades, it has been a great trend for research and industrial application of marine compounds and biotechnology (Bhatnagar and Kim, 2010; Dyshlovoy and Honecker, 2015; Jha and Zi-rong, 2004; Kang et al., 2015; Mayer et al., 2013; Suleria et al., 2015). Among the large spectrum of marine organisms, microalgae represent a promising resource for blue technologies, due to their rapid growth and usually simple nutriment requirements need. Furthermore, many microalgal species are able to grow on saline water or wastewater, this represents an invaluable advantage, considering freshwater resources are becoming scarce. Microalgae are usually very versatile and able to acclimate to various and changing environments. They offer the opportunity to discover novel molecules or produce known molecules at lower cost. Furthermore, the nature and amount of their secondary metabolites can be manipulated through changing culture conditions, due to a tremendous phenotypic plasticity. Many valuable compounds can be extracted from microalgae, including among others pigments, fatty acids, lipids, proteins, polysaccharides, vitamins or minerals (Encarnacao et al., 2015; Shimizu, 2003; Singh et al., 2005; Talero et al., 2015). If an important research effort in microalgal biotechnology is made to promote the production of biofuels (Ahmad et al., 2011; Ghasemi et al., 2012; Halim et al., 2012), the important variety of compounds produced by microalgae enables a broad spectrum of applications such as pharmaceutical, cosmetics, human and animal nutrition, environmental restoration and protection or bioenergy (Barra et al., 2014; Cadoret et al., 2012; Priyadarshani and Biswajit, 2012; Raposo et al., 2013a; Spolaore et al., 2006; Yaakob et al.,

2014). Several compounds have shown potent biological activities, such as antioxidant, anticoagulant, anti-inflammatory, antimicrobial or antitumoral (Encarnacao et al., 2015; Raposo et al., 2013b, 2015a; Singh et al., 2005). The possible use of these compounds as a source of prebiotics, nutraceuticals, chemopreventive agents or antimicrobial drugs was investigated and has demonstrated promising results (Amaro et al., 2011; Raposo et al., 2015b, 2016; Smith et al., 2010; Talero et al., 2015). Microalgae are also valuable for their production of a diverse range of pigments such as chlorophyll, phicobiliproteins or carotenoids that can be used as dyes for food industry or cosmetic.

The earliest and most common use of microalgae is aquaculture. They have been used as food source and feed additive to promote the growth of various aquatic animals, especially for the larvae of bivalves and prawns and for the live food used to feed the larvae of finfish and crustaceans (Borowitzka, 1997). Microalgae are not only essential as a food source but they also permit to improve the quality of aquaculture stock. For example, the carotenoid astaxanthin, especially abundant in the green microalga *Haematococcus pluvialis*, can be supplied to give or increase color to the flesh of salmon and trout (Lorenz and Cysewski, 2000) and the blue pigment marennine produced by the diatom *Haslea ostrearia* gives a blue-green color on the gills and labial palps of oysters, increasing their market value (Gastineau et al., 2014b).

The so-called "green-water" and "pseudo green-water" techniques do not refer to *H. ostrearia* cultures, but to natural phytoplankton populations in outdoor ponds and to the regular addition of selected microalgae to provide favorable and continuous nutrition to larvae or to the live food, respectively (Muller-Feuga, 2000; Neori, 2010; Papandroulakis et al., 2001). Both have proven multiple benefits over the "clear water" system, for which a series of external filters allows to maintain the water quality, in terms of survival and growth of several animal species (Naas et al., 1992; Reitan et al., 1997; Supamattaya et al., 2005; Van der Meeren et al., 2007). Indeed, "green-water" cultures help to provide food with high nutritional value with chemical and digestive stimulants, and to improve and stabilize the quality of the culture medium (Cahu et al., 1998; Palmer et al., 2007). Such culture techniques allow increasing the good animal health and their resistance to diseases, thanks

not only to a better nutritional immunology (Marques et al., 2006), but also to the production of antimicrobial compounds by some microalgae. The “green-water” culture, and especially the “pseudo green-water” culture present many advantages as they allow direct supply of nutrients, are easy to manage, environmentally friendly and could lower the use of antibiotics in rearing systems (Tendencia and dela Peña, 2003).

Considering the remarkable biodiversity of microalgae and the improvement in culture, screening and extraction techniques, it is likely these microorganisms will represent an important source of new products in the future as part as blue technology. So far, bioactive compounds from cyanobacteria have been more studied than those from eukaryotic microalgae, probably due to their simpler culture methods, and have been the subject of several recent papers (Burja et al., 2001; Costa et al., 2012; Gupta et al., 2013; Vijayakumar and Menakha, 2015).

The present work is thus an update of previous works (Amaro et al., 2011; Bhatnagar and Kim, 2010; Borowitzka, 1995), and a complement to recent review on freshwater microalgae (Pradhan et al., 2014). It aims to present the available information about the biological activities of eukaryotic microalgae, by focusing on their (i) antibacterial, (ii) antifungal and (iii) antiviral properties, with a focus on the activity against human pathogens and their potential application in aquaculture against various microbial diseases.

2 ANTIBACTERIAL ACTIVITY FROM MICROALGAE

2.1 Antibacterial activity from microalgae against human pathogenic bacteria

The increasing resistance of pathogenic bacteria against a significant number of antibiotics, with consequences in human health and human mortality, had been a great concern for the past decades and had forced the efforts to find new antibacterial substances. Some bacteria may indeed cause several serious diseases in humans and some others can also provoke foodborne illness inducing moderate to severe nausea, vomiting and diarrhea. Since the pioneer work of Pratt in 1944, which demonstrated the activity of the green alga *Chlorella* against several Gram-positive and Gram-negative bacteria (Pratt et al., 1944), the interest for antibacterial compounds from microalgae has been growing. Numerous studies followed to detect compounds with antibacterial activity in microalgae, either to develop new drugs against bacterial infections, or to develop additives for food preservations.

Large screening programs have thus been conducted to assess the potential antibacterial activity of various microalgal extracts against pathogenic and foodborne bacteria. Numerous microalgal species from distinct taxonomical groups originating from various areas (Mudimu et al., 2014; Ördög et al., 2004; Pane et al., 2015), mainly from marine environment (Chang et al., 1993; Duff et al., 1966; Kellam and Walker, 1989; Ohta et al., 1993; Srinivasakumar and Rajashekhar, 2009; Viso et al., 1987), but also from freshwater environment (Cannell et al., 1988; Katircioglu et al., 2005), or even from the soil (Safonova and Reisser, 2005) were shown to have potent antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 2.1). As screening studies can sometimes include hundreds of different microalgae (Cannell et al., 1988; Kellam and Walker, 1989; Ördög et al., 2004), Table 2.1 only presents the microalgae with the highest antibacterial activity or the wider spectrum of activity from these screenings.

It appeared from these studies that the production of antibiotics is largely dependent on the microalgal species (Bhagavathy et al., 2011). The presence of antibiotic agents can vary widely between different species from a same class, even if some studies presume that the antibacterial activity is predominantly found in the members from the classes Bacillariophyceae and Chrysophyceae (Duff et al., 1966; Kellam and Walker, 1989). The antibacterial activity can also differs between ecotypes of a same species. Indeed, the green microalga *Dunaliella sp.* isolated from high pollution waters was proved to be more active against bacteria than its ecotypes isolated from low pollution waters (Lustigman, 1988).

Table 2.1. Antibacterial activity from microalgae against human pathogenic bacteria.

Microalgae species	Antibacterial compound/fraction	Gram + bacteria growth inhibition	Gram – bacteria growth inhibition	References
Green microalgae				
Chlamydomonas reinhardtii	Aqueous or methanolic and exanolic extracts	*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis*	*Escherichia coli, Pseudomonas aeruginosa, Salmonella thyphi*	(Ghasemi et al., 2007)
Chlorella minutissima	Ethanolic extracts	*S. aureus*	*E. coli, P. aeruginosa*	(Ördög et al., 2004)
Chlorella pyrenoidosa	Various organic solvent extracts	*B. subtilis, S. aureus*	*E. coli, P. aeruginosa*	(Abedin and Taha, 2008)
Chlorella vulgaris	Chlorellin	*B. subtilis, S.aureus, Streptococcus pyogenes*	*E. coli, P. aeruginosa*	(Pratt et al., 1944)
Chlorella vulgaris	Aqueous or methanolic and hexanolic extracts	*B. subtilis, S. aureus, S. epidermidis*	*E. coli, P. aeruginosa, S. thyphi*	(Ghasemi et al., 2007)
Chlorococcum HS-101	alpha-linolenic acid	*B. subtilis, Bacillus cereus, S. aureus,* MRSA	*E. aerogenes*	(Chang et al., 1993; Ohta et al., 1993, 1995)
Chlorococcum humicola	Various organic solvent extracts and purified pigments (carotenoid, chlorophyll)	*B. subtilis, S. aureus*	*E. coli, P. aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae, Vibrio cholerae*	(Bhagavathy et al., 2011)
Desmococcus olivaceus	Ethanolic extracts	*S. aureus*	*E. coli, P. aeruginosa*	(Ördög et al., 2004)
Dunaliella primolecta	Polyunsatured fatty acids: alpha-linolenic acid	*B. cereus, B subtilis, S. aureus,* MRSA	*Enterobacter aerogenes*	(Chang et al., 1993; Ohta et al., 1995)
Dunaliella salina	Indolic derivative, polyunsaturated fatty acids, beta-ionone and neophytadiene	*S.aureus*	*E. coli, P.aeruginosa*	(Herrero et al., 2006; Mendiola et al., 2008; Pane et al., 2015)
Dunaliella sp.	Lysed cells	*S. epidermidis, Micrococcus luteus*	*Proteus vulgaris*	(Lustigman, 1988)
Haematococcus pluvialis	Short-chain fatty acids	*S.aureus*	*E. coli*	(Rodríguez-Meizoso et al., 2010; Santoyo et al., 2009)
Klebsormidium sp.	Pellet	*B. subtilis*	Ne	(Mudimu et al., 2014)
Pseudokirchneriella subcapitata	Methanolic extracts	*S.aureus*	*P.aeruginosa*	(Pane et al., 2015)
Scenedesmus obliquus	Long chain fatty acids	*S. aureus*	*E. coli, P. aeruginosa, Salmonella sp.*	(Guedes et al., 2011)

Table 2.1 *Cont.*

Microalgae species	Antibacterial compound/fraction	Gram + bacteria growth inhibition	Gram – bacteria growth inhibition	References
Green microalgae				
Scenedesmus quadricauda	Various organic solvent extracts	*B. subtilis, S. aureus*	*E. coli, P. aeruginosa*	(Abedin and Taha, 2008)
Scenedesmus sp.	Ethanolic extracts	*S. aureus*	*E. coli, P. aeruginosa*	(Ördög et al., 2004)
Red microalgae				
Porphyridium aerugineum	Phycobiliproteins	*S. aureus, S. pyogenes*	Nt	(Najdenski et al., 2013)
Porphyridium purpureum	Methanolic extracts	*B. subtilis*	*E. coli*	(Mudimu et al., 2014)
Porphyridium sordidum	Pellet	*B. subtilis*	*E. coli, Pseudomonas fluorescens*	(Mudimu et al., 2014)
Rhodella reticulata	Exopolysaccharides	*S. aureus, B. cereus, S. pyogenes*	Ne	(Najdenski et al., 2013)
Diatoms				
Asterionella glacialis	Whole cell	*S. aureus, S. epidermidis, M. luteus, Sarcina sp.*	*E. coli*	(Viso et al., 1987)
Attheya longicornis	Methanolic extracts	*S. aureus*, MRSA	Ne	(Ingebrigtsen et al., 2015)
Chaetoceros muelleri	Unsaturated fatty acid-containing lipidic fractions (triglycedrides and DPA)	*B. subtilis, S. aureus*	*E. coli*	(Mendiola et al., 2007; Sánchez-Saavedra et al., 2010)
Navicula delognei (Parlibellus delognei)	transphytol ester, hexadecatetraenoic and octadecatetraenoic acids	*S. aureus, S.epidermidis*	*S.typhimurium, P.vulgaris*	(Findlay and Patil, 1984)
Phaeodactylum tricornutum	eicosapentaenoic acid (EPA), palmitoleic and hexadecatrienoic acids (HTA)	*B. cereus, Bacillus weihenstephanensi, S. aureus, S. epidermidis*, MRSA	Ne	(Desbois et al., 2008, 2009)
Rhizosolenia alata	Various organic solvent extracts	*B. subtilis, S. aureus*	*E. coli, P. aeruginosa, P. vulgaris, S. typhi, V. cholerae*	(Venkatesan, R. et al., 2007)
Thalassiothrix frauenfeldii	Non-axenic culture and organic solvent extracts	*B. subtilis, S. aureus*	*E. coli, P. aeruginosa, S. paratyphi, S. typhi, V. cholerae*	(Walter, C.S and Mahesh, R, 2000)
Skeletonema costatum	Aqueous and organic extracts	*B. subtilis, S. aureus*	*P. aeruginosa*	(Cooper et al., 1983)
Skeletonema costatum	Various organic solvents extracts	*S. aureus, S. peoria, S. fecalis, S. pyogenes*	Ne	(Duff et al., 1966)
Haptophytes				
Isochrysis galbana	Chlorophyll a derivatives : pheophytin a and chlorophyllide a	*S. aureus, Streptococcus faecalis, S. pyogenes, Micrococcus sp.*	Nt	(Bruce et al., 1967; Duff et al., 1966)

Ne = No effect of the microalgal compound against the bacteria tested; Nt = Not tested; MRSA = Methicillin resistant S. aureus.

2.1.1 Toward improving extraction techniques

Beside the microalgal species, the presence of the antibacterial compound in the microalgal extracts is also highly dependent on the solvent used during the extraction. As the biological activity is rarely found in aqueous extracts (Cannell et al., 1988; Venkatesan, R. et al., 2007; Walter, C.S and Mahesh, R, 2000), it seems that compounds with an activity against foodborne and human pathogenic bacteria in microalgae are mostly hydrophobic and can be more readily extracted with organic solvents. Some authors found that antibacterial activity was generally found in methanol extracts (Cannell et al., 1988; Mudimu et al., 2014), while some other studies described a better extraction using acetone (Venkatesan, R. et al., 2007; Walter, C.S and Mahesh, R, 2000), benzene and ethyl acetate extracts (Bhagavathy et al., 2011) or petroleum ether and hexane (Herrero et al., 2006).

Other techniques have been tested to extract bioactive compounds from microalgae, such as the supercritical CO_2 extracts, pressurized liquid extraction (PLE) or subcritical water extraction (SWE). These techniques are considered as “greener” than the traditional techniques as they do not need large quantities of organic solvents, allow a faster extraction and are more selective toward the compounds of interest (Herrero et al., 2013). Supercritical CO_2 method allowed obtaining lipid fractions from *Chaetoceros muelleri* with antibacterial activity against *Staphyloccocus aureus* and *Escherichia coli* (Mendiola et al., 2007), while a classic extraction with solvents such as hexane, dichloromethane and methanol did not demonstrate any activity against *E. coli* (del Pilar Sanchez-Saavedra, M. et al., 2010). PLE and SWE permitted to extract antimicrobial agents from *H. pluvialis* in the red phase with good efficiency (Herrero et al., 2006; Rodríguez-Meizoso et al., 2010; Santoyo et al., 2009). SWE presents the advantage not to require the use of toxic solvent, and low temperatures such as 50°C can allow a good extraction. SWE could therefore represent an interesting green technique to obtain extracts for natural ingredients, and particularly for the food industry.

2.1.2 Diversity of antibacterial compounds extracted from microalgae

In some studies, the antibacterial compounds present in the organic extracts were characterized. These bioactive compounds can be pigments, such as phycobiliproteins (Najdenski et al., 2013) or chlorophyll derivatives (Bruce et al., 1967; Jørgensen, 1962), but they are most of time free fatty acids. Short chain fatty acids from *H. pluvialis* (Rodríguez-Meizoso et al., 2010; Santoyo et al., 2009) and long chain fatty acids from *Scenedesmus obliquus* (Guedes et al., 2011) present antibacterial activity against *E. coli* and *S. aureus*. The polyunsaturated fatty acids from *Chlorococcum* strain HS-101 and *Dunaliella primolecta* demonstrated antibacterial activity against the methicillin-resistant *S. aureus* (MRSA), a bacteria causing infections that kill thousands of people per year and which is highly resistant to conventional antibiotics (Ohta et al., 1995). Desbois et al. also found fatty acids from the diatom *Phaeodactylum tricornutum* with a very potent antibacterial activity against the MRSA and have characterized three different unsaturated fatty acids involved in the antibacterial activity: the polyunsaturated fatty acid eicosapentaenoic acid (EPA), the monounsaturated fatty acid palmitoleic acid (PA) and the relatively unusual polyunsaturated fatty acid hexadecatrienoic acid (HTA ; Desbois et al., 2008, 2009). It has also been observed that the fusiform morphotype of this diatom produced greater levels of EPA, PA and HTA compared to its oval morphotype (Desbois et al., 2010).

In natural environments, fatty acids are released when the microalgal cell loses its integrity and they seem to be involved in an 'activated' defence mechanism to protect an algal population against grazing predators (Jüttner, 2001) and when pathogenic bacteria are in the vicinity of the algae (Smith et al., 2010). Moreover, it has previously been shown that fatty acids possess bactericidal properties against a diverse range of bacteria (Kabara et al., 1972). The exact mechanism with which the bioactive compounds exert their antibacterial activity is not yet fully elucidated, but it seems that bacterial cellular membranes would be the main site of action (Galbraith and Miller, 1973). There are some evidences of deleterious effects of fatty acids in the bacterial membrane, causing a cell leakage, a reduction of nutrient intake and a reduction of the cellular respiration (Smith et al., 2010). The

antibacterial action of fatty acids may also be mediated by the inhibition of bacterial fatty acid synthesis (Zheng et al., 2005).

These mechanisms could explain why Gram-positive bacteria are more susceptible to microalgae bioactive compounds than Gram-negative bacteria. In fact, the bacterial growth inhibition is generally lower when microalgae are tested against Gram-negative bacteria, and in some cases the tested extracts do not present any bactericidal effect. As examples, the phycobiliproteins and exopolysaccharides from the red microalgae *Porphyridium aerugineum* and *Rhodella reticulata* respectively, were active against the Gram-positive bacteria *S. aureus* and *Streptoccocus pyogenes* but presented no effect against the Gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa* (Najdenski et al., 2013). The diatom *P. tricornutum* did not demonstrated antibacterial effect against these two Gram-negative bacteria either, whereas a good antibacterial activity against the gram-positive MRSA was observed (Desbois et al., 2009). Thus, the difference in sensitivities between Gram–positive and Gram–negative bacteria may be due to their complex membrane permeability, making the penetration and the bactericidal action of the compound more difficult.

The potent activity of microalgal compounds, especially free fatty acids, against various bacteria straightens further development in the search for drugs and food preservatives from microalgae. Bacterial resistance to free fatty acids has not been encountered yet (Petschow et al., 1996; Sun et al., 2003), so their exploitation in medicine deserves to be further investigated (Desbois et al., 2009). Furthermore, as consumers tend to avoid synthetic preservatives, microalgae could be good candidates as natural sources against food-borne pathogens.

2.2 Use of microalgae against pathogenic bacteria in aquaculture

Bacteria are the main cause of infections in intensive aquaculture of finfish and shellfish worldwide (Austin, 2012), occurring as well in hatcheries and nurseries as in rear and grow-out ponds. These infections can lead to serious mass mortalities and imply considerable economic losses. A wide range of bacteria are known to infect farmed and wild species with minor to severe consequences on health and survival. Among the important bacterial diseases in finfish we can cite those caused by *Aeromonas sp.* and *Pseudomonas sp.,* inducing hemorrhages in many fresh water species, or *Vibrio sp.* inducing vibrioses in marine fish species (Austin and Austin, 2007). *Vibrio* are able to infect a wide variety of hosts (Vandenberghe et al., 2003) and are also the most common and harmful shrimp pathogenic bacteria, with luminous species such as *Vibrio harveyi* involved in mass mortalities in shrimp hatcheries (Aguirre-Guzmán et al., 2004).

Antibiotics have been largely used in intensive fish farming but, as for humans and terrestrial animals, bacterial resistance in aquaculture is increasing and most antibiotics are no longer effective (Fernández-Alarcón et al., 2010; Miranda and Zemelman, 2002). The presence of drug residues in tissues of aquatic animals and the risk of transferring resistant bacteria to humans have led to a great concern about the use of synthetic antibiotics for public health. Strict regulations have thus been established, and a few antibiotics are now licensed in aquaculture. Various alternative and natural compounds are available today to control aquatic pathogenic bacteria, mainly derived from plants. They present the advantages of decreasing the side effects observed with synthetic antibiotics and being less expansive (Citarasu, 2012). In this context, several microalgae species have been investigated for their antibacterial activity *in vitro,* and in co-culture with pathogenic bacteria, and some studies have also been conducted *in vivo* with the “green water” technique and using microalgae as food supplement (Table 2.2).

Table 2.2. Antibacterial activity from microalgae against diseases in aquaculture.

Microalgae species	Compound/fraction tested	Target bacteria/antibacterial effect	References
***In vitro* experiments**			
Chaetoceros lauderi	Whole cell	*Vibrio anguillarum, Aeromonas salmonicida*	(Viso et al., 1987)
Dunaliella tertiolecta	Aqueous extract	*Vibrio campbellii*	(González-Davis et al., 2012)
Euglena viridis	Organic solvent extracts	*Aeromonas hydrophila, Edwardsiella tarda,, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida Vibrio alginolyticus, V. anguillarum, Vibrio fluvialis, Vibrio harveyi, Vibrio parahaemolyticus*	(Das et al., 2005)
Haslea karadagensis	Purified pigment (intra- and extracellular forms)	*Polaribacter irgensii, Pseudoalteromonas elyakowii, Vibrio aestuarianus*	(Gastineau et al., 2012c)
Haslea ostrearia	Purified marennine (intra- and extracellular forms)	*P. irgensii, P. elyakowii, V. aestuarianus*	(Gastineau et al., 2012b)
	Purified marennine (intracellular form)	*V. anguillarum*	(Pouvreau, 2006)
	Purified marennine (extracellular form)	*Vibrio splendidus*	(Gastineau et al., 2014b)
Phaeodactylum tricornutum	Aqueous and organic extracts	*Alcaligenes cupidus, Alteromonas communis, Alteromonas haloplanktis, Vibrio fischeri, V. parahaemolyticus*	(Cooper et al., 1983)
	Polyunsaturated free fatty acid	*Listonella. anguillarum, M. luteus, Photobacterium sp.*	(Desbois et al., 2009)
Skeletonema costatum	Aqueous and Organic extracts	*A. cupidus, A. communis, Pseudomonas marina, V. fischeri, V. parahaemolyticus*	(Cooper et al., 1983)
	Organic and purified extracts	*L. anguillarum, Vibrio mytili T, Vibrio spp. S322, Vibrio spp. VRP*	(Naviner et al., 1999)
	Aqueous extracts	*Vibrio campbellii*	(González-Davis et al., 2012)
Stichochrysis immobilis	Microalgal homogenates	*Xanthomonas sp. 1, Flavobacterium sp., Pseudomonas sp. Strain 101*	(Berland et al., 1972)
Tetraselmis suecica	Microalgal supernatant and microalgal homogenates of a commercial spray-dried preparation	*A. A. hydrophila, A. salmonicida, Serratia liquefaciens, V. alginolyticus, V. anguillarum, V. parahaemolyticus, Vibrio salmonicida, Vibrio vulnificus, Yersinia ruckeri*	(Austin and Day, 1990; Austin et al., 1992)
Co-culture experiments			
Chaetoceros calcitrans	Axenic culture	*V. harveyi*	(Lio-Po et al., 2005)
Chlorella minutissima	Axenic culture	*V. alginolyticus, V. anguillarum, Vibrio lentus, V. parahaemolyticus, Vibrio scophthalmi, V. splendidus,*	(Kokou et al., 2012)
Chlorella sp.	Axenic culture	*V. harveyi*	(Tendencia and dela Peña, 2003)
Isochrysis galbana	Non-axenic culture	*V. alginolyticus, V. campbellii, V. harveyi*	(Molina-Cárdenas et al., 2014)
Isochrysis sp.	Axenic culture	*V. alginolyticus, V. lentus, V. splendidus, V. scophthalmi, V. parahaemolyticus, V. anguillarum*	(Kokou et al., 2012)
Nannochloropsis sp.	Axenic culture	*V. alginolyticus, V. anguillarum, V. lentus, V. parahaemolyticus, V. scophthalmi, V. splendidus*	(Kokou et al., 2012)
Nitzchia sp.	Axenic culture	*V. harveyi*	(Lio-Po et al., 2005)
S. costatum	Exometabolites in the culture fluid	*Listeria monocytogenes*	(Terekhova et al., 2009)
	Axenic culture	*Pseudomonas sp., Vibrio sp.*	(Kogure et al., 1979)
Tetraselmis chui	Axenic culture	*V. alginolyticus, V. anguillarum, V. lentus, V. parahaemolyticus, V. scophthalmi, V. splendidus*	(Kokou et al., 2012)

Table 2.2. *Cont.*

Microalgae species	Compound/fraction tested	Target bacteria/antibacterial effect	References
***In vivo* experiments**			
C. minutissima	30 min incubation of enriched *Artemia metanauplii*	Decrease of the bacterial load in *Artemia* and diminution of presumptive *Vibrio*	(Makridis et al., 2006)
D. tertiolecta	Daily diet of *Artemia franciscana*	Protection against *V. campbellii* and *V. proteolyticus*	(Marques et al., 2006)
H. ostrearia	Incubation of *Mytilus edulis* and *Placopecten magellanicus* larvae with supernatant containing extracellular pigments	Higher survival and physiological conditions of larvae challenged with *V. splendidus*	(Turcotte et al., 2016)
Tetraselmis sp.	4 h incubation of *Artemia franciscana*	Diminution of associated bacteria, better bacterial diversity and the flora less dominated by *V. alginolyctus*	(Olsen et al., 2000)
T. suecica	Food supplement for the Atlantic salmon *Solmo salar*	Reduction of *A. hydrophila, A. salmonicida, S. liquefaciens, V. anguillarum, V. salmonicida, Y. ruckeri* infections, reduction of bacterial populations in water tanks and increase of the microbial communities in the digestive tract	(Austin et al., 1992)
	Food supplement for the broodstock and partial live larvae feed for the white prawn *Fenneropenaeus inducus*	Reduction of *Vibrio* numbers in water tank, better egg hatching rate and survival rate of the larvae	(Regunathan C. and Wesley S.G., 2004)

2.2.1 Benefits of the "green-water" technique against bacterial diseases in aquaculture

The "green water" technique has demonstrated beneficial effects on health, survival rate and resistance of different organisms (Cahu et al., 1998; Marques et al., 2006; Naas et al., 1992; Papandroulakis et al., 2001; Reitan et al., 1997; Supamattaya et al., 2005; Van der Meeren et al., 2007). Addition of the microalga *Isochrysis galbana* allowed a better viability and a faster grow of the turbot *Scophthalmus maximus* larvae as well as a lower proliferation of opportunistic bacteria (Salvesen et al., 1999). The green microalga *Tetraselmis suecica* first showed good antibacterial activity *in vitro* against several *Vibrio* species (Austin and Day, 1990) and was then proved to reduce *in vivo* the number of various bacterial species in the water tank of the Atlantic salmon *Salmo salar* (Austin et al., 1992). Adding *T. suecica* also reduced the number of *Vibrio* species in broodstock gut, eggs and larvae of the white prawn *Fenneropenaeus indicus*, resulting in improved egg hatching and larval survival (Regunathan C. and Wesley S.G., 2004). A better survival and physiological conditions of the blue mussel *Mytilus edulis* larvae and the scallop *Placopecten magellanicus* larvae were observed when

incubated with supernatant containing marennine, the extracellular blue pigment of the diatom *H. ostrearia*, at concentrations as low as 0.1 mg/L (Turcotte et al., 2016). The use of the diatoms *Skeletonema costatum* and *Chaetoceros calcitrans* to feed *Penaeus monodon* larvae allowed a growth inhibition of the luminous bacteria *Vibrio harveyi* (Lavilla-Pitogo et al., 1998). However, this seemed mainly due to the microbial flora associated with the diatoms rather than their metabolic products. Indeed, the effectiveness of the “green water” culture in preventing bacterial infections and outbreaks can also be attributed to the presence of antibacterial factors in the bacterial, fungal and phytoplankton microbiota associated with this culture technique (Lio-Po et al., 2005).

Yet, several co-culture experiments with axenic microalgae demonstrated the ability of some species to produce and release compounds with potent activity against pathogenic bacteria. Axenic cultures of *Chlorella minutissima, Tetraselmis chui, Isochrysis sp.* and *Nannochloropsis sp.* limited the growth of various *Vibrio* species (Kokou et al., 2012), and growth of the luminous bacteria *V. harveyi* highly decreased when co-cultured with pure *Chlorella sp.* (Tendencia and dela Peña, 2003)*, C. calcitrans* or *Nitzschia sp.* (Lio-Po et al., 2005).

Finally, genetically modified microalgae were also used *in vivo*. In a recent study, better growth, resistance to bacteria and survival rate were observed in the shrimp postlarvae *P. monodon* fed with “fusant” *Chlorella* and *Dunaliella* (Kusumaningrum and Zainuri, 2015). The so-called “fusant” microalgae resulted from the generation of a unique cell through somatic hybridization by fusion of the two protoplasts. This fusion technology is especially used to transfer agronomically useful traits to plants (Davey et al., 2005) and would allow an improvement of valuable metabolites production from these two microalgae. Another genetically modified microalga tested in “green water” systems is the transgenic line of *Nannochloropsis oculata,* able to produce the antimicrobial bovine lactoferricin (LFB) peptide. These LFP-containing transgenic microalgae were developed and tested as food supplement for the medaka fish *Oryzias latipes* infected with *Vibrio parahaemolyticus*, which displayed a significantly better survival rate after 24h (Li and Tsai, 2009).

2.2.2 Microalgae to improve the live-food quality

Microalgae also show advantages for the live food quality (Marques et al., 2006; Øie et al., 1994), by reducing the number of associated pathogenic bacteria such as *Vibrio* and allowing a lower risk of transmission to fish larvae. Daily addition of *Dunaliella tertiolecta* to feed the brine shrimp *Artemia franciscana*, considered as an essential part of the live food chain for the culture of fish and shellfish larvae, conferred a full protection against *Vibrio campbellii* and *Vibrio proteolyticus* (Marques et al., 2006). A 4 h incubation of *A. franciscana* with the microalgae *Tetraselmis sp.* resulted in a diminution by 75 % of associated bacteria, with a better bacterial diversity and the flora less dominated by *Vibrio alginolyticus* (Olsen et al., 2000). Similar observations were made by Makridis et al. with incubation of *Artemia* with *T. chui* and *C. minutissima* (Makridis et al., 2006). These authors proposed that the reduction of *Vibrio* cells in *Artemia* cultures could either be due to compounds released by microalgae or to Gram-positive associated bacteria.

2.2.3 *In vitro* efficiency of microalgal compounds against marine bacteria

Several *in vitro* studies demonstrated the ability of various microalgae to produce antibacterial compounds effective against relevant marine pathogenic bacteria. The whole cells of *Chaetoceros lauderi* (Viso et al., 1987), supernatant and homogenates of *T. suecica* (Austin and Day, 1990; Austin et al., 1992) or homogenates from *Stichochrysis immobilis* (Berland et al., 1972) induced a growth inhibition of various marine bacteria. However, Berland et al. noted that limited attention should be paid to antibacterial activity obtained with broken cells, as in natural conditions substances synthesized have to be released into the water to target another organism (Berland et al., 1972). Microalgae producing antibacterial compounds that are not released in the medium cannot indeed be considered gainful in “green water” techniques, but these compounds can be highly useful in the design of novel drugs.

Only a few compounds with activity against marine bacteria have been characterized, such as the polyunsaturated free fatty acid in *P. tricornutum*, identified as ecosapentaenoic acid (Desbois et al., 2009). An antibacterial activity was also demonstrated *in vitro* with the

blue pigment of the two diatoms *H. ostrearia* and *Haslea karadagensis.* These blue pigments can be observed in the apex of the microalgae (intracellular form) but can also be released in the medium (extracellular form). The purified forms of these pigments (Pouvreau et al., 2006b) have been shown to inhibit the growth of several marine bacteria including *Vibrio* species such as *Vibrio splendidus* or *Vibrio aestuarianus* (Gastineau et al., 2012b, 2012c, 2014b; Pouvreau, 2006), involved in oyster mass mortality (Garnier et al., 2008).

For instance, a series of experiment were conducted to assess the spectrum of activity of purified extracellular marennine, the pigment produced by *H. ostrearia,* against pathogenic *Vibrio* species. The growth of *V. splendidus* was slowed down when exposed to a concentration of marennine up to 10 µg mL^{-1} and seemed totally inhibited with a concentration of 1 mg mL^{-1} (Figure 2.1).

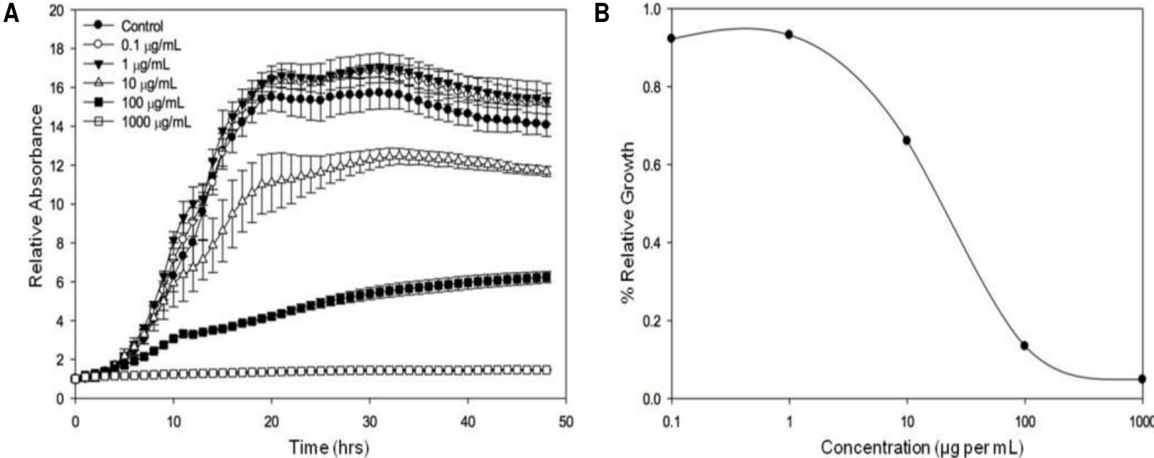


Figure 2.1. Growth inhibition of *Vibrio splendidus* by purified marennine, the blue pigment produced by *Haslea ostrearia*. **(A)** *V. splendidus* was grown over night at 25°C, cells were then washed with sterile water 2 times and adjusted to an OD_{600}=0.5. Cells were added to wells with marennine in the following concentrations: 0, 0.1, 1, 10, 100 and 1000 µg mL^{-1}. Kinetics were run at OD_{600} for 48 h, with measurements taken every 30 min (n=3). **(B)** Relative values were graphed in order to account for the absorbance differences due to the pigment. The effective concentration reducing bacteria growth rate by 50 %, EC_{50}, was estimated at 19.14 µg mL^{-1} (Standard error: 6.736; Pasetto et al., unpublished).

In another series of experiments, sensitivity of various strains of *V. aestuarianus, Vibrio coralliilyticus* and *Vibrio tubiashii* to purified extracellular marennine was assessed by exposing the bacteria to marennine concentration ranging from 1 to 100 µg mL^{-1}. For all the three species, after 48 h, the higher the marennine concentration, the higher bacterial growth inhibition (*data not shown*), confirming the antibacterial activity of the pigment produced by the diatom *H. ostrearia*. Moreover, when comparing different ecotypes within a same *Vibrio* species, the sensitivity of the strains exposed to marennine could be significantly different (Figure 2.2). Complementary experiments are under way in order to confirm and precise the biological effect of marennine on these *Vibrio* species and to highlight the variability in sensitivity of different strains within a same species.

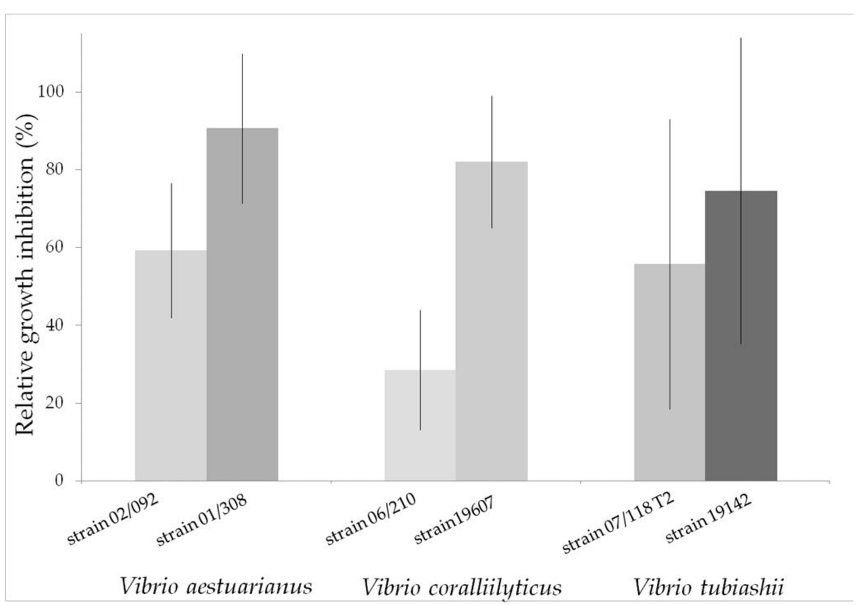


Figure 2.2. Relative growth inhibition of three *Vibrio* species: *V. aestuarianus, V. coralliilyticus, V. tubiashii,* after a 48 h exposition to purified extracellular marennine, produced by the diatom *Haslea ostrearia.* Each strain was grown over night in a Mueller-Hinton Broth medium at 22°C and their concentration was then adjusted to an OD_{600}=0.1. Bacterial cultures were exposed for 48 h to marennine at a concentration of 100 µg mL^{-1} before OD measurement. The relative growth inhibition was assessed in comparison with the growth of the control, not exposed to marennine. A difference of sensitivity between the two *V. aestuarianus* and *V. coralliilyticus* strains was observed. Results are means ± SD, for two separate experiments conducted using triplicates (ANOVA statistical test, p-value 0.01 and 7.10^{-4} respectively; Falaise et al., unpublished).

2.2.4 Interactions between microalgae and marine bacteria

The antibacterial mechanisms of microalgae are still unclear and the bioactive compounds released by the cells could either be bactericidal or prevent the bacterial multiplication. The very rapid growth inhibition of various *Vibrio* species in co-culture with *Chlorella sp., Isochrysis sp.* or *Nannochloropsis sp.* with no recovery after few days (Kokou et al., 2012; Tendencia and dela Peña, 2003) allows considering a bactericidal action of the extracellular substances produce by some microalgae (Molina-Cárdenas et al., 2014). Austin et al. also observed *in vitro* a prompt inhibition of several *Vibrio* species by *T. suecica* and noticed a very rapid decrease in bacterial mobility with an elongation and vacuolisation of the cells in less than 20 min. Though, a reduction of the inhibitory activity was observed after only 5 h. It was suggested that the bioactive substance could have been denatured or adsorbed by some bacterial cells, allowing others to grow (Austin and Day, 1990; Austin et al., 1992).

In some cases, the antibacterial activity of microalgae can be induced by the presence of bacteria in the vicinity of the microalgae, or can be constitutive and always present in the algal culture medium (Safonova and Reisser, 2005). The constitutive production of antibacterial exometabolites by some microalgae was highlighted with the growth diminution of *Listeria monocytogenes* in co-culture with the cell-free culture media of *S. costatum* (Terekhova et al., 2009).

Microalgae can influence marine bacteria in different ways. They can either inhibit or stimulate bacterial growth, or have no apparent effect, depending on the target bacteria (Berland et al., 1972; Kogure et al., 1979). As an example, the diatom *S. costatum* inhibits the growth of *Pseudomonas* and *Vibrio* in co-culture, but enhances the growth of *Flavobacterium* (Kogure et al., 1979). The production of antibacterial compounds by microalgae such as lipids or fatty acids varies according to the taxonomic group, the growth conditions, the available nutrients and their concentration in the medium, the light intensity, the temperature or the pH. The development stage of the algal culture is also highly significant as it is assumed that various secondary metabolites are produced and released in the medium at different growth phases (Borowitzka, 1995). Terekhova et al. showed that only the exometabolites produced by *S. costatum* during the middle steady-state growth

phase presented an antibacterial activity against *L. monocytogenes* while compounds released during the exponential growth phase had no effect on these bacteria (Terekhova et al., 2009). Cooper et al. have also demonstrated the direct relation between cell growth phase and antibacterial activity, and showed that *P. tricornutum* presented a better activity against a wide spectrum of marine bacteria during the exponential growth phase compared to the stationary phase, while the reverse relationship was found for *S. costatum* (Cooper et al., 1983).

3 3. ANTIFUNGAL ACTIVITY FROM MICROALGAE

3.1 Antifungal activity from microalgae against human pathogens

Searching for antifungal compounds from microalgae started much later than screening for antibacterial activity. As a matter of fact, fungi have been considered as harmful human pathogens since the 70's, when mortality induced by fungal infections and the frequency of nosocomial mycoses increased in hospitalized patients. Increase of fungal infections was mainly due to therapies that depress patients' immune system such as the use of intensive and aggressive chemotherapy regimens, the expansion of organ transplant programs and the spread of the AIDS epidemic. (Alangaden, 2011; Anaissie and Bodey, 1989). The incidence of invasive aspergillosis (induced by *Aspergillus* species), and the number of cases of candidemia (an infection caused by *Candida* species), have been rising inexorably from that time (Manuel and Kibbler, 1998; Wey SB et al., 1988). The growing use of antifungal agents in recent years has led to the development of drug resistance (Accoceberry and Noël, 2006; Ghannoum and Rice, 1999). Thus, there is a need for novel drugs and several study were recently conducted to find fungicide activity from marine natural products against human pathogenic fungi (Cheung et al., 2014; El Amraoui et al., 2014; Hong et al., 2015; Shishido et al., 2015; Wang et al., 2015b; Xu et al., 2015), including antifungal agents from microalgae (Table 2.3).

Table 2.3. Antifungal activity from microalgae.

Microalgae species	Antifungal compounds/fraction	Target microorganims	References
Green microalgae			
Chlorella vulgaris	Microalgal supernatant	Yeast: *Candida kefyr* Mold: *Aspergillus fumigatus, Aspergillus niger*	(Ghasemi et al., 2007)
Chlorella pyrenoidosa	Organic solvent extracts	Yeast: *Candida albicans, Saccharomyces cerevisiae.* Mold: *Aspergillus flavus, A. niger, Penicillium herquei.*Other: *Alternaria brassicae, Fusarium moniliforme, Helminthosporium sp.*	(Abedin and Taha, 2008)
Chlorococcum humicola	Organic solvent extracts and pigments: beta carotene, Chlorophyll a and Chlorophyll b	Yeast: *C. albicans.* Mold: *A.flavus, A.niger*	(Bhagavathy et al., 2011)
H. luteoviridis	Microalgal supernatant	Yeast: *C.albicans*	(Mudimu et al., 2014)
Haematococcus pluvialis	Short-chain fatty acids	Yeast: *C.albicans*	(Santoyo et al., 2009)
Scenedesmus quadricauda	Organic solvent extracts	Yeast: *C. albicans, S. cerevisiae.* Mold: *A. flavus, A. niger, P. herquei.* Other: *A. brassicae, F. moniliforme, Helminthosporium sp.*	(Abedin and Taha, 2008)
Red microalgae			
Porphyridium aerugineum	Phycobiliproteins	Yeast: *C. albicans*	(Najdenski et al., 2013)
Porphyridium purpureum	Microalgal supernatant	Yeast: *C. albicans*	(Mudimu et al., 2014)
Rhodella reticulata	Exopolysaccharides	Yeast: *C. albicans*	(Najdenski et al., 2013)
Diatoms			
Chaetoceros lauderi	Polysaccharides	Mold: *A. fumigatus, Blastomyces dermatitidis, Emmonsia parva, Madurella mycetomi, Sprorothrix schenckii.* Dermatophyte: *Epidermophyton floccosum, Microsporum audouini, Microsporum canis, Microsporum ferrugineum, Microsporum gypseum, Microsporum nanum, Microsporum persicolor, Trichophyton spp.*	(Gueho et al., 1977; Pesando et al., 1979a; Viso et al., 1987)
Chaetoceros muelleri	Lipidic fractions: triglycerides, DPA	Yeast: *C. albicans*	(Mendiola et al., 2007)
Haslea karadagensis	Purified pigment (intra- and extracellular forms)	*Corollospora maritima, Lulworthia sp., Dendryphiella salina*	(Gastineau et al., 2012c)
Thalassiothrix frauenfeldii	culture filtrates and organic solvent extracts	Yeast: *C. albicans, Candida glabrata, Candida krusei, Candida tropicalis, Cryptococcus neoformans.* Mold: *A. niger*	(Walter, C.S and Mahesh, R, 2000)
Dinoflagellates			
Amphidinium sp.	Polyols: karatungiols A(1)	Mold: *A. niger*	(Washida et al., 2006)
Gambierdiscus toxicus	Gambieric acids A and B forms	Mold: *A. fumigatus, A. niger, Aspergillus oryzae, Penicillium citrinum, Penicillium chrysogenum, Penicilliumvariotii.* Dermatophyte: *E. floccosum, T. mentagrophytes*	(Nagai et al., 1993)
Goniodoma pseudogonyaulax	Goniodomin A (polyether macrolide)	Yeast: *C. albicans, C. neoformans, S. cerevisiae.* Mold: *Penicillium sp.* Dermatophyte*: T. mentagrophytes*	(Murakami et al., 1988; Sharma et al., 1968)
Prorocentrum lima	Polyethers	Yeast: *Candida rugosa.* Mold: *A. niger, Penicillium funiculosum*	(Nagai et al., 1990)

There are less screening activities for microalgal fungicides than for bactericides, and most of the studies focus not only on the antifungal activity but also on antibacterial activities (Abedin and Taha, 2008; Bhagavathy et al., 2011; Gastineau et al., 2012c; Gauthier, 1969; Katircioglu et al., 2005; Mudimu et al., 2014; Nagai et al., 1990; Najdenski et al., 2013;

Ördög et al., 2004; Viso et al., 1987; Walter, C.S and Mahesh, R, 2000). As for antibacterial activity, antifungal activity varies widely depending on microalgal species, type of solvent used to extract the compound and the microorganism tested. It does not seem to be a taxonomic trend for the antifungal activity, and the capability to produce antifungal compounds would have evolved independently of phylogenetic relationship in microalgae (Mudimu et al., 2014). However, Pesando et al. noticed a significant activity of the genus *Chaetoceros* (Pesando et al., 1979b) and Kellam et al. indicated also that marine microalgae showed more potential in the search for new antifungal agents than do freshwater species (Kellam et al., 1988).

As illustrated on Table 2.3, several antifungal compounds from various microalgae have been characterized. Polysaccharides with high molecular weight were identified in the diatom *C. lauderi*. They presented a large spectrum of activity against dermatophytes, moulds and phyto-fungi, but no activity was detected against the yeasts tested (Gueho et al., 1977; Pesando et al., 1979a; Viso et al., 1987). Gambieric acids from the dinoflagellate *Gambierdiscus toxicus* had also an antifungal activity against several dermatophytes and moulds but had not activity against yeasts like *Candida albicans* or *Saccharomyces cerevisiae* (Nagai et al., 1993). The diatom *Thalassiothrix frauenfeldii* was meanwhile active against yeasts and moulds but not against dermathophytes (Walter, C.S and Mahesh, R, 2000). Other compounds such as pigments like beta-carotene, chlorophyll-a and chlorophyll-b from *Chlorococcum humicola* (Bhagavathy et al., 2011), or phycobiliproteins from *Porphyridium aerugineum* have also demonstrated antifungal activities (Najdenski et al., 2013). The polyene-polyhydroxy metabolites amphidinols were extracted from the dinoflagellate *Amphidinium klebsii* (Houdai et al., 2004; Satake et al., 1991). Polyenes are metabolites known for having a potent antifungal activity as they target the biosynthetic pathway of ergosterols, found in fungi membranes (Ghannoum and Rice, 1999). These few results illustrate that the search for novel antifungal compounds from microalgae has not been greatly developed so far, although an increasing number of fungi display drug resistance phenomenon.

3.2 Potential use of microalgae against fungal diseases in aquaculture

An increase in fungal infections has been observed in the last few decades with the modernization and intensification of aquaculture at an industrial scale, resulting in huge loss of aquaculture industries. Fungal infections in aquaculture may cause severe diseases and mortality events leading to economical impacts. They are often considered secondary to other factors or pathogens such as consequences of water quality problems, fish trauma by rough handling or temperature shock, bacterial diseases or parasites (Yanong, 2003). Several fungi are known to induce diseases by developing in the skin and the gills of the infected fish or in eggs, or by producing toxins (Noga, 2011). Indeed, mycotoxins can provoke many disorders and can accumulate in fish tissues, representing a risk for public health (Anater et al., 2016).

Chemicals used to treat infected animals are limited and, due to the increasing resistance of fungi against conventional drugs, environmental legislations and consumer's safety, the alternative ‘herbal formulations’ and alternative save and cheap methods have become of renewed interest (Trakranrungsie, N., 2011). Studies have been conducted in order to find new antimycotics of natural origin that may have no harmful effect either on fish, fish eggs, human health or on ecosystems, such as plant extracts (Hussein et al., 2002; Mori et al., 2002; Udomkusonsri et al., 2007) or essential oils (Hussein et al., 2000; Mousavi et al., 2009).

So far, very few studies have been conducted to assess the antifungal activity from microalgae against pathogenic fungi in aquaculture systems (Table 2.3). Gastineau et al. have demonstrated *in vitro* the antifungal activity of the pigment produced by the diatom *H. karadagensis* against three marine fungal species *Corollospora maritima, Dendryphiella salina, Lulworthia sp.*, which can be involved in the phenomenon of biofouling (Gastineau et al., 2012c). Organic extracts of the green microalgae *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* have demonstrated antifungal activity against *Fusarium monofiliform* (Abedin and Taha, 2008) reported to cause black gill disease in shrimps

(Rhoobunjongde et al., 1991). This result is of great interest as *Fusarium sp.* were recently shown to produce toxins that accumulate in fish (Tolosa et al., 2014). *Aspergillus fumigatus* is also a fungus susceptible to produce toxins and it thus represents a potential contaminant for seafood, and particularly for marine bivalves (Grovel et al., 2003). It can be inhibited by compounds produced by *C. lauderi* (Viso et al., 1987), *G. toxicus* (Nagai et al., 1993), and *Chlorella vulgaris* (Ghasemi et al., 2007).

More generally, fungi from the taxa *Fusarium* and *Aspergillus* are found in many countries in water, sediments and marine invertebrates, and their presence in shellfish farming areas evidences the necessity to pay attention to shellfish contamination by such fungi (Matallah-Boutiba et al., 2012). Furthermore, reports of fungi causing deleterious effects are frequently related (Karthikeyan and Gopalakrishnan, 2014; Karthikeyan et al., 2015), which encourage a larger screening of microalgae for the production of novel antifungal compounds.

4 ANTIVIRAL ACTIVITY FROM MICROALGAE

4.1 Antiviral activity from microalgae against human pathogenic viruses

Viral pathogens are the leading cause of human diseases and mortality worldwide. Treatments to attack the pathogenic virus or its replication directly are difficult to design, as they can have adverse effects on the infected host cells (Kitazato K et al., 2007). Thus, treatments against diseases caused by virus are limited, and resistances to these available treatments demonstrate the need for new medicines (Yasuhara-Bell and Lu, 2010). Many drugs exhibiting selective inhibition of mammalian originate from synthetic organic chemicals or from natural products, for instance secondary metabolites in plants (Kitazato K et al., 2007). Along with the development of “blue” technology and extraction improvement, there is a growing interest in marine-derived antiviral compounds. Thus, thousands of compounds from various marine organisms such as algae, bacteria, fungi, marine invertebrates or sponges have been screened (Cheung et al., 2014; Yasuhara-Bell and Lu, 2010; Moussa et al., 2015; Vo et al., 2011) and some of them have demonstrated antiviral activities and are commercially available.

Potential antiviral activity from algal compounds has been first demonstrated in the 50’s by Gerber et al. who observed that the polysaccharides extracted from *Gelidium cartilagenium* afforded protection for embryonated eggs against influenza B and mumps viruses (Gerber et al., 1958), and the first brood-based studies of seaweeds for their antiviral substances started in the 70’s (Ehresmann et al., 1977). Screenings for antiviral compounds from macroalgae are still predominant (Bouhlal Rhimou, 2010; Luescher-Mattli, 2003; Schaeffer and Krylov, 2000; Soares et al., 2012; Wijesekara et al., 2011), but the interest for antiviral compounds from microalgae and cyanobacteria rapidly increased as they also present relevant antiviral activities and are easier to culture. Cyanobacteria are promising sources of antiviral compounds, and their simple growth needs make them good candidates for the production of antiviral agents at an industrial scale (Arif et al., 2012; Vijayakumar and

Menakha, 2015). The sulphated polysaccharide isolated from the cyanobacteria *Spirulina platensis*, named spirulan, has demonstrated potent antiviral activity against the herpes simplex virus type 1 (HSV-1) and also against the human immunodeficiency virus type 1 (HIV-1 ; Hayashi et al., 1996). A spirulan-like molecule isolated from *Arthrospira platensis* has also been indicated to possess antiviral activities against these two viruses, with absence of cytotoxic effects (Rechter et al., 2006).

Several studies have been conducted to test microalgae compounds against human pathogenic viruses (Table 2.4). Antiviral compounds extracted from microalgae are mainly polysaccharides. Their mechanisms of action against viruses are not fully understood but it seems that they can inhibit different stages of the viral infection, such as the adhesion, the penetration or the replication. Polysaccharides have gained interest in the biomedical and pharmaceutical industries as they are easily available in nature and most of them are nontoxic, safe, biodegradable and biocompatible (Ahmadi et al., 2015). However, numerous polysaccharides with antiviral activities were not developed for clinical use. The reasons are probably the very high molecular weights of some polysaccharides, preventing them to pass through the different barriers of the body and the incapacity of enzymes to digest these large and complex molecules, leading to their accumulation in the body and to potential cytotoxic effects (Huleihel et al., 2001).

Table 2.4. Antiviral activity from microalgae.

Microalgae species	Antiviral compound and cytotoxicity (µg mL^{-1})	Target virus	Mechanism of action and efficiency (µg mL^{-1})	References
Green microalgae				
Chlorella vulgaris	Polysaccharide-rich fraction CC_{50} >1600 (Vero cells)	HSV-1	Inhibit attachment, replication IC_{50} = 61	(Santoyo et al., 2010)
Dunaliella primolecta	Pheophorbide-like compound Not cytotoxic (Vero cells)	HSV-1	Inhibit adsorption, invasion MIC = 5 (totally inhibit the CPE)	(Ohta et al., 1998)
Dunaliella salina	Short chain fatty acids, β-ionone, neophytadiene, phytol, palmitic and α-linolenic acids CC_{50} = 1711 (Vero cells)	HSV-1	Inhibit infectivity IC_{50} = 85	(Santoyo et al., 2011)
Haematococcus pluvialis	Polysaccharide-rich fraction CC_{50} = 1867 (Vero cells)	HSV-1	Inhibit attachment, penetration, replication IC_{50} = 99	(Santoyo et al., 2011)
Red microalgae				
Porphyridium cruentum	Sulphated exopolysaccharide Not cytotoxic at 100 (HeL cells)	HSV-1 HSV-2 Vaccina	Inhibit penetration, replication EC_{50} (HSV-1) = 34 EC_{50} (HSV-2) = 12 EC_{50} (Vaccina) = 12	(Raposo et al., 2014)
Porphyridium purpureum	Exopolysaccharide Not cytotoxic at 500 (HEp-2 cells)	Vaccina	Interaction with free viral particles IC_{50} = 0.65	(Radonić et al., 2011)
Porphyridium sp.	Sulphated polysaccharide Not cytotoxic at 250 (Vero cells) and 2000 (*in vivo* in rats)	HSV-1	*In vitro*: inhibit adsorption, replication CPE_{50} = 1 *In vivo*: prevent the development of symptoms at 100	(Huleihel et al., 2001, 2002)
		HSV-2 VZV	Inhibit adsorption, replication CPE_{50} (HSV-2) = 5 CPE_{50} (VZV) = 0.7	
	Purified polysaccharide Not cytotoxic at 1000 (NIH/3T3 cells)	MuSV/MuLV MuSV-124	Inhibit the production of retroviruses in the cells RT_{50} reduction = 5 Inhibit cell transformation ffu_{50} protection = 10	(Talyshinsky et al., 2002)
Diatoms				
Haslea karadagensis	Purified pigment: intra- and extracellular forms CC_{50}(Int) = 87 CC_{50}(Ext) > 200 (Vero cells)	HSV-1	Inhibit infection, cell destruction EC_{50}(Int) = 62 EC_{50}(Ext) = 23	(Gastineau et al., 2012c)
Haslea ostrearia	Purified pigment: intra- and extracellular forms CC_{50}(Int) > 200 (Vero cells) CC_{50}(Ext) = 107 (Vero cells)	HSV-1	Inhibit infection, cell destruction EC_{50}(Int) = 24 EC_{50}(Ext) = 27	(Gastineau et al., 2012c)
	Water soluble extract CC_{50} > 200 (Vero and MT-4 cells)	HSV-1	Inhibit replication EC_{50} = 14	(Bergé et al., 1999)
Navicula directa	Sulphated polysaccharide: Navicülan CC_{50}(HSV-1) = 3800 (Vero cells) CC_{50}(HSV-2) = 3800 (Vero cells) CC_{50}(IFV-A)= 5400 (MDCK cells) CC_{50}(HIV-1) = 4000 (HeLA cells)	HSV-1 HSV-2 IFV-A HIV-1	Inhibit adhesion, penetration IC_{50}(HSV-1) = 14 IC_{50}(HSV-2) = 7.4 IC_{50}(IFV-A) = 170 IC_{50}(HIV-1) = 53	(Lee et al., 2006)

Table 2.4. *Cont.*

Microalgae species	Antiviral compound and cytotoxicity (µg mL^{-1})	Target virus	Mechanism of action and efficiency (µg mL^{-1})	References
Dinoflagellates				
Cochlodinium polykrikoides	Extracellular sulphated polysaccharides: A1 and A2 CC_{50}(HIV-1) > 100 (MT-4 cells) CC_{50}(IFV-A) > 100 (MDCK cells) CC_{50}(IFV-B) > 100 (MDCK cells) CC_{50}(RSV-A) > 100 (Hep-2 cells) CC_{50}(RSV-B) > 100 (Hep-2 cells)	HIV-1 IFV-A IFV-B RSV-A RSV-B	Inhibit replication and the CPE IC_{50} (HIV-1) = 1.7 IC_{50}(IFV-A) = 0.45 – 1 IC_{50}(IFV-B) = 7.1 – 8.3 IC_{50}(RSV-A) = 2 – 3 IC_{50}(RSV-B) = 0.8	(Hasui et al., 1995)
	A1 CC_{50} > 100 (HMV-2 cells)	HSV-1	IC_{50} = 4.5	
	A2 CC_{50} > 100 (HMV-2 cells)	PFluV-2	IC_{50} = 0,8	
Gyrodinium impudicum	Purified sulphated exopolysaccharide: p-KG03 CC_{50} = 3,4 (MT-4 cells) CC_{50} = 59,9 (Vero cells) CC_{50} > 1000 (HeLa cells)	EMCV	Inhibit the development of the CPE, suppress tumor cell growth EC_{50} = 27	(Yim et al., 2004)
	Not in MDCK cells CC_{50} > 100	IFV-A IFV-B	Inhibit adsorption EC_{50}(IFV-A) = 0.19-0.48 EC_{50}(IFV-B) = 0.26	(Kim et al., 2012)

EMCV: encephalomyocarditis virus; HIV-1: human immunodeficiency virus type 1; HSV-1: Herpes simplex virus type 1; HSV-2: herpes simplex virus type 2; IFV-A: influenza A virus; IFV-B: influenza B virus; MuLV: murine leukemia virus; MuSV-124: murine sarcoma virus; RSV: respiratory syncytal virus; VZV: varicella zoster virus. CC_{50}: concentration that kills 50 % of the infected cells; CPE_{50}: concentration that offers 50 % protection against the cytopathic effect; EC_{50}: concentration requires to inhibit 50 % of the virus-induced cytopathic effects (CPE); ffu_{50}: concentration that offers 50 % protection against the formation of foci of malignant cells; IC_{50}: concentration that inhibits 50 % of the virus infection; MIC: minimum inhibitory concentration; RT_{50}: concentration that offers 50 % reduction of reverse transcriptase activity.

Some sulphated polysaccharides present a broad antiviral spectrum against enveloped viruses. Naviculan, extracted from the diatom *Navicula directa*, or A1 and A2 extracted from *Cochlodinium polykrikoides* demonstrated to potent antiviral activity against several enveloped viruses such as HIV-1, HSV-1 or influenza virus type A (IFV-A ; Hasui et al., 1995; Lee et al., 2006). The sulphated polysaccharide p-KG03 extracted from *Gyrodinium impudicum* did not demonstrate antiviral activity against HSV-1 and HSV-2, but presented a good activity against the encephalomyocarditis RNA virus (EMCV ; Yim et al., 2004), and against several strains of influenza viruses with an efficiency comparable to some

existing drugs (Kim et al., 2012). Antiviral activities of microalgae against HSV type 1 and 2 are the most studied (Bergé et al., 1999; Gastineau et al., 2012b, 2012c; Hasui et al., 1995; Huleihel et al., 2001, 2002; Lee et al., 2006; Ohta et al., 1998; Santoyo et al., 2010, 2011). More than one third of the world population is affected by HSV-1 or HSV-2, infections that cause infectious diseases such as oral and genital herpes (Moussa et al., 2015). The efficiency and the low toxicity of some of the microalgal compounds tested attest their advantageous use as antiviral agents. They could help to control viral diseases occurring in humans, but also in animal species with economic value.

4.2 Potential use of microalgae against viruses in aquaculture

Aquaculture production undergoes numerous viral diseases, which can affect organism health and survival rate, and can sometimes lead to mass mortality. Viral diseases are spreading and are consequently important limiting factors for the expansion of aquaculture (Ahne, 1994). Many different viruses, from various virus families, are known to infect farmed species, such as finfish, crustaceans or molluscs (Ahne, 1994; Bower et al., 1994; Crane and Hyatt, 2011; Elston, 1997; Farley, 1978). As a few relevant examples, we can cite the infectious pancreatic necrosis virus (IPNV), isolated from a very wide host range among finfish (Crane and Hyatt, 2011), the yellow head virus (YHV) and the white spot syndrome virus (WSSV) causing important losses in shrimp culture, or the oyster herpesvirus-1 (OsHV-1), leading to high mortality in marine bivalves and which can be transmitted between different bivalve species (Arzul et al., 2001). It appears that diseases induced by RNA viruses are the highest cause of ecological and socio-economical impacts in European farmed finfish (Gomez-Casado et al., 2011). Viruses in aquacultured species are either established for decades or are newly emerging because of the intensification of farming practices that facilitates rapid transmission of diseases.

Viral diseases in aquaculture are challenging to manage.They are difficult to treat directly and a few, if any, efficacious treatments are available other than destroying all

organisms in infected farms and avoiding their movements to and from infected areas. In some particular cases, vaccination is used in farmed finfish, mainly to treat trout and salmon (Gomez-Casado et al., 2011; Sommerset et al., 2005). Most of these vaccines are made with inactivate virus, which are less subject to strict regulatory constraints compared to activated vaccines, but might not be highly effective. Furthermore, vaccines have more often to be delivered by intra-peritoneal injections, which require fish to be anesthetised and can be laboured. The vaccination issue did not arise in invertebrates, as it was widely assumed that, unlike vertebrates, they do not have the capacity to develop long-term acquired immunity. Nevertheless, there are evidences for specific immune memory in some invertebrates (Loker et al., 2004) such as crustaceans (Rowley and Pope, 2012), and several studies have demonstrated the antiviral protection of shrimp by “vaccination” (Johnson et al., 2008; Venegas et al., 2000). However, vaccination has not shown any effect on bivalve molluscs and drug treatments can hardly be considered for these species, usually grown in an open environment (Arzul and Renault, 2002). The lack of bivalve cell lines has limited the isolation of bivalve viruses, and even if some of them can be isolated in fish cell lines, the unavailability of specific tools is a major scientific lock to make significant progress in bivalve virology (Elston, 1997).

Plant and herbal extracts with activity against viral diseases in aquaculture production have recently been reviewed by Sivasankar et al. (Sivasankar et al., 2015). Some extracts have already been successfully tested *in vivo*, such as the plant extract of *Cyanodon dactylon* against the WSS virus of the shrimp *P. monodon* (Balasubramanian et al., 2008). Plant extracts, acting as immunostimulants, have the advantage of being easily delivered by oral administration and may be eco-friendly as they are biodegradable.

In contrast, very few studies have been conducted to assess the antiviral activity of microalgae against virus in aquaculture. Some polysaccharide extracts of various microalgae have been tested against the viral hemorrhagic septicaemia virus (VHSV; Fabregas et al., 1999), a virus of economic importance afflicting over 50 species of fresh water and marine fish including salmonid fish (Crane and Hyatt, 2011). Endocellular extracts of *Porphyridium cruentum*, *D. tertiolecta*, *Ellipsoidon sp.*, *Isochrysis galbana var Tiso* and *Chlorella*

autotrophica inhibited the viral infection of VHSV *in vitro* in epithelioma papulosum cyprinid (EPC) cells. Concentrations lower than 2 µg of extracts per mL of *P. Cruentum* and *D. tertiolecta* were sufficient to detect an antiviral activity. Exocellular extracts of these algal species were also able to inhibit the viral infection, except for *I. galbana var Tiso.* This study has also demonstrated that there is no correlation between the content of sulphated polysaccharides of each microalga and its capacity to inhibit the viral replication. Thus, the observed antiviral effects would be due to different polysaccharide molecular species with differences in molecular size.

A higher resistance to the WSSV was observed in the tiger shrimp *P. menodon* reared in a "green water" system using commercially available extracts of *Dunaliella salina* (Supamattaya et al., 2005). Culture bath treatments with the microalga *C. minutissima* significantly reduced the mortality of *Epinephelus marginatus* showing signs of Viral Encephalopathy and Retinopathy (VER ; Katharios et al., 2005). These results indicate that the control of the disease is probably due to the antiviral effect of *C. minutissima* cultures, which thus needs to be further investigated using *in vitro* testing of water-soluble algal extracts.

Viral diseases are increasingly spreading, causing great economic loss for aquaculture industries. No effective treatment has yet been developed, as vaccination possibility is limited and chemical drugs are gradually avoided because of their toxicity and potential residue accumulation. Plant and herbal extracts have shown potent antiviral activity against several pathogenic viruses in aquaculture. They could be promising candidates for antiviral agents as part of an environmentally friendly and sustainable aquaculture, although their production and delivery processes could be limiting factors. In this context, the use of microalgae as a source of antiviral agents should be further studied in the future, as water-soluble compounds present in algal supernatants could be a valuable alternative.

5 CONCLUSION

The diversity of microalgae is immense, with species, genera or even classes being discovered every year. On the estimated millions of existing species, about 30 000 have been described, but only a dozen are cultivated in a large scale for biotechnological applications. The main obstacle for their commercial exploitation remains the production cost, but it should be bypassed by the optimization of mass culturing conditions. Microalgae are promising source of high-valued products, and their application as antimicrobials are only in its onset. The development of novel drugs with no microbial resistance and the use of environmentally friendly antibiotics in a context of sustainable aquaculture are needed. The efficiency of various microalgal compounds against human or aquatic pathogens is very encouraging and there is no doubt that their exploitation and application will expand.

CHAPITRE III.

EFFECTS OF MARENNINE SOLUTIONS ON THE GROWTH OF MARINE *VIBRIO*

CHAPITRE III.

Partie 1 :

Complex relationships between the blue pigment marennine and marine bacteria of the genus *Vibrio*

Chapitre III. Effects of marennine solutions on the growth of marine *Vibrio*. Partie 1 : Complex relationships between the blue pigment marennine and marine bacteria of the genus *Vibrio*.

Ce travail a bénéficié de l'expertise d'Adèle James[2-3], Marie-Agnès Travers[4], Marie Zanella[1], Myriam Badawi[1] et Jean-Luc Mouget[1].

Ces expérimentations, conduites au laboratoire MMS Le Mans, ont pu être réalisées grâce à la mise à disposition par Frédérique Le Roux[2-3] et Marie-Agnès Travers[4] de diverses souches de *Vibrio.*

Les résultats de ce chapitre ont été présentés lors d'une communication orale au congrès de la *British Phycological Society* (PBS) à Oban (Écosse) les 7-10 janvier 2019.

Cet article a été publié dans le journal *Marine Drugs* en Mars 2019.

Affiliations des différents contributeurs de ce travail :

[1]Laboratoire Mer Molécule Santé (EA 2160/FR CNRS 3473 IUML), Le Mans Université, France ; [2]Unité Physiologie Fonctionnelle des Organismes Marins (UMR 6539 CNRS/UBO/IRD/Ifremer), Plouzané, France ; [3]Laboratoire de Biologie Intégrative des Modèles Marins, Sorbonne Universités (UPMC Paris 06/CNRS/UMR 8227), Station Biologique de Roscoff, France ; [4]Laboratoire de Génétique et de Pathologie des Mollusques Marins Ifremer (RBE/SG2M/LGPMM/Ifremer), Station La Tremblade, France.

Abstract

Marennine, the water-soluble blue pigment produced by the marine diatom *Haslea ostrearia* is known to display antibacterial activities. Previous studies demonstrated a prophylactic effect of marennine on bivalve larvae challenged with a pathogenic *Vibrio splendidus*, suggesting that the blue *Haslea* is a good candidate for applications in aquaculture as a source of natural antimicrobial agent. Indeed, the genus *Vibrio* is ubiquitous in aquaculture ecosystems and regular events of pathogenic invasion causes among the biggest losses worldwide. To better characterize the effects of marennine on *Vibrios*, a panel of 30 *Vibrio* strains belonging to 10 different species was tested, including bivalve pathogenic species (*e.g. Vibrio crassostreae, Vibrio harveyi*). *Vibrio* strains were first exposed to 10 and 25 µg mL^{-1} of Blue Water (BW), a concentrated culture supernatant of *H. ostrearia* containing marennine. This screening evidenced a great diversity in responses, from growth stimulation to a total inhibition, at both the interspecific or intraspecific level. In a second series of experiments, 10 *Vibrio* strains were exposed to BW at concentrations ranging from 5 to 80 µg mL^{-1}. Highest concentrations of BW did not systematically result in highest growth inhibition as hormetic responses – opposite effects regarding the concentration – were occasionally evidenced. Relationships between marennine and *Vibrio* strains appear more complex than expected and justify further study, in particular on the mechanisms of action, before considering applications as a natural prophylactic or antibiotic agent in aquaculture.

Key words: antibacterial activity; diauxie; *Haslea*; hormesis; marennine; *Vibrio*

1 INTRODUCTION

The marine diatom *Haslea ostrearia* is characterized by the production of a specific blue-green pigment, named marennine. This water-soluble pigment accumulates at the apices of the cells before its release in the surrounding environment (Nassiri et al., 1998). *H. ostrearia* is a ubiquitous diatom and is of special interest in the Atlantic French coast (*e.g.* Marennes Bay, Bourgneuf Bay) where blooms in oyster ponds induce the greening of oyster gills that increases the market value of bivalves. Blue diatoms other than *H. ostrearia* have been identified in the last decade such as *Haslea karadagensis* (Black Sea; Gastineau et al., 2012a), *Haslea provincialis* (Mediterranean Sea; Gastineau et al., 2016) and more recently *Haslea nusantara* (Java Sea; Prasetiya et al., in press). All these species produce blue pigments whose spectral characteristics slightly differ from marennine and are named marennine-*like* pigments in the absence of more specific determination (*e.g.* Gastineau et al., 2012a). Despite an increasing knowledge on blue *Haslea* biodiversity and distribution, questions still remain about these blue pigments and their functions for the algae. Indeed, marennine or marennine-like pigments are highly complex molecules and their chemical structure remains undetermined. Some glycosidics units attached to one or various aromatic rings have been evidenced, but the exact nature of the chromophore is still unknown yet (Pouvreau et al., 2006a).

Regarding the function of the pigment for the microalga, the significant release of blue pigments by *Haslea* species in seawater (in the range of 1–15 µg mL^{-1} in oyster ponds; Turpin et al., 2001) combined with an increasing number of evidences that marennine interacts with different marine organisms could advocate for a protective or a competitive role. Indeed, allelopathic effects were demonstrated toward various microalgal species (Pouvreau et al., 2007; Prasetiya et al., 2016) as well as antimicrobial effects against several marine bacteria and fungi (reviewed in Falaise et al., 2016b). More particularly, *in vitro* experiments demonstrated antibacterial effects of marennine against various marine bacteria, including strains from the *Vibrio* genus such as *Vibrio anguillarium* (Pouvreau, 2006), *Vibrio aestuarianus* (Gastineau et al., 2012b) *or Vibrio splendidus* (Gastineau et al., 2014b). The *Vibrio* genus is genetically and metabolically highly diverse and several species

have been described as pathogenic for shellfish (Travers et al., 2015). Major pathogens found in hatcheries or in fields belong to *Splendidus*, *Coralliilyticus*, *Harveyi* clade or to *V. aestuarianus* and *V. tapetis* species. However, it is important to consider ecological populations as all strains of a same species do not share colonization and toxicity characteristics and thus are not pathogenic. Furthermore, different strains of a same *Vibrio* species presented distinct sensitivities toward marennine (Falaise et al., 2016b). Hence, bacterial response to marennine exposure can be species-, and strain-dependent. This biological activity seems to be intrinsic to blue *Haslea* species as antibacterial activities were also demonstrated with the marennine-like pigment produced by *H. karadagensis* against *V. aestuarianus* and other species of interest in aquaculture (Gastineau et al., 2012c).

At the sight of such results, the use of blue *Haslea* and marennine has been considered for aquaculture applications. Marennine biological activities have thus been investigated *in vivo*, using blue mussel and giant scallop larvae exposed to a concentrated supernatant of *H. ostrearia* culture enriched in extracellular marennine (Turcotte et al., 2016). Low concentrations of this Blue Water (BW) solution significantly increased larval survival when challenged with a pathogenic *V. splendidus* strain (Turcotte et al., 2016). This result is very promising in aquaculture for shellfish and fish larval health, but a better characterization of the interactions between marennine and pathogenic bacteria is needed. Thus the present work aims to increase further our knowledge about antimicrobial activity of *H. ostrearia* blue pigment, by assessing the effects of marennine on different species and strains of the genus *Vibrio* that are threatening aquaculture sustainability.

2 MATERIALS AND METHODS

2.1 *Vibrio* strains

Thirty *Vibrio* strains belonging to 10 species were tested for their sensitivity toward Blue Water (BW), a concentrated supernatant of *H. ostrearia* culture containing the extracellular marennine. *Vibrio chagasii* (strain #11, #12, #13), *Vibrio crassostreae* (#51, #52, #53), *Vibrio fortis* (#7, #8, #9), *Vibrio harveyi* (#21, #22, #23), *Vibrio orientalis* (#1, #2, #3), *Vibrio splendidus* (#90, #91, #93), *V. tasmaniensis* (#112, #113, #114), *Vibrio sp.* (isolated from oyster tissues; #36, #37, #38), *V sp*. (isolated from sea water; #90, #91, #33) strains were provided by the Genomics of *Vibrio* team (*Laboratoire de Biologie Intégrative des Modèles Marins (LBI2M)*, station biologique de Roscoff, France) and were previously described in (Bruto et al., 2017). *Vibrio aestuarianus* strains (#07/115, #12/016, #03/008) were provided by the *Laboratoire de Génétique et de Pathologie des Mollusques Marins (LGPMM), Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER*; La Tremblade, France).

2.2 *Vibrio* exposure to Blue Water (BW) solutions

The susceptibility of the *Vibrio* strains to BW was assessed with the method described in the Clinical and Laboratory Standards Institute (CLSI) antimicrobial microdilution guidelines (Clinical and Laboratory Standards Institute (CLSI), 2012). Bacterial inocula at a defined concentration were exposed to different BW concentrations in 96 well microplates with flat bottom and cover (BrandTech™BRAND*plates*™pureGrade™ S 96-well Microplates, Fisher Scientific). Bacterial growth was monitored by Optical Density (OD) measurements with a microplate spectrophotometer (xMark, Bio-Rad). Bacterial growth was then recorded using *Microplate Manager 6* Software (MPM6) with OD measurement (at 600 nm, to avoid the absorbance peak of marennine around 677 nm; Pouvreau et al., 2006a) of each well every 30 min for 24 h at ambient temperature.

2.2.1 Preparation of bacterial inocula

Vibrio strains were kept at -80 ℃ in 25 % glycerol. Broth cultures were prepared with an autoclaved cation-adjusted Mueller-Hinton Broth media (CaMHB; Thermo Fisher Scientific) by addition of 1 % NaCl (pH 7.5 ± 0.2; salinity 32) and agar media prepared with an autoclaved cation-adjusted Nutrient Agar (CaNA; Biokar) by addition of 2.3 % NaCl (final pH 7.5 ± 0.2; salinity 32). Prior to the antibacterial assays, each *Vibrio* strain was inoculated in CaMHB from the -80 ℃ sample, incubated overnight at 25 ℃ under moderate agitation (130 rpm) and isolated on CaNA Petri dishes. After 1 day incubation at 25 ℃, plates containing the isolated colonies were kept at 4 ℃ for no more than a week. Three different colonies per Petri dish were inoculated in CaMHB (biological replicates, n=3) and grown overnight at ambient temperature. The next day, the OD (630 nm) of bacteria in the broth culture was measured (V-10 Plus, Onda Spectrophotometer) and the absorbance was adjusted at 0.1 by dilution in CaMHB. To obtain the bacterial inoculum, the solution was further diluted by 1/100 in CaMHB as recommended by the CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2012). The bacterial inocula were exposed to BW in the microplates within 15 min after the dilution.

2.2.2 Blue Water (BW) production

The growth of the 30 *Vibrio* strains exposed to Blue Water (BW) solutions was studied over a 24 h period. BW was prepared from a concentrated supernatant of *H. ostrearia* culture containing the extracellular marennine and was produced at the *Station aquicole de Pointe-au-Père, Institut des Sciences de la Mer à Rimouski - Université du Québec à Rimouski* (ISMER - UQAR; Québec, Canada) during spring 2017. *H. ostrearia* was cultured in 100 L circular and flat bottom photobioreactors with filtered sea water (temperature: 20 ℃; salinity 28) at high irradiance (180 µmol photons m^{-2} s^{-1}), in a 14/10 h light/dark cycle for 3 weeks, until marennine concentration reached around 6–7 µg mL^{-1} as described in (Turcotte et al., 2016). The supernatant was then collected and concentrated *ca*. 20 times by ultrafiltration (double cut off 3–30 kDa) as described in (Pouvreau et al., 2006b) for a final estimated concentration of *ca.* 120 µg mL^{-1} (pH 7.7 ± 0.2; salinity 0) and stored in the dark at

4 °C. BW concentration was assessed with spectrophotometric measurements (UV/Vis Lambda 25 Perkin Elmer spectrophotometer, UV Winlab software) on a syringe filtered BW solution (0.2 µm; Sarstedt) and 1 cm path length quartz cuvettes using the Beer-Lambert equation (ε_{677}=12.13 L $g^{-1}cm^{-1}$) as proposed by (Pouvreau et al., 2006b). Prior to the antibacterial experiments, the BW stock solution was syringe-filtered on 0.4 µm, the salinity and the pH were respectively adjusted to be similar with the CaMHB at 32 and 7.5 ± 0.2 by addition of NaCl and HCl 0.1 M. BW dilutions were prepared with sterile ultra pure water + NaCl (pH 7.5 ± 0.2; salinity 32). The BW solutions at different concentrations were then syringe-filtered through 0.2 µm and kept at 4 °C.

2.2.3 Antibacterial essay

In a first series of experiments, the 30 *Vibrio* strains corresponding to 10 different species were screened and exposed to three BW concentrations: 0 (control); 10 and 25 µg mL^{-1}. In a second series of experiments, 10 *Vibrio* strains presenting different patterns of sensitivity to marennine were exposed to a dilution range of BW: 0; 5; 10; 25; 50; 70 and 85 µg mL^{-1}. For the *screening* experiment, the final volume in each well of the microplates was 100 µL with a final ratio of 1:1 (v/v) bacterial inoculum: BW. In the *dilution range* experiment, to reach concentrations as high as 70 and 85 µg mL^{-1} with a BW stock solution of 117 µg mL^{-1}, the final volume in each well was adjusted to 200 µL with a final ratio of 1:4 (v/v) bacterial inoculum: BW. Microplates were first filled with the BW solutions and bacterial inocula were then added in each well using a single channel electronic micropipette (Eppendorf Research Pro 50-1000 µL). After being completed, microplates were sealed with parafilm and placed in the microplate spectrophotometer for the 24 h run. Experiments were conducted in triplicates with technical triplicates for each condition. A negative control was also run per microplate with only BW and CaMHB.

2.3 Growth curve analyzes and statistics

Bacterial growth kinetics were analyzed using *R 3.5.1- software.* For the screening experiment, the OD (600 nm) data obtained over the 24 h run were fitted with a bi-phasic logistic growth equation defined as below:

$$f(x) = \frac{k_1}{1+e^{-r_1(x-x_1)}} + \frac{k_2}{1+e^{-r_2(x-x_2)}}.$$

Interpretable metric parameters were then obtained, such as the maximum possible population size in a particular environment for the first phase of growth (“k1” parameter) and for the second phase of growth (“k2” parameter), or the growth rate (“r1” and “r2” parameters). The “k2” parameter was chosen in the screening experiment to study the effects of BW on the *Vibrio* strains. For the concentration range experiments, the “k” parameter was also studied but growth curves were fitted with a simple logistic growth equation as it was not possible to fit growth curves of a same strain under the different BW exposures with the same bi-phasic logistic growth equation. Growth curves were analyzed with the *R* package *Growth Curver* (Sprouffske and Wagner, 2016).

R software was also used for statistical analyzes. Shapiro-Wilkinson test was used to verify data normality and Fisher test for the homogeneity of variance. There was no need to perform data transformation. Differences between treatments were assessed with One-Way ANOVAs and post hoc Tukey’s pairwise multiple comparison tests were used to determine differences between pairs. Unless specified data are expressed as mean ± standard error (SE).

3 RESULTS

3.1 Different patterns of *Vibrio* growth curves evidenced by the screening experiment

The growth curves of the 30 *Vibrio* strains exposed to BW at 0, 10 or 25 µg mL^{-1} were recorded for 24 h (Supplementary Figure 3.1). Typical growth patterns selected from the 30 strains tested are presented in Figure 3.1. *Vibrio* strains in the CaMHB growth media presented a diauxic growth characterized by two distinct exponential phases (Monod, 1949). A diauxic growth is typically observed when bacteria grow in a medium containing two different sources of nutrients (*e.g.*, sugars). The diauxic lag phase was particularly marked for the three *V. harveyi* strains tested (Figure 3.1C) and to a lesser extent to the other tested strains of *V. orientalis, V. fortis, V. chagasii, V. crassostreae* (Supplementary Figure 3.1).

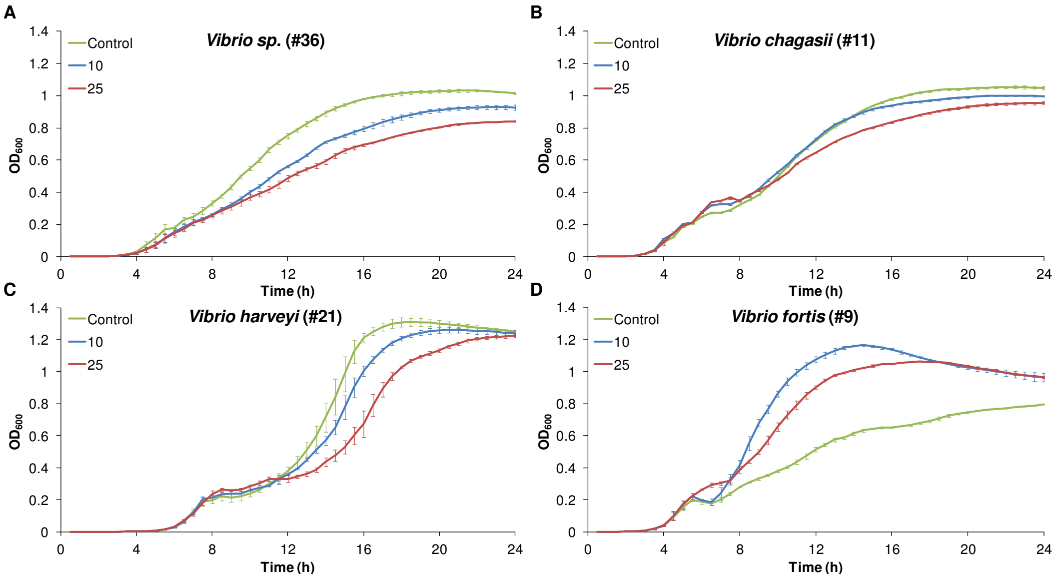
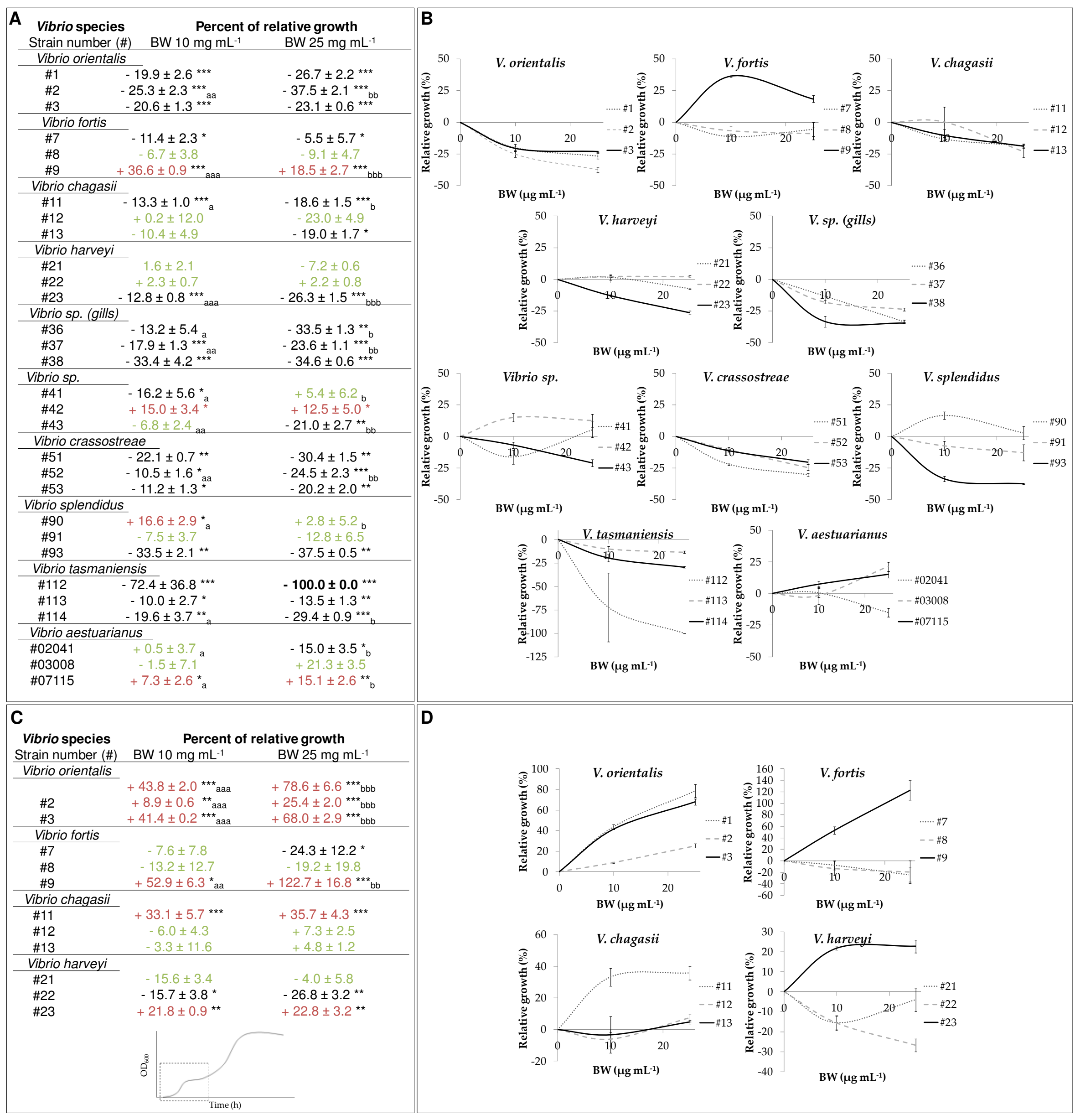


Figure 3.1. Growth kinetics of four *Vibrio* strains (#) exposed to 0, 10 or 25 µg mL^{-1} of Blue Water (BW) over a 24 h period with growth characteristic features observed for the 30 *Vibrio* strains tested in the screening experiments. BW exposure inhibited the growth of **(A)** *Vibrio sp.* #36, **(B)** *Vibrio chagasii* #11 and **(C)** *Vibrio harveyi* #21 but stimulated the growth of **(D)** *Vibrio fortis* #9. Vibrios presented a diauxic growth characterized by two distinct exponential growth phases with a diauxic lag phase in between. Results are means ± SE (n=3).

Exposure to BW at 10 and 25 µg mL^{-1} affected the growth of 80 % of the *Vibrio* strains tested over a 24 h period and had varying effects on the maximum bacterial population size (Figure 3.2A-B). An inhibiting dose dependant effect was observed for 10 strains: *V. orientalis* #3, *V. chagasii* #12 and #13, *V. harveyi* #23, *V. sp.* #36, #37, #43, *V. crassostreae* #52, *V. tasmaniensis* #114 and *V. aestuarianus* #02/041. For 9 other strains, the inhibiting effect of BW was similar under 10 and 25 µg mL^{-1}: *V. orientalis* #1 and #2, *V. fortis* #7, V. sp. #38, *V. crassostreae* #51 and #52, *V. splendidus* #93 and *V. tasmaniensis* #112 and #113. A growth stimulating effect was observed for *V. fortis* #9 with 36.6 ± 0.9 % of stimulation under 10 µg mL^{-1} of BW exposure, and the stimulating effect was reduced to 18.5 ± 2.7 % under 25 µg mL^{-1}. The growth stimulation of *V. aestuarianus* #07/115 was higher at 25 than 10 µg mL^{-1} and was comparable between both concentrations for *V. sp.* #42. For 2 strains the growth was modified under BW exposure at 10 µg mL^{-1} while no effect was observed under 25 µg mL^{-1} exposure: V. sp. #41 with a growth inhibition and V. splendidus #90 with a growth stimulation. For 6 strains, no differences were found under BW exposure on the maximum population size: *V. fortis* #8, *V. splendidus* #91, *V. aestuarianus* #03/008, *V. chagasii* #12 even if an inhibiting trend was observed but which not appeared statistically significant, and *V. harveyi* #21 and #22. Although BW had no effect on the maximum population size of *V. harveyi* #21 and #22, it can be noted that the growth rate of these strains was inhibited with 30.1 ± 1.1 % and 37.8 ± 2.3 % of inhibition for V. harveyi #21 and 38.1 ± 1.6 % and up to 57.2 ± 1.2 % of inhibition for *V. harveyi* #22 under 10 and 25 µg mL^{-1} respectively (see growth curves in Figure 3.1C and Supplementary Figure 3.1).

Although most *Vibrio* strains exhibited a growth inhibition under BW exposure over a 24 h period, growth stimulation was sometimes observed during the first phase of growth. The maximum population size was thus also studied during the first growth phase for some strains that presented a diauxic growth with a marked diauxic lag phase (*i.e. V. orientalis*, *V. fortis*, *V. chagasii*, *V. harveyi*) and the percentage of relative growth was calculated (Figure 3.2C-D).



A

Vibrio species Strain number (#)	Percent of relative growth BW 10 mg mL^{-1}	 BW 25 mg mL^{-1}
Vibrio orientalis		
#1	- 19.9 ± 2.6 ***	- 26.7 ± 2.2 ***
#2	- 25.3 ± 2.3 ***$_{aa}$	- 37.5 ± 2.1 ***$_{bb}$
#3	- 20.6 ± 1.3 ***	- 23.1 ± 0.6 ***
Vibrio fortis		
#7	- 11.4 ± 2.3 *	- 5.5 ± 5.7 *
#8	- 6.7 ± 3.8	- 9.1 ± 4.7
#9	+ 36.6 ± 0.9 ***$_{aaa}$	+ 18.5 ± 2.7 ***$_{bbb}$
Vibrio chagasii		
#11	- 13.3 ± 1.0 ***$_{a}$	- 18.6 ± 1.5 ***$_{b}$
#12	+ 0.2 ± 12.0	- 23.0 ± 4.9
#13	- 10.4 ± 4.9	- 19.0 ± 1.7 *
Vibrio harveyi		
#21	1.6 ± 2.1	- 7.2 ± 0.6
#22	+ 2.3 ± 0.7	+ 2.2 ± 0.8
#23	- 12.8 ± 0.8 ***$_{aaa}$	- 26.3 ± 1.5 ***$_{bbb}$
Vibrio sp. (gills)		
#36	- 13.2 ± 5.4 $_{a}$	- 33.5 ± 1.3 **$_{b}$
#37	- 17.9 ± 1.3 ***$_{aa}$	- 23.6 ± 1.1 ***$_{bb}$
#38	- 33.4 ± 4.2 ***	- 34.6 ± 0.6 ***
Vibrio sp.		
#41	- 16.2 ± 5.6 *$_{a}$	+ 5.4 ± 6.2 $_{b}$
#42	+ 15.0 ± 3.4 *	+ 12.5 ± 5.0 *
#43	- 6.8 ± 2.4 $_{aa}$	- 21.0 ± 2.7 **$_{bb}$
Vibrio crassostreae		
#51	- 22.1 ± 0.7 **	- 30.4 ± 1.5 **
#52	- 10.5 ± 1.6 *$_{aa}$	- 24.5 ± 2.3 ***$_{bb}$
#53	- 11.2 ± 1.3 *	- 20.2 ± 2.0 **
Vibrio splendidus		
#90	+ 16.6 ± 2.9 *$_{a}$	+ 2.8 ± 5.2 $_{b}$
#91	- 7.5 ± 3.7	- 12.8 ± 6.5
#93	- 33.5 ± 2.1 **	- 37.5 ± 0.5 **
Vibrio tasmaniensis		
#112	- 72.4 ± 36.8 ***	**- 100.0 ± 0.0** ***
#113	- 10.0 ± 2.7 *	- 13.5 ± 1.3 **
#114	- 19.6 ± 3.7 **$_{a}$	- 29.4 ± 0.9 ***$_{b}$
Vibrio aestuarianus		
#02041	+ 0.5 ± 3.7 $_{a}$	- 15.0 ± 3.5 *$_{b}$
#03008	- 1.5 ± 7.1	+ 21.3 ± 3.5
#07115	+ 7.3 ± 2.6 *$_{a}$	+ 15.1 ± 2.6 **$_{b}$

C

Vibrio species Strain number (#)	Percent of relative growth BW 10 mg mL^{-1}	 BW 25 mg mL^{-1}
Vibrio orientalis		
	+ 43.8 ± 2.0 ***$_{aaa}$	+ 78.6 ± 6.6 ***$_{bbb}$
#2	+ 8.9 ± 0.6 **$_{aaa}$	+ 25.4 ± 2.0 ***$_{bbb}$
#3	+ 41.4 ± 0.2 ***$_{aaa}$	+ 68.0 ± 2.9 ***$_{bbb}$
Vibrio fortis		
#7	- 7.6 ± 7.8	- 24.3 ± 12.2 *
#8	- 13.2 ± 12.7	- 19.2 ± 19.8
#9	+ 52.9 ± 6.3 *$_{aa}$	+ 122.7 ± 16.8 ***$_{bb}$
Vibrio chagasii		
#11	+ 33.1 ± 5.7 ***	+ 35.7 ± 4.3 ***
#12	- 6.0 ± 4.3	+ 7.3 ± 2.5
#13	- 3.3 ± 11.6	+ 4.8 ± 1.2
Vibrio harveyi		
#21	- 15.6 ± 3.4	- 4.0 ± 5.8
#22	- 15.7 ± 3.8 *	- 26.8 ± 3.2 **
#23	+ 21.8 ± 0.9 **	+ 22.8 ± 3.2 **

Figure 3.2. (A) Relative maximum population size (%) of *Vibrio* strains exposed over a 24 h period to concentrations of 10 and 25 µg mL^{-1} of Blue Water (BW). **(B)** Graphical illustrations of the dose-response results presented in (A). **(C)** Relative maximum population size (%) after the first growth phase (see insert) for some of the *Vibrio* strains presenting a diauxic growth curve and exposed to 10 and 25 mg mL^{-1} of BW. **(D)** Graphical illustrations of the screening experiment results presented in (C). Significant growth inhibitions are presented in black, growth stimulations in red and no observed effects in green. Asterisks (*) indicate a statistical difference with the control and letters (A.B) significant differences between the two BW concentrations tested. Values are means ± SE (n=3).

The first growth phase of *V. orientalis* #1, #2 and #3 and *V. fortis* #9 was dose-dependently stimulated under BW exposure. Bacterial growth during the first phase was also stimulated by BW for *V. chagasii* #11 and *V. harveyi* #23 but no differences were found between exposures to 10 and 25 µg mL^{-1}. A growth inhibition was observed during the first growth phase for *V. harveyi* #22 and *V. fortis* #7 while no effect of BW was recorded for *V. fortis* #8, *V. chagasii* #12 and #13 and *V. harveyi* #21 during the first growth phase (Figure 3.2C-D).

3.2 Experiment with BW concentration range

Different dose-response patterns were observed depending on the strain tested, including linear response and “U shape” responses (Figure 3.3). *V. fortis* #8, *V. orientalis* #3, *V. aestuarianus* #02/041, *V. harveyi* #23 presented a linear dose-response curve with increasing growth inhibition along with an increasing concentration exposure (Figure 3.3A-B and Supplementary Figure 3.2). For *V. fortis* #8 a significant inhibitory effect was observed from 5 µg mL^{-1} with 5.1 ± 0.5 % of growth inhibition (*p*-value < 0.001) up to 64.6 ± 0.7 % at 85 µg mL^{-1} with an IC_{50} (concentration at which the response is inhibited by 50 %) around 50 µg mL^{-1} (Figure 3.3A). An increasing inhibitory effect was also observed for *V. orientalis* #3 from 5 µg mL^{-1} (*p*-value < 0.001) up to 50 µg mL^{-1}, but there was no difference in growth inhibition between 50 µg mL^{-1} and 85 µg mL^{-1} with a maximum inhibition level of about 37 % (Supplementary Figure 3.2). A No Observed Effect Level (NOEL) was noted for *V. aestuarianus* #02/041 between 0 µg mL^{-1} and 25 µg mL^{-1} (*p*-value 0.351) and the first inhibitory effect was evidenced at 50 µg mL^{-1} with 12 ± 0.2 % of inhibition (*p*-value 0.015) while the maximum effect was observed at 85 µg mL^{-1} with 23.4 ± 1.7 % of inhibition (Supplementary Figure 3.2). For *V. harveyi* #23, the diauxic lag phase was particularly marked and the growth response changed with BW concentration and time. Growth curves were thus analyzed as two independent phases of growth with phase 1 from 0 to 9 h and phase 2 from 9h to 24 h (Figure 3.3B). For the first growth phase a significant growth stimulation was observed starting from 5 µg mL^{-1} (*p*-value < 0.001). The second growth

phase presented a dose dependent inhibition with a total blockage of the growth at the highest concentrations tested (*i.e.* 50, 70 and 85 µg mL^{-1}) and an important increase of the diauxic lag phase for bacteria exposed to 25 µg mL^{-1}, 7 h *vs* 2 h for the control.

The growth of *V. sp.* 38, *V. chagasii* #13, *V. crassostreae* #53 and *V. splendidus* #90 was also inhibited by the BW concentrations tested but the dose-response curves expressed in function of the control presented a “U” shape, with the growth inhibitory effect first increasing, then decreasing with increasing BW concentrations (Figure 3.3C and Supplementary Figure 3.2). For *V. sp.* #38 the maximum effect was observed at the concentration 50 µg mL^{-1} with 51.7 ± 0.6 % of growth inhibition (Figure 3.3C). However, the relative growth inhibition decreased to 37.1 ± 1.1 % at 70 µg mL^{-1} and was similar to the inhibition obtained at 25 µg mL^{-1} (*p*-value 0.971). Same observations were made for *V. chagasii* #13 and *V. crassostreae* #53 with the inhibitory effect significantly higher at 50 µg mL^{-1} than at 70 µg mL^{-1} (*p*-values < 0.005). For *V. splendidus* #90 the percentage of maximum inhibition was 33.3 ± 1.5 % at 70 µg mL^{-1}, and the inhibition was significantly lower, 23.0 ± 0.7 % at 85 µg mL^{-1} (*p*-value 0.004; Supplementary Figure 3.2).

The strains *V. tasmaniensis* #114 and *V. fortis* #9 presented a hormetic dose-response curve with opposite effects depending on the BW concentration. For *V. tasmaniensis* #114 the maximum inhibitory effect was observed between 5 µg mL^{-1} and 10 µg mL^{-1} with about 23 % of inhibition, and decreased until reaching the NOEL between 50 µg mL^{-1} and 70 µg mL^{-1} (Supplementary Figure 3.2). *V. fortis* #9 presented a growth stimulation at the lower concentrations tested, with a maximum growth stimulation of 53.0 ± 0.1 % at 5 µg mL^{-1}, then a decrease with increasing BW concentration. The NOEL was reached at 50 µg mL^{-1} and the growth inhibition increased dose-dependently with 31.4 ± 0.6 % of inhibition at 85 µg mL^{-1} (Figure 3.3D).

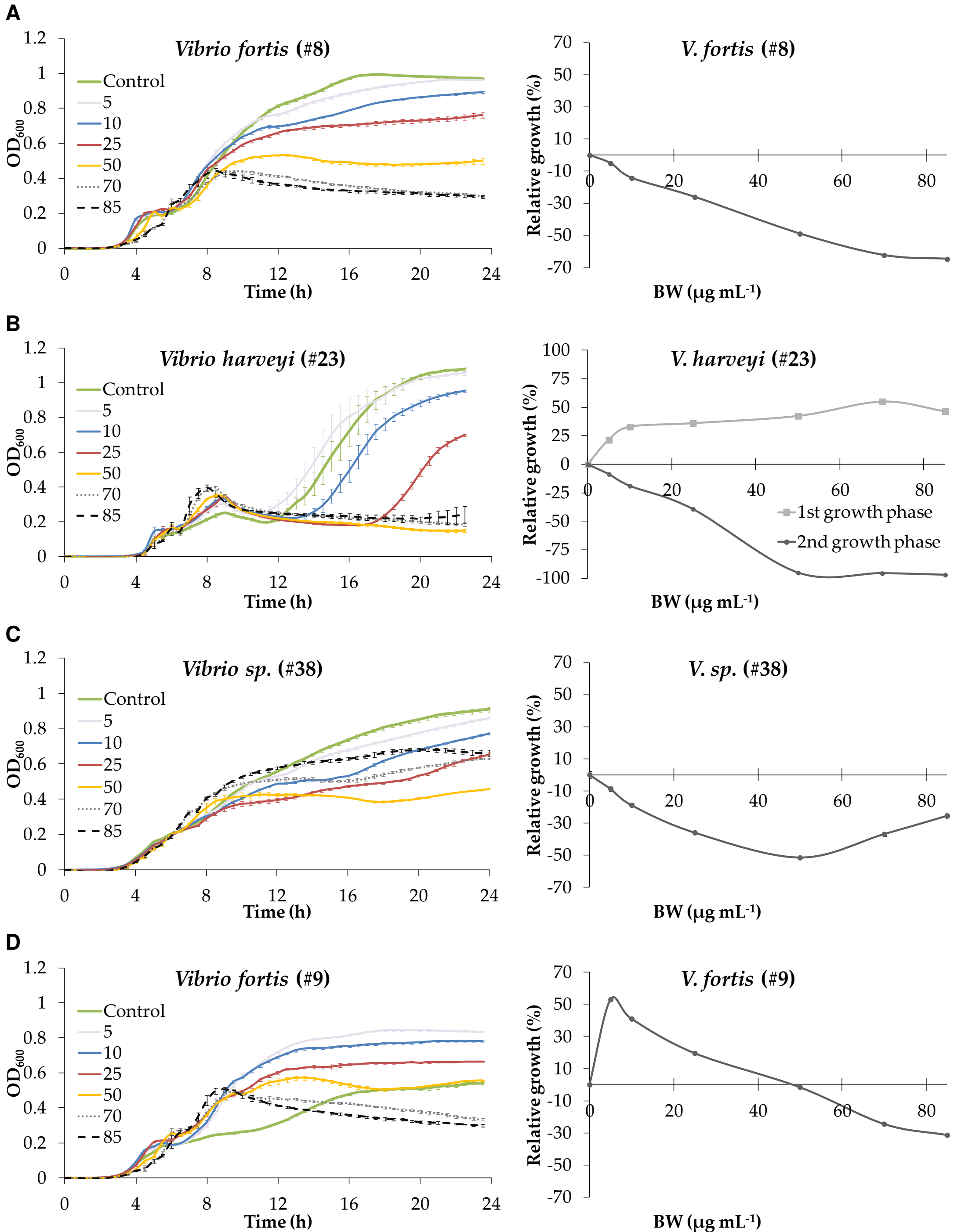


Figure 3.3. Growth kinetics and dose-response curves of *Vibrio* strains exposed to a concentration range (µg mL^{-1}) of Blue Water (BW) over a 24 h period presenting: **(A,B)** a linear response, **(C)** a "U shape" response or **(D)** a hormetic response. Growth of *V. harveyi* #23 was analyzed in two independent phases, with phase 1 from 0 to 9 h and phase 2 from 9 to 24 h. Values in the growth curves are means ± SD (n=3).

4 DISCUSSION

The effects of marennine on several *Vibrio* strains with different ecological and virulence properties have been investigated by monitoring bacterial growth over a 24 h period after addition of BW in the culture medium. The general trend for all the experiments is that BW affected most of the *Vibrio* strains tested (80 %), but the results are contrasting. Indeed, BW either inhibited or stimulated the growth of the *Vibrio* strains.

As BW solution (*i.e.* the concentrated supernatant of *H. ostrearia* containing marennine) was used in the present work and not a purified form of the pigment, it could be questioned if the observed effects were induced by marennine or by other molecules possibly present in the non-axenic culture medium (*e.g.* exopolysaccharides (EPS) from *Haslea* or epibiont bacteria). However, previous studies conducted with purified extracellular marennine (EMn) on marine bacteria, including *Vibrio* species, confirmed that the pigment itself did exert an antibacterial activity (Falaise et al., 2016b; Gastineau et al., 2012b, 2014b; Pouvreau, 2006). Also, if blue *Haslea* species should be cultured at large scale to ensure the production of marennine solutions for applications as antimicrobials in aquaculture, BW represents the much easier and cheaper production process in comparison with the purified pigment.

The observed effect of BW on vibrios was species- but also strain-dependent, as for a same species, the sensitivity of distinct strains could significantly differ. This is in accordance with previous observations *on V. aestuarianus, V. coralliilyticus*, *V. tubiashii* exposed to purified marennine (Falaise et al., 2016b). Moreover, it seemed difficult to establish a correlation between the effects of BW and the strain ecological characteristics such as sampling season, distribution (sea water fractions and oyster tissues) or the virulence of the strain toward bivalves (Supplementary Table 3.1).

However, the present work considerably extends our understanding of the variability of marennine effect on bacteria of the *Vibrio* genus. The diversity observed in growth responses was especially noteworthy in two species, *V. fortis* on the one hand, with a growth inhibition for strains #7 and #8 and important growth stimulation for strain #9, and

V. tasmaniensis on the other hand, with a total inhibition of strain #112 but no effect observed on the strain #113.

Also, a diauxic growth was evidenced for all the *Vibrio* strains tested, a phenomenon commonly observed when bacteria are grown in a medium containing two types of carbon sources (*e.g.*, sugars) and characterized by two distinct exponential phases with a diauxic lag phase in between (Monod, 1949). *Vibrio* diauxic growth is not extensively evidenced in the literature and was mainly illustrated using *V. cholerae* (Bag, 1974; Bhattacharya et al., 2018; Chatterjee et al., 2017) but also *V. alginolyticus* (Vine et al., 2004). It is worth noting that complete growth kinetics are rarely recorded for antibacterial assays as most experiments conducted to screen bioactive compounds against marine *Vibrio* species used the disc diffusion method (Banerjee et al., 2013; Cavallo et al., 2013; Das et al., 2005), as the ones conducted with marennine and marennine-like pigments on *V. aestuarianus* (Gastineau et al., 2012b, 2012c). The effect of purified marennine was previously tested with the microdilution method on another *V. tasmaniensis* strain (Falaise et al., 2016b; Gastineau et al., 2014b) and no obvious diauxic growth was observed then, which could be explained by the use of a different growth media (marine broth media *vs* CaMHB in the present study), a different time scale or a different treatment of data.

Bacterial diauxic growth has long been considered as a bacterial phase of enzymatic acclimation to metabolize a different type of sugar, however, there are now growing evidences that this phenomenon could rather correspond to the presence of two bacterial subpopulations in the isogenic culture with different phenotypic adaptation and growth strategy (Solopova et al., 2014). Based on this new hypothesis, diauxic growth curves in the present study could evidence the existence of different subpopulations of *Vibrio* strains; the Type 1 that can divide quickly by using the most suitable carbon source to sustain its growth, and the Type 2 that grows more slowly but that would be able to metabolize a different carbon source. According to Solopova et al. (2014), the Type 1 subpopulation, that cannot switch to an alternative metabolic pathway, will stop dividing possibly because of the low energy state of cells (Solopova et al., 2014). This phenotype heterogeneity has been recently evidenced in *V. cholerae* (Chatterjee et al., 2017) and would result from the bet

hedging strategy, an evolutionary strategy that allows colonies to cope with fluctuating environments (Ackermann, 2015; Grimbergen et al., 2015; Martins and Locke, 2015). In the present work, although bacterial growth was globally inhibited over the 24 h of the essay, a growth stimulation of the subpopulation Type 1 was sometimes observed under BW exposure (*e.g. V. orientalis*, *V. chagasii*, *V. harveyi* strains). It can be hypothesized that the subpopulation Type 1 would be able to metabolize part of the sugars constituting the carbon skeleton of the marennine molecule or, if any, other EPS present in BW solution to sustain its growth, or that the stimulation would result from overcompensation mechanisms by the bacteria under BW exposure, a mechanism that can be observed when cells undergo a disruption in homeostasis (Calabrese, 2013). It may also be argued that, when exposed to BW, it is the growth of subpopulation Type 2 that was mainly affected by the BW as illustrated by the total absence of growth of *V. harveyi* #23 at the highest concentrations tested (from 50 to 80 µg mL^{-1}).

A global observation from the results shows that the dose-response curves of most *Vibrio* strains did not follow a linear or threshold model with growth inhibition increasing with concentrations. Indeed, some curves presented a “U-shape” response regarding the antibacterial effect that decreased with increasing BW exposure (*e.g. V. chagasii* #13 or *V. sp* #38). On top of this, opposite effects were observed at low and high concentrations, a phenomenon known as hormesis (Calabrese and Baldwin, 2002). Indeed, the lowest BW concentration tested (5 µg mL^{-1}) highly stimulated the growth of *V. fortis* #9 up to 50 % while the growth was significantly inhibited at higher concentrations (from 50 to 80 µg mL^{-1}). Hormetic dose-response relationships have raised a growing awareness in toxicological and ecotoxicological studies and have been extensively documented over the past two decades in different models as plants, algae or fungi (Belz and Duke, 2014; Calabrese and Baldwin, 2001; Cedergreen et al., 2006). Mechanisms of hormesis are not yet clearly understood and it seems that only a subset of compounds with specific cellular mechanisms would mediate hormetic responses (Kendig et al., 2010; Lushchak, 2014). Moreover, hormetic effects are challenging to observe in laboratory conditions, being dependent on various factors such as endpoint measurements or growth conditions (Calabrese and Baldwin, 1997). In the present

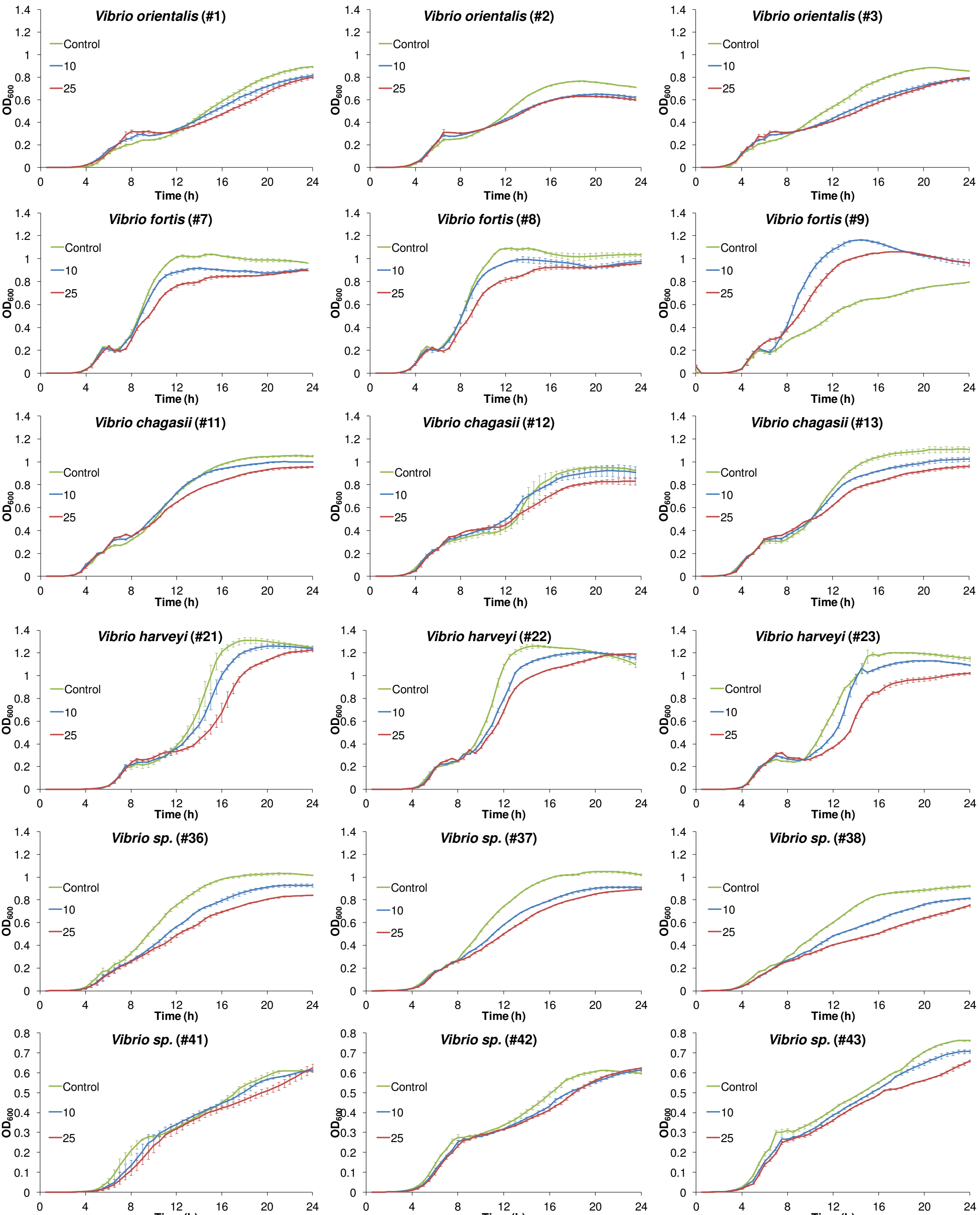
study, hormetic response of *Vibrios* to BW was observed in the CaMHB medium, a culture medium that was previously reported to promote the observation of hormesis in *Escherichia coli* exposed to antibiotics, while the hormetic effect was not observed in Luria-Bertani (LB) culture media (Wang et al., 2017).

The mechanisms of action responsible for the antibacterial activity of marennine remain little explored. So far, the *Haslea provincialis* purified blue pigment was demonstrated to interact with the outer membrane of the Gram – bacteria *E. coli*, rendering it more rigid (Tardy-Laporte et al., 2013). Recent studies conducted on *V. cholerae* also evidenced a disruption of the bacterial membrane integrity and a deformation of the cell architecture by antibacterial agents such as polyphenols (Bhattacharya et al., 2018) or nanoparticles (Sarwar et al., 2016). For now, it is still unclear how marennine and *Vibrio* interact *in vivo* and if the pigment has a direct effect on *Vibrio* growth or if it could decrease its pathogenicity by fixing on cell membranes, which both could explain better survival of bivalve larvae exposed to marennine and challenged with *V. splendidus* (Turcotte et al., 2016).

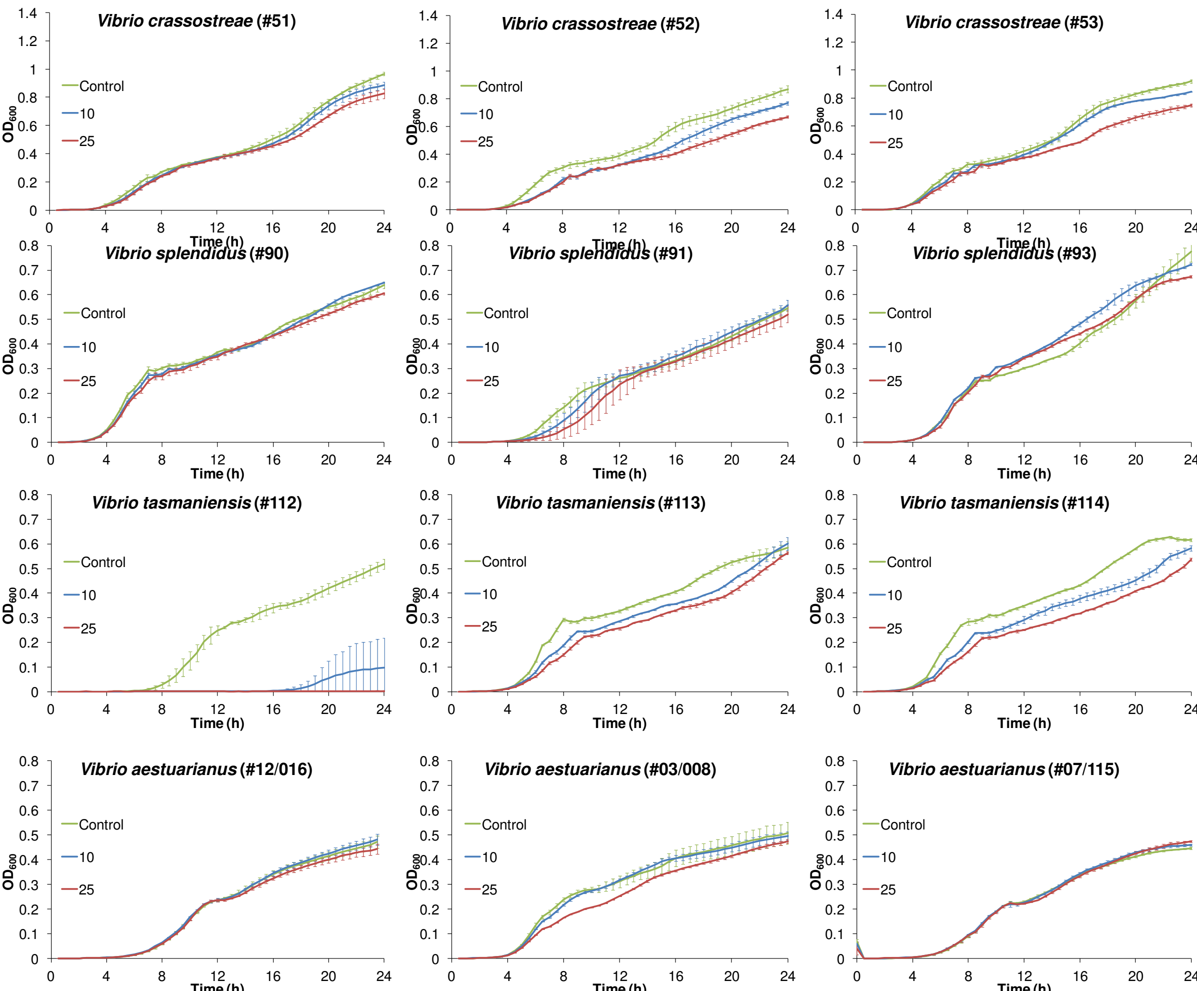
5 CONCLUSION

In conclusion, the present work evidences much more complex interactions between marennine and *Vibrios* than a standard linear correlation between the dose and the effect. Moreover, the antibacterial activity of marennine is dependent on the species, the strain, and also, possibly, on the population of bacteria, suggesting that the blue pigment would act on specific targets. Marennine antibacterial mechanisms and low dose stimulation phenomenon will have to be better understood before considering any application of *Haslea* and marennine as antimicrobials in aquaculture.

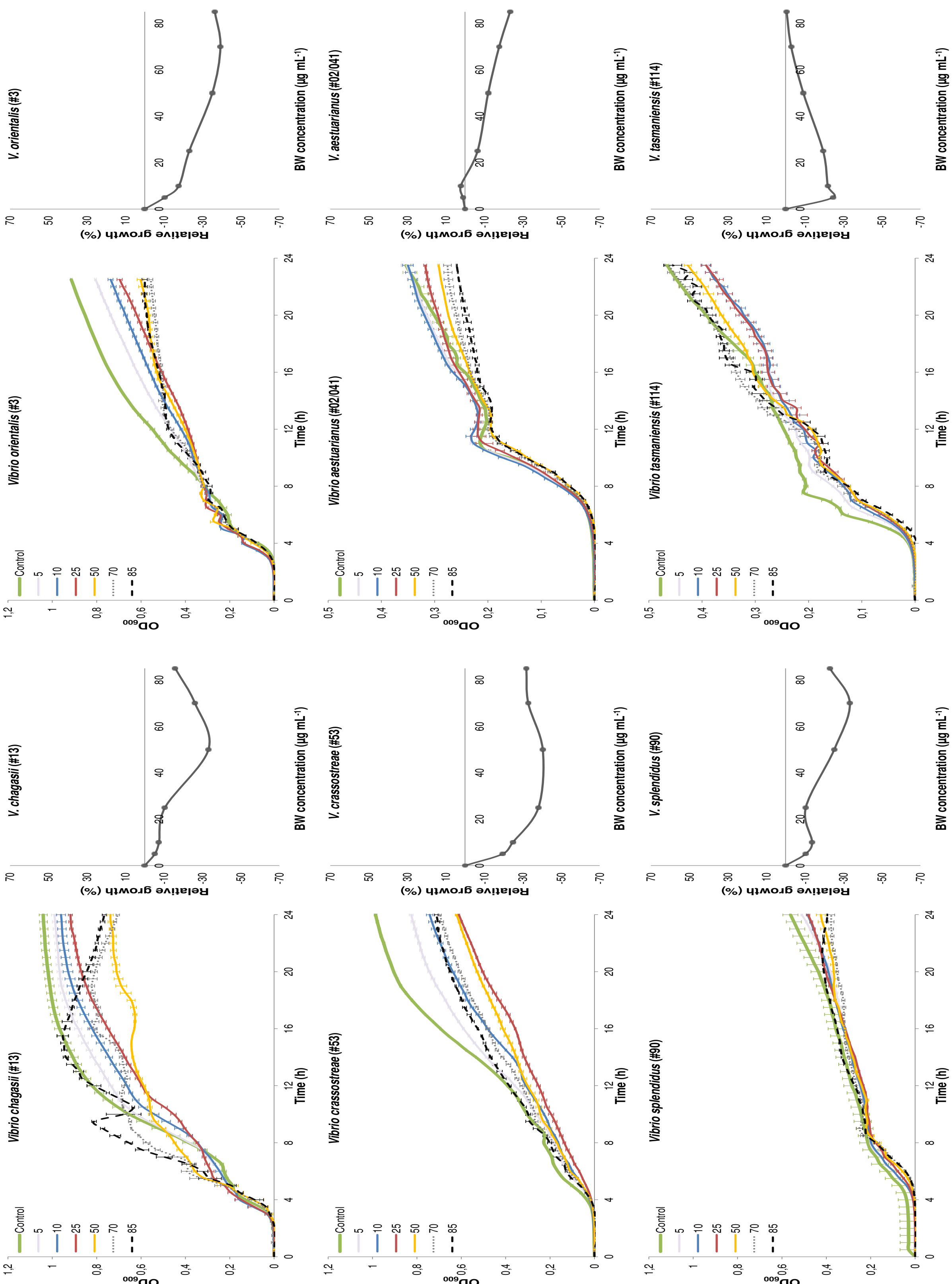
Supplementary Figure 3.1. Growth curves of 30 Vibrio strains (#) from 10 different species exposed over a 24 h period to 0, 10 or 25 µg mL-1 of Blue Water (BW), the concentrated supernatant of Haslea ostrearia containing the extracellular marennine. Results are means ± SE (n=3).



Supp. Fig. 3.1 Cont.



Supplementary Figure 3.2. Growth kinetics and dose-response curves of Vibrio strains exposed to a concentration range (µg mL-1) of Blue Water (BW) over a 24 h period. Values are means ± SD (n=3).



Supplementary Table 3.1. Preferential distribution, virulence to oysters and Blue Water (BW) effect over a 24 h period and over the first phase of growth of the 30 *Vibrio* strains tested.

Vibrio species	Preferential distribution														Virulence	BW effect at 10 µg mL-1	
	Sampling season				Sea Water (SW) Fraction				Oyster				Summary				
strain number (#)	Early spring	Late spring	Early summer	Late summer	> 60µm	60-5µm	5-1 µm	<1 µm	Gills	Digestive gland	Hemolymph	Total	SW	Oyster		Global Effect	1st GP stimulation
V. orientalis																	
#1				x	x								x			-	x
#2				x		x							x			-	x
#3				x									x			-	x
V. fortis																	
#7				x								x		x		-	
#8				x						x				x		0	
#9				x						x				x		+	x
V. chagasii																	
#11				x								x		x		-	x
#12				x								x		x		0	
#13				x								x		x		0	
V. harveyi																	
#21			x						x					x		0	
#22			x								x			x		0	
#23				x								x		x		-	x
V. sp (oyster gills)																	
#36			x				x						x			-	
#37			x					x					x			-	
#38				x					x					x		-	
V. sp																	
#41			x		x								x			-	
#42				x		x							x			+	
#43		x			x								x			0	
V. crassostreae																	
#51			x									x		x	vir	-	
#52			x									x		x	vir	-	
#53				x								x		x	vir	-	
V. splendidus																	
#90	x				x								x			+	
#91	x					x							x			0	
#93	x								x					x		-	
V. tasmaniensis																	
#112		x							x					x		-	
#113		x							x					x		-	
#114	x											x		x		-	
V. aestuarianus																	
#07/115													oyster		vir	0	
#02/041													oyster			0	
#03/008													type souche			+	

+: relative growth stimulation; -: relative growth inhibition; 0: no effect on growth

CHAPITRE III.

Partie 2 :

Co-culture of *Haslea ostrearia* with an oyster virulent *Vibrio* strain

Chapitre III. Effects of marennine solutions on the growth of marine *Vibrio*. Partie 2 : Co-culture of *Haslea ostrearia* with an oyster virulent *Vibrio* strain.

Les travaux présentés dans la partie 2 de ce chapitre ont été conduits dans le cadre du stage de Master 2 de l'étudiante Intan Chandra Dewi, co-encadrée avec Jean-Luc Mouget entre janvier et juin 2017. Un protocole de co-culture *Haslea-Vibrio* a été imaginé et mis en place pour ces travaux dont les résultats ont été présentés par Intan en juillet 2017 lors d'une soutenance orale intitulée « *Antibacterial activity from the supernatant containing extracellular marennine produced by the marine diatom* Haslea ostrearia *».* Les résultats produits au cours de ce stage ont également été présentés au congrès de l'*International Society of Applied Phycology* (ISAP) à Nantes (France), les 18-23 juin 2017.

Abstract

The effect of marennine, the water-soluble blue pigment produced by the diatom *Haslea ostrearia*, was investigated on the growth of an oyster-virulent strain of *Vibrio aestuarianus* in co-culture. To trigger *H. ostrearia* marennine production, cultures were exposed to high irradiance level and the growth of *V. aestuarianus* was compared between cultures under a high light treatment (HL) and cultures under a low light treatment (LL). In HL conditions, the growth of *H. ostrearia* and marennine production per cell was significantly higher in comparison with the LL condition but the growth of *V. aestuarianus* did not appear different between the two conditions. Addition of *Vibrio* in the cultures had no effect on the growth of the microalgae or on marennine production but lowered the associated bacteria (AB) concentration (*i.e.* bacteria naturally present in the non-axenic *H. ostrearia* cultures), highlighting complex interactions between AB, *Vibrio* and *Haslea* cells. From this experiment, the effect of *H. ostrearia* and marennine on *V. aestuarianus* growth in co-culture could not have been determined. Complementary experiments should be conducted with axenic cultures of *H. ostrearia* to overcome the interactions between AB and *Vibrio*, but no culture conditions are yet defined to sustain the growth of the microalgae without its associated microbiota.

Keywords: *Haslea ostrearia*; *Vibrio aestuarianus*; marennine; co-culture

1 INTRODUCTION

Bacterial species of the genus *Vibrio* are involved in diseases and mass mortality events in shellfish cultures (Travers et al., 2015) with species such as *V. aestuarianus* particularly virulent for oysters (Garnier et al., 2007). As the use of antibiotics is limited due to increasing bacterial resistance and chemical residues in sea food tissues, alternative natural compounds are developed to decrease risk for public health, especially compounds derived from plants (Citarasu, 2012). Another strategy promotes the use of microalgae for the biocontrol of pathogenic bacteria. For instance, antibacterial effects have been evidenced with microalgae from different phyla (Bacillariophytes, Chlorophytes, Haphtophytes, etc.), either *in vitro* using algal extracts or in vivo using algae co-cultured with bacteria, including *Vibrio* species (reviewed in Falaise et al., 2016b). The diatom *Haslea ostrearia,* which produces the water-soluble blue pigment marennine, has already been shown to display antibacterial activities *in vitro*, especially against various *Vibrio* species such as *V. aestuarianus* (Gastineau et al., 2012b)*, V. anguillarum* (Pouvreau, 2006) and *V. splendidus* (Gastineau et al., 2014; Falaise et al 2016b). This diatom has long been known to be of great interest in oyster farming as marennine is also responsible for the “greening” of oyster gills that increases their market value by 20 %. The possible use of *H. ostrearia* and its pigment marennine as natural antibiotics has received more attention. Indeed a recent study evidenced the effects of marennine on a large panel of *Vibrio* strains, with cases of growth stimulation or growth inhibition under marennine exposure and demonstrated that the interactions between the blue pigment and *Vibrios* could be very complex (see Chapter III Part. 1). However, no *in vivo* studies have been conducted to assess the effects of *H. ostrearia* on the *Vibrio* growth while the understanding of such interaction is much needed before considering the use of *Haslea* and marennine in aquaculture.

In the present work, an oyster virulent strain of *V. aestuarianus* was co-cultured with *H. ostrearia*. Two different light treatments were applied to the cultures: a high light (HL) treatment and a low light (LL) treatment in order to enhance microalgal growth and marennine production under HL condition (Figure 3.4; Mouget et al., 1999). The effect of

marennine on the growth of the Vibrio strain was also investigated *in vitro* using Blue Water (BW), a concentrated supernatant of *H. ostrearia* cultures.

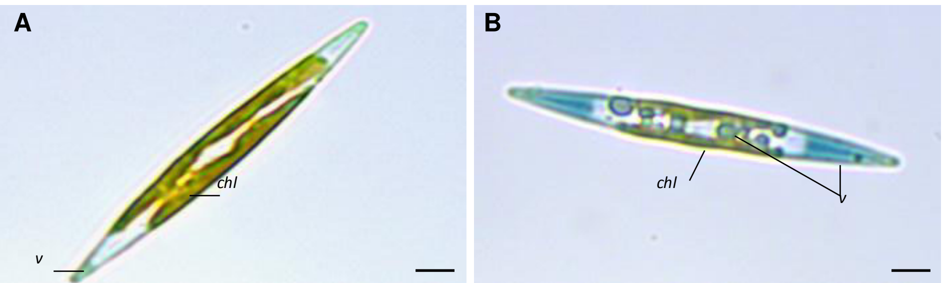


Figure 3.4. Observation of *Haslea ostrearia* cells under light microscopy (Gx400). **(A)** *H. ostrearia* cell under low light treatment (LL; 80 µmol m^{-2} s^{-1}). Chloroplasts (chl) occupy a large part of the cell and cytoplasmic vesicles containing marennine (v) are very reduced. **(B)** *H. ostrearia* under high light treatment (HL; 580 µmol m^{-2} s^{-1}). Chloroplasts (chl) are reduced, many cytoplasmic vesicles containing marennine (v) are observable, with those at the ends of the cell particularly developed. Scale bars 10 µm.

2 RESULTS AND DISCUSSION

The growth of *V. aestuarianus* was not inhibited *in vitro* by marennine from BW solution at the tested concentrations (Figure 3.5). However, it remained interesting to investigate the effect of *H. ostrearia* on *V. aestuarianus* in co-cultures, not only because the strain 12/016 is of particular interest due to its virulence to oysters, but also because the interactions *in vivo* would include those with associated bacteria (AB). The interactions between algae and bacteria could thus differ and be much more complex in comparison with the ones occurring *in vitro*.

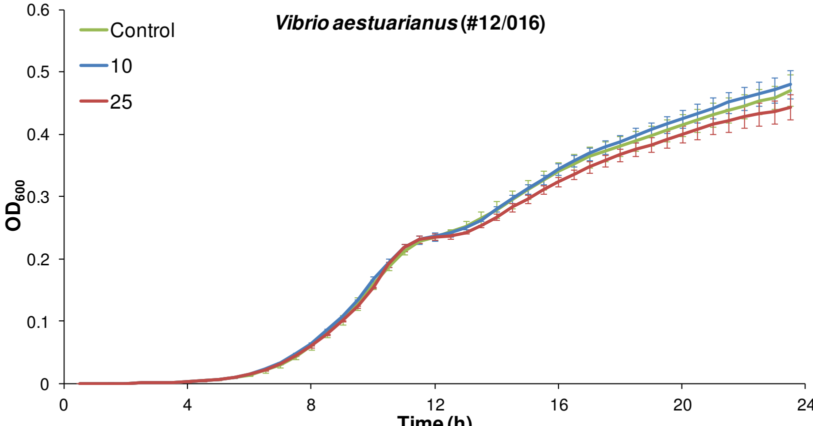


Figure 3.5. Growth kinetic of *Vibrio aestuarianus* (strain 12/016) over a 24 h period under Blue Water (BW) exposure (µg mL^{-1}). BW had no effect on *V. aestuarianus* growth with 6.8 ± 2.9 % of relative inhibition at the higher concentration tested (*p*-value 0.4). Results are means ± SE (n=3).

In the co-culture experiment, *Haslea* cultures under HL condition had a higher growth rate in comparison with those exposed to LL condition (Figure 3.6A; 0.45 ± 0.02 *vs.* 0.38 ± 0.01 d^{-1}; *p*-value < 0.001) and produced significantly more marennine over a 3 d period (Figure 3.6B; 2.70 ± 0.41 *vs* 1.30 ± 0.39 µg mL^{-1}; *p*-value < 0.001). The higher marennine concentration under HL condition was not only explained by a higher *H. ostrearia* cell concentration in the cultures (Figure 3.6C; Adj R^2 0.681; *p*-value < 0.001) but also by a *ca.* 40 % increase in marennine production per cell in HL *vs* LL (Figure 3.6D; 4.69 ± 0.4 10^{-5} *vs* 2.83 ± 0.8 10^{-5} µg $cell^{-}1$; *p*-value < 0.001). Marennine production per cell obtained in the present work under LL treatment are in the range of those assessed by Prasetiya et al. (2016) at 100 µmol photons m^{-2} s^{-1} under similar culture conditions.

Addition of *V. aestuarianus* in the cultures had no effect on *H. ostrearia* concentration under HL or LL treatments (Figure 3.6A; *p*-values 0.98 and 0.09 respectively). A previous study demonstrated that the growth of *Isochrysis galbana* was highly stimulated when co-cultured with *V. alginolyticus, V. campbellii* and *V. harveyi* and it was suggested that the interaction between the diatom and *Vibrio spp.* generates compounds that could be used as nutrients, or by inducing the synthesis of growth factors (Molina-Cárdenas and Sánchez-Saavedra, 2017). However, the authors could not conclude if the antibacterial effect of *I. galbana* against *Vibrio* was due to fatty acid production by the diatom or by some other interactions between *Vibrio* and the microalgal associated microbiota of the non-axenic culture.

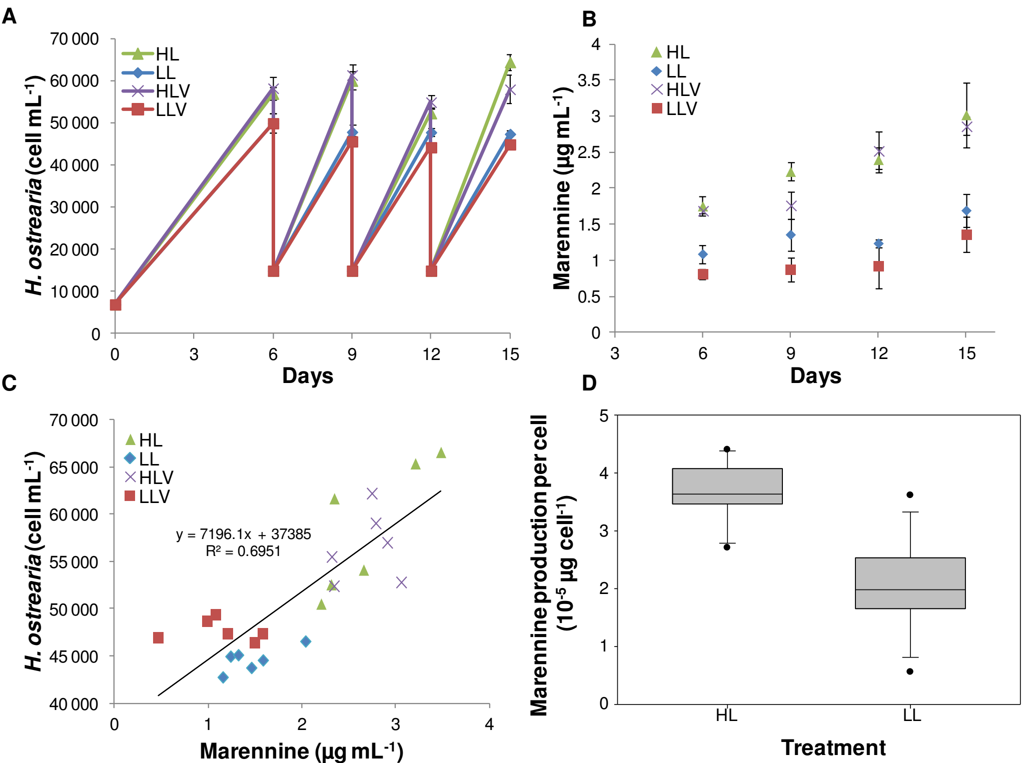


Figure 3.6. *Haslea ostrearia* cultures under different light treatments: high light (HL) and low light (LL) and co-cultured with *Vibrio aestuarianus* (HLV and LLV). **(A)** *H. ostrearia* concentration (cell mL^{-1}) in a semi-continuous culture mode and sub-cultured every 3 days (d 6, d 9 and d 12). **(B)** Extracellular marennine concentration (µg mL^{-1}) in the culture media of the four different treatments. **(C)** Correlation between *H. ostrearia* concentration (cell mL^{-1}) and marennine concentration (µg mL^{-1}). **(D)** Marennine production per *H. ostrearia* cell (µg $cell^{-1}$). Results in (A) and (B) are means ± SE (n=3) and graphics in (C) and (D) gather results from d 12 and d 15 (n=6).

Concentration of AB was similar under HL and LL treatments (Figure 3.7A; *p*-value 0.325) while it could have been expected an upper AB load in HL condition due to the higher *H. ostrearia* cell concentration. The number of AB per microalgal cell was thus lower under HL condition with 2.9 ± 0.8 AB $cell^{-1}$ *vs* 4.6 ± 0.8 AB $cell^{-1}$ for the LL condition (Figure 3.7B; *p*-value < 0.001). It can be hypothesis that a competition for nutrients occurred under HL condition between diatoms and AB, or between *Vibrio* and AB (see below), while the lower microalgal concentration under LL condition allowed the AB to develop further. It seems unlikely that the higher marennine concentration under HL condition decreased the AB growth, as these bacteria that grow naturally in the presence of *H. ostrearia* should be rather insensitive to marennine. It can however be hypothesized that microalgal cells under LL condition, that synthesize less marennine, would produce other kinds of compounds (*e.g.* exopolysaccharides (EPS)) that sustain and promote the growth of AB. Indeed, little is known about the *Haslea* production of compounds other than marennine under different light treatment, but a research project is currently implementing to investigate *Haslea* EPS production (international postdoc project, RFI FFT – Bluedimary).

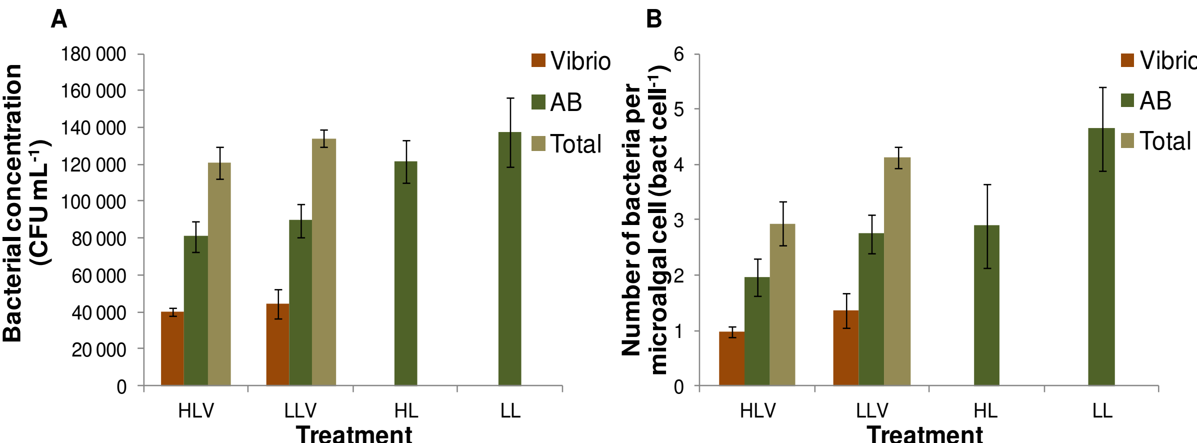


Figure 3.7. (A) Bacterial concentration (CFU mL^{-1}) in *H. ostrearia* cultures under different light treatments: High Light (HL) or Low Light (LL) and in co-culture with *Vibrio aestuarianus* (HLV, LLV). The total bacterial concentration (Total) was calculated after the associated bacteria (AB) concentration and the *V. aestuarianus* (Vibrio) concentration. **(B)** Number of bacteria per *H. ostrearia* cell (bacteria $cell^{-1}$) in the four different treatments. Results are means ± SE and gather data from d 12 and d 15 (n=6).

It was also noteworthy that the AB load was significantly lowered in the presence of *V. aestuarianus* under HL and LL conditions (Figure 3.7A; *p*-values 0.002 and < 0.001 respectively), that could suggest a competition between the epibiont bacteria and the opportunistic *Vibrio*. The number of *Vibrio* per *H. ostrearia* cell was significantly lower under HL condition in comparison with the LL condition (Figure 3.7B; *p*-value 0.013). However, it cannot be deduced that this effect resulted from a higher marennine concentration under HL condition but it would rather result from complex interactions such as competition between AB, *Vibrio* and *Haslea* cells. The influence of the light treatment on the *V. aestuarianus* strain tested was assessed without record of difference of light intensity on the bacterial growth (*data not shown*). This is in accordance with a work conducted by Kokou et al. (2012) in which the growth of six different *Vibrio* species was not influenced by light treatments. Same authors demonstrated that the growth of *Vibrio spp.* was inhibited when co-cultured with axenic microalgal cultures under light or dark treatment suggesting that the observed antibacterial effect was not caused by oxygen radicals produced during photosynthesis by the microalgae (Kokou et al., 2012).

The total bacterial concentration and total number of bacteria per cell was similar under HL conditions in the presence or in the absence of *Vibrio* (Figure 3.7A-B; *p*-values 0.964 and 0.999 respectively). Same observations on the total bacterial concentration and total number of bacteria per cell could be made under LL conditions in the presence or in the absence of *Vibrio* (Figure 3.7A-B; *p*-values 0.742 and 0.413 respectively). This would correspond to the maximal bacterial carrying capacity of the culture under the different light treatment.

3 CONCLUSION

In conclusion, the present work does not allow demonstrating an inhibiting effect of marennine on *V. aestuarianus* growth in co-culture with *H. ostrearia,* mainly because of the complex interactions between AB, *Haslea* cells and *Vibrio*. Addition of *Vibrio* to the cultures did not result in a higher marennine concentration, while it could have been expected that the release of this antimicrobial pigment would be triggered by the microalga under bacterial exposure. However, the *Vibrio* inoculum was low and the total bacterial concentration was similar in the absence or presence of *Vibrio*. To assess whether marennine released is triggered by the presence of bacteria the experiment should be conducted with axenic *Haslea* cultures and various inoculum concentrations tested. For instance, running a co-culture experiment with axenic cultures remains challenging at the experimental level as previous attempts of axenisation led either to culture death or to a significant reduction of cell growth and marennine production. As *H.* ostrearia, such as other diatoms, cannot sustain growth without the nutrients provided by the associated bacteria (Amin et al., 2012; Fuentes et al., 2016; Lépinay et al., 2016), maintaining axenic cultures would only be considered with the development of an optimal culture media with additional nutrient to compensate for the absence of AB (*e.g.* vitamins). Although this study does not answer to the initial issue, it provides new data on *H. ostrearia* cultures such as the quantification of marennine production per cell under different light treatments, and also on the interaction between *H. ostrearia* and its AB, with lower AB concentration at higher *Haslea* densities. This information will be particularly relevant for future culture axenisation assays in which high light conditions should be favored to promote *Haslea* growth and slow down the AB growth.

4 MATERIALS AND METHODS

In the present work, a co-culture experiment was conducted between *H. ostrearia* (strain NCC 495; isolated in Bourgneuf bay, France) and an oyster-virulent *V.* aestuarianus strain (strain 12/016; provided by RBE - SG2M - LGPMM, IFREMER station La Tremblade, France) to assess the marennine effects on *V. aestuarianus* growth. *H. ostrearia* was cultured under two different light treatments: a high light treatment (HL; 580 µmol.m^{-2}.s^{-1}) and a low light treatment (LL; 80 µmol.m^{-2}.s^{-1}) in order to obtain high marennine concentrations in *H. ostrearia* cultures under the HL condition and low marennine concentration under the LL condition (Figure 3.4).

The effect of different light treatments on *V. aestuarianus* growth was previously assessed and no differences were recorded (*data not shown*). *H. ostrearia* was cultured in 250 mL Erlenmeyer flasks containing 100 mL of sterile sea water (SSW) prepared from a commercial sea salt mix (Instant Ocean, Aquarium Systems®; pH 7.7 ± 0.2; salinity 32 ppm) with enrichment solutions as described in Mouget et al. (2009). Three flasks were placed under each light treatment in a control temperature room (16 °C) with illumination provided by cool-white fluorescent tubes in a 14/10 h light/dark cycle. *V. aestuarianus* strain was maintained at -80 °C in glycerol 50 % (v/v), cultured on Nutrient Broth (NB) + NaCl (Biokar) and colonies isolated on Nutrient Agar 2 % (NA) + NaCl (Biokar; final pH and salinity for NB and NA: 7.7 ± 0.2 and 32 ppm). The co-culture experiment was run over a 15 d period on a semi-continuous mode with subcultures conducted every 3 d (on d 6, d 9 and d 12) with *H. ostrearia* concentration adjusted to 15.000 cell mL^{-1}. The semi continuous mode was used to maintain cells in exponential growth phase, to ensure working on cells always in the same physiological stage, without any nutrient deficiency due to competition or light limitation due to self-shading. *V. aestuarianus* was added to 3 flasks per light treatment on d 0 at a final concentration in the culture of 5.10^{3} CFU mL^{-1}. *V. aestuarianus* concentration was determined with Optical Density measurements (OD; 630 nm) after establishing a calibration curve (OD *vs* CFU mL^{-1}). Before each subculture, *H. ostrearia* concentration was assessed using an Nageotte hemocytometer and marennine concentration was determined on cell-free

culture supernatant (syringe filtered through 0.2 µm, Sarstedt) using a spectrophotometer (UV/Vis Lambda 25 Perkin Elmer spectrophotometer, UV Winlab software) and the Beer-Lambert's equation (ε_{677}=12.13 L $g^{-1}cm^{-1}$) as proposed by Pouvreau et al. (2006c). Bacterial concentration was estimated by counting the CFU on Petri dish after plating the samples with an automatic plater (easySpiral Pro ®, Intersciences) and incubation at 27 °C over a 24 h period. Selective agar growth media were used to discriminate between *V. aestuarianus* and *H. ostrearia* associated bacteria (AB) colonies (NA + NaCl media for *V. aestuarianus* and NA + SSW for AB). For the result treatments, only data from d 12 and d 15 were taken into account, which correspond to a stabilized system with significantly more marennine under HL in comparison with the LL condition.

Also, prior to the co-culture experiment, the effect of marennine was assessed *in vitro* on the *V. aestuarianus* growth following the same protocol described in Chapter III Part. 2, using a Blue Water (BW) solution (*i.e.* a concentrated supernatant of *H. ostrearia* containing the extracellular marennine).

Statistical analyses were conducted on *SigmaPlot 12.3* software. Differences between treatments were assessed with One-Way ANOVAs. Normality was tested by the Shapiro-Wilk test and the assumption of homoscedasticity of variance with Fisher's test (F-test). Post hoc Tukey's pairwise multiple comparison tests were used to determine differences between means. Unless specified, alpha value used was 0.05.

CHAPITRE IV.

NEW INSIGHTS INTO THE EFFECTS OF MARENNINE SOLUTIONS ON MARINE ORGANISMS

CHAPITRE IV.

Partie 1 :

Harmful or harmless: biological effects of marennine on marine organisms

Chapitre IV. New insights into the effects of marennine solutions on marine organisms. Partie 1 : Harmful or harmless: biological effects of marennine on marine organisms

Les travaux présentés dans ce chapitre ont en partie été conduits à la station biologique de Roscoff (Roscoff, France) sous l'encadrement de Patrick Cormier[1], à l'Institut des sciences de la mer (Rimouski, Canada) sous l'encadrement de Réjean Tremblay[2] et au LGPMM, Ifremer (La Tremblade, France) sous l'encadrement de Cyrille François[3]. Ce chapitre a également bénéficié des expertises de Céline Audet[2], Jean-Sébastien Deschênes[2], François Turcotte[2], Andreas Seger[4], Gustaaf Hallegraeff[4], Niels Lindquist[5], Damien Sirjacobs[6], Sylvie Gobert[7-8], Pierre Lejeune[8], Vincent Demoulin[6] et Jean-Luc Mouget[9].

Les résultats de ce chapitre ont été présentés lors de communications orales au congrès *Physiomar* à Cambridge (Angleterre) les 18-22 septembre 2017, au congrès *International Conference on Harmful Algae* (ICHA) à Nantes (France) les 21-26 octobre 2018 et au congrès de la *British Phycological Society* (PBS) à Oban (Écosse) les 7-10 janvier 2019.

L'article présenté dans cette partie a été publié dans le journal *Aquatic Toxicology* le 17 janvier 2019.

Affiliations des différents contributeurs de ce travail :

[1]Laboratoire de Biologie Intégrative des Modèles Marins, Sorbonne Universités (UPMC Paris 06/CNRS/UMR 8227), Station Biologique de Roscoff, France ; [2]Institut des Sciences de la Mer, Université du Québec à Rimouski, Canada ; [3]Laboratoire de Génétique et de Pathologie des Mollusques Marins (RBE/SG2M/LGPMM/Ifremer), Station La Tremblade, France ; [4]Institute for Marine and Antarctic Studies (IMAS), Université de Tasmanie, Australie ; [5]Institute of Marine Sciences, Université de Caroline du Nord, Etats-Unis ; [6]INBIOS-PHYTOSYSTEMS, Liège Université, Belgique ; [7]Laboratoire d'Océanologie Biologique, Liège Université, Belgique ; [8]Station de Recherche Sous-marines et Océanographiques (STARESO), Calvi, France ; [9]Laboratoire Mer Molécule Santé (EA 2160/FR CNRS 3473 IUML), Le Mans Université, France

Abstract

Marennine is a water-soluble blue-green pigment produced by the marine diatom *Haslea ostrearia*. The diatom and its pigment are well known from oyster farming areas as the source of the greening of oyster gills, a natural process increasing their market value in Western France. Blooms of blue *Haslea* are also present outside oyster ponds and hence marine organisms can be exposed, periodically and locally, to significant amounts of marennine in natural environments. Due to its demonstrated antibacterial activities against marine pathogenic bacteria (*e.g. Vibrio*) and possible prophylactic effects toward bivalve larvae, marennine is of special interest for the aquaculture industry, especially bivalve hatcheries. The present study aimed to provide new insights into the effects of marennine on a large spectrum of marine organisms belonging to different phyla, including species of aquaculture interest and organisms frequently employed in standardised ecotoxicological assays. Different active solutions containing marennine were tested: partially purified Extracellular Marennine (EMn), and concentrated solutions of marennine present in *H. ostrearia* culture supernatant; the Blue Water (BW) and a new process called Concentrated Supernatant (CS). Biological effects were meanwhile demonstrated in invertebrate species for the three marennine-based solutions at the highest concentrations tested (*e.g.*, decrease of fertilization success, delay of embryonic developmental stages or larval mortality). Exposure to low concentrations did not impact larval survival or development and even tended to enhance larval physiological state. Furthermore, no effects of marennine were observed on the fish gill cell line tested. Marennine could be viewed as a Jekyll and Hyde molecule, which possibly affects the earliest stages of development of some organisms but with no direct impacts on adults. Our results emphasize the need to determine dosages that optimize beneficial effects and critical concentrations not to be exceeded before considering the use of marennine in bivalve or fish hatcheries.

Key words: diatom; *Haslea ostrearia*; marennine; marine organisms; natural bioactive compound

1 INTRODUCTION

Haslea ostrearia is a cosmopolitan marine pennate diatom that synthesizes and releases a water-soluble blue-green pigment called marennine. This species can bloom erratically in oyster ponds in Western France (Baie de Marennes-Oléron, Baie de Bourgneuf), a phenomenon that has long been known to be responsible for the greening of oyster gills. This phenomenon is of economic interest for the French oyster farming industry as the market value of green oyster is significantly increased (by 20 to 30 %) due to changes in the organoleptic properties of the oysters and the scarcity of the product, blooms in ponds being an erratic phenomenon. In the last decade, new species of blue *Haslea* have been discovered, some of them producing marennine-like pigments chemically distinct from the originally described marennine molecule (Gastineau et al., 2012a, 2016). Blooms of blue *Haslea* are also observed in natural environments, such as in the Mediterranean Sea and East coast of the USA (Figure 4.1). Moreover, oysters with green gills have long been observed worldwide, *e.g.*, in Great Britain (Sprat, 1667), Denmark (Petersen, 1916), the USA (Mitchell and Barney, 1917) and Australia (Hallegraeff and Mouget, *personal communications*). Furthermore, the greening of gills has been reported in other organisms, such as polychaetes, crabs, littorina, mussels (Ranson, 1927), sea-anemones (Gaillon, 1820), scallops and cockles (Gastineau et al., 2018), illustrating that many marine organisms can be exposed to blue *Haslea* populations and marennine-like pigments not only in artificial (oyster) ponds, but also in natural environments.

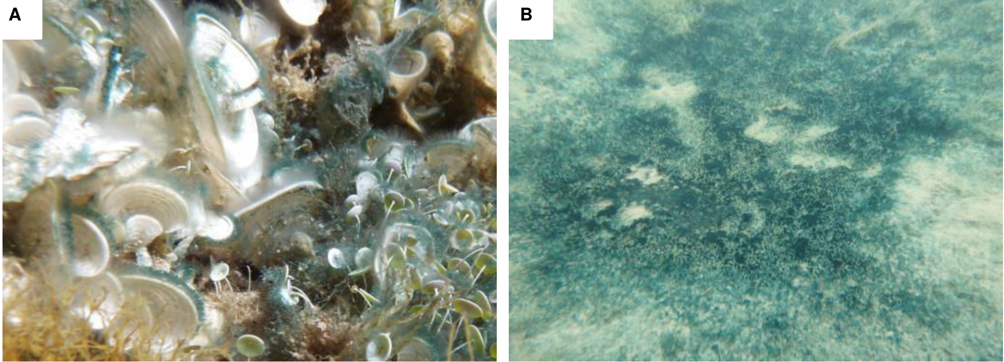


Figure 4.1. Underwater pictures of natural blue *Haslea* blooms forming biofilms observed **(A)** in macro-algae *Padina sp.* in the Mediterranean Sea, Corsica (France) and **(B)** on sediments in the Beaufort Strait, North Carolina (United States).

Although our knowledge of the blue *Haslea* biodiversity has recently increased, little is known about the chemical properties of marennine and marennine-like pigments or their functions for the microalgae. Marennine is thought to be produced *via* a cytoplasmic synthesis pathway (Nassiri et al., 1998) and transiently accumulates at the cell apices (intracellular form of the pigment, IMn). Marennine is excreted from the cells, possibly by exocytosis via small vesicles, which collapse and release an extracellular form of marennine (EMn). The two forms of the pigment differ in their UV-visible spectral characteristics and molecular mass (Pouvreau et al., 2006a). Marennine is a complex molecule composed of glycosidic units (Gastineau et al., 2014b) attached to one or various aromatic rings (Pouvreau et al., 2006a). A protocol to obtain a purified form of EMn or IMn has been developed (Pouvreau et al., 2006b) and several authors have proposed different methods to estimate marennine concentration in solution, despite incomplete knowledge of its chemical structure (Pouvreau et al., 2006c; Robert et al., 2002).

Various studies have demonstrated that marennine (as purified molecule or raw extract) has multiple biological activities, such as antioxidant (Pouvreau et al., 2008), antiproliferative (Carbonnelle et al., 1998; Gastineau et al., 2012b), antiviral (Bergé et al., 1999; Gastineau et al., 2012b) and antibacterial (Falaise et al., 2016b; Gastineau et al., 2012b, 2014b). It has also been shown that marennine possesses allelopathic properties, limiting the growth of various microalgae (Pouvreau et al., 2007; Prasetiya et al., 2016). The biological activities of marennine are species- and even strain-dependent in the case of bacteria (Falaise et al., 2016b), suggesting that marennine could act on specific molecular targets. Tests conducted with Gram-negative bacteria have demonstrated activity of the marennine-like pigment produced by *Haslea provincialis* (Gastineau et al., 2016) and marennine produced by *H. ostrearia*, on the lipopolysaccharidic cell membrane of *Escherichia coli* (Tardy-Laporte et al., 2013) and of *Vibrio splendidus* (Bouhlel et al., 2018), rendering it more rigid.

In line with these results, particularly those demonstrating the capacity of marennine to limit the proliferation of certain pathogenic marine bacteria (Falaise et al., 2016b; Gastineau et al., 2012b, 2014b), further research has confirmed the protective effect of

marennine on the giant scallop (*Placopecten magellanicus*) and blue mussel (*Mytilus edulis*) larvae when challenged with *V. splendidus* (Turcotte et al., 2016). The same study also revealed that a 20 d exposure of mussel larvae to low concentrations of marennine (0.1 µg mL^{-1}) provided a significantly higher survival rate than the control, although exposure to higher concentrations of marennine (1 µg mL^{-1}) resulted in 100 % larval mortality. Even brief exposure to marennine has been demonstrated to result in behavioural, physiological and biochemical changes that were still present eight weeks after exposure (Prasetiya et al., 2017).

These results underpin the interest to further study the effects of marennine on early developmental stages of marine organisms, to identify which are possibly sensitive to marennine, as well as defining exposure concentrations and durations that would be beneficial to species of aquaculture interest. The present work presents a broad panel of experiments conducted to provide an overview of the biological effects of marennine solutions on various marine organisms belonging to different phyla. The organisms were selected based on aquaculture interest or their established role in ecotoxicological model assays. Different solutions containing marennine, prepared from *H. ostrearia* culture supernatants, were used in the exposures: the purified EMn (Pouvreau et al., 2006b), the Blue Water (BW; (Turcotte et al., 2016) and a newly patented Concentrated Supernatant (CS; see Materials). Although EMn represents the most purified form of marennine currently available, BW and CS are of particular interest if the use of marennine-based solutions is considered at larger scales than laboratory experiments due to their easier and cheaper method of production. Marennine exposures were conducted on embryos and/or larvae of the mollusc *M. edulis*, the crustacean *Chthamalus bisinuatus*, the chordate *Pseudopleuronectes americanus* and the echinoderms *Sphaerechinus granularis* and *Paracentrotus lividus*. The effects of the blue pigment solutions were also investigated *in vitro* on the fish gill cell line RTgill-W1, on the oyster *Crassostrea gigas* haemocytes, and on prokaryotic models with bacteria of the genus *Vibrio*, providing a broad view of the diversity of marennine effects on marine organisms.

2 MATERIALS AND METHODS

Purified form of extracellular marennine (EMn), Blue Water (BW) and Concentrated Supernatant (CS) were all obtained from *H. ostrearia* culture supernatant (Table 4.1), but the process to obtain BW and CS is faster and has a better yield in comparison with purified EMn. BW and CS are not purified marennine *per se*, but they allow preparing concentrated solutions of marennine needed to run dose-response experiments between *Haslea* pigment and target organisms. Experiments are presented in the Results section in the following order: 1) Experiments using the BW solution, 2) CS solution, and 3) purified EMn solution.

Table 4.1. Summary of the marennine based solutions and marine organisms used in the present study.

Marennine based solutions	Production process	UV-visible spectra	Method of quantification	Marine organisms tested and studied effect
Blue Water **(BW)**	Ultrafiltration of *H. ostrearia* culture medium (3-30kDa)	Absorbance / 300 400 500 600 700 / Wavelength (nm)	ε_{677}=12.13 L $g^{-1}cm^{-1}$ (Pouvreau et al. 2006c)	Mussel *M. edulis*, barnacle *C. bisinuatus* and winter flounder *P. americanus*: Larval survival and/or development
				Bacterial species of the *Vibrio* genus: Bacterial growth
Concentrated Supernatant **(CS)**	Innovative process (patent n°1872316)	Absorbance / 300 400 500 600 700 / Wavelength (nm)	ε_{669}=17.2 L $g^{-1}cm^{-1}$ (Robert et al. 2002)	Bacterial species of the *Vibrio* genus: Bacterial growth
			ε_{677}=12.13 L $g^{-1}cm^{-1}$ (Pouvreau et al. 2006c)	Sea urchin *S. granularis*: Fertilization, early embryonic development
Purified Extracellular Marennine **(EMn)**	- Ultrafiltration of *H. ostrearia* culture medium (3-30kDa) - Anion-Exchange chromatography - Dialysis & freeze-drying (Pouvreau et al. 2006c)	Absorbance / 300 400 500 600 700 / Wavelength (nm) Adapted from Pouvreau et al. (2006)	ε_{677}=12.13 L $g^{-1}cm^{-1}$ (Pouvreau et al. 2006c)	Preliminary assays with EMn on the sea urchins *S. granularis* and *P. lividus*: Fertilization Oyster *C. gigas* haemocytes: Cytotoxicity
			Weighing of the purified EMn dried powder	Fish gill cell line: Cytotoxicity

2.1 Preparation of the purified extracellular marennine (EMn), Blue Water (BW) and Concentrated Supernatant (CS)

The Blue Water (BW) and purified extracellular marennine (EMn) were produced at the *Station aquicole de Pointe-au-Père* (Québec, Canada) and provided by the *Institut des sciences de la mer de Rimouski-Université du Québec à Rimouski* (ISMER-UQAR; Québec, Canada). The production process of BW and purified EMn were previously described (Turcotte et al., 2016; Pouvreau et al., 2006b). Briefly, *H. ostrearia* strains (NCC 136), isolated from Bourgneuf Bay (France) and provided by NCC (Nantes Culture Collection), were cultured in 100 L photobioreactors until the extracellular marennine concentration reached a maximum of 6 to 8 µg mL^{-1}. Marennine concentration was determined on cell-free culture supernatant (filtered through Sarstedt 0.2 µm syringe filters) using a spectrophotometer (Cary 100 Bio UV-Visible, Agilent Technologies) and the Beer-Lambert's equation (ε_{677}=12.13 L $g^{-1}cm^{-1}$) as proposed by Pouvreau et al. (2006c). The BW was then obtained by concentration of the culture supernatant containing EMn by ultrafiltration (double cut off 3-30 kDa; (Turcotte et al., 2016). To obtain the purified EMn, the BW was further treated by an anion-exchange chromatography process and the fraction collected was dialyzed and freeze-dried (Pouvreau et al., 2006b). BW and purified EMn were stored in the dark at 4 °C and -20 °C respectively.

Concentrated Supernatant (CS) was produced in the *Mer Molécule Santé* (MMS) and *Institut des Molécules et Matériaux du Mans* (IMMM) laboratories (Le Mans, France). A strain of *H. ostrearia* (NCC 495) was batch cultured in 500 mL Erlenmeyers flask containing 250 mL of autoclaved sea water prepared from a commercial sea salt mix (Instant Ocean, Aquarium Systems®; pH 7.6 ± 0.2; salinity 32) with an enrichment solution as described in Mouget et al. (2009). Microalgal cultures were maintained in a 16 °C temperature-controlled room at an irradiance of 200 µmol photon m^{-2} s^{-1}, with illumination provided by cool-white fluorescent tubes in a 14/10 h light/dark cycle. At the beginning of the stationary growth phase the culture supernatant containing EMn was collected by decantation of the microalgal

cells and subsequent vacuum filtrations through 15 µm (150 mm Filter paper, Fisher Scientific®) and 1.2 µm (37 mm glass microfiber filters, Whatman®). Filtered culture supernatant was collected in 1 L glass bottles and the EMn was then concentrated using an innovative technique recently patented (patent n° 1 872 316). The resulting solution was then dialyzed using a 2 kDa dialysis membrane (Spectra/Por®6, Spectrum®). Dialysis tubes were placed in ultra-pure water tanks for 3 d under agitation with the water changed every 24 h. The dialyzed solution was then ultra-filtered (double cut-off 1 kD-30 kDa; Kros Flo® Research IIi TFF System and Kros Flo® Automatic Backpressure valve, Spectrum®) and further concentrated by evaporation with a Rotavapor (Vacuum controller CVC2, Vacuubrand®; Rotary Elevator, Heating Bath Hei-Vap, Base Hei-Vap ML Adv/Pre, Heidolph®). The pH of the resulting CS was neutralized if required to 7.5 ± 0.2 by addition of NaOH 0.1 M. The CS was stored one week in the dark at 4 °C until use. The concentration of marennine in the CS was determined using a spectrophotometer and the Beer-Lambert equation (ε_{669}=17.2 L $g^{-1}cm^{-1}$) as proposed by (Robert et al., 2002). UV-Vis spectra of the different marennine based solutions were conducted using quartz cuvettes with 1 cm path length (UV/Vis Lambda 25 Perkin Elmer spectrophotometer, UV Winlab software).

2.2 Exposure of the mussel *Mytilus edulis* larvae to BW

Adult mussels were obtained in summer 2017 from a farm in the Magdalen Islands (47° 25'N, 61° 50'W, Quebec, Canada), characterized by pure *M. edulis* populations (Myrand et al., 2009) and conditioned for spawning in the *Station aquicole de Pointe-au-Père* (ISMER-UQAR; Québec, Canada) facilities for one month. Mussels were maintained in 180 L tanks in 1 µm filtered seawater at 20 °C flowing at 1 L min^{-1} and were continuously fed with a mixture of *Pavlova lutherii*, *Isochrysis galbana*, and *Nannochloropsis oculata* (ratio 1:1:1) supplied with a peristaltic pump at constant flow to maintain food load at 0.5 mg L^{-1} as described in Hennebicq et al. (Hennebicq et al., 2013). Before spawning, a dozen individuals were removed from the tank and washed by gently rubbing the shell with a brush and diluted bleach and finally rinsed with filtered, UV sterilized sea water. Spawning was

induced by successive thermal shocks from 10 to 25 °C. Fertilized eggs were transferred to 60 L tanks at densities of 10 eggs μL^{-1} and embryos were maintained at 18 °C for 48 h. D-larvae were collected by filtration of the water through a 20 µm mesh screen and transferred to 60 L rearing tanks at densities of 10 D-larvae mL^{-1}. Every 2-3 d, tanks were washed and sterilized and the larvae fed with a mixture of *P. lutherii*, *I. galbana* and *Chaetoceros gracilis* at a final concentration of 90 cells μL^{-1} (30 cell μL^{-1} for each component of the diet; Turcotte et al., 2016).

Mytilus edulis embryos were collected prior to the transfer to the embryogenesis tank, and D-larvae prior to the transfer to the rearing tank (48h post fertilization). Veliger larvae were collected in the rearing tanks (14 d post fertilization) by filtration of the water through a mesh screen. Embryos and larvae were exposed to BW in 12-well flat bottom sterile microplates with each well containing 3 mL of diluted BW and 10-15 embryos or 10-15 larvae per well. Five concentrations of marennine from BW were tested: 0, 0.1, 0.5, 1 and 5 µg mL^{-1}. Marennine concentration from BW was estimated as described in section 2.1 and Table 4.1. BW was syringe-filtered through 0.2 µm and diluted in sterile sea water. Microplates were kept in a controlled temperature room at 18 °C without addition of food during the time of the experiment. The mortality of embryos and veliger larvae was assessed using a light microscope (Olympus BX41; W. Carsen Co., Ltd., Don Mills, ON, Canada) coupled to a digital camera (Evolution VF Color, MediaCybernetics, Silver Spring, MD, USA). The percentages of swimming and motionless D-larvae were assessed by observation of the microplates using an inverted microscope (Axiovert 100, Zeiss). Larval size was measured with Image Pro-Express (Media Cybernetics) after addition of formaldehyde to the wells. A minimum of 10 larvae were counted per well with at least 3 wells per concentration tested.

2.3 Exposure of the barnacle *Chthamalus bisinuatus* larvae to BW

Experiments were conducted in June 2015 in the *Center for Marine Biology of Sao Paulo University* (CEBIMar/USP; Sao Paulo, Brazil). Spawners were harvested by collecting rock

fragments in the intertidal zone of Calhetas Beach (23°49’ 28”S, 45°25’11”W). The fragments were transferred to the laboratory and placed in seawater pumped from the adjacent bay, as described in Kasten and Flores (Kasten and Flores, 2013). A light source was placed above the tanks containing the breeders to recreate the natural photoperiod. Broodstock was submerged for 30 min every 12.4 h to simulate the natural effect of the tide and stimulate spawning. After a first submersion, the water used to submerge the broodstock was siphoned and filtered to harvest the larvae. The larvae were placed in filtered seawater and isolated using a dissecting microscope to obtain a number of larvae sufficient to run the experiment. Larvae were placed individually in the wells of a 96-well microplate in 2 mL of seawater containing 0, 0.05 or 0.1 µg mL^{-1} of marennine from BW without food to test only the potential toxicity of BW. The BW was prepared and the concentration estimated as described in section 2.1 (Table 4.1). Four larvae were tested at each concentration. The microplate was placed on a stirring plate and no food was provided during the experiment. Larvae were observed once a day and survival recorded over 9 d. The experiment was repeated once with spat from a different production.

2.4 Exposure of the winter flounder *Pseudopleuronectes americanus* larvae to BW

Larvae were reared as described by(Khemis et al., 2003) in 57 L cylindro-conical polyethylene tanks and fed rotifers from d 4 to d 26 (Fraboulet et al., 2010). Rotifers were fed a cocktail of microalgal concentrates (1:1:1 *N. oculata*: *I. galbana*: *P. lutherii*, Instant algae, REED Mariculture) and a SELCO food supplement (1 g M^{-1} rotifers, INVE Aquaculture Nutrition, Gransville, UT, USA). The larvae were kept in green water (addition of *N. oculata* culture directly to basins). From d 2 to d 14, two concentrations of marennine from BW were tested on the larvae, 0.05 and 0.1 µg mL^{-1}, with three tanks per treatment (n = 3). BW preparation, as described in section 2.1 (Table 4.1), was added to the tanks in the morning, when water flow was cut to feed the larvae. Water flow was restarted at the end of the day (for a total of about 8 h of exposure) and the BW was gradually evacuated from the tanks

with the flow of water outlet. A dose of BW was given every two days, for a total of seven treatments per tank for the duration of the experiment. Procedures were the same for the control treatments, without the addition of BW in the control tanks. The effect of BW treatment on bacterial load and larval size was evaluated on d 2, d 6, d 10, and d 14, and the size and physiological condition (assessment of energy reserves) of the larvae were determined at the end of the experiment, *i.e.* at d 14 (12 d of treatment). Standard length was measured on formaldehyde-preserved larval pictures as described in (Hjörleifsson and Klein-MacPhee, 1992) using a dissecting microscope (Olympus SZ61) coupled to a digital camera (Evolution VF; Media Cybernetics) and Image Pro-Plus measurement software 5.0 (Media Cybernetics). Lipids were extracted in a 2:1 mixture of dichloromethane: methanol according to (Folch et al., 1957), the lipid classes (triglyceride [TAG], sterol [ST], acetone mobile polar lipids [AMPL], and phospholipids [PL]) concentrations were quantified by TLC-FID as described by (Parrish, 1987), and chromatograms analyzed using PeakSimple v3.21 software (SRI Inc.). To estimate the potential effect of BW on bacterial load in rearing water, bacterial analyzes were carried out using a flow cytometer on water samples taken from the tanks and frozen with glutaraldehyde according to (Seychelles et al., 2011).

2.5 Exposure of the sea urchins *Sphaerechinus granularis* and *Paracentrotus lividus* to EMn and CS

A first series of assays were conducted in May 2016 with purified EMn on *S. granularis* and *P. lividus*, model animals frequently used for *in vivo* ecotoxicological bioassays (Buttino et al., 2016; Pinsino et al., 2010). Sea urchins were collected in the Brest area (France) and obtained from the *Centre de Ressources Biologiques Marines* (CRBM) at the *Roscoff Biological Station* (Roscoff, France). Other experiments on sea urchins were run in March 2017 using CS rather than purified EMn due to its limited production, and the species *S. granularis* because it is available throughout the year.

Marennine-based solutions were prepared by weighing purified EMn freeze-dried powder or after the estimation of CS concentration as described in section 2.1 (Table 4.1).

Solutions were diluted in 0.22 µm Millipore-filtered seawater (FSW) and the final solutions were syringe filtered through 0.2 µm. Sea urchin spawning was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in FSW, rinsed twice by centrifugation (2.000 *g*, 2 min) and re-suspended in FSW for a final 2 % (v/v) egg solution containing 0.1 % (v/v) glycine. Sperm was kept dry at 4 °C until use. Experiments took place in a temperature-controlled room at 16 °C.

Effects of purified EMn and CS were first assessed on fertilization. Eggs were transferred to a 24-well culture plate and incubated during 10 min in marennine-based solutions prior to addition of sperm. Solutions at different concentrations were added in wells containing 1 mL of the egg suspension. Only FSW was added for the control condition. pH strips were used to ensure that the pH did not vary between wells of the different concentrations tested. For fertilization, 50 µL of dry sperm was diluted in 1 mL of FSW shortly before use and 4 µL of diluted sperm added per mL of egg suspension. Observation of the culture plate under phase contrast inverted microscopy allowed the determination of the fertilization rate by counting at least 60 eggs per well (n = 3 wells per concentration tested). For experiments conducted with CS, unfertilized eggs exposed to the highest CS concentrations were rinsed 3 times in FSW using a benchtop centrifuge for 5 to 10 seconds and exposed to sperm for fertilization as described above. Two hours post fertilization, eggs exposed to CS were fixed on a DNA fluorescent stain (Hoechst fixative: Bisbenzymide 0.1 µg mL^{-1}; methanol 75 %, glycerol 25 %) and observed under a fluorescence microscope (ApoTome, Zeiss).

The effects of CS were also assessed post fertilization (kinetics of first cleavage and early embryonic development). For fertilization, diluted sperm was added in a 50 mL tube containing 25 mL of eggs suspended in FSW (2 % (v/v) egg suspension). When egg batches exhibited greater than 90 % fertilization, sperm was removed by centrifugation in Heraeus Labofuge centrifuge (2.000 *g*, swinging bucket rotor 2 min). Fertilized eggs were transferred to a 24-well plate and CS solutions at 1; 10; 50 and 100 µg mL^{-1} added 10 min post fertilization. The percentage of dividing eggs was determined under phase contrast inverted light microscope; at least 60 eggs per well were counted. Three different sea urchin couples

were used to replicate the experiment. For the "washed" condition, embryos from 3 wells per concentration tested were rinsed 6 h post fertilization as described earlier. Culture plates were maintained under constant agitation at 16 °C and embryos were observed 8, 48 and 72 h post fertilization with a Leica DMi8 inverted microscope and pictures acquisition done with LASX software. For each observation time, a 75 µL sample of each well was transferred to a glass slide and pictures of the developmental stages predominantly observed were taken. For post hatching developmental stages, a 1 µL drop of Janus green was added to the samples. Bright Field microscopy technique (BF) was applied for embryos 8 h post fertilization and a Differential Interference Contrast technique (DIC) for embryos 48 h and 72 h post fertilization.

2.6 Exposure of *Vibrio* species to BW and CS

Experiments were conducted during spring 2018 in *MMS* laboratory (Le Mans, France). BW and CS were prepared as described in section 2.1 (Table 4.1), and the concentration of marennine was determined with spectrophotometric measurements using the Beer-Lambert law with the specific extinction coefficient (ε_{677}=12.13 L $g^{-1}cm^{-1}$) proposed by (Pouvreau et al., 2006a). CS and BW were diluted with sterile ultra-pure water. Salinity and pH were adjusted by addition of NaCl and 0.1 M of NaOH or HCl (pH 7.5 ± 0.2; salinity 32) and the solutions were then syringe-filtered through 0.22 µm (Sarstedt). The three *Vibrio* strains tested, *Vibrio chagasii* (strain 8T3_5), *Vibrio crassostreae* (strain 8T2_1) and *Vibrio sp.* (strain 7G1_11) were previously identified (Bruto et al., 2017) and kindly provided by the *Laboratory of Integrative Biology of Marine Models* (CRBM/Roscoff, France). Bacterial strains were kept at -80 °C in 25 % glycerol, inoculated in Mueller Hinton Broth (MHB) + 1 % NaCl (pH 7.5 ± 0.2; salinity 32) and incubated overnight at 25 °C. Isolations were done on Petri dishes containing agar prepared with Nutrient Agar (Biokar) + 2.3 % NaCl (pH 7.5 ± 0.2; salinity 32). The plates were incubated at 25 °C for one day. The antibacterial activity of BW and CS was assessed according to a method described in the Clinical and Laboratory Standards Institute (CLSI) antimicrobial microdilution guidelines (Clinical and

Laboratory Standards Institute (CLSI), 2012). The day prior to the experiment, three different colonies per Petri dish were inoculated in broth media and grown overnight at ambient temperature. Wells of a 96-well sterile microplate with cover and flat bottom were filled with 50 µL of either CS or BW at a final concentration of 10 µg mL-1 (or sterile saline water for the controls) and 50 µL of a bacterial inoculate. A negative control was also run with sterile saline water and broth media without bacteria to ensure that no contamination occurred during the experiment. To prepare the bacterial inoculate the optical density (OD) of the broth culture was measured at 630 nm (V-10 Plus Onda Spectrophotometer), the OD was adjusted to 0.1 by dilution in MHB + 1 % NaCl and the solution was then further diluted by 1/100 as recommended by the CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2012). Microplates were inserted in a microplate spectrophotometer (xMark Bio-Rad) for a 20 h run at ambient temperature. Growth was managed with microplate Manager 6 Software by taking the OD in each well every 30 min at 600 nm.

2.7 Exposure of fish gill cell lines to EMn

Experiments took place in August 2017 at the Institute for Marine and Antarctic Studies (IMAS; Hobart, Australia). The gill epithelium cell line RTgill-W1 was obtained from the American Type Culture Collection (ATTC; and originally isolated from the Rainbow trout *Oncorhyncis mykiss*). The cell line was maintained and exposures to purified EMn conducted as described in Dorantes-Aranda et al. (2011) in conventional 96-well plates. Gill cells were seeded into a flat-bottom 96-well plate (655180, Greiner) at 2.5 x 10^5 cells mL^{-1} in L-15 medium (L-1518, Sigma) and allowed to attach for 48 h in the dark. Confluence of cell cultures was verified 12 h before experimental exposure and the L-15 medium replaced by L-15/ex (Schirmer et al., 1997). The concentration of the original purified EMn solution was estimated (540 µg mL^{-1} in 50 % methanol) as described in section 2.1 (Table 4.1), and the solution was diluted in L-15/ex by factors of 1 x 10^{-1}, 5 x 10^{-2}, 1 x 10^{-2}, 5 x 10^{-3}, 1x 10^{-3}, 5 x 10^{-4} and 1 x 10^{-4}, yielding final exposure concentrations of 0-54 µg mL^{-1}. Methanol was added to all dilutions to achieve a constant final MeOH concentration of 5 % across all

treatments, including the non-toxic control (L-15/ex). Gill cells were exposed to these solutions for 2 h at 20 ± 1 ˚C in the dark (quadruplicate wells per concentration). After the exposure, wells were rinsed twice with saline phosphate buffer (100 µL per well) and incubated for a further 2 h in the dark with 100 µL of 5 % resazurin viability stain in L-15/ex medium. Metabolic reduction of resazurin to resorufin by the gill cells was measured in a microplate reader (Fluostar Omega, BMG Labtech) at excitation and emission wavelengths of 540 and 590 nm, respectively. Results are expressed as percentage viability of the nontoxic control (5 % MeOH in L-15/ex).

2.8 Exposure of the oyster *Crassostrea gigas* haemocytes to EMn

Pacific oysters *C. gigas* were harvested in 2015-2016 in the hatchery of *Laboratoire de Génétique et de Pathologie des Mollusques Marins* (LGPMM; La Tremblade, France) in raceways supplied with a constant flow of seawater enriched with phytoplankton (*Skeletonema costatum*, *Tetraselmis suecica, I. galbana* and *C. gracilis*). They were maintained in safe conditions, free of known infectious pathogens. The shell of 20 adult oysters was broken with metal clamps and haemolymph withdrawn from the adductor muscle sinus (1 to 1.5 mL of haemolymph per oyster) using 1 mL sterile plastic syringes equipped with a needle (0.90 mm x 25 mm). The haemolymph of the different individuals was pooled, filtered through 60 µm nylon mesh and held on ice to prevent haemocyte aggregation (Auffret and Oubella, 1997). Haemocytes were observed under light microscopy using a Malassez-cell. Haemocytes were then exposed to different concentrations of purified EMn: 0, 1, 50 and 100 µg mL^{-1}. The purified EMn was obtained as described in section 2.1 (Table 4.1) and the solutions were prepared by weighing of the EMn dried powder on an analytical balance (Sartorius Entris®) and by dilution on sterile sea water. Purified EMn solutions were added to the haemolymph (1:1 ratio) for a final haemocyte concentration of 2.10^4 cells mL^{-1}. Haemocytes were exposed to EMn during 1, 3 or 6 h and mortality was quantified using 200 µL of cell suspension. Cells were incubated in the dark for 30 min on ice

with 50 µL of Propidium Iodide (PI, 1.0 g L^{-1}, Interchim), a fluorescent DNA/RNA-specific dye that only permeates through the membranes of dead cells and stains the nucleic acids. Haemocyte samples were analyzed with flow cytometry using an EPICS XL 4 (Beckman Coulter) and red fluorescence following the protocol of (Morga et al., 2009). Based on size discrimination, only haemocytes were taken into account with 5.000 events counted per sample. Results were depicted as cell cytograms and reported as log scale fluorescence levels. Data were analyzed with *Flowing Software 2*.

2.9 Statistics

Statistical analyses were run using *SigmaPlot 12.3* software for Windows. Differences between treatments were assessed with One-Way or Two-Ways ANOVAs. Normality was tested by the Shapiro-Wilk test and the assumption of homoscedasticity of variance with Fisher's test (F-test) and/or verified visually by the spread of residuals, as suggested by (Quinn and Keough, 2002). Post hoc Tukey's pairwise multiple comparison tests were used to determine differences between means. Unless specified, alpha value used was 0.05.

3 RESULTS

3.1 Differences in the solutions containing the extracellular marennine

BW and CS presented the same UV-Vis spectral characteristics in comparison with purified EMn (Pouvreau et al., 2006a), with one peak around 670 nm in the visible region and two others in the UV region around 250 and 320 nm. Different coefficients of extinction were used to assess the extracellular marennine concentration depending on the preparation and concentration process and on the series of experiments (summarized in Table 4.1). Despite a difference in calculated concentrations of about 30 – 40 % resulting from the use of either the apparent extinction coefficient proposed by (Robert et al., 2002) or the one proposed by (Pouvreau et al., 2006c), the solutions tested had comparable concentration ranges and activities as described below.

3.2 Effects of BW on *Mytilus edulis* larvae

After a 48 h exposure of embryos to BW, the percentage of larvae that reached the D-larval stage and the mortality rate were assessed (Figure 4.2A). The three lowest concentrations tested did not delay larval development; however, when embryos were exposed to 1 µg mL^{-1} of BW, the percentage of D-larvae was significantly lower (22 ± 1 % of D-larvae compared to 52 ± 2 % for the control; *p*-value = 0.026). Exposure to 1 µg mL^{-1} significantly increased the mortality rate, with 47 ± 1 % of mortality while no mortality was recorded in control condition. Exposure to 1 µg mL^{-1} of BW significantly delayed the development with a D-larvae mean length of 88 ± 7 µm compared to 122 ± 1 µm for the control (*p*-value < 0.001; Figure 4.2B). At 5 µg mL^{-1}, none of the embryos survived after a 48 h exposure to BW (Figure 4.2A).

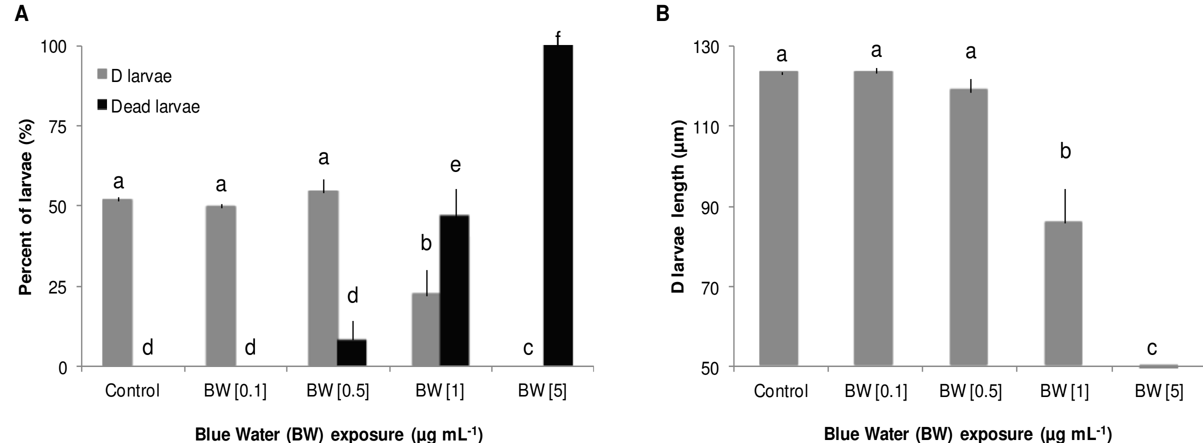


Figure 4.2. Exposure of the mussel *Mytilus edulis* embryos to different concentrations (µg mL-1) of Blue Water (BW) over a 48 h period. **(A)** Percentage of embryos that reached the D-larval stage (grey bars) and percentage of mortality (black bars). **(B)** Length of the larvae that reached the D stage (µm). Values are means ± SE (n=3). Error bars with different lower case letters are significantly different.

In a second series of experiments, D-larvae were exposed to BW in order to assess the effects on larval swimming (Figure 4.3A). After 24 h in BW, the percentage of swimming larvae exposed to 1 µg mL^{-1} was significantly lowered (80 ± 9 % compared to 100 ± 0 % for the control; *p*-value < 0.001). D-larvae exposed to 5 µg mL^{-1} of BW were totally motionless, with the velum highly coloured with marennine (Figure 4.3B). After 72 h in BW, the percentage of swimming D-larvae exposed to 1 µg mL^{-1} of BW decreased significantly in comparison with the day one with only 45 ± 2 % of swimming larvae (*p*-value 0.012).

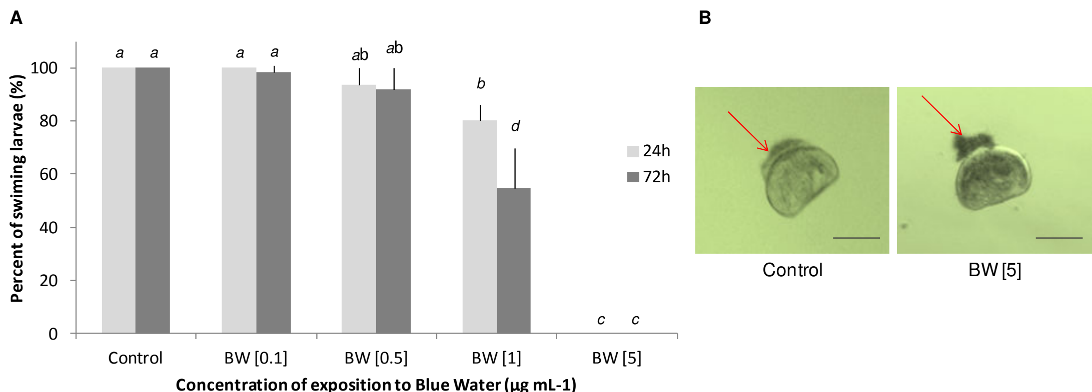


Figure 4.3. Exposure of the mussel *Mytilus edulis* D-larvae to different concentrations (µg mL^{-1}) of Blue Water (BW). **(A)** Percentage of swimming D-larvae exposed during 24 h (light grey bars) and 72 h (dark grey bars) to BW. Values are means ± SE (n=6). Error bars with different lower case letters are significantly different. **(B)** Observation of D-larvae of the control condition (left picture) and exposed to 5 µg mL^{-1} of BW (right picture) after 24 h under light invert microscopy. D-larvae exposed to 5 µg mL^{-1} of BW were totally motionless with marennine agglutinated on the velum (arrow). Scale bars: 100µm.

When veliger larvae were exposed over a 5 d period to BW (Figure 4.4A), a significant increase in mortality was only observed at a concentration of 5 µg mL^{-1}, with 64 ± 7 % of dead veliger larvae compared to 7 ± 6 % of mortality in control (*p*-value < 0.001). Larval length only differed for treatments of 0.5 and 1 µg mL^{-1} compared to 5 µg mL^{-1} of BW (Figure 4.4B; *p*-values 0.036 and 0.030 respectively).

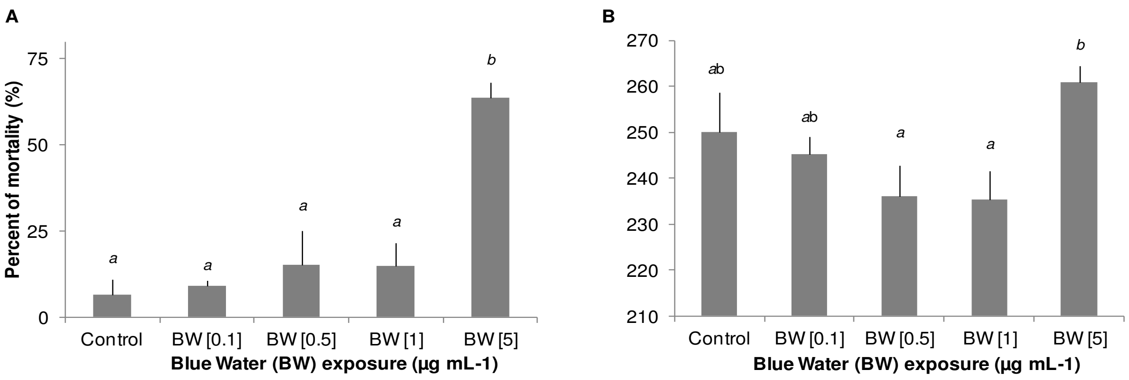


Figure 4.4. Mussel *Mytilus edulis* veliger larvae exposed to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 5 d period. **(A)** Percentage of veliger larvae mortality and **(B)** veliger larvae mean length after BW exposure. Values are means ± SE (n=3). Error bars with different lower case letters are significantly different.

3.3 Effect of BW on the barnacle *Chthamalus bisinuatus* larvae

The survival rate of *C. bisinuatus* larvae was measured at two concentrations of BW, 0.05 and 0.1 µg mL^{-1} over a 9 d period, the maximum period to maintain barnacle larvae without food. At both concentrations, exposure to BW had no significant effect on *C. bisinuatus* larval survival rate in comparison with the control (Figure 4.5; *p*-value 0.1427).

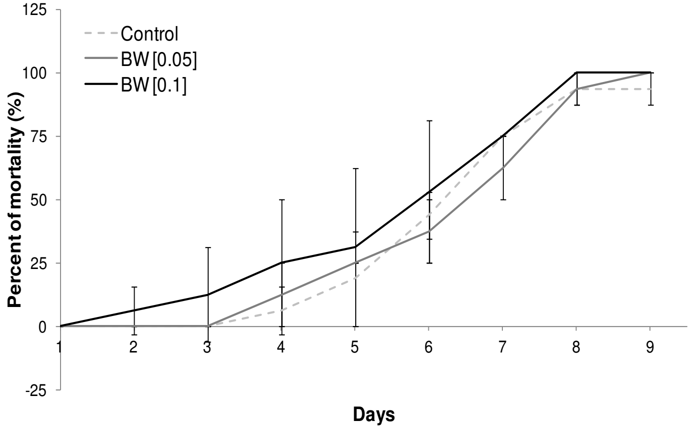
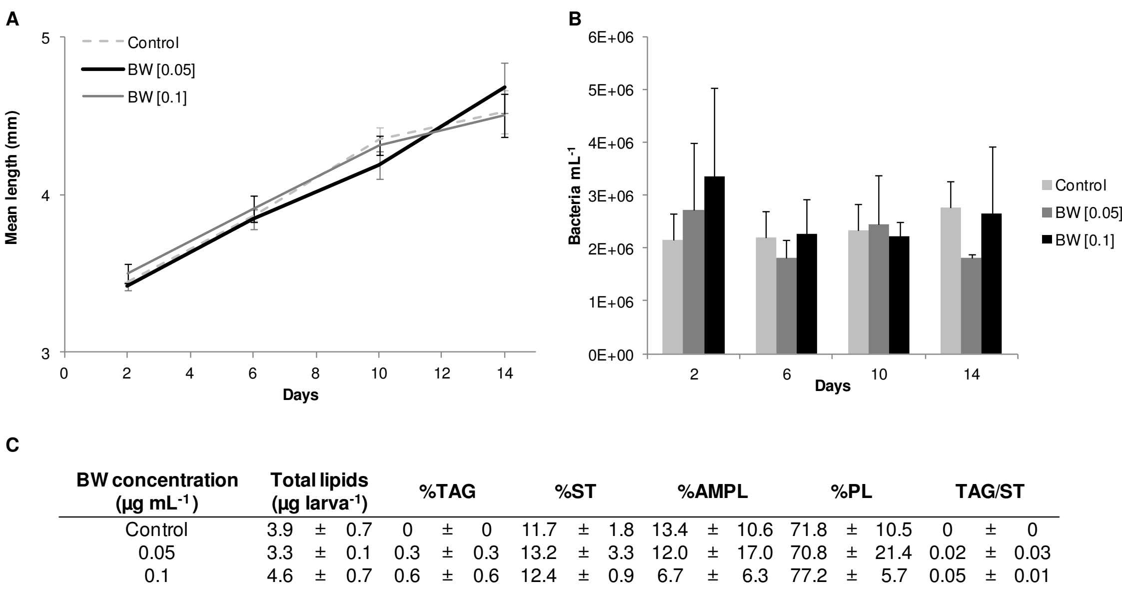


Figure 4.5. Percentage of mortality of the barnacle Chthamalus bisinuatus larvae exposed to different concentrations (µg mL-1) of Blue Water (BW) over a 9 d period. Results are means ± SE (n=4).

3.4 Effect of BW on the winter flounder *Pseudopleuronectes americanus* larvae

The addition of BW at 0.05 and 0.1 µg mL^{-1} had no effect on larval growth (*p*-value 0.287). Indeed, the three groups experienced a growth of 0.1 mm d^{-1} from d 2 to d 14 (Figure 4.6A). The bacterial load remained comparable in all treatments (*p*-value 0.868), demonstrating that, at such concentration, marennine had no impact on bacterial development in fish rearing tanks (Figure 4.6B). Energy reserves in larvae treated with marennine seemed to increase with 4.6 ± 0.7 µg $larva^{-1}$ under 0.1 µg mL^{-1} of BW exposure *vs* 3.9 ± 0.7 µg $larva^{-1}$ for the control, but the difference from the control was not significant due to the high variability among tanks (total lipids, *p*-value 0.1092; triacyglycerol/sterol (TAG/ST) ratio, *p*-value 0.0767). However, the larvae showing the highest lipid content, and the only ones containing TAG, were sampled from the marennine treated tanks (Figure 4.6C).



BW concentration (µg mL⁻¹)	Total lipids (µg larva⁻¹)	%TAG	%ST	%AMPL	%PL	TAG/ST
Control	3.9 ± 0.7	0 ± 0	11.7 ± 1.8	13.4 ± 10.6	71.8 ± 10.5	0 ± 0
0.05	3.3 ± 0.1	0.3 ± 0.3	13.2 ± 3.3	12.0 ± 17.0	70.8 ± 21.4	0.02 ± 0.03
0.1	4.6 ± 0.7	0.6 ± 0.6	12.4 ± 0.9	6.7 ± 6.3	77.2 ± 5.7	0.05 ± 0.01

Figure 4.6. Winter flounder *Pseudopleuronectes americanus* larvae exposed to different concentration (µg mL^{-1}) of Blue Water (BW) over a 14 d period. **(A)** Mean lengths (mm) of *P. americanus* larvae. **(B)** Bacterial load (bacteria mL^{-1}) in larval tanks and **(C)** larval energy reserves: percentages of triglyceride (TAG), sterol (ST), acetone mobile polar lipids (AMPL), and phospholipids (PL) and TAG/ST ratio. Results are means ± SE (n=3).

3.5 Effects of CS and purified EMn on *Sphaerechinus granularis* and *Paracentrotus lividus*

A first series of experiments was conducted on the sea urchins *S. granularis* and *P. lividus* to assess if purified EMn had an effect on fertilization and first cleavage of the egg. In both species, fertilization was totally blocked at 50 and 100 µg mL^{-1} and the phenotypes of embryos exposed to 10 µg mL^{-1} were either delayed in comparison with the control or abnormal (*e.g.,* flattened). Four different incubation periods were tested, from 0 to 20 min prior to the addition of purified EMn, but the effects on fertilization were similar. A 10 min incubation of sperm prior to fertilization was also conducted, but the fertilization rate remained unaffected with 100 % fertilization observed even when sperm was incubated in EMn at 100 µg mL^{-1} (*data not shown*). It was checked if purified EMn prevented fertilization or if the absence of fertilization membrane around the eggs was caused by an effect of EMn on the elevation of fertilization membrane steps. To do so, a calcium ionophore (*i.e.*, a

chemical that can activate echinoderm eggs by a release of intracellular calcium ions and inducing the membrane elevation ; Steinhardt and Epel, 1974) was added in EMn incubated eggs. For all concentrations of EMn tested, from 1 to 100 µg mL^{-1}, the calcium ionophore induced the egg activation demonstrating that purified EMn did not interfere with the elevation of the fertilization membrane steps (*data not shown*).

Incubation of *S. granularis* eggs with CS prior to the addition of sperm also exhibited a dose-dependent effect on fertilization (Figure 4.7A). Exposure to 10 µg mL^{-1} significantly lowered the fertilization success with 85 ± 8 % of fertilization compared to 99 ± 2 % for the control condition (*p*-value 0.032). When eggs were incubated in 50 µg mL^{-1}, no fertilization occurred although the sperm was highly active around the eggs (Figure 4.7B). When eggs previously exposed to 50 µg mL^{-1} of CS were rinsed and removed in FSW without CS, the fertilization rate was similar to the control condition (*p*-value 0.58) demonstrating that the effect of CS on *S. granularis* eggs and fertilization was reversible (Figure 4.7A).

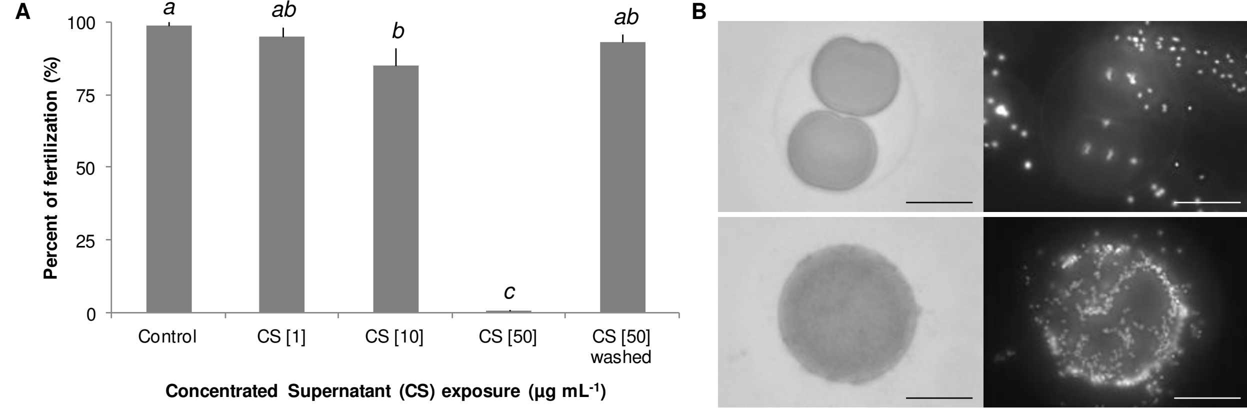


Figure 4.7. Sea urchin *Sphaerechinus granularis* eggs exposed to different concentrations (µg mL^{-1}) of *Haslea ostrearia* Concentrated Supernatant (CS) before fertilization. **(A)** Fertilization rate and **(B)** observation under phase contrast (left panels) and fluorescence (right panels) microscopy (Gx400) of *S. granularis* eggs of the control condition (upper raw) and exposed to CS at 50 µg mL^{-1} (lower raw) 2 h post exposure to CS. Eggs were fixed in a DNA fluorescent stain (Hoechst fixative) to observe cell nuclei of the control dividing egg and spermatozoa agglutinated around the unfertilized egg exposed to 50 µg mL^{-1} of CS. Values in (A) are means ± SE (n=3). Error bars with different lower case letters are significantly different. Scale bars in (B): 25 µm.

Incubation of newly fertilized *S. granularis* eggs with CS demonstrated a dose dependent effect on the kinetics of first cleavage (Figure 4.8A) and on the early embryonic development (Figures 4.8B-C). The two lower concentrations tested, 1 and 10 µg mL^{-1}, had no effect on the kinetics of first cleavage: as for the control, divisions started 95 min post fertilization with embryos dividing symmetrically and synchronously, and at 180 min post fertilization all embryos reached at least the 2-cell stage. At higher incubation concentrations, a dose-dependent effect of CS was observed with fewer dividing eggs, asymmetrical and asynchronous divisions. At 180 min post fertilization, less than 85 % and 50 % of embryos incubated in 50 and 100 µg mL^{-1} of CS, respectively, started to divide and the others quickly degenerated. Similar observations were made for the 3 series of experiments.

Eight hours post fertilization, embryos of the control and those incubated in the presence of CS (1 and 10 µg mL^{-1}) reached the blastula stage (Figure 4.8B) while cell division for embryos incubated in 50 and 100 µg mL^{-1} was quickly halted after a few and incoherent divisions or no division at all. Twenty-four hours post fertilization, the gastrula stage was observed for the control embryos and those exposed to 1 µg mL^{-1} while embryos incubated in 10 µg mL^{-1} were still at the blastula stage and did not hatch (*data not shown*). Forty-eight hours post fertilization, embryos incubated in 1 and 10 µg mL^{-1} exhibited a dose dependent developmental delay with embryos still at the blastula stage and at the early prism stage, respectively, while control embryos reached the late prism stage (Figure 4.8C). Three days post fertilization, the early pluteus stage was observed in controls while the early prism stage and hatched blastula stage were observed in those incubated in 1 and 10 µg mL^{-1} of CS, respectively. Washed embryos were able to recover; embryos initially exposed to 1 µg mL^{-1} presented similar developmental stages to the control, 48 and 72 h post fertilization and the development of embryos initially exposed to 10 µg mL^{-1} and rinsed 6 h post fertilization was still slightly delayed in comparison with the control but more advanced than in embryos incubated in the CS.

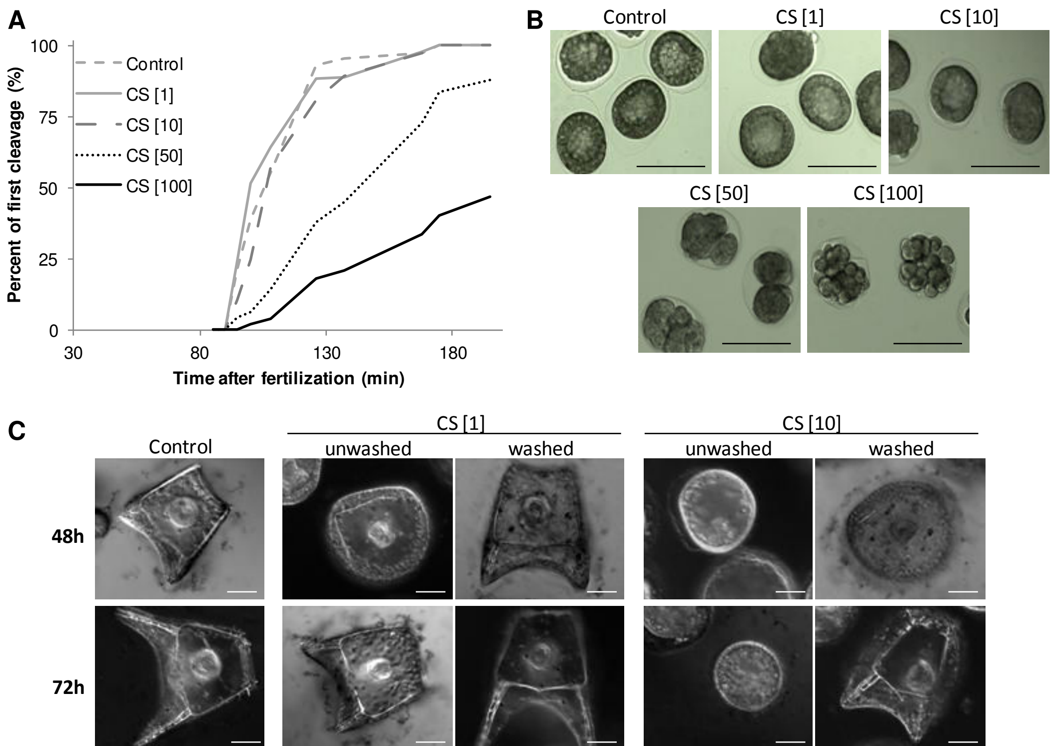


Figure 4.8. Exposure of newly fertilized sea urchin *Sphaerechinus granularis* eggs to different concentrations (µg mL^{-1}) of concentrated supernatant (CS) of *Haslea ostrearia* culture. **(A)** Kinetic of first cleavage of *S. granularis* eggs exposed to the CS 10 min post fertilization. **(B)** Observation of *S. granularis* embryos under phase contrast microscopy 8 h post exposure to CS (Gx200) and **(C)** 48 h (top raw) and 72 h (lower raw) post exposure to the CS (Gx400). Embryos of the "unwashed" conditions were still incubated in the CS while embryos of the "washed" condition were rinsed 6 h post exposure to the CS. The kinetics of first cleavage presented in (A) is representative of 3 replicate experiments. Views in (B) and (C) are representative of the observed stages, scale bars: 50 µm.

3.6 Antibacterial effects of BW and CS on *Vibrio* species

This series of experiments aimed to compare the antibacterial activity of two different solutions of marennine, BW and CS. The bacteria *V. chagasii, V. crassostreae* and *V. sp.* were exposed to 10 µg mL^{-1} of BW and CS for 20 h and relative growth inhibition in comparison with the control was estimated (Figure 4.9). *Vibrio sp* was the most sensitive strain with 37 ± 2 % and 40 ± 1 % of growth inhibition when exposed to 10 µg mL^{-1} of BW and CS, respectively. The growth of *V. chagasii* exposed to BW and CS also decreased

significantly, with percent inhibition as a fraction of the control of 9 ± 1 % (*p*-value < 0.001) and 6 ± 2 % (*p*-value 0.003), respectively. *V. crassostreae* was the least sensitive strain, although growth was also significantly lower than in controls when exposed to BW (5 ± 2 % of inhibition, *p*-value 0.034) and to CS (6 ± 2 % of inhibition, *p*-value 0.020). For each strain tested, growth inhibition induced by BW was statistically similar to the growth inhibition induced by CS (*p*-values > 0.05) indicating that the two different solutions had similar antibacterial effects at 10 µg mL^{-1}.

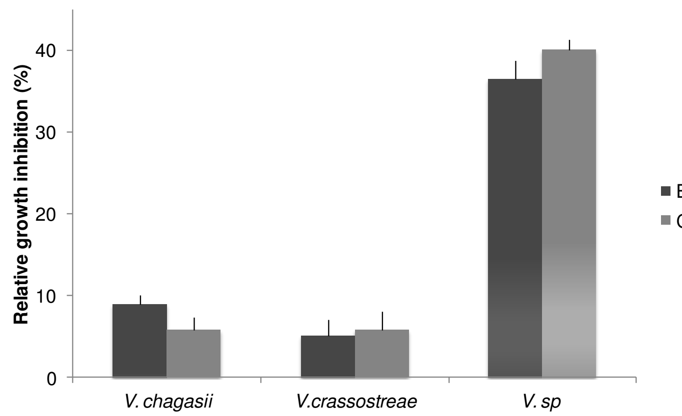


Figure 4.9. Relative growth inhibition of the bacteria *Vibrio chagasii, Vibrio crassostreae* and *Vibrio sp.* exposed to 10 µg mL^{-1} of Blue Water (BW) and 10 µg mL^{-1} of Concentrated Supernatant (CS) over a 24 h period. Values are means ± SE (n=3).

3.7 Effects of EMn on fish gill cell line RTgill-W1

After 2 h exposure to purified EMn, marennine showed no significant effect on fish gill cell viability in the concentration range tested (0 - 54 µg mL^{-1}; Figure 4.10).

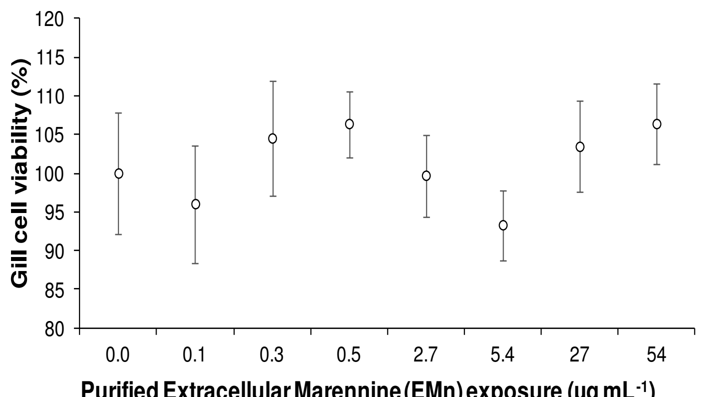


Figure 4.10. Percentage of viable cells of the fish gill cell line Rtgill-W1 exposed to different concentrations (µg mL^{-1}) of purified Extracellular Marennine (EMn). Values are means ± SE (n=4).

3.8 Effects of EMn on *Crassostrea gigas* haemocytes

After an exposure of *C. gigas* haemocytes to 1 µg mL^{-1} of purified EMn, the percentage of lysed cells was not significantly different from the control, 6 ± 1 % compared to 5 ± 1 %, respectively (Figure 4.11). The percentage of lysed haemocytes increased in a dose dependent way at 50 (8 ± 1 %, *p*-value 0.009) and 100 µg mL^{-1} (10 ± 1 %; *p*-value < 0.001). Different durations of exposure were tested (1, 3 and 6 h) without any difference observed in number of lysed cell suggesting that the effects on haemocytes result from the immediate contact with the BW (*data not shown*).

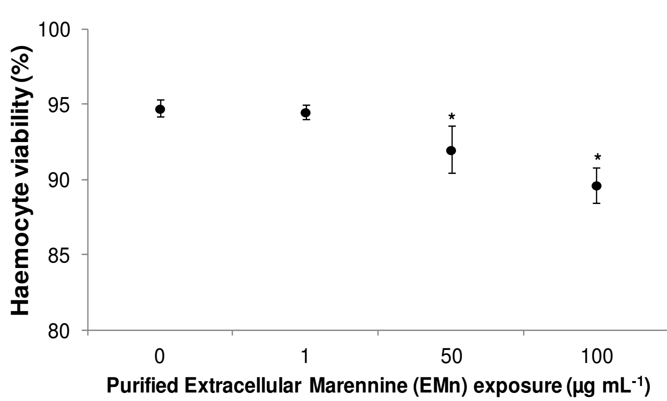


Figure 4.11. Percentage of viable oyster *Crassostrea gigas* hemocytes exposed to different concentrations (µg mL^{-1}) of purified Extracellular Marennine (EMn). Values are means and SE (n=6).

4 DISCUSSION

This work represents a broad panel of experiments conducted in different laboratories to study the biological effects of marennine on various marine model organisms of aquaculture interest. The results confirm that independently of the mode of preparation, solutions containing marennine can exert biological activities, ranging from growth inhibition of pathogens to the death of bivalve larvae. The effects observed depend on the species, the life cycle stage and concentrations of marennine used. The results are summarized in Table 4.2 and raise two questions regarding: 1) limitations of the study due to diversity of marennine solutions for the marine environment and potential applications in aquaculture.tested, and 2) interpretation of data in view of importance of these biological activities

Table 4.2. Summary of the marennine based solutions effects on the marine organisms tested.

Marennine based solutions	Marine organisms tested	Summary of the observed effects
Blue Water **(BW)**	Mussel *M. edulis*	Higher mortality and developmental delay for embryos from 1 µg mL^{-1} Higher mortality for veliger larvae from 5 µg mL^{-1} Decreased of motility for D-larvae from 1 µg mL^{-1} No observed effects at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
	Barnacle *C. bisinuatus*	No observed effect on larval survival at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
	Winter flounder *P. americanus*	No observed effect on larval and development at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
Blue Water **(BW)** / Concentrated Supernatant **(CS)**	Bacterial species of the genus *Vibrio*	Growth inhibition at 10 µg mL^{-1} Similar effects observed with BW solutions and CS solutions
Concentrated Supernatant **(CS)**	Sea urchin *S. granularis*	Decreased of the fertilization rate from 10 µg mL^{-1} and delay of the embryonic development from 1 µg mL^{-1} Reversible effects after rinsing the eggs and/or the embryos Similar effects on fertilization observed with CS solutions ad EMn solutions on *S. granularis* and *P. lividus*
Purified Extracellular Marennine **(EMn)**	Sea urchins *S. granularis* and *P. lividus*	
	Fish gill cell line Rtgill-W1	No observed effect on cell viability from 0 to 54 µg mL^{-1}
	Oyster C. *gigas* haemocytes	No observed effect up to 1 µg mL^{-1}

4.1 Limitations of the study

This work brings together the results of different experiments, conducted over a three-year period, in different laboratories and using three different marennine-based solutions (purified EMn, BW and CS), as well as different methods to quantify marennine concentrations. Moreover, cultures of *H. ostrearia* were grown on different media, which also may have impacted marennine production and quality. The first series of experiments using purified EMn and CS exposed against *S. granularis* and *P. lividus,* showed similar effects on sea urchin egg fertilization. Furthermore, growth inhibition induced by BW and CS was comparable within the same *Vibrio* species at 10 µg mL^{-1}. We conclude that no important modification in the biological activities therefore resulted from differences in preparation of marennine solutions.

The concentration ranges tested could vary by about 30 – 40 %, depending on the extinction coefficient used. Various marennine quantification methods have been published so far, using an extinction coefficient determined from raw extract (Robert et al., 2002) or purified marennine (Pouvreau et al., 2006c), or weighing of purified marennine dried powder (Prasetiya et al., 2017; Turcotte et al., 2016 and Gastineau et al., 2012, respectively). As the absolute quality and quantity of the pigment in different solutions could not be clearly assessed without a complete characterization of this pigment, our results provided a range of concentrations showing biological activities against various marine organisms. While the higher concentrations (50 and 100 µg mL^{-1}) provide clues about doses with deleterious effects, the lower concentrations (from 0.01 to 10 µg mL^{-1}) are more ecologically relevant. In natural environments, marennine measurements indicate a range of 1 - 10 µg mL^{-1} (Turpin et al., 2001), and natural blooms of blue *Haslea* have been recorded worldwide (Figure 4.1). *Haslea* blooms are regularly observed in Calvi Bay (Corsica, France) as part of seaweed surveys. These blooms, which mostly occur in spring, result in the development of a blue-green biofilm at the surface of the thallus of seaweeds (*i.e., Padina, Halopteris, Acetabularia*), on short turfs, and to a lower extent on sediments and rocks (STAtion de REcherche Sous-marines et Océanographique - STARESO and Liège University;

V. Demoulin, D. Sirjacobs, S. Gobert and P. Lejeune, *personal communications*). Blue Haslea can display very large patches reaching several square meters and be observed at depths ranging from 2 to 8 meters. Such extensive blooms are also annually recorded from November through March in central and southern coastal estuaries of North Carolina (USA), in particular along the central coast from lower Core Sound westward across the lower North River estuary and Back Sound to the western portions of Bogue Sound. These blooms of a blue *Haslea* possibly different from *H. ostrearia* do not seem to be associated with the development of biofilms on *Padina* sp. thalli, however they cause a wide-spread greening of oysters, which could underpin emerging *green oyster* industries in the USA (N. Lindquist, *personal communications*).

Thus it can be inferred that, at the microenvironment scale, high concentrations of marennine could be encountered, and that many marine organisms can periodically and locally face acute marennine exposure. All laboratory experiments to study biological activity of marennine lasted hours or days, and the cumulated effect of long-lasting exposure to low concentrations of marennine has not yet been tested. Greening of oyster gills can be viewed as a cumulative, longer term effect. According to green oyster producers, the greening in oyster ponds can occur in a matter of days, depending on *Haslea* growth and density. In the laboratory, EMn rather than IMn was preferentially responsible for greening, which proved to be time dependent and long-lasting (Gastineau et al., 2014b, 2018). However, green oysters have also been observed in natural open environments, in absence of any record of *Haslea* blooms, for instance in oyster leases in Australia (New South Wales and Tasmania). This means that marennine could have biological activities in the long-term, from subacute and chronic exposure, and further work is required to better assess the impact of this pigment in the natural environment.

4.2 The unpredictable "Jekyll and Hyde", good and bad nature of marennine

On the one hand, our results showed that exposure to the tested marennine solutions could lead to adverse effects against many marine animals, depending on the exposure dose. Concentrations from 1 µg mL^{-1} significantly lowered the survival rate of *M. edulis* embryos and locomotion of the D-larvae. Significantly higher mortality rates were observed for veliger larvae exposed to 5 µg mL^{-1} of BW while no mortality was recorded at 1 µg mL^{-1}, which contrasts with results of a previous study where exposure of veliger larvae to 1 µg mL^{-1} of BW led to 100 % mortality, but the duration of that experiment was 4 times longer, 20 d *vs* 5 d (Turcotte et al., 2016). Significant dose-response effects of marennine were also observed with the sea urchin model. While high concentrations (50 and 100 µg mL^{-1}) resulted in a total blockage of fertilization and embryonic development, lower concentrations (1 and 10 µg mL^{-1}) of CS induced a developmental delay of *S. granularis* embryos. Previous works also have demonstrated the effects of a water-soluble extract of the diatom *Thalassiosira rotula* on cell division of the sea urchin *P. lividus*, with a blockage of cell division at the higher doses tested and a delay and abnormal development of embryos at lower doses (Buttino et al., 1999). Intact diatom cells of six different species could inhibit the egg first cleavage of the sea urchins *Strongylocentrotus droebachiensis* and *Echinus acutus* (Gudimova et al., 2016). Most interestingly, our results indicated that even at high concentrations, the effects of CS on fertilization and embryonic development were reversible after rinsing the eggs or embryos, suggesting non- toxicity of the CS, or inability of marennine to act at the cell surface, or to penetrate the cell and exert long-term effects. This confirms previous observations of the effects of water-soluble diatom extracts against various marine invertebrates, including the sea urchin *Psammechinus miliaris* (Caldwell et al., 2002).

The present results could suggest an inability of marennine to bind tightly at the cell surface if considering the reversible effects observed in sea urchins, and to penetrate the cell and exert irreversible damages given the high molecular weight of the molecule (> 10 kDa;

Pouvreau et al. 2006b). Marennine could however link to cell surface or membrane receptors and trigger stress responses or even apoptotic signals by inducing changes in cell metabolism. Furthermore, the variability of BW and CS effects observed at both the interspecific and intraspecific levels on the three *Vibrio* species tested, but also those previously shown with *V. aestuarianus*, *V. coralliilyticus* and *V. tubiashii* strains (Falaise et al., 2016b) could reflect differences in membrane receptor affinities and in mechanisms of action. Such variability should be considered before application of marennine in aquaculture as an antibacterial agent. However, more data is necessary to confirm these hypotheses.

On the other hand, despite observed deleterious effect at the higher concentrations tested, very low concentrations (0.05 and 0.1 µg mL^{-1}) of BW did not exhibit a negative effect on the barnacle *C. bisinuatus* survival nor on the winter flounder *P. americanus.* Both flounder growth rate and bacterial load in the water remained unaffected throughout the marennine exposure (0.05 and 0.1 µg mL^{-1}), and physiological condition (energetic reserves) improved in response to marennine treatment. These results compare with no observable effect of marennine (0-54 µg L^{-1}) on the viability (metabolic activity) of the rainbow trout gill cell line RTgill-W1. Although the gill cell line exposure was conducted for only 2 h, results provide a strong indication that marennine does not impair fish gill function at the concentration ranges tested here. These results are further supported by (Turcotte et al., 2016) who demonstrated that low exposure to BW (0.1 µg mL^{-1}) enhanced survival and physiological condition of *M. edulis* and *P. magellanicus* larvae. In addition, our results showed that low concentrations of EMn (1 µg mL-1) did not present a cytotoxic effect against haemocytes of adult oysters. Even though cytotoxic effects were evidenced at the highest concentrations (50 and 100 µg mL-1), the point of inflection could not be determined with the concentration range tested. However, it should be emphasized that even after centuries of production of green oysters, no negative impacts on adult oysters, nor green oyster consumers have been reported.

Despite the fact that diatoms are traditionally considered a high quality food source enhancing growth and survival of many marine organisms, they can produce secondary metabolites that function as grazing deterrents (Ianora and Miralto, 2010). Most of these

secondary metabolites are polyunsaturated fatty acids (PUFAs) and polyunsaturated aldehydes (PUAs) with a special focus on oxylipins (Caldwell, 2009). Effects of diatom aldehydes on reproductive and fertilization success or embryonic development of various organisms such as crustaceans, echinoderms or arthropods have been widely studied (Caldwell, 2009; Ianora and Miralto, 2010). Aldehyde production is a diatom chemical defense strategy to limit growth of their grazers (Ban et al., 1997; Pohnert, 2005), but other studies reported the absence of a correlation between diatom aldehyde and reproductive biology and early development of marine invertebrates, suggesting the involvement of other unidentified diatom metabolites (Poulet et al., 2006; Wichard et al., 2008). Unlike aldehydes, that are likely to exert their activity following diatom cell destruction and ingestion, the water-soluble marennine may target a larger spectrum of marine organisms and not only diatom grazers.

5 CONCLUSION

In conclusion, the present work demonstrates that *H. ostrearia* supernatant containing marennine represents a biologically active water-soluble solution with potential effects on various marine organisms. More studies are required to better estimate the long term impacts of blue *Haslea* blooms in natural environments, facilitated by laboratory studies, investigating chronic exposure to low concentrations of marennine. If the use of *H. ostrearia* supernatant is considered in hatcheries as a preventive or curative anti-infectious agent, concentrated solutions such as BW or CS could be of interest in order to control the delivered marennine doses. It is likely that the adverse effects of marennine only target the early and more succeptable fragile developmental stages such as embryos and larvae, depending on the exposure concentration, whereas adults remain unaffected.

CHAPITRE IV.

Partie 2 :

Preliminary results on marennine effects on the mussel *Mytilus edulis* larvae

Chapitre IV. New insights into the effects of marennine solutions on marine organisms. Partie 2 : Preliminary results on marennine effects on the mussel *Mytilus edulis.*

Les travaux présentés dans la Partie 2 de ce chapitre ont été conduits à l'Institut des Sciences de la Mer à Rimouski (ISMER, Canada) et à la station aquicole de l'ISMER à Pointe-au-Père durant les mois de mai-août 2017, de novembre 2017 et de mai 2018. Ces travaux ont bénéficié des expertises de Réjean Tremblay, Nathalie Gauthier et François Turcotte pour les pontes de moules, élevages larvaires et production de *Blue Water* ; de Karine Lemarchand et Kim Doiron pour les challenges bactériens ; et de Bertrand Genard et Renée Gagné pour les expérimentations de biologie moléculaire.

Les résultats de ce chapitre ont été présentés lors de communications orales au congrès *Physiomar* à Cambridge (Angleterre) les 18-22 septembre 2017, au congrès *International Conference on Harmful Algae* (ICHA) à Nantes (France) les 21-26 octobre 2018 et au congrès de la *British Phycological Society* (PBS) à Oban (Écosse) les 7-10 janvier 2019.

Abstract

Marennine is the water-soluble blue pigment produced by the marine diatom *Haslea ostrearia*. This pigment displays biological effects on micro-organisms, such as growth inhibition of marine bacteria of the *Vibrio* genus or allopathic activities against several microalgae. Moreover a previous study showed that mussel larvae exposed to 0.5 µg mL^{-1} of Blue Water (BW), a concentrated marennine solution, had a better physiological condition and survival when challenged with a pathogenic *Vibrio splendidus* strain. However, higher exposure doses led to larval mortality. The present work aimed to assess the effects of BW exposure on the expression of some genes in the mussel *Mytilus edulis* larvae. Three different successive larval developmental stages were tested: veliger, pediveliger and metamorphosed larvae, and four BW concentrations: 0, 0.5, 1 and 5 µg mL^{-1}. A challenge with *V. splendidus* on veliger larvae was also conducted to reproduce the prophylactic effects previously observed, but the opportunistic bacteria did not induce a significant larval mortality. A 3 d exposure to BW induced a significant larval mortality at the higher concentration tested, with the early developmental stages (veliger, pediveliger) more sensitive than the later ones (metamorphosed larvae). Transcriptomic analyses confirmed that BW induced stress and enhanced a defense responses in veliger larvae with a higher expression of the four target genes tested: Cathepsin B, Superoxide Dismutase, ATP Synthase Beta and Heat Shock Protein 70. This work represents, to our knowledge, the first analysis of gene expression in *M. edulis* larvae and the first molecular evidences of marennine effects. Also, the present work proposes two reliable reference genes (Elongation Factor 1 and Tubulin) with good stability in the three developmental stages tested that could be used in future studies.

Key words: *Haslea ostrearia;* diatom; marennine; bioactive compound; *Mytilus edulis*; gene expression

1 INTRODUCTION

Diatoms can produce secondary metabolites with toxigenic effects on various marine organisms such as grazers, other algae or microbes (Ianora and Miralto, 2010). Most of the diatom-derived compound studied so far are short chain polyunsaturated aldehydes (PUAs) and other oxygenated fatty acid degradation products collectively named oxylipins (Caldwell, 2009). Their deleterious effects on fertilization, embryonic development or larval fitness were demonstrated in various marine organisms and mostly on copepods, which are the major diatom feeders in planktonic ecosystems (Adolph et al., 2004; Caldwell et al., 2002; Romano et al., 2010). More recently, biomolecular studies evidenced the effects of diatom PUAs and oxylipins on the expression of genes involved in apoptosis, detoxification process or stress from copepod and sea urchin models (Lauritano et al., 2016; Varrella et al., 2016a). However, as several studies reported the absence of a correlation between diatom PUAs or oxylipins production and the observed adverse effects, the involvement of other cytotoxic diatom-derived metabolites were suggested (Poulet et al., 2006; Wichard et al., 2008).

In the present work, the effects of marennine were investigated on the expression of some genes in the mussel *Mytilus edulis* larvae. Marennine is the biologically active blue pigment produced by the marine diatom *Haslea ostrearia*. Although *H. ostrearia* has never been considered as a harmful diatom, there are growing evidences that this diatom and its water-soluble pigment can impair growth or survival of marine organisms and, unlike PUAs or oxylipins, not only diatom-grazers (see Chapter IV. Part 1). Allelopathic activities were also demonstrated, marennine being able to inhibit the growth of several microalgal species (Pouvreau et al., 2007; Prasetiya et al., 2016), as well as neutral, positive or negative interactions with a large panel of bacteria of the genus *Vibrio* (see Chapter III. Part 1). Moreover, marennine can affect the behavior (*e.g.* valve opening frequency) or the physiology (*e.g.* clearance rate, oxygen consumption) of mussel and oyster juveniles (Prasetiya et al., 2017), and can also impair embryonic development of sea urchins (see Chapter IV. Part 1). But, despite potential deleterious effects of marennine, a study conducted by Turcotte et al. (2016) demonstrated a prophylactic activity of the pigment on the blue mussel *M. edulis* and on the giant scallop *Placopecten magellanicus* larvae.

Exposure to low concentration (0.5 µg mL^{-1}) of Blue water (BW), a concentrated marennine solution, significantly improved the survival of larvae challenged with a virulent strain of *Vibrio splendidus* over a 3 d period. It was found that the larvae exposed to BW had a better physiological condition state, characterized by a higher accumulation of triacylglycerol (TAG), an energetic lipid reserve (Gallager et al., 1986). As the bacterial concentration was not different between the two treatments *BW + Vibrio* and *Vibrio*, it was suggested that BW reduced bacterial pathogenicity and did not inhibit the growth of *Vibrio* strain. As bacterial exposure triggers an oxidative stress in bivalve larvae (Genard et al., 2012), it was suggested that the antioxidant properties of the pigment (Pouvreau et al., 2008) could afford a protective action to mussel larvae. However, the same study showed that exposure of larvae to 0.1 µg mL^{-1} of BW over a 20 d period tended to improve larval survival but that exposure to 1 µg mL^{-1} led to 100 % larval mortality, highlighting the tiny limit between beneficial and deleterious effects of the pigment on bivalve larvae (Turcotte et al., 2016).

The present work aimed to assess the molecular effects of BW on the mussel *M. edulis* larvae at different developmental stages: veliger (17 days post fertilization, dpf), pediveliger (23 dpf) and metamorphosed (30 dpf). Larvae were exposed over a 3 d period to different BW doses: 0, 0.5, 1 and 5 µg mL^{-1}. Gene expression of Cathepsin B (CTPB), Superoxide Dismutase (SOD), ATP Synthase Beta (ASB) and Heat Shock Protein 70 (HSP70) was measured by real time quantitative PCR (RT-qPCR) to evidence the effects of BW exposure on larval defense and stress response. CTPB, SOD, ASB and HSP70 genes were respectively chosen for their role in pathogen recognition, oxidative stress response, energy metabolism and cytoprotection. Also, bacterial challenges with veliger larvae and *Vibrio* were conducted in order to reproduce the prophylactic effects of BW observed in Turcotte et al. (2016) and assess the resulting effects on gene expression.

2 Results and discussion

2.1 *Mytilus edulis* larvae challenged with *Vibrio*

Veliger larvae were challenged with *V. splendidus* and exposed to BW at 0.5 µg mL^{-1} in order to induce a significant larval mortality in the presence of *Vibrio* and to reproduce BW prophylactic effect observed in Turcotte et al. (2016). Veliger larvae were also challenged with a strain of *V. aestuarianus*, known for its virulence to oyster, but never tested on mussel larvae. However, none of the *Vibrio* strain induced a significant mortality to veliger larvae (Figure 4.12; *p*-value 0.076). While the virulence of *V. aestuarianus* on *M. edulis* mortality was unsettled, a significant mortality rate was expected with *V. splendidus* as up to 30 % of mortality was recorded in Turcotte et al. (2016) under the same experimental design.

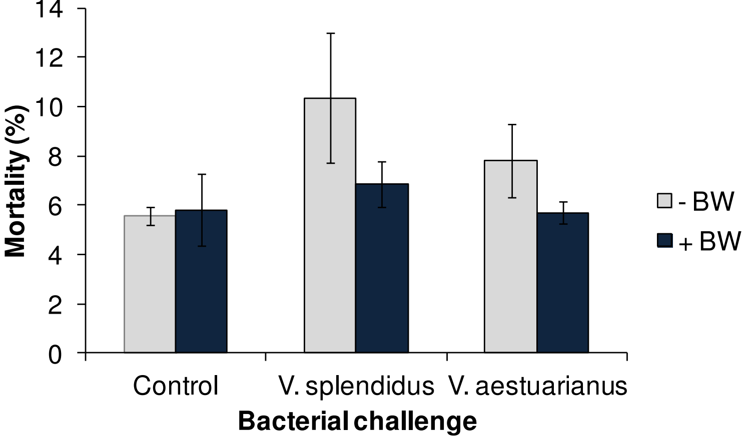


Figure 4.12. Percentage of mortality of *Mytilus edulis* veliger larvae over a 3 d exposure period to Blue Water (BW) at 0.5 µg mL^{-1} and challenged with *Vibrio splendidus* or *Vibrio aestuarianus*. Results are means ± SE (n=3).

Several hypotheses could explain the resistance of veliger larvae to *V. splendidus* in the present work. First, *V. splendidus* is an opportunistic bivalve pathogen that exerts its virulence only under particular environmental conditions (Liu et al., 2016). Moreover, larvae used for the experiment were particularly tough as the embryogenesis success of the larval batch (*i.e.* percentage of embryos that reached the larval stage) was 73 %, while the

standard embryogenesis success generally obtained in the aquaculture station is about 50 – 60 % (R. Tremblay, *personal communications*). For the second bacterial challenge trial conducted with *V. splendidus* only, the embryogenesis success of larvae was 57 %, but the bacteria did neither induce a significant mortality in veliger larvae (Figure 4.13-insert, *p*-value 0.225) suggesting that *V. splendidus* strain was not virulent for *M. edulis* larvae under these experimental conditions.

No differences in the expression of HSP70 (*p*-value 0.903), SOD (*p*-value 0.112), ASB (*p*-value 0.079) or CTPB (*p*-value 0.302) were thus observed for veliger larvae challenged with *V. splendidus* (*data not shown)*.

2.2 ***Mytilus edulis*** **exposure to different BW concentrations**

2.2.1 Effect of BW on *Mytilus edulis* larval mortality

BW exposure at different concentrations had a significant effect on larval mortality, depending on the developmental stage of larvae (Figure 4.13, *p*-value dose x developmental stage interaction 0.003) with 44.5 ± 2.7 % of relative mortality for veliger, 35.1 ± 1.9 % for pediveliger and 27.8 ± 3.5 % for metamorphosed larvae after a 3 d exposure to 5 µg mL^{-1} of BW. Veliger larvae exposed to 5 µg mL^{-1} of BW presented a higher mortality rate than pediveliger larvae (*p*-value 0.009) and metamorphosed larvae (*p*-value < 0.001). Also, pediveliger larvae had a higher mortality rate than the metamorphosed larvae (*p*-value 0.046), demonstrating that early developmental stages are more sensitive to marennine than late developmental stages.

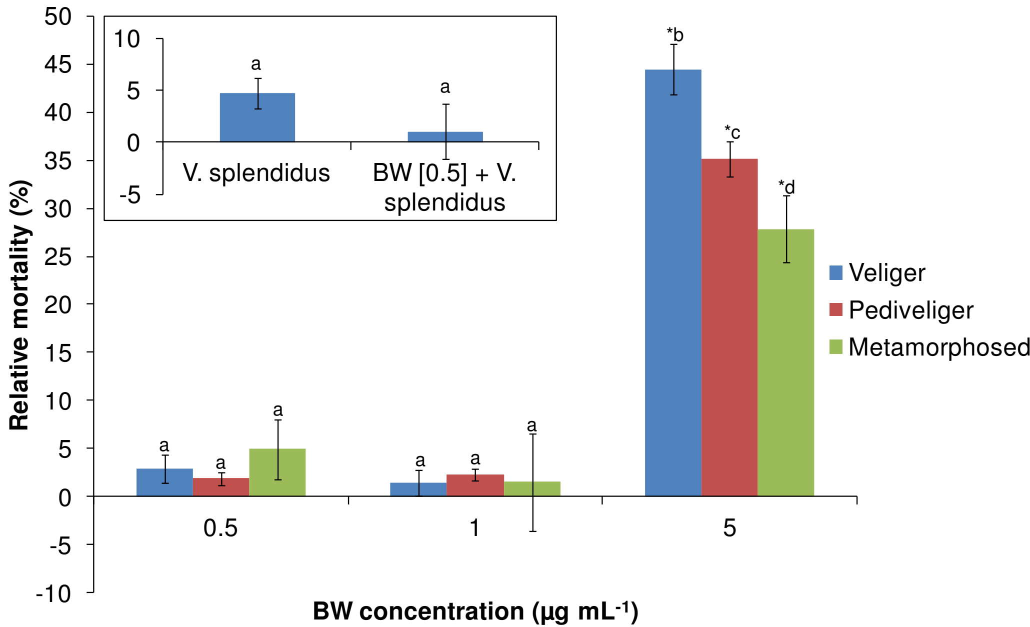


Figure 4.13. Percentage of relative mortality of *Mytilus edulis* veliger, pediveliger and metamorphosed larvae over a 3 d exposure period to different Blue Water (BW) concentrations (µg mL^{-1}) and of veliger larvae challenged with *Vibrio splendidus* (insert). Results are means ± SE (n=3). Error bars with different lower case letters in are significantly different and asterisks indicate significant difference with the control (*p*-value < 0.05).

2.2.2 Molecular response of *Mytilus edulis* larvae under BW exposure

BW concentration enhanced the expression levels of the HSP70 gene (Figure 4.14A, *p*-value 0.004). Also, a tendency for early developmental stage to express more HSP70 in comparison with late developmental stages was observed but did not appear significant because of the high variability between replicates (*p*-value 0.128). A higher HSP70 expression was also observed in the sea urchin *Paracentrotus lividus* embryos and larvae after exposure to diatom-derived oxilipins, with differential expression level depending on the developmental stage (Varrella et al., 2016b). Also, a higher expression of HSP70 genes was previously observed in two different *Mytilus* species under thermal stress (Lockwood et al., 2010). The up-regulation of HSP70 in the present work confirmed that larvae exposed to BW were subjected to stress, activating this gene as a first defense system.

The relative expression of SOD varied significantly depending on the BW concentration and the larval developmental stage (Figure 4.14B, *p*-value dose x

developmental stage interaction < 0.001). The expression of SOD was significantly higher in veliger larvae exposed to BW at 5 µg mL^{-1} in comparison with pediveliger and metamorphosed larvae (*p*-values < 0.001 and < 0.001 respectively). However, no difference in SOD expression was found between pediveliger and metamorphosed larvae (*p*-value 0.900). The higher activity levels of SOD suggest the activation of antioxidant defense that occurs before mortality events as it was previously observed on oyster larvae.

A dose effect of BW was also observed for the CTPB gene (Figure 4.14C) with an up-regulation under high BW exposure (*p*-value 0.003), but no differences were found between larval stages (*p*-value 0.527). CTPB plays crucial roles in host defense system against pathogen infection in molluscs and the overexpression of this gene was previously demonstrated in oysters infected with a parasite (Morga et al., 2009).

The expression of ASB was also activated under high BW exposure (Figure 4.14D; *p*-value 0.013) with no related effect of the larval developmental stage (*p*-values 0.848). ASB (or ATPB) is part of the large family of genes coding for different enzymes that participate in formation of ATP. In molluscs, immune stimulation results in an increased demand of energy to sustain cell defense response (*e.g.* phagocytosis, cytotoxicity; Coyne, 2011). The activation of ATPB expression would reflect the extra demand for ATP required by cells to sustain the immune defense under BW exposure. Expression of ATPB was also shown to be up-regulated in oyster exposed to pesticides (Tanguy et al., 2005).

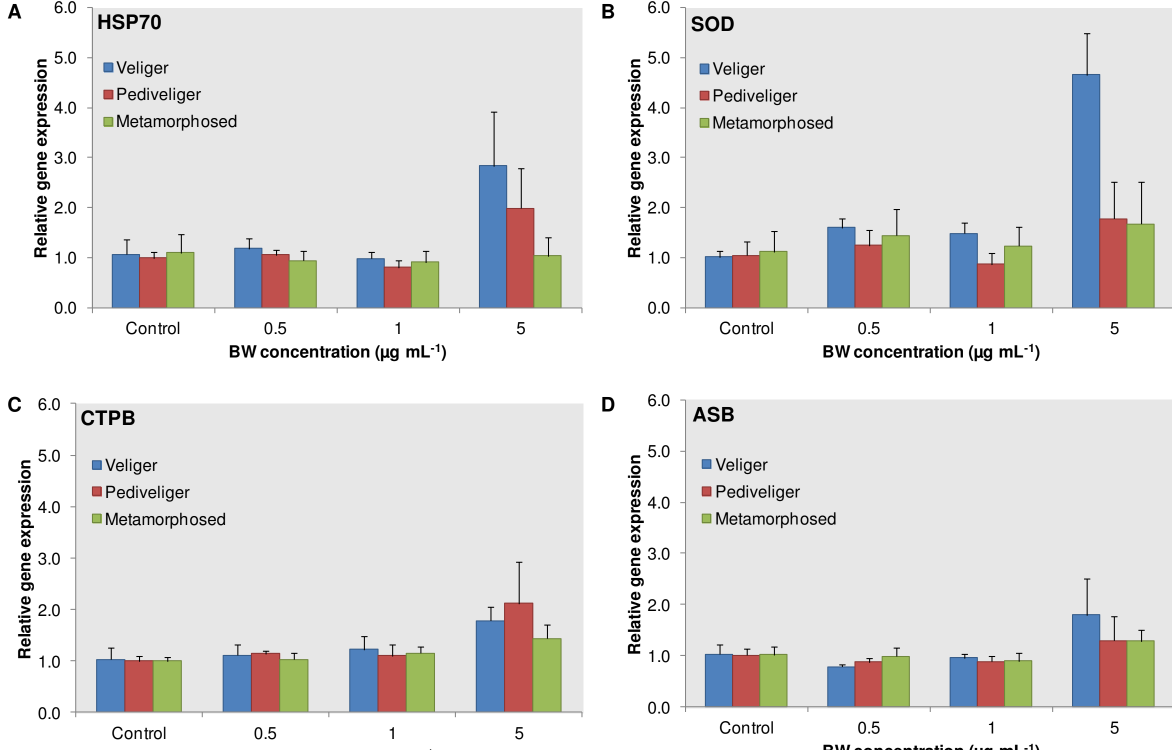


Figure 4.14. Relative expression of **(A)** HSP70, **(B)** SOD, **(C)** CTPB and **(D)** ASB genes in *Mytilus edulis* veliger, pediveliger and metamorphosed larvae exposed over a 3 d exposure period to different concentrations (µg mL^{-1}) of Blue Water (BW). Relative gene expression data were estimated by comparison with the corresponding level from larvae of the *Control* treatment at each developmental stage. Results are means ± SE (n=3).

Several studies have been conducted to assess the molecular responses of mussels subjected to stress such as heat stress (Mohamed et al., 2014; Negri et al., 2013), insecticides (Dondero et al., 2010) or micro-plastics (Avio et al., 2015) with the study of a hundred target genes in some works (Lockwood et al., 2010). However, these studies have been conducted on adult mussels, and only a very few transcriptomic analyses were conducted on mussel larvae. In a work conducted by Navarro et al. (2011), the response to a metallic stress of a dozen genes was investigated in adult and larvae of the zebra mussel but differences in gene expression levels of larvae were only observed for 2 genes: HSP70 and Metallothionein. To our knowledge, the present work represents the first attempt of molecular response analysis in *M. edulis* larvae. The study of gene expression in bivalve larvae can be complicated because of the need to work with pools of thousand individuals to obtain sufficient RNA concentrations and the impossibility to remove larval shells, which increases

the risk to obtain poor RNA quality extractions. Also, only a few gene sequences specific to *M. edulis* larvae are available on gene data banks. Moreover, several genes are only punctually expressed in larvae depending on the developmental stage. Indeed, some genes involved in muscular and neuronal development or in biomineralization are only expressed in veliger and pediveliger larvae but not in metamorphosed ones and *vice versa* (Bassim et al., 2014). Also, stable reference genes have to be defined to obtain reliable results, which can be challenging as the stability of housekeeping gene can vary between developmental stages, as demonstrated in Cubero-Leon et al. (2012) during the different stages of gametogenesis in *M. edulis*. In the present work, we proposed two housekeeping genes with stable expressions over the three different developmental stages studied (*i.e.* TUB and EF1). These two housekeeping genes could therefore be good reference gene candidates for further transcriptomic studies on *M. edulis* larvae.

3 CONCLUSION

Our results demonstrate that BW from 5 µg mL^{-1} induces a significant mortality in *M. edulis* larvae at each of the three developmental stages tested, with veliger larvae particularly sensitive in comparison with pediveliger and metamorphosed larvae. The effects of BW were evidenced at a molecular level with the up-regulation of genes involved in cytoprotection (HSP70), oxidative stress (SOD), energy metabolism (ASB) and pathogen recognition (CTPB). These molecular effects were mainly demonstrated for veliger larvae while no differences in gene expression were observed in metamorphosed larvae, suggesting that different stress response and immune defense are activated in larvae depending on the developmental stage. Although several other primers for potential target genes were designed for the present study (*e.g.* glutathione S-transferase for the detoxification response, P53 for the apoptotic response, or catalase for the antioxidative response) but they did not present a satisfactory efficiency and could not be tested. The analysis of an extensive number of target genes in future studies should allow to further identifying marennine molecular targets and the defense systems activated by larvae. Indeed, in recent works several dozens of target genes were investigated in the sea urchin *P. lividus* after exposure to diatom-derived PUAs and oxylipins and allowed identifying cellular mechanisms and part of the chemical defensome (*e.g.* network of genes that afford protection) activated by the sea urchin at different developmental stages (Marrone et al., 2012; Varrella et al., 2014, 2016a).

So far, this work represents the first molecular evidence of marennine effects on the mussel *M. edulis* larvae. Four target genes were found to be up-regulated in veliger larvae under BW exposure but no molecular target of the blue pigment was defined in metamorphosed larvae. Additional studies on a larger number of target genes may provide some clues about BW mechanisms of action and about the differential defense systems activated by larvae depending on the developmental stage.

4 MATERIALS AND METHODS

4.1 Experimental design

4.1.1 *Mytilus edulis* rearing procedure and exposure to BW

Details about *M. edulis* spawning conditioning, spawning and larval rearing are given in the Chapiter IV Part 1 of this manuscript. Briefly, this work was conducted in the *Station aquicole de Pointe-au-Père* (ISMER-UQAR; Québec, Canada) in 2017. After fertilization, larvae were reared in 60 L filtered sea water (FSW) tanks at densities of 8 D-larvae μL^{-1}. Tanks were washed and sterilized every 2-3 d and the larvae fed with a mixture of *Pavlova lutherii*, *Isochrisis galbana* and *Chaetoceros gracilis*. Veliger larvae were collected in the rearing thanks 17 dpf, pediveliger larvae 23 dpf and metamorphosed larvae 30 dpf. Larvae of each developmental stage were collected on a 53 µm square mesh filter and re-suspended in 2 L of FSW. Larval concentration and size was estimated under light microscope (Olympus BX41; W. Carsen Co., Ltd., Don Mills, ON, Canada) coupled to a digital camera (Evolution VF Color, MediaCybernetics, Silver Spring, MD, USA), and the lengths of at least 30 larvae were measured to ensure size homogeneity with Image Pro-Express (Media Cybernetics) after addition of 2 µL of formaldehyde 37 % in 50 µL sample. Larvae were transferred in a 2.5 L Erlenmeyer filled with FSW (9 larvae mL^{-1}) and exposed to BW at the final concentrations of 0, 0.5, 1 or 5 µg mL^{-1} over a 3 d period in a 20°C controlled temperature room (n=3 Erlenmeyers per treatment). BW was prepared as described in the Chapter IV. Part 1 of this manuscript, stored in the dark at 4°C and syringe-filtered through 0.2 µm before use.

4.1.2 *Mytilus edulis* challenged with *Vibrio* species

A challenge with two *Vibrio* strains was also conducted with veliger larvae only. Veliger larvae were exposed to 0.5 µg mL^{-1} of BW and to *V. splendidus* and *V. aestuarianus*. Challenges were conducted as described above in triplicates with six treatments: *Control*,

BW, *V. splendidus*, *V. splendidus + BW*, *V. aestuarianus* and *V. aestuarianus + BW*. The strain *V. splendidus* 7SHRW was isolated from sediments of Hillsborough River, Prince Edward Island (Gulf of Saint Lawrence, Canada) and was shown to induce significant mortality of blue mussel and scallop larvae. The strain *V. aestuarianus* 02/041 was isolated from oyster tissues and provided by the *Laboratoire de Génétique et de Pathologie des Mollusques Marins* (LGPMM; La Tremblade, France). *Vibrio* strains were kept at - 80℃, streaked onto Marine Agar plates to ensure purity and cultured in 20 mL Marine Broth medium (Difco, Lawrence, KS, USA) at 25℃ for 24 h prior to use. Bacterial cultures were centrifuged, the supernatant discarded and the pellets re-suspended in 10 mL of sterile physiologic water (9 ‰ NaCl; 3x5 min at 5000 g). Optical density (OD_{600}) of the bacterial suspension was measured to determine the bacterial concentration and Erlenmeyers were inoculated at an initial concentration of 1.10^6 bacteria mL^{-1} or with sterile physiologic water only for treatments without *Vibrio*. Another trial with *V. splendidus* only was conducted on veliger larvae at the same time as the BW exposure experiment presented in section 3.1.1. Veliger larvae were challenged with *V. splendidus* at a final concentration of 2.10^6 bacteria mL^{-1} and exposed to 0.5 µg mL^{-1} of BW and the mortality rate and gene expression in the different treatments were assessed.

4.2 Sample collection

After a 72 h period, larvae were collected by filtration using a 52 µm mesh screen, rinsed with FSW and re-suspended in 200 mL of FSW. To estimate the mortality and larval size, the larval suspension was homogenized; 3x1 mL samples per treatment were transferred in Eppendorf micro-tubes containing 100 µL of formaldehyde 37 % and the samples were subsequently observed under light microscopy. The larval suspension was then filtered on a 0.35 µm filter with filtration system connected to a manual pump. Larvae were collected on the filter with a scalpel, transferred to a cryotube and frozen in liquid nitrogen before storage at - 80℃. Larvae were grinded in the mixer Mill MM400 (Retsch) for 15 s at 30 freq s^{-1} and the obtained powder transferred in Eppendorf microtubes, shortly plunged in liquid nitrogen

and stored at - 80 °C before RNA extraction. Total RNA was extracted for each replicate using E.Z.N.A® Mollusc RNA Kit Protocol (OMEGA bio-tek) according to the manufacturer's protocol. RNA was re-suspended in sterile Diethyl pyrocarbonate (DEPC) water and both quality and quantity were assessed (ratio 260/280 for quantity and 260/230 for quality) with a NanoVue™ Plus (GE Healthcare) spectrophotometer. Total RNA samples were then diluted to a final concentration of 200 ng μL^{-1} in RNase-free water. RNA quality was further estimated with migration on a 2 % agarose gel containing the Invitrogen™ SYBER™ Safe™ DNA gel stain (Fisher). Migration bands were observed after 1 h migration at 100 V on Alphamger HP under UV and fluorescence SYBER gold filters. Ten µg of total RNA extracted for each treatment were reverse transcribed using the Quantitect® Reverse Transcription Kit (Qiagen) and following the manufacturer's protocol.

4.3 Studied genes and RT-qPCR

Mytilus edulis nucleotide sequences for reference and target genes were obtained from the GenBank database, designed on *Primer3* (v.0.4.0) online software and the primers quality checked with *Oligo Calc* (v.3.27) online software. Three genes were selected as putative housekeeping genes: tubulin (TUB), elongation factor 1 (EF1) and the ribosomal protein subunit 28S (28S), and four target genes: cathepsin B (CTPB), superoxide dismutase (SOD), ATP Synthase Beta (ASB) and Heat Shock protein 70 (HSP70) chosen for their role in pathogen recognition, oxidative stress, energy metabolism and cytoprotection, respectively (Table 4.3). The specificity of PCR products was checked for the 7 genes by DNA sequencing of fragments amplified with AmpliTaq Gold® 360 Master Mix (Applied Biosystems). Real-time amplification reactions were carried out in a final volume of 20 µL containing 10 µL of Fast SYBR™ Green Master Mix (Fisher), 8 µL of diluted cDNA (1/200) and 500 nM of each primer. Reactions were run in triplicates and a control lacking cDNA template was included in qPCR analysis to determine the specificity of target cDNA amplification. Amplification was detected with a 7900 HT Fast Real Time PCR System (Applied Biosystems). Thermocycling was performed using the following conditions:

polymerase activation at 95℃ for 20 s followed by amplification and quantification cycles repeated 40 times (95℃ for 1 s, 60℃ for 20 s). Following the amplification, a melting curve analysis was carried out for each sample.

Table 4.3. Details of genes and primers used in the study.

Genes	Label	Function	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
Tubulin	TUB	House Keeper	TCCAACCGGATTCAAGGTCG	TCAACATGCAGACGGCTCTC	95
Elongation Factor 1	EF1	House Keeper	AAGCAGCTGAGATGGGAAAA	CCACAAAGCAATGTCAATGG	90
Ribosomal protein subunit 28S	28S	House Keeper	TCCCCTAGTAATGGCGAATG	TAGACGTCCCAAACACCACA	90
Cathepsin B	CTPB	Pathogen recognition	ACATCATGGAAGGCTGGTGA	AGTTTCATGTGCTCTGGGGTT	107
Superoxyde dismutase	SOD	Oxydative stress	GAGAACACGGTTTCCACGTT	CACCATGGGTCTTTCCAAAT	96
ATP Synthase Beta	ASB	Energy metabolism	GCTCCAGCTACAACATTCGC	CCCAACTCAGCAATACCACG	68
Heat Shock Protein 70	HSP70	Cytoprotection	GTGCTGTCCGACGACTTAGA	ACCTGGCTCTTGTGATGCTT	132

The relative level of target gene expression was based on a comparative method (Livak and Schmittgen, 2001; Pfaffl, 2001). The threshold value (Ct) was determined for each target gene as the number of cycles at which the fluorescence curve entered exponential phase. The relative quantification value of a sample was expressed as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (target sample)$-\Delta Ct$ (reference sample) and $\Delta Ct = Ct$ (target gene)$-Ct$ (housekeeping gene). All of the reference genes were amplified for all larval developmental stages and all treatments. Larvae of the *Control* treatment from each developmental stage were used as reference samples to determine treatment differences. Reference genes TUB and EF1 were found to have the lowest variation during the experiment with *BestKeeper* software (Pfaffl et al., 2004) and were then chosen as the housekeeping genes for the rest of the analyses.

4.4 Statistical analysis

Two-way ANOVA (treatment x larval developmental stage) was used to compare the effect of each treatment on the mortality and the relative gene expression at the different developmental stages. Statistical analyses were conducted on SigmaPlot 12.3. Normality was tested by the Shapiro-Wilk test and the assumption of homoscedasticity of variance with Fisher's test (F-test). Post hoc Tukey's pairwise multiple comparison tests were used to determine differences between means. Statistical significance was accepted at *p*-value < 0.05.

CHAPITRE V.

DISCUSSION GÉNÉRALE ET PERSPECTIVES

1 CONTEXTE DES TRAVAUX DE RECHERCHE

Les travaux de ce doctorat s'inscrivent dans le cadre du projet européen G*Ha*NA (*The Genus* Haslea*, New marine resources for blue biotechnology and Aquaculture*, H2020-MSCA-RISE-2016) et tout particulièrement dans l'axe de recherche visant à explorer les applications potentielles des diatomées du genre *Haslea* et de leur pigment bleu dans le secteur de l'aquaculture, et notamment de la conchyliculture.

L'aquaculture est un secteur en pleine expansion dont la croissance annuelle mondiale moyennait 6 % entre 2000 et 2016 (FAO, 2018a). Alors que la proportion d'animaux issus de la pêche en milieu naturel tend à diminuer, la production d'animaux d'élevage en fermes aquacoles ne cesse d'augmenter depuis ces dix dernières années en réponse à la hausse de la demande de la consommation mondiale (FAO, 2018b). Le tonnage annuel des productions aquacoles est estimé à 80 millions en 2016 (contre 90 millions pour les captures en milieu naturel), dont 17,1 millions de tonnes pour les mollusques. L'exploitation des mollusques, comprenant majoritairement les bivalves, est le 3ème secteur d'élevage le plus rentable (29,2 milliards US$ de revenus en 2016) après ceux des poissons et de crustacés (FAO, 2018b). Cependant, l'essor de l'aquaculture et l'intensification des pratiques d'élevage ont été accompagnés par la propagation d'épidémies dans les zones d'exploitation (d'origine endémique ou bien exotique) ainsi qu'à l'émergence de nouveaux agents infectieux. De plus, les conditions de culture parfois intensives induisent des stress aux animaux et affaiblissent leur résistance face aux infections (Eissa et Wang, 2016 ; Lin et al., 2018). Ces maladies, causées essentiellement par des bactéries, des virus et des champignons, peuvent affecter les productions en écloseries et en bassins d'élevage larvaire mais également s'épandre dans les installations extérieures et engendrent alors des pertes économiques importantes pour les éleveurs (FAO, 2018b).

Le recourt aux antibiotiques a été rapidement un moyen communément utilisé pour éradiquer ou limiter les propagations d'épidémies dans les exploitations aquacoles (Henriksson et al., 2015 ; Romero et al., 2012). Cependant, les utilisations récurrentes et

parfois massives de ces produits ont conduit à une diminution de leur efficacité de par le développement de résistances des microorganismes ciblés (Santos et Ramos, 2018 ; Watts et al., 2017). De plus, l'utilisation d'antibiotiques chimiques soulève des enjeux de santé publique avec une préoccupation des consommateurs concernant la présence de résidus de xénobiotiques dans leurs aliments (Okocha et al., 2018).

Afin de renforcer le développement d'une aquaculture durable, plus respectueuse de l'environnement et du consommateur, des alternatives aux antibiotiques chimiques telles que l'utilisation d'additifs alimentaires ou d'antibiotiques naturels sont développées (Dawood et al., 2018). Les additifs alimentaires (*e.g.* probiotiques, plantes médicinales), sont utilisés depuis plus de trois décennies en aquaculture mais connaissent une utilisation accrue depuis le début des années 2000 (Zorriehzahra et al., 2016). Ces additifs peuvent être administrés par ajout direct dans la colonne d'eau, ou délivrés directement aux animaux en les nourrissant avec des organismes ayant reçu, eux-mêmes, une alimentation supplémentée, ou encore par l'intermédiaire de granulés contenant la substance d'intérêt (Jahangiri et Esteban, 2018).

Les additifs alimentaires ont démontré de nombreux bénéfices sur les animaux d'élevage ainsi que sur la qualité du milieu, et permettraient notamment de préserver la santé générale des animaux en exerçant des propriétés immunostimulantes et antistress (Dawood et al., 2018 ; Jahangiri et Esteban, 2018 ; Van Hai, 2015). Parmi les additifs alimentaires connaissant une utilisation accrue on retrouve les probiotiques (*i.e.* microorganismes, morts ou vivants, agissant selon différents modes d'actions et conférant des effets bénéfiques à l'hôte et à son environnement), représentés majoritairement par des bactéries mais également par des levures et des microalgues (Banerjee et Ray, 2017 ; Zorriehzahra et al., 2016).

2 BILAN DES EFFETS BIOLOGIQUES DE LA MARENNINE

Le **Chapitre II** de ce manuscrit développe ainsi les potentielles utilisations des microalgues comme probiotiques en aquaculture en présentant leurs propriétés antibactériennes, antifongiques ou immunostimulantes. Les microalgues sont essentielles aux élevages aquacoles car elles représentent une source alimentaire pour les animaux des stades larvaires à adulte (Guedes et Malcata, 2012 ; Roy et Pal, 2015). Ainsi, l'utilisation dans les élevages de certaines microalgues ayant des effets biologiques peuvent apporter des bénéfices multiples en 1) contribuant à l'apport d'une nourriture de qualité, 2) en permettant d'améliorer les conditions physiologiques et la croissance des animaux, et enfin 3) en limitant les infections microbiennes.

Parmi les microalgues aux propriétés bioactives présentées dans cette revue de littérature, un intérêt tout particulier a été apporté aux diatomées du genre *Haslea*. Le pigment bleu produit par certaines espèces, et spécialement la marennine produite par *H. ostrearia,* représente d'ores et déjà un intérêt économique dans le secteur de l'ostréiculture. La couleur bleue-verte que prennent les branchies des huîtres suite à une exposition au pigment augmente en effet la valeur marchande des bivalves. Différentes études ont démontré *in vitro* les activités biologiques de la marennine et des pigments « de type marennine » produits par d'autres espèce d'*Haslea* bleues avec des effets inhibiteurs sur la croissance de bactéries (Gastineau et al., 2012b, 2014b ; Pouvreau, 2006) ou de champignons (Gastineau et al., 2012c) nuisibles en aquaculture, ainsi que des activités antivirales (Bergé et al., 1999 ; Gastineau et al., 2012c), antiprolifératives (Carbonnelle et al., 1998) et également antioxydantes (Pouvreau et al., 2008). Une étude conduite par Turcotte et al. (2016) démontrant clairement les effets prophylactiques du pigment à faible dose sur des larves de moule et de pétoncle exposées à une souche de *Vibrio* pathogène a conduit à considérer l'utilisation potentielle de la marennine dans le secteur de l'aquaculture et à développer cette approche dans mon travail de thèse. Des résultats originaux présentés dans le Chapitre II de mon mémoire démontrent effectivement une action inhibitrice de la marennine extracellulaire purifiée (EMn) sur la croissance de diverses souches de *Vibrio* avec des effets espèces-dépendants mais également souches-dépendants.

Dans le **Chapitre III** de ce manuscrit les effets de la marennine sur la croissance de souches bactériennes du genre *Vibrio* ont davantage été étudiés. Les vibrions sont les bactéries les plus fréquemment impliquées lors d'épisodes de mortalité associés à des infections bactériennes chez les bivalves (Travers et al., 2015). Un panel de 30 souches de *Vibrio* appartenant à 10 espèces différentes a été testé pour sa sensibilité à la marennine, certaines d'entre elles (*e.g. V. crassostreae*) reconnues comme virulentes pour les huîtres. La croissance des souches exposées à la marennine sous forme de *Blue Water* (BW, surnageant de culture d' *Haslea* concentré) a été suivie par mesures spectrophotométriques pendant 24 h. Ce *screening* a permis de mettre en évidence la variabilité de réponses entre les différentes souches exposées à 10 et 20 µg mL^{-1} de BW. Une exposition à la BW a modifié la croissance de 80 % des souches testées : 67 % des souches ont eu une croissance significativement inhibée par la BW et 13 % des souches ont présenté une stimulation de croissance. Les effets de la BW sont donc apparus dépendants des espèces testées, mais également des souches au sein d'une même espèce, confirmant ainsi les résultats préliminaires présentés dans le Chapitre II. Dans une seconde série d'expérimentations, une sélection de 10 souches a été exposée à une gamme de concentration de BW allant de 0 à 80 µg mL^{-1}. Les courbes dose-réponse obtenues ont mis en évidence des effets non linéaires de la marennine : l'augmentation des concentrations d'exposition n'a pas systématiquement conduit à une augmentation de l'effet inhibiteur. En effet, des courbes dose-réponse en forme de cloche inversée (ou en forme de « U ») ont été observées pour certaines souches. Un phénomène d'hormèse a également pu être mis en évidence chez la souche *V. fortis* #9, caractérisé par une stimulation de la croissance à la plus faible concentration testée (croissance de la souche stimulée à plus de 50 %), et par une inhibition de la croissance aux plus fortes concentrations d'exposition. La réponse des *Vibrio* à la marennine est apparue complexe et imprévisible, étant dépendante des souches étudiées ainsi que des concentrations testées. Bien qu'ayant exercé un effet sur la croissance de la majorité des souches testées, la BW n'a pas présenté d'effets antibactériens puissants. En effet, l'inhibition de la croissance relative des souches se situait aux alentours de 10 à 30 %, à l'exception de la souche *V. tasmaniensis* #112 dont la

croissance a été totalement inhibée. De plus, pour considérer une application de la marennine en aquaculture pour limiter les infections microbiennes, il faudrait au préalable s'assurer de l'innocuité du pigment, voire de ses effets bénéfiques, sur les animaux exposés.

Enfin, très peu de données concernant les effets de la marennine *in vivo* sur des animaux marins sont disponibles dans la littérature. Turcotte et al. (2016) ont montré les effets prophylactiques de la BW à faible doses (0,5 µg mL^{-1}) chez les larves de la moule *Mytilus edulis* et du pétoncle *Placopecten magellanicus* exposées durant 3 jours à une souche virulente de *V. splendidus* (Turcotte et al., 2016). La même étude a également montré un meilleur taux de survie des larves exposées à 0,1 µg mL^{-1} de BW comparativement au contrôle sur une période de 20 jours. Cependant, une exposition à 1 µg mL^{-1} de BW a conduit à la mortalité de la totalité des larves, démontrant les effets ou bien bénéfiques (≤ 0,5 µg mL^{-1}), ou bien nocifs (≥ 1µg mL^{-1}) de la marennine en fonction de la dose considérée. Par la suite, Prasetiya et al. (2017) ont démontré qu'une brève exposition à 2 µg mL^{-1} de EMn (forme du pigment externalisée) sur des juvéniles de moule *M. edulis* et d'huître *Crassostrea virginica* induisait sur le long terme (8 semaines) des modifications comportementales (diminution de la fréquence d'ouverture des valves) ou encore physiologiques (diminution de la croissance).

Ainsi, le **Chapitre IV** explore les effets de la marennine sur divers organismes marins, d'intérêt en aquaculture ou représentant des modèles écotoxicologiques pertinents. Les essais conduits sur les oursins *Paracentrotus lividus* et *Sphaerechinus granularis* ont montré des effets de la marennine concentrée (CS) sur le taux de fécondation à partir de 10 µg mL^{-1} et également des effets sur le développement embryonnaire chez *S. granularis* exposé à des concentrations ≥ 1 µg mL^{-1}. Des concentrations de BW ≥ 1 µg mL^{-1} ont également induit une forte mortalité et un retard de développement des embryons de la moule *M. edulis* ainsi qu'une diminution de la motilité des larves. De même, la sensibilité des larves de moule à la BW est apparue dépendante de leur stade de développement avec les larves véligères (larves âgées de 17 jours) significativement plus sensibles que les larves pédivéligères (23 j), elles-mêmes plus sensibles que les larves métamorphosées (30 j). Aucun effet nocif de la BW n'a cependant été observé sur les embryons et larves de moule

aux concentrations 0,1 et 0,5 µg mL^{-1}. Ces faibles concentrations n'ont également pas eu d'impact sur la survie des larves du pétoncle *Chthamalus bisinuatus* ni sur le développement des larves de la plie *Pseudopleuronectes americanus*. L'état physiologique des larves de plie exposées à 1 µg mL^{-1} de BW a même été amélioré (augmentation des réserves énergétiques), rejoignant les résultats obtenus par Turcotte et al. (2016) sur les larves de moule. La marennine purifiée (EMn) à 1 µg mL^{-1} n'a pas non plus eu d'effet sur les hémocytes de l'huître adulte *Crassostrea gigas* ni sur la viabilité de lignées cellulaires branchiales de poissons jusqu'à des concentrations ≥ 50 µg mL^{-1}. Les résultats montrent donc que la marennine peut, selon la dose considérée, présenter des effets nocifs sur les stades de développement précoces et fragiles (embryons, larves) alors que les stades adultes exposés aux mêmes concentrations ou à des concentrations supérieures ne sembleraient pas affectés.

Pour une application de la marennine en aquaculture, les doses de pigment à délivrer devront donc être estimées en fonction des organismes exposés et également en fonction de leur stade de développement. La maîtrise des doses administrées ne peut pas s'envisager par ajout direct de cultures d'*Haslea* bleues dans les bassins d'élevages mais plutôt par l'ajout de marennine sous forme de solutions, de gélules ou de granules de concentrations connues. Le développement d'additifs alimentaires à base de marennine destinés aux élevages aquacoles pourrait être envisagé à échelle industrielle grâce au procédé de récolte du pigment développé au cours de ce doctorat (brevet n°1 872 316), qui présente un meilleur rendement et un faible coût comparativement à ceux précédemment développés (Pouvreau, 2006 ; Turcotte et al., 2016). Il a été montré dans le Chapitre IV de ce manuscrit que la marennine concentrée (CS) obtenue avec ce procédé conserve ses activités biologiques avec des effets comparables à ceux de la BW ou de la EMn. Cependant, les effets prophylactiques de la marennine concentrée (CS) resteraient à démontrer.

3 Valorisation de la marennine en aquaculture : limites actuelles et perspectives de recherche

La Figure 5.1 propose une vue d'ensemble des bénéfices et contraintes de l'utilisation de la marennine en aquaculture avec pour exemple les interactions entre le pigment bleu, des bactéries pathogènes et des bivalves. Des pistes de réflexion sur les modes d'action de la marennine ainsi que des perspectives de recherche en vue d'une valorisation potentielle du pigment dans le secteur de l'aquaculture sont discutées ci-après :

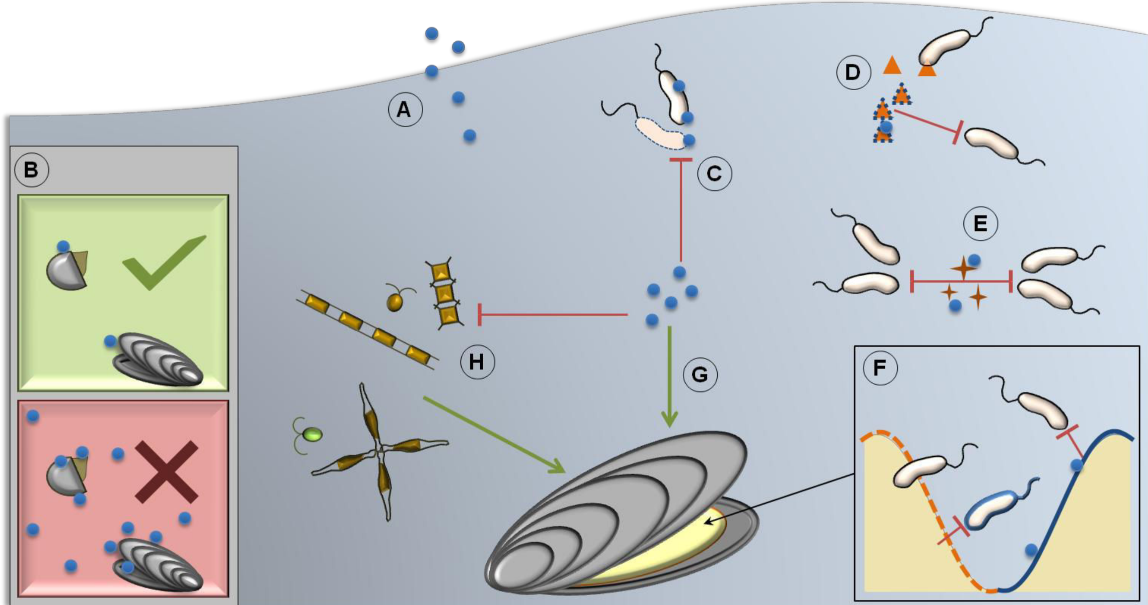


Figure 5.1. Exemple d'utilisation de la marennine pour lutter contre une infection bactérienne dans un bassin d'élevage de bivalves. **(A)** Pour une application de la marennine en aquaculture les doses administrées devront être maîtrisées, comme par exemple en délivrant le pigment sous forme de granulés afin d'éviter tout effet nocif sur les animaux élevés et notamment **(B)** sur les jeunes stades de développement particulièrement sensibles. La marennine pourrait interférer sur la croissance des bactéries : **(C)** rôle direct sur les membranes bactériennes ; **(D)** rôle indirect en formant des complexes avec des nutriments du milieu ; **(E)** rôle indirect en perturbant la communication (quorum sensing) des bactéries. **(F)** La marennine pourrait diminuer le pouvoir infectieux des bactéries et **(G)** exercer un effet prophylactique sur les animaux élevés. **(H)** Le pigment possédant des activités allélopathiques, les microalgues utilisées pour nourrir les animaux dans les bassins devront être choisies en fonction de leur sensibilité à la marennine. Schéma C. Falaise©.

Afin de maîtriser les concentrations administrées dans les bassins d'élevages, les doses de marennine pourraient être délivrées par granulés (Figure 5.1A) et devraient être ajustées en fonction des animaux élevés et de leur stade de développement. Les stades de développement précoces (larves, juvéniles), étant particulièrement sensibles, ne devraient pas être exposés à des concentrations ≥ 0,5 µg mL^{-1} pour éviter tout effet adverse sur leur survie et leur développement (Figure 5.1B).

La marennine pourrait exercer un effet direct sur la croissance des bactéries et pourrait ainsi limiter la prolifération bactérienne (Figure 5.1C). Les mécanismes d'actions exactes du pigment sur les bactéries ne sont pas encore déterminés mais, étant donné le poids moléculaire estimé de la marennine extracellulaire (environ 10 kDa ; Pouvreau et al., 2006), il paraît plus vraisemblable que la molécule ne pénètre pas les cellules mais agisse plutôt au niveau de la surface membranaire des bactéries. Des travaux conduits avec le pigment de type marennine purifié produit par *Haslea provincialis* ont d'ailleurs permis de mettre en évidence une interaction du pigment avec la membrane externe de la bactérie Gram (-) *Escherichia coli* (Tardy-Laporte et al., 2013). Récemment, une étude conduite avec de la marennine purifiée et une souche de *V. splendidus* a démontré un effet du pigment sur la fluidité de la membrane bactérienne. Il semblerait ainsi que la marennine induise une rigidification des membranes de la souche de *V. splendidus* testée en interagissant avec des lipopolysaccharides présents sur la membrane externe (Bouhlel et al., article soumis). La marennine agirait donc au niveau de la surface membranaire des *Vibrio*, pourrait se lier spécifiquement à certains récepteurs et déclencher des réponses de stress ou des signaux apoptotiques en induisant des modifications du métabolisme cellulaire. La marennine pourrait donc cibler des récepteurs membranaires particuliers, dont la présence et la fréquence pourraient varier entre les différentes espèces de *Vibrio* et également entre souches d'une même espèce (Biosca et al., 1993 ; Wang et al., 2015a ; Zhu et al., 2013), ce qui pourrait expliquer la variabilité de réponses des souches de *Vibrio* exposées à la marennine observée dans le Chapitre III de ce manuscrit.

La marennine pourrait également avoir un effet indirect sur la croissance des bactéries en limitant leur accès à certains nutriments (Figure 5.1D). Bien que sa structure

chimique ne soit pas encore déterminée, il semblerait que la marennine soit une molécule complexe composée de plusieurs groupements phénols (vraisemblablement des anthocyanes ; responsables de l'activité biologique), et d'un squelette glucidique (L. Zebiri, communication personnelle). Il a été démontré que les polyphénols et anthocyanes peuvent se lier et former des complexes avec des peptides, des sucres ou encore des métaux (Cai et al., 1989 ; Charlton et al., 2002 ; Dunbar, 2002 ; Fernandes et al., 2014) pouvant ainsi rendre indisponibles certains nutriments du milieu aux bactéries. Jakobek L. (2015) a également montré que l'interaction des polyphénols avec des sucres, des lipides ou des protéines conduisait à la formation de complexes modifiant l'activité biologique des phénols. Les interactions de la marennine avec les nutriments du milieu de culture pourraient donc être étudiées afin d'estimer si elles limitent la disponibilité des nutriments pour les bactéries et également d'évaluer si elles induisent une modification des activités biologiques du pigment. On peut également se poser la question de savoir si certaines bactéries sont capables d'assimiler les sucres du squelette carboné de la marennine, modifiant ainsi sa structure chimique et ses propriétés biologiques, ce qui pourrait expliquer, par ailleurs, certains cas de stimulation de croissance observés au Chapitre III.

Un autre mode d'action potentiel de la marennine qui demeure inexploré est l'effet sur le quorum sensing (QS) bactérien (Figure 5.1E). La perturbation du QS (*i.e.* système de communication qui permet aux bactéries de réguler un large éventail d'activités physiologiques ; Miller et Bassler, 2001), est une stratégie anti-infectieuse étudiée dans le domaine de l'aquaculture (Pérez-Sánchez et al., 2018 ; Tinh et al., 2008 ; Zorriehzahra et al., 2016). Diverses molécules, telles que les polyphénols, peuvent perturber le QS bactérien et limiter la prolifération bactérienne (Huber et al., 2014 ; Shukla et Bhathena, 2016 ; Zhu et al., 2011). Dans le secteur de l'aquaculture, la furanone extraite d'une algue rouge a démontré des résultats prometteurs en agissant comme antagoniste du QS de vibrions pathogènes, réduisant ainsi les mortalités de crevettes et de truites (Defoirdt et al., 2006 ; Rasch et al., 2004). De ce fait, il se pourrait que la marennine interagisse avec des molécules de communication bactérienne ou bien perturbe la réception du signal par les bactéries en bloquant par exemple des récepteurs membranaires.

Une hypothèse sur les effets biologiques de la marennine actuellement mise à l'essai est la capacité du pigment à diminuer le pouvoir infectieux des bactéries (Figure 5.1F). Pour provoquer une maladie, un organisme pathogène doit être capable de coloniser les surfaces de l'hôte et de pénétrer dans ses tissus (Casadevall et Pirofski, 1999). La marennine fixée sur les membranes bactériennes ou sur les tissus (*e.g.* branchies) des bivalves pourrait ainsi représenter une barrière aux infections bactériennes. Des tests ont récemment été mis en place afin d'évaluer si des huîtres aux branchies préalablement verdies seraient moins sensibles aux infections bactériennes que les animaux aux branchies non verdies.

Ainsi, les effets prophylactiques de la marennine représentent sans doute la facette la plus prometteuse du pigment pour une valorisation en aquaculture (Figure 5.1G). La BW à faible concentration (0,5 µg mL^{-1}) permet une amélioration de l'état physiologique (*i.e.* augmentation des réserves énergétiques) des larves de moule et de pétoncle (Turcotte et al., 2016) et de la plie (Chapitre IV. de ce manuscrit). Cette faible concentration de BW n'a pas eu d'effet sur la concentration bactérienne dans les bassins d'élevage mais a procuré une meilleure résistance des larves de moule et de pétoncle face à une souche pathogène de *V. splendidus* (Turcotte et al., 2016). Le même protocole expérimental a récemment permis de démontrer l'effet protecteur de la marennine sur les larves de moules en modifiant la composition de leur microbiote intestinal (Latour et al., 2018). Il semblerait donc intéressant d'étudier davantage les effets prophylactiques et immunostimulants de le marennine. Les bivalves, et les invertébrés de façon générale, ne possèdent pas de système immunitaire *stricto sensu* mais possèdent une mémoire immunitaire et peuvent mettre en place des réponses cellulaires (hémocytaires) et humorales pour faire face à des microorganismes envahisseurs (*e.g.* limitation de l'entrée du microbe, résistance ou tolérance à la maladie ; Rowley et Powell, 2007). En plus d'améliorer l'état physiologique des animaux (Turcotte et al., 2016) et de prévenir les infections en modifiant la composition du microbiote intestinal (Latour et al., 2018), la marennine pourrait également stimuler la mise en place de processus tels que la libération de peptides antimicrobiens ou autres

mécanismes cellulaires de défense (*e.g.* phagocytose, encapsulation) permettant à l'animal de se protéger des agents infectieux (Smith, 2016 ; Soudant et al., 2013).

Enfin, les activités allélopatiques de la marennine devront également être prises en considération pour une application en aquaculture (Figure 5.1H). En effet, il a été montré que le pigment réduisait la croissance de microalgues fréquemment utilisées pour nourrir les bivalves telles que *Skeletonema costatum* ou *Chaetoceros calcitrans* (Prasetiya et al., 2016), et donc la co-culture de ces espèces avec une *Haslea* bleue, ou en présence de marennine en solution dans le milieu, devrait être évitée. En revanche, d'autres espèces comme *Phaeodactylum tricornutum*, *Tetraselmis suecica* ou *Isochrysis galbana* (Prasetiya et al., 2016 ; Turcotte et al., 2016) semblent insensibles à la présence de marennine et devraient donc être privilégiées.

En conclusion principale de ce travail de thèse, nous pouvons affirmer que la marennine présente des effets biologiques significatifs qui encouragent son utilisation dans le secteur de l'aquaculture. Administré à faibles doses, le pigment ne permettrait pas de réduire la prolifération des bactéries dans les bassins d'élevage mais pourrait conférer aux animaux exposés une meilleure résistance aux agents infectieux. Cependant, les doses d'exposition devront être maîtrisées pour ne pas nuire au développement ou à la survie des animaux, et particulièrement ceux des stades larvaires ou juvéniles. La définition de la structure chimique de la molécule permettra d'explorer davantage les mécanismes d'action du pigment et également d'étendre les secteurs de valorisation potentiels de la marennine.

RÉFÉRENCES BIBLIOGRAPHIQUES

- A -

Abedin, R., and Taha, H. (2008). Antibacterial and antifungal activity of cyanobacteria and green microalgae. Evaluation of medium components by Plackett-Burman design for antimicrobial activity of *Spirulina platensis*. Glob. J. Biotechnol. Biochem. *3*, 22–31.

Accoceberry, I., and Noël, T. (2006). Antifongiques : cibles cellulaires et mécanismes de résistance. Thérapie *61*, 195–199.

Ackermann, M. (2015). A functional perspective on phenotypic heterogeneity in microorganisms. Nat. Rev. Microbiol. *13*, 497–508.

Adolph, S., Bach, S., Blondel, M., Cueff, A., Moreau, M., Pohnert, G., Poulet, S.A., Wichard, T., and Zuccaro, A. (2004). Cytotoxicity of diatom-derived oxylipins in organisms belonging to different phyla. J. Exp. Biol. *207*, 2935–2946.

Aguirre-Guzmán, G., Mejia Ruíz, H., and Ascencio, F. (2004). A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. Aquac. Res. *35*, 1395–1404.

Ahmad, A.L., Yasin, N.H.M., Derek, C.J.C., and Lim, J.K. (2011). Microalgae as a sustainable energy source for biodiesel production: A review. Renew. Sustain. Energy Rev. *15*, 584–593.

Ahmadi, A., Zorofchian Moghadamtousi, S., Abubakar, S., and Zandi, K. (2015). Antiviral potential of algae polysaccharides isolated from marine sources: A review,. BioMed Res. Int. *2015*.

Ahne, W. (1994). Viral infections of aquatic animals with special reference to Asian aquaculture. Annu. Rev. Fish Dis. *4*, 375–388.

Alangaden, G.J. (2011). Nosocomial fungal infections: epidemiology, infection control, and prevention. Infect. Dis. Clin. North Am. *25*, 201–225.

AlgaeBase (2018). World-wide electronic publication, National University of Ireland, Galway.

Allard, W.G., Belt, S.T., Massé, G., Naumann, R., Robert, J.M., and Rowland, S. (2001). Tetra-unsaturated sesterterpenoids (Haslenes) from *Haslea ostrearia* and related species. Phytochemistry *56*, 795–800.

Amaro, H.M., Guedes, A.C., and Malcata, F.X. (2011). Science against microbial pathogens: communicating current research and technological advances (A. Mendez-Vilas).

Amin, S.A., Parker, M.S., and Armbrust, E.V. (2012). Interactions between diatoms and bacteria. Microbiol. Mol. Biol. Rev. *76*, 667–684.

Anaissie, E., and Bodey, G.P. (1989). Nosocomial fungal infections. Old problems and new challenges. Infect. Dis. Clin. North Am. *3*, 867–882.

Anater, A., Manyes, L., Meca, G., Ferrer, E., Luciano, F.B., Pimpão, C.T., and Font, G. (2016). Mycotoxins and their consequences in aquaculture: A review. Aquaculture *451*, 1–10.

Arif, J.M., Farooqui, A., Siddiqui, M.H., Al-Karrawi, M., Al-Hazmi, A., and Al-Sagair, O.A. (2012). Chapter 5. Novel bioactive peptides from cyanobacteria: Functional, biochemical, and biomedical significance. In Studies in Natural Products Chemistry, Atta-ur-Rahman, ed. (Elsevier), pp. 111–161.

Armbrust, E.V. (2009). The life of diatoms in the world's oceans. Nature *459*, 185–192.

Arzul, I., and Renault, T. (2002). Herpèsvirus et bivalves marins. Virologie *6*, 169–174.

Arzul, I., Renault, T., Lipart, C., and Davison, A.J. (2001). Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. J. Gen. Virol. *82*, 865–870.

Auffret, M., and Oubella, R. (1997). Hemocyte Aggregation in the Oyster *Crassostrea gigas*: *In Vitro* Measurement and Experimental Modulation by Xenobiotics. Comp. Biochem. Physiol. -- Part Physiol. *3*, 705–712.

Austin, B. (2012). Infectious Disease in Aquaculture: Prevention and Control (Elsevier).

Austin, B., and Austin, D.A. (2007). Bacterial fish pathogens: disease of farmed and wild fish (Springer Science & Business Media).

Austin, B., and Day, J.G. (1990). Inhibition of prawn pathogenic *Vibrio spp.* by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture *90*, 389–392.

Austin, B., Baudet, E., and Stobie, M. (1992). Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. J. Fish Dis. *15*, 55–61.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., and Regoli, F. (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut. *198*, 211–222.

- B -

Bachrach, E., and Simonet, M. (1936). Le phénomène du bleuissement chez les diatomées. Rev. Trav. Inst. Pêch. Marit. *9*, 113–116.

Bag, J. (1974). Glucose inhibition of the transport and phosphoenolpyruvate-dependent phosphorylation of galactose and fructose in *Vibrio cholerae*. J. Bacteriol. *118*, 764–767.

Balasubramanian, G., Sarathi, M., Venkatesan, C., Thomas, J., and Hameed, A.S.S. (2008). Studies on the immunomodulatory effect of extract of *Cyanodon dactylon* in shrimp, *Penaeus monodon*, and its efficacy to protect the shrimp from white spot syndrome virus (WSSV). Fish Shellfish Immunol. *25*, 820–828.

Ban, S., Burns, C., Castel, J., Chaudron, Y., Christou, E., Escribano, R., Umani, S.F., Gasparini, S., Ruiz, F.G., Hoffmeyer, M., et al. (1997). The paradox of diatom-copepod interactions. Mar. Ecol. Prog. Ser. *157*, 287–293.

Banerjee, G., and Ray, A.K. (2017). The advancement of probiotics research and its application in fish farming industries. Res. Vet. Sci. *115*, 66–77.

Banerjee, S., Kim, L.M., Shariff, M., Khatoon, H., and Yusoff, F.M. (2013). Antibacterial activity of neem (*Azadirachta indica*) leaves on *Vibrio spp.* isolated from cultured shrimp. Asian J. Anim. Vet. Adv. *8*, 355–361.

Barra, L., Chandrasekaran, R., Corato, F., and Brunet, C. (2014). The challenge of ecophysiological biodiversity for biotechnological applications of marine microalgae. Mar. Drugs *12*, 1641–1675.

Bassim, S., Tanguy, A., Genard, B., Moraga, D., and Tremblay, R. (2014). Identification of *Mytilus edulis* genetic regulators during early development. Gene *551*, 65–78.

Bavestrello, G., Arillo, A., Calcinai, B., Cattaneo-Vietti, R., Cerrano, C., Gaino, E., Penna, A., and Sarà, M. (2000). Parasitic diatoms inside antarctic sponges. Biol. Bull. *198*, 29–33.

Bellou, S., Triantaphyllidou, I.-E., Aggeli, D., Elazzazy, A.M., Baeshen, M.N., and Aggelis, G. (2016). Microbial oils as food additives: recent approaches for improving microbial oil production and its polyunsaturated fatty acid content. Curr. Opin. Biotechnol. *37*, 24–35.

Belz, R.G., and Duke, S.O. (2014). Herbicides and plant hormesis. Pest Manag. Sci. *70*, 698–707.

Bennett, A.G. (1920). On the occurrence of diatoms on the skin of whales. Proc R Soc Lond B *91*, 352–357.

Benoiston, A.-S., Ibarbalz, F.M., Bittner, L., Guidi, L., Jahn, O., Dutkiewicz, S., and Bowler, C. (2017). The evolution of diatoms and their biogeochemical functions. Philos. Trans. R. Soc. B Biol. Sci. *372*.

Bergé, J.-P., Bourgougnon, N., Alban, S., Pojer, F., Billaudel, S., Chermann, J.-C., Robert, J.M., and Franz, G. (1999). Antiviral and anticoagulant activities of a water-soluble fraction of the marine diatom *Haslea ostrearia*. Planta Med. *65*, 604–609.

Berland, B.R., Bonin, D.J., Cornu, A.L., Maestrini, S.Y., and Marino, J.-P. (1972). The antibacterial substances of the marine alga *Stichochrysis immobilis* (chrysophyta). J. Phycol. *8*, 383–392.

Bhagavathy, S., Sumathi, P., and Jancy Sherene Bell, I. (2011). Green algae *Chlorococcum humicola*-a new source of bioactive compounds with antimicrobial activity. Asian Pac. J. Trop. Biomed. *1*, S1–S7.

Bhatnagar, I., and Kim, S.-K. (2010). Immense essence of excellence: marine microbial bioactive compounds. Mar. Drugs *8*, 2673–2701.

Bhattacharya, D., Ghosh, D., Bhattacharya, S., Sarkar, S., Karmakar, P., Koley, H., and Gachhui, R. (2018). Antibacterial activity of polyphenolic fraction of Kombucha against *Vibrio cholerae*: targeting cell membrane. Lett. Appl. Microbiol. *66*, 145–152.

Biosca, E.G., Garay, E., Toranzo, A.E., and Amaro, C. (1993). Comparison of outer membrane protein profiles of *Vibrio vulnificus* biotypes 1 and 2. FEMS Microbiol. Lett. *107*, 217–222.

Bocat, L. (1907). Sur la marennine de la diatomée bleue; comparaison avec la phycocyanine. Comptes Rendus Seances Société Biol. Ses Fil. *62*, 1073–1075.

Boon, D.D. (1977). Coloration in bivalves: A review. J Food Sci *42*, 1008–1012.

Borowitzka, M.A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. J. Appl. Phycol. *7*, 3–15.

Borowitzka, M.A. (1997). Microalgae for aquaculture: Opportunities and constraints. J. Appl. Phycol. *9*, 393–401.

Bory de Saint-Vincent, J.B. (1826). Dictionnaire Classique d'Histoire Naturelle (Paris, France: Rey et Gravier).

Bosc, G., and Lair, P.A. (1817). Huître. In Nouveau Dictionnaire d'Histoire Naturelle Appliquée Aux Arts, à l'agriculture, à l'économie Rurale et Domestique, à La Médecine, Etc. Tome XV, (Paris, France: Deterville), p.

Bouhlal Rhimou, R.H. (2010). Antiviral activity of the extracts of Rhodophyceae from Morocco. Afr. J. Biotechnol. *9*, 7968–7975.

Bouhlel, Z., Deschênes, J.-S., Mouget, J.-L., Tremblay, R., and Marcotte, I. (article soumis). Insight into the interaction mechanism of marennine with *Vibrio splendidus* as seen by *in vivo* 2H solid-state NMR.

Bouhlel, Z., Turcotte, F., Warschawski, D.E., Arnold, A., Lemarchand, K., Deschênes, J.-S., Marcotte, I., and Tremblay, R. (2018). Marennine, a potential alternative antibiotic to polymyxine in aquaculture. (Québec (Canada)), p.

Bower, S.M., Sharon E. McGladdery, and Iola M. Price (1994). Synopsis of infectious diseases and parasites of commercially exploited shellfish. Annu. Rev. Fish Dis. *4*, 1–199.

Boyce, R.W., and Herdman, W.A. (1896). The possible infectivity of the oyster, and upon the green disease in oysters. Comm Br Assoc Adv Sci *66*, 663–669.

Brauch, J.E., Zapata-Porras, S.P., Buchweitz, M., Aschoff, J.K., and Carle, R. (2016). Jagua blue derived from *Genipa americana* L. fruit: A natural alternative to commonly used blue food colorants? Food Res. Int.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., and Dunstan, G.A. (1997). Nutritional properties of microalgae for mariculture. Aquaculture *151*, 315–331.

Bruce, D.L., Duff, D.C., and Antia, N.J. (1967). The identification of two antibacterial products of the marine planktonic alga *Isochrysis galbana*. J. Gen. Microbiol. *48*, 293–298.

Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivesse, S., Alunno-Bruscia, M., Polz, M.F., and Le Roux, F. (2017). *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. ISME J. *11*, 1043–1052.

Buchweitz, M. (2016). Chapter 17. Natural Solutions for Blue Colors in Food. In Handbook on Natural Pigments in Food and Beverages, (Woodhead Publishing), pp. 355–384.

Burja, A.M., Banaigs, B., Abou-Mansour, E., Grant Burgess, J., and Wright, P.C. (2001). Marine cyanobacteria—a prolific source of natural products. Tetrahedron *57*, 9347–9377.

Buttino, I., Miralto, A., Ianora, A., Romano, G., and Poulet, S.A. (1999). Water-soluble extracts of the diatom *Thalassiosira rotula* induce aberrations in embryonic tubulin organisation of the sea urchin *Paracentrotus lividus*. Mar. Biol. *134*, 147–154.

Buttino, I., Hwang, J.-S., Romano, G., Sun, C.-K., Liu, T.-M., Pellegrini, D., Gaion, A., and Sartori, D. (2016). Detection of malformations in sea urchin plutei exposed to mercuric chloride using different fluorescent techniques. Ecotoxicol. Environ. Saf. *123*, 72–80.

- C -

Cadoret, J.-P., Garnier, M., and Saint-Jean, B. (2012). Microalgae, functional genomics and biotechnology. Adv. Bot. Res. *64*, 285–341.

Cahu, C.L., Zambonino Infante, J.L., Péres, A., Quazuguel, P., and Le Gall, M.M. (1998). Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: effect on digestive enzymes. Aquaculture *161*, 479–489.

Cai, Y., Gaffney, S.H., Lilley, T.H., and Haslam, E. (1989). Carbohydrate - Polyphenol Complexation. In Chemistry and Significance of Condensed Tannins, R.W. Hemingway, J.J. Karchesy, and S.J. Branham, eds. (Boston, MA: Springer US), pp. 307–322.

Calabrese, E.J. (2013). Hormetic mechanisms. Crit. Rev. Toxicol. *43*, 580–606.

Calabrese, E.J., and Baldwin, L.A. (1997). A quantitatively-based methodology for the evaluation of chemical hormesis. Hum. Ecol. Risk Assess. Int. J. *3*.

Calabrese, E.J., and Baldwin, L.A. (2001). Hormesis: A Generalizable and Unifying Hypothesis. Crit. Rev. Toxicol. *31*, 353–424.

Calabrese, E.J., and Baldwin, L.A. (2002). Defining hormesis. Hum. Exp. Toxicol. *21*, 91–97.

Caldwell, G.S. (2009). The influence of bioactive oxylipins from marine diatoms on invertebrate reproduction and development. Mar. Drugs *7*, 367–400.

Caldwell, G.S., Olive, P.J.W., and Bentley, M.G. (2002). Inhibition of embryonic development and fertilization in broadcast spawning marine invertebrates by water soluble diatom extracts and the diatom toxin 2-trans,4-trans decadienal. Aquat. Toxicol. Amst. Neth. *60*, 123–137.

Cannell, R.J.P., Owsianka, A.M., and Walker, J.M. (1988). Results of a large-scale screening programme to detect antibacterial activity from freshwater algae. Br. Phycol. J. *23*, 41–44.

Carbonnelle, D., Pondaven, P., Morancais, M., Masse, G., Bosch, S., Jacquot, C., Briand, G., Robert, J., and Roussakis, C. (1998). Antitumor and antiproliferative effects of an aqueous extract from the marine diatom *Haslea ostrearia* (Simonsen) against solid tumors: lung carcinoma (NSCLC-N6), kidney carcinoma (E39) and melanoma (M96) cell lines. Anticancer Res. *19*, 621–624.

Casadevall, A., and Pirofski, L.A. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infect. Immun. *67*, 3703–3713.

Cavallo, R., Acquaviva, M., Stabili, L., Cecere, E., Petrocelli, A., and Narracci, M. (2013). Antibacterial activity of marine macroalgae against fish pathogenic *Vibrio* species. Open Life Sci. *8*, 646–653.

Cedergreen, N., Streibig, J.C., Kudsk, P., Mathiassen, S.K., and Duke, S.O. (2006). The occurrence of hormesis in plants and algae. Dose-Response Publ. Int. Hormesis Soc. *5*, 150–162.

Chang, T., Ohta, S., Ikegami, N., Miyata, H., Kashimoto, T., and Kondo, M. (1993). Antibiotic substances produced by a marine green alga, *Dunaliella primolecta*. Bioresour. Technol. *44*, 149–153.

Charlton, A.J., Baxter, N.J., Khan, M.L., Moir, A.J.G., Haslam, E., Davies, A.P., and Williamson, M.P. (2002). Polyphenol/peptide binding and precipitation. J. Agric. Food Chem. *50*, 1593–1601.

Chatterjee, T., Chatterjee, B.K., and Chakrabarti, P. (2017). Modelling of growth kinetics of Vibrio cholerae in presence of gold nanoparticles: effect of size and morphology. Sci. Rep. *7*, 9671.

Cheung, R.C.F., Wong, J.H., Pan, W.L., Chan, Y.S., Yin, C.M., Dan, X.L., Wang, H.X., Fang, E.F., Lam, S.K., Ngai, P.H.K., et al. (2014). Antifungal and antiviral products of marine organisms. Appl. Microbiol. Biotechnol. *98*, 3475–3494.

Citarasu, T. (2012). Natural antimicrobial compounds for use in aquaculture. In Infectious Disease in Aquaculture: Prevention and Control, (Brian Austin), pp. 419–457.

Clinical and Laboratory Standards Institute (CLSI) (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Document M07-A9. Approved Standard—Ninth Edition (Wayne, PA, USA).

Cooper, S., Battat, A., Marsot, P., and Sylvestre, M. (1983). Production of antibacterial activities by two Bacillariophyceae grown in dialysis culture. Can. J. Microbiol. *29*, 338–341.

Costa, M., Costa-Rodrigues, J., Fernandes, M.H., Barros, P., Vasconcelos, V., and Martins, R. (2012). Marine cyanobacteria compounds with anticancer properties: A review on the implication of apoptosis. Mar. Drugs *10*, 2181–2207.

Coyne, V.E. (2011). The importance of ATP in the immune system of molluscs. Invertebr. Surviv. J.

Crane, M., and Hyatt, A. (2011). Viruses of Fish: An Overview of Significant Pathogens. Viruses *3*, 2025–2046.

Croll, D.A., and Holmes, R.W. (1982). A note on the occurence of diatoms on the feathers of diving seabirds. The Auk *99*, 765–766.

- D -

Darwin, C. (1859). On the Origin of Species by Means of Natural Selection (London, England: J. Murray).

Das, B.K., Pradhan, J., Pattnaik, P., Samantaray, B.R., and Samal, S.K. (2005). Production of antibacterials from the freshwater alga *Euglena viridis* (Ehren). World J. Microbiol. Biotechnol. *21*, 45–50.

Davey, M.R., Anthony, P., Power, J.B., and Lowe, K.C. (2005). Plant protoplasts: status and biotechnological perspectives. Biotechnol. Adv. *23*, 131–171.

Davidovich, N.A., Mouget, J.-L., and Gaudin, P. (2009). Heterothallism in the pennate diatom *Haslea ostrearia* (Bacillariophyta). Eur. J. Phycol. *44*, 251–261.

Dawood, M.A.O., Koshio, S., and Esteban, M.Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: A review. Rev. Aquac. *10*, 950–974.

Defoirdt, T., Crab, R., Wood, T.K., Sorgeloos, P., Verstraete, W., and Bossier, P. (2006). Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates. Appl Env. Microbiol *72*, 6419–6423.

del Pilar Sanchez-Saavedra, M., Licea-Navarro, A., and Bernaldez-Sarabia, J. (2010). Evaluation of the antibacterial activity of different species of phytoplankton.

Desbois, A.P., Lebl, T., Yan, L., and Smith, V. (2008). Isolation and structure characterization of two antibacterial free fatty acids from marine diatom, *Phaeodactylum tricornutum*. Appl. Microbiol. Biotechnol. *81*, 755–764.

Desbois, A.P., Mearns-Spragg, A., and Smith, V.J. (2009). A fatty acid from the diatom *Phaeodactylum tricornutum* is antibacterial against diverse bacteria including multi-resistant *Staphylococcus aureus* (MRSA). Mar. Biotechnol. *11*, 45–52.

Desbois, A.P., Walton, M., and Smith, V.J. (2010). Differential antibacterial activities of fusiform and oval morphotypes of *Phaeodactylum tricornutum* (Bacillariophyceae). J. Mar. Biol. Assoc. U. K. *90*, 769–774.

Deschamps, P., and Moreira, D. (2012). Reevaluating the green contribution to diatom genomes. Genome Biol. Evol. *4*, 683–688.

Dondero, F., Negri, A., Boatti, L., Marsano, F., Mignone, F., and Viarengo, A. (2010). Transcriptomic and proteomic effects of a neonicotinoid insecticide mixture in the marine mussel (*Mytilus galloprovincialis*, Lam.). Sci. Total Environ. *408*, 3775–3786.

Dorantes-Aranda, J.J., Waite, T.D., Godrant, A., Rose, A.L., Tovar, C.D., Woods, G.M., and Hallegraeff, G.M. (2011). Novel application of a fish gill cell line assay to assess ichthyotoxicity of harmful marine microalgae. Harmful Algae *10*, 366–373.

Duff, D.C.B., Bruce, D.L., and Antia, N.J. (1966). The Antibacterial Activity of Marine Planktonic Algae. Can. J. Microbiol. *12*, 877–884.

Dunbar, R.C. (2002). Metal cation binding to phenol: DFT comparison of the competing sites. J. Phys. Chem. A *106*, 7328–7337.

Dyshlovoy, S.A., and Honecker, F. (2015). Marine compounds and cancer: where do we stand? Mar. Drugs *13*, 5657–5665.

- E -

Ehresmann, D.W., Deig, E.F., Hatch, M.T., DiSalvo, L.H., and Vedros, N.A. (1977). Antiviral Substances from California Marine Algae1. J. Phycol. *13*, 37–40.

Eissa, N., and Wang, H.-P. (2016). Transcriptional stress responses to environmental and husbandry stressors in aquaculture species. Rev. Aquac. *8*, 61–88.

El Amraoui, B., El Amraoui, M., Cohen, N., and Fassouane, A. (2014). Anti-*Candida* and anti-*Cryptococcus* antifungal produced by marine microorganisms. J. Mycol. Médicale J. Med. Mycol. *24*, 149–153.

Elston, R. (1997). Special topic review : bivalve mollusc viruses. World J Microbiol Biotechnol *13*, 393–403.

Encarnacao, T., Pais, A.A.C.C., Campos, M.G., and Burrows, H.D. (2015). Cyanobacteria and microalgae: A renewable source of bioactive compounds and other chemicals. Sci. Prog. *98(2)*, 145–168.

Eriksen, N.T. (2008). Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. Appl. Microbiol. Biotechnol. *80*, 1–14.

- F -

Fabregas, J., García, D., Fernandez-Alonso, M., Rocha, A.I., Gómez-Puertas, P., Escribano, J.M., Otero, A., and Coll, J.M. (1999). In vitro inhibition of the replication of haemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV) by extracts from marine microalgae. Antiviral Res. *44*, 67–73.

Falaise, C., Gastineau, R., Poulin, M., Leignel, V., Hardivillier, Y., Widowati, I., Syakti, A.D., Lemieux, C., Turmel, M., and Mouget, J.-L. (2016a). *Haslea nusantara sp.* nov., a novel species of blue diatom from Indonesia (24th International Diatom Symposium, Québec (Canada)).

Falaise, C., François, C., Travers, M.-A., Morga, B., Haure, J., Tremblay, R., Turcotte, F., Pasetto, P., Gastineau, R., Hardivillier, Y., et al. (2016b). Antimicrobial compounds from eukaryotic microalgae against human pathogens and diseases in aquaculture. Mar. Drugs *14*, 159.

Falaise, C., Dewi, I.C., Hellio, C., Bourgougnon, N., and Mouget, J.-L. (2018). Chapter 12. Anticancer, antiviral, antibacterial and antifungal properties in microalgae. In Levine I., Fleurence J. (Eds). Microalgae in Health and Disease Prevention, (Elsevier Science, Amsterdam, Netherlands), p.

Falasco, E., Bona, F., Badino, G., Hoffmann, L., and Ector, L. (2009). Diatom teratological forms and environmental alterations: a review. Hydrobiologia *623*, 1–35.

FAO (2018a). La situation mondiale des pêches et de l'aquaculture 2018. Atteindre les objectifs de développement durable (Rome: Licence: CC BY-NC-SA 3.0 IGO.).

FAO (2018b). FAO Aquaculture Newsletter (FAN) April 2018.

FAO/WHO (2001). Health and nutritional properties of probiotics in food including powder milk with liver lactic acid bacteria. (Food and Agriculture Organization and World Health Organization Joint report).

Farley, C. (1978). Viruses and virus like lesions in marine molluscs. Mar Fish Rev *40*, 18–20.

FDA (2016). http://www.fda.gov.

Fernandes, A., Brás, N.F., Mateus, N., and de Freitas, V. (2014). Understanding the molecular mechanism of anthocyanin binding to pectin. Langmuir *30*, 8516–8527.

Fernández-Alarcón, C., Miranda, C.D., Singer, R.S., López, Y., Rojas, R., Bello, H., Domínguez, M., and González-Rocha, G. (2010). Detection of the floR gene in a diversity of florfenicol resistant Gram-negative bacilli from freshwater salmon farms in Chile. Zoonoses Public Health *57*, 181–188.

Field, C.B., Behrenfeld, M.J., Randerson, J.T., and Falkowski, P. (1998). Primary production of the biosphere: integrating terrestrial and oceanic components. Science *281*, 237–240.

Findlay, J.A., and Patil, A.D. (1984). Antibacterial constituents of the diatom *Navicula delognei*. J. Nat. Prod. *47*, 815–818.

Folch, J., Lees, M., and Stanley, G.H.S. (1957). A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. J. Biol. Chem. *226*, 497–509.

Fraboulet, E., Lambert, Y., Tremblay, R., and Audet, C. (2010). Assessment of paternal effect and physiological cost of metamorphosis on growth of young winter flounder *Pseudopleuronectes americanus* juveniles in a cold environment. J. Fish Biol. *76*, 930–948.

Frick, D. (2003). The coloration of food. Rev. Prog. Color. Relat. Top. *33*, 15–32.

Fuentes, J.L., Garbayo, I., Cuaresma, M., Montero, Z., González-del-Valle, M., and Vílchez, C. (2016). Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. Mar. Drugs *14*.

- G -

Gagneux-Moreaux, S., Moreau, C., Gonzalez, J.-L., and Cosson, R.P. (2007). Diatom artificial medium (DAM): a new artificial medium for the diatom *Haslea ostrearia* and other marine microalgae. J. Appl. Phycol. *19*, 549–556.

Gaillon, B. (1820). Des huîtres vertes, et des causes de cette coloration. J. Phys. Chim. D'histoire Nat. Arts *91*, 222–225.

Galbraith, H., and Miller, T.B. (1973). Physicochemical effects of long chain fatty acids on bacterial cells and their protoplasts. J. Appl. Bacteriol. *36*, 647–658.

Gallager, S.M., Mann, R., and Sasaki, G.C. (1986). Lipid as an index of growth and viability in three species of bivalve larvae. Aquaculture *56*, 81–103.

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., and Nicolas, J.-L. (2007). Evidence for the involvement of pathogenic bacteria in summer mortalities of the pacific oyster *Crassostrea gigas*. Microb. Ecol. *53*, 187–196.

Garnier, M., Labreuche, Y., and Nicolas, J.-L. (2008). Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. Syst. Appl. Microbiol. *31*, 358–365.

Gastineau, R. (2011). Biodiversité, reproduction et phylogénie des diatomées bleues du genre *Haslea* et valorisation de leurs pigments de type marennine. Université du Maine.

Gastineau, R., Davidovich, N.A., Bardeau, J.-F., Caruso, A., Leignel, V., Hardivillier, Y., Jacquette, B., Davidovich, O.I., Rincé, Y., Gaudin, P., et al. (2012a). *Haslea karadagensis* (Bacillariophyta): a second blue diatom, recorded from the Black Sea and producing a novel blue pigment. Eur. J. Phycol. *47*, 469–479.

Gastineau, R., Pouvreau, J.-B., Hellio, C., Morançais, M., Fleurence, J., Gaudin, P., Bourgougnon, N., and Mouget, J.-L. (2012b). Biological activities of purified marennine, the blue pigment responsible for the greening of oysters. J. Agric. Food Chem. *60*, 3599–3605.

Gastineau, R., Hardivillier, Y., Leignel, V., Tekaya, N., Morançais, M., Fleurence, J., Davidovich, N., Jacquette, B., Gaudin, P., Hellio, C., et al. (2012c). Greening effect on oysters and biological activities of the blue pigments produced by the diatom *Haslea karadagensis* (Naviculaceae). Aquaculture *368–369*, 61–67.

Gastineau, R., Davidovich, N., Hansen, G., Rines, J., Wulff, A., Kaczmarska, I., Ehrman, J., Hermann, D., Maumus, F., Hardivillier, Y., et al. (2014a). Chapter 15. - *Haslea ostrearia*-like Diatoms: Biodiversity out of the Blue. In Advances in Botanical Research, N. Bourgougnon, ed. (Academic Press), pp. 441–465.

Gastineau, R., Turcotte, F., Pouvreau, J.-B., Morançais, M., Fleurence, J., Windarto, E., Prasetiya, F.S., Arsad, S., Jaouen, P., Babin, M., et al. (2014b). Marennine, promising blue pigments from a widespread *Haslea* diatom species complex. Mar. Drugs *12*, 3161–3189.

Gastineau, R., Hansen, G., Davidovich, N.A., Davidovich, O., Bardeau, J.-F., Kaczmarska, I., Ehrman, J.M., Leignel, V., Hardivillier, Y., Jacquette, B., et al. (2016). A new blue-pigmented hasleoid diatom, *Haslea provincialis*, from the Mediterranean Sea. Eur. J. Phycol. *51*, 156–170.

Gastineau, R., Prasetiya, F.S., Falaise, C., Cognie, B., Decottignies, P., Morancais, M., Méléder, V., Davidovich, N.A., Turcotte, F., Tremblay, R., et al. (2018). Chapter 16. Marennine-Like Pigments: Blue Diatom of Green Oyster Cult? In Blue Biotechnology. Production and Use of Marine Molecules., (Wiley-VCH, Weinheim, Germany: Stéphane La Barre and Stephen S. Bates), pp. 529–551.

Gaudin, P., Lebeau, T., and Robert, J.-M. (2006). Microalgal cell immobilization for the long-term storage of the marine diatom *Haslea ostrearia*. J. Appl. Phycol. *18*, 175–184.

Gauthier, M.J. (1969). Activité antibactérienne d'une Diatomée marine : *Asterionella notata* (Grun). Rev Intern Océan. Méd *25–26*, 103–165.

Genard, B., Moraga, D., Pernet, F., David, E., Boudry, P., and Tremblay, R. (2012). Expression of candidate genes related to metabolism, immunity and cellular stress during massive mortality in the American oyster *Crassostrea virginica* larvae in relation to biochemical and physiological parameters. Gene *499*, 70–75.

Geneves, L., Choussy, M., Barbier, M., Neuville, D., and Daste, P. (1976). Ultrastructure et composition pigmentaire comparées des chromatophores de la diatomee *Navicula ostrearia* (Gaillon) Bory normale et bleue. Comptes Rendus Hebd. Seances Ser. Sci. Nat.

Gerber, P., Dutcher, J.D., Adams, E.V., and Sherman, J.H. (1958). Protective Effect of Seaweed Extracts for Chicken Embryos Infected with Influenza B or Mumps Virus. Exp. Biol. Med. *99*, 590–593.

Ghannoum, M.A., and Rice, L.B. (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin. Microbiol. Rev. *12*, 501–517.

Ghasemi, Y., Moradian, A., Mohagheghzadeh, A., Shokravi, S., and Morowvat, M.H. (2007). Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: Characterization of antimicrobial activity of *Chroococcus dispersus*. J. Biol. Sci. *7 (6)*, 904–910.

Ghasemi, Y., Rasoul-Amini, S., Naseri, A.T., Montazeri-Najafabady, N., Mobasher, M.A., and Dabbagh, F. (2012). Microalgae biofuel potentials (Review). Appl. Biochem. Microbiol. *48*, 126–144.

Gomez-Casado, E., Estepa, A., and Coll, J.M. (2011). A comparative review on European-farmed finfish RNA viruses and their vaccines. Vaccine *29*, 2657–2671.

González-Davis, O., Ponce-Rivas, E., Sánchez-Saavedra, M.D.P., Muñoz-Márquez, M.-E., and Gerwick, W.H. (2012). Bioprospection of microalgae and cyanobacteria as biocontrol agents against *Vibrio campbellii* and their use in white shrimp *Litopenaeus vannamei* culture. J. World Aquac. Soc. *43*, 387–399.

Grelon, M. (1978). Saintonge, Pays des Huîtres Vertes (La Rochelle, France: Rupella).

Grimbergen, A.J., Siebring, J., Solopova, A., and Kuipers, O.P. (2015). Microbial bet-hedging: the power of being different. Curr. Opin. Microbiol. *25*, 67–72.

Grovel, O., Pouchus, Y.F., and Verbist, J.-F. (2003). Accumulation of gliotoxin, a cytotoxic mycotoxin from *Aspergillus fumigatus*, in blue mussel (*Mytilus edulis*). Toxicon Off. J. Int. Soc. Toxinology *42*, 297–300.

Gudimova, E., Eilertsen, H.C., Jørgensen, T.Ø., and Hansen, E. (2016). *In vivo* exposure to northern diatoms arrests sea urchin embryonic development. Toxicon *109*, 63–69.

Guedes, A.C., and Malcata, F.X. (2012). Nutritional value and uses of microalgae in aquaculture. Aquaculture *10*, 59–78.

Guedes, A.C., Barbosa, C.R., Amaro, H.M., Pereira, C.I., and Malcata, F.X. (2011). Microalgal and cyanobacterial cell extracts for use as natural antibacterial additives against food pathogens. Int. J. Food Sci. Technol. *46*, 862–870.

Gueho, E., Pesando, D., and Barelli, M. (1977). Propriétés antifongiques d'une diatomeé *Chaetoceros lauderi* ralfs C C. Mycopathologia *60*, 105–107.

Guiry, M.D. (2012). How many species of algae are there? J. Phycol. *48*, 1057–1063.

Gupta, V., Ratha, S.K., Sood, A., Chaudhary, V., and Prasanna, R. (2013). New insights into the biodiversity and applications of cyanobacteria (blue-green algae)—Prospects and challenges. Algal Res. *2*, 79–97.

- H -

Halim, R., Danquah, M.K., and Webley, P.A. (2012). Extraction of oil from microalgae for biodiesel production: A review. Biotechnol. Adv. *30*, 709–732.

Hallegraeff, G.M., Bolch, C.J.S., Hill, D.R.A., Jameson, I., LeRoy, J.-M., McMinn, A., Murray, S., de Salas, M.F., and Saunders, K. (2010). Algae of Australia: Phytoplankton of Temperate Coastal Waters (Melbourne: CSIRO Publishing).

Hamm, C.E., Merkel, R., Springer, O., Jurkojc, P., Maier, C., Prechtel, K., and Smetacek, V. (2003). Architecture and material properties of diatom shells provide effective mechanical protection. Nature *421*, 841–843.

Hanic, L.A., Sekimoto, S., and Bates, S.S. (2009). Oomycete and chytrid infections of the marine diatom *Pseudo-nitzschia pungens* (Bacillariophyceae) from Prince Edward Island, Canada. Botany *87*, 1096–1105.

Harrison, P.J., Waters, R.E., and Taylor, F.J.R. (1980). A broad spectrum artificial sea water medium for coastal and open ocean phytoplankton. J. Phycol. *16*, 28–35.

Hasui, M., Matsuda, M., Okutani, K., and Shigeta, S. (1995). *In vitro* antiviral activities of sulfated polysaccharides from a marine microalga (*Cochlodinium polykrikoides*) against human immunodeficiency virus and other enveloped viruses. Int. J. Biol. Macromol. *17*, 293–297.

Hayashi, T., Hayashi, K., Maeda, M., and Kojima, I. (1996). Calcium spirulan, an inhibitor of enveloped virus replication, from a blue-green alga *Spirulina platensis*. J. Nat. Prod. *59*, 83–87.

Hennebicq, R., Fabra, G., Pellerin, C., Marcotte, I., Myrand, B., and Tremblay, R. (2013). The effect of spawning of cultured mussels (*Mytilus edulis*) on mechanical properties, chemical and biochemical composition of byssal threads. Aquaculture *410–411*, 11–17.

Henriksson, P.J.G., Troell, M., and Rico, A. (2015). Antimicrobial use in aquaculture: Some complementing facts. Proc. Natl. Acad. Sci. *112*, E3317–E3317.

Herrero, M., Ibáñez, E., Cifuentes, A., Reglero, G., and Santoyo, S. (2006). *Dunaliella salina* microalga pressurized liquid extracts as potential antimicrobials. J. Food Prot. *69*, 2471–2477.

Herrero, M., Mendiola, J.A., Plaza, M., and Ibañez, E. (2013). Chapter 35. Screening for Bioactive Compounds from Algae. In Advanced Biofuels and Bioproducts, J.W. Lee, ed. (Springer New York), pp. 833–872.

Hjörleifsson, E., and Klein-MacPhee, G. (1992). Estimation of live standard length of winter flounder Pleuronectes americanus larvae from formalin-preserved, ethanol-preserved and frozen specimens. Mar. Ecol. Prog. Ser. *82*, 13–19.

Hoagland, K.D., Roemer, S.C., and Rosowski, J.R. (1982). Colonization and community structure of two periphyton assemblages, with emphasis on the diatoms (Bacillariophyceae). Am. J. Bot. *69*, 188–213.

Hoek, C., Hoeck, H.V. den, Mann, D., and Jahns, H.M. (1995). Algae: An Introduction to Phycology (Cambridge University Press).

Hong, J.-H., Jang, S., Heo, Y.M., Min, M., Lee, H., Lee, Y.M., Lee, H., and Kim, J.-J. (2015). Investigation of marine-derived fungal diversity and their exploitable biological activities. Mar. Drugs *13*, 4137–4155.

Houdai, T., Matsuoka, S., Matsumori, N., and Murata, M. (2004). Membrane-permeabilizing activities of amphidinol 3, polyene-polyhydroxy antifungal from a marine dinoflagellate. Biochim. Biophys. Acta BBA - Biomembr. *1667*, 91–100.

Huber, B., Eberl, L., Feucht, W., and Polster, J. (2014). Influence of polyphenols on bacterial biofilm formation and quorum-sensing. Z. Für Naturforschung C *58*, 879–884.

Huleihel, M., Ishanu, V., Tal, J., and Arad, S. (2001). Antiviral effect of red microalgal polysaccharides on Herpes simplex and Varicella zoster viruses. J. Appl. Phycol. *13*, 127–134.

Huleihel, M., Ishanu, V., Tal, J., and Arad, S. (2002). Activity of *Porphyridium sp.* polysaccharide against Herpes simplex viruses *in vitro* and *in vivo*. J. Biochem. Biophys. Methods *50*, 189–200.

Hussein, M.M.A., Wada, S., Hatai, K., and Yamamoto, A. (2000). Antimycotic activity of eugenol against selected water molds. J. Aquat. Anim. Health *12*, 224–229.

Hussein, M.M.A., El-Feki, M.A., Hatai, K., and Yamamoto, A. (2002). Inhibitory effects of thymoquinone from *Nigella sativa* on pathogenic *Saprolegnia* in fish. Biocontrol Sci. *7*, 31–35.

- I -

Ianora, A., and Miralto, A. (2010). Toxigenic effects of diatoms on grazers, phytoplankton and other microbes: a review. Ecotoxicol. Lond. Engl. *19*, 493–511.

Ingalls, A.E., Whitehead, K., and Bridoux, M.C. (2010). Tinted windows: The presence of the UV absorbing compounds called mycosporine-like amino acids embedded in the frustules of marine diatoms. Geochim. Cosmochim. Acta *74*, 104–115.

Ingebrigtsen, R.A., Hansen, E., Andersen, J.H., and Eilertsen, H.C. (2015). Light and temperature effects on bioactivity in diatoms. J. Appl. Phycol. *28*, 939–950.

- J -

Jahangiri, L., and Esteban, M.Á. (2018). Administration of probiotics in the water in finfish aquaculture systems: A review. Fishes *3*, 33.

Jakobek, L. (2015). Interactions of polyphenols with carbohydrates, lipids and proteins. Food Chem. *175*, 556–567.

Jha, R.K., and Zi-rong, X. (2004). Biomedical compounds from marine organisms. Mar. Drugs *2*, 123–146.

Johnson, K.N., van Hulten, M.C.W., and Barnes, A.C. (2008). “Vaccination” of shrimp against viral pathogens: Phenomenology and underlying mechanisms. Vaccine *26*, 4885–4892.

Jørgensen, E.G. (1962). Antibiotic substances from cells and culture solutions of unicellular algae with special reference to some chlorophyll derivatives. Physiol. Plant. *15*, 530–545.

Jorgensen, J.H., Hindler, J.F., Reller, L.B., and Weinstein, M.P. (2007). New Consensus Guidelines from the Clinical and Laboratory Standards Institute for Antimicrobial Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Clin. Infect. Dis. *44*, 280–286.

Jüttner, F. (2001). Liberation of 5,8,11,14,17-Eicosapentaenoic Acid and Other Polyunsaturated Fatty Acids from Lipids as a Grazer Defense Reaction in Epilithic Diatom Biofilms. J. Phycol. *37*, 744–755.

- K -

Kabara, J.J., Swieczkowski, D.M., Conley, A.J., and Truant, J.P. (1972). Fatty acids and derivatives as antimicrobial agents. Antimicrob. Agents Chemother. *2*, 23–28.

Kaczmarska, I., Poulíčková, A., Sato, S., Edlund, M.B., Idei, M., Watanabe, T., and Mann, D.G. (2013). Proposals for a terminology for diatom sexual reproduction, auxospores and resting stages. Diatom Res. *28*, 263–294.

Kang, H.K., Seo, C.H., and Park, Y. (2015). Marine peptides and their anti-infective activities. Mar. Drugs *13*, 618–654.

Karthikeyan, V., and Gopalakrishnan, A. (2014). A novel report of phytopathogenic fungi *Gilbertella persicaria* infection on *Penaeus monodon*. Aquaculture *430*, 224–229.

Karthikeyan, V., Selvakumar, P., and Gopalakrishnan, A. (2015). A novel report of fungal pathogen *Aspergillus awamori* causing black gill infection on *Litopenaeus vannamei* (pacific white shrimp). Aquaculture *444*, 36–40.

Kasten, P., and Flores, A.A.V. (2013). Disruption of endogenous tidal rhythms of larval release linked to food supply and heat stress in an intertidal barnacle. Mar. Ecol. Prog. Ser. *472*, 185–198.

Katharios, P., Papadakis, I.., Prapas, A., Dermon, C.., Ampatzis, K., and Divanach, P. (2005). Mortality control of viral encephalopathy and retinopathy in 0+ grouper *Epinephelus marginatus* after prolonged bath in dense *Chlorella minutissima* culture. Bull. Eur. Assoc. Fish Pathol. *25*, 28–31.

Katircioglu, H., Beyatli, Y., Aslim, B., Yüksekdag, Z., and Atici, T. (2005). Screening for antimicrobial agent production of some microalgae in freshwater. Internet J. Microbiol. *2*.

Kellam, S.J., and Walker, J.M. (1989). Antibacterial activity from marine microalgae in laboratory culture. Br. Phycol. J. *24*, 191–194.

Kellam, S.J., Cannell, R.J.P., Owsianka, A.M., and Walker, J.M. (1988). Results of a large-scale screening programme to detect antifungal activity from marine and freshwater microalgae in laboratory culture. Br. Phycol. J. *23*, 45–47.

Kendig, E.L., Le, H.H., and Belcher, S.M. (2010). Defining Hormesis: Evaluation of a Complex Concentration Response Phenomenon. Int. J. Toxicol. *29*, 235–246.

Khemis, I.B., Audet, C., Fournier, R., and Noüe, J.D.L. (2003). Early weaning of winter flounder (*Pseudopleuronectes americanus* Walbaum) larvae on a commercial microencapsulated diet. Aquac. Res. *34*, 445–452.

Kim, M., Yim, J.H., Kim, S.-Y., Kim, H.S., Lee, W.G., Kim, S.J., Kang, P.-S., and Lee, C.-K. (2012). *In vitro* inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03. Antiviral Res. *93*, 253–259.

Kitazato K, Wang Y, and Kobayashi N (2007). Viral infectious disease and natural products with antiviral activity. Drug Discov. Ther. *1*, 14–22.

Kociolek, J.P., and Hamsher, S.E. (2017). Chapter 12. Diatoms: By, with and as Endosymbionts. In Algal and Cyanobacteria Symbioses, (Martin Grube, Joseph Seckbach, Lucia Muggia), pp. 371–397.

Kociolek, J.P., Spaulding, S.A., and Lowe, R.L. (2015a). Chapter 16. Bacillariophyceae: The Raphid Diatoms. In Freshwater Algae of North America (Second Edition), J.D. Wehr, R.G. Sheath, and J.P. Kociolek, eds. (Boston: Academic Press), pp. 709–772.

Kociolek, J.P., Theriot, Edward C., Williams, David M., Julius, Matthew, Stoermer, Eugene F., and Kingston, J.C. (2015b). Chapter 15. Centric and Araphid Diatoms. In Freshwater Algae of North America (Second Edition), J.D. Wehr, R.G. Sheath, and J.P. Kociolek, eds. (Boston: Academic Press), pp. 653–708.

Kogure, K., Simidu, U., and Taga, N. (1979). Effect of *Skeletonema costatum* (Grev.) Cleve on the growth of marine bacteria. J. Exp. Mar. Biol. Ecol. *36*, 201–215.

Kokou, F., Makridis, P., Kentouri, M., and Divanach, P. (2012). Antibacterial activity in microalgae cultures. Aquac. Res. *43*, 1520–1527.

Kooistra, W.H., De Stefano, M., Mann, D.G., and Medlin, L.K. (2003). The phylogeny of the diatoms. Prog. Mol. Subcell. Biol. *33*, 59–97.

Kusumaningrum, H.P., and Zainuri, M. (2015). Detection of bacteria and fungi associated with *Penaeus monodon* postlarvae mortality. Procedia Environ. Sci. *23*, 329–337.

- L -

La Barre, S., and Bates, S.. (2018). Chapter 27. Marine Biominerals with a Biotechnological Future. In Blue Biotechnology. Production and Use of Marine Molecules, (Weinheim, Germany: Stéphane La Barre and Stephen S. Bates), pp. 855–912.

Lankester, E.R. (1886). On green oysters. Q J Microsc Sci *26*, 71–94.

Latour, J.C.-T., Bernier, S.-B., Doiron, K., Tremblay, R., and Lemarchand, K. (2018). Protective effect of marennine as probiotic against pathogen-challenge of *Mytilus edulis* larvae in aquaculture: role of the larvae microbiota (Oregon, USA).

Lauritano, C., Romano, G., Roncalli, V., Amoresano, A., Fontanarosa, C., Bastianini, M., Braga, F., Carotenuto, Y., and Ianora, A. (2016). New oxylipins produced at the end of a diatom bloom and their effects on copepod reproductive success and gene expression levels. Harmful Algae *55*, 221–229.

Lavilla-Pitogo, C.R., Albright, L.J., and Paner, M.G. (1998). Will microbial manipulation sustain the ecological balance in shrimp (*Penaeus monodon*) hatcheries? In In: Flegel, TW (Ed.). Advances in Shrimp Biotechnology. Proceedings to the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, 11-14 November 1998, Chiengmai, Thailand. Bangkok, Thailand: National Center for Genetic Engineering and Biotechnology. Pp. 185-192, (National Center for Genetic Engineering and Biotechnology), p.

Lee, J.J. (2011). Diatoms as Endosymbionts. In The Diatom World, J. Seckbach, and P. Kociolek, eds. (Dordrecht: Springer Netherlands), pp. 437–464.

Lee, J.-B., Hayashi, K., Hirata, M., Kuroda, E., Suzuki, E., Kubo, Y., and Hayashi, T. (2006). Antiviral sulfated polysaccharide from *Navicula directa*, a diatom collected from deep-sea water in Toyama bay. Biol. Pharm. Bull. *29*, 2135–2139.

Lefebvre, K.A., and Robertson, A. (2010). Domoic acid and human exposure risks: A review. Toxicon *56*, 218–230.

Lépinay, A., Capiaux, H., Turpin, V., Mondeguer, F., and Lebeau, T. (2016). Bacterial community structure of the marine diatom *Haslea ostrearia*. Algal Res. *16*, 418–426.

Li, S.-S., and Tsai, H.-J. (2009). Transgenic microalgae as a non-antibiotic bactericide producer to defend against bacterial pathogen infection in the fish digestive tract. Fish Shellfish Immunol. *26*, 316–325.

Lin, W., Li, L., Chen, J., Li, D., Hou, J., Guo, H., and Shen, J. (2018). Long-term crowding stress causes compromised nonspecific immunity and increases apoptosis of spleen in grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. *80*, 540–545.

Lio-Po, G.D., Leaño, E.M., Peñaranda, M.M.D., Villa-Franco, A.U., Sombito, C.D., and Guanzon Jr., N.G. (2005). Anti-luminous *Vibrio* factors associated with the 'green water' grow-out culture of the tiger shrimp *Penaeus monodon*. Aquaculture *250*, 1–7.

Liu, R., Chen, H., Zhang, R., Zhou, Z., Hou, Z., Gao, D., Zhang, H., Wang, L., and Song, L. (2016). Comparative transcriptome analysis of *Vibrio splendidus* JZ6 reveals the mechanism of its pathogenicity at low temperatures. Appl. Environ. Microbiol. *82*, 2050–2061.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif *25*, 402–408.

Lockwood, B.L., Sanders, J.G., and Somero, G.N. (2010). Transcriptomic responses to heat stress in invasive and native blue mussels (genus *Mytilus*): molecular correlates of invasive success. J. Exp. Biol. *213*, 3548–3558.

Loker, E.S., Adema, C.M., Zhang, S.-M., and Kepler, T.B. (2004). Invertebrate immune systems – not homogeneous, not simple, not well understood. Immunol. Rev. *198*, 10–24.

Lorenz, R.T., and Cysewski, G.R. (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends Biotechnol. *18*, 160–167.

Losic, D., Mitchell, J.G., and Voelcker, N.H. (2009). Diatomaceous Lessons in Nanotechnology and Advanced Materials. Adv. Mater. *21*, 2947–2958.

Luescher-Mattli, M. (2003). Algae, a possible source for new drugs in the treatment of HIV and other viral diseases. Curr. Med. Chem. - Anti-Infect. Agents *2*, 219–225.

Lushchak, V.I. (2014). Dissection of the Hormetic Curve: Analysis of Components and Mechanisms. Dose-Response *12*, 466–479.

Lustigman, B. (1988). Comparison of antibiotic production from four ecotypes of the marine alga, *Dunaliella*. Bull. Environ. Contam. Toxicol. *40*, 18–22.

- M -

Mackay, A.W., Jones, V.J., and Battarbee, R.W. (2003). Chapter 20. Approaches to Holocene Climate Reconstruction using Diatoms. In Global Change in the Holocene, (London), pp. 294–309.

Makridis, P., Costa, R.A., and Dinis, M.T. (2006). Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia metanauplii*. Aquaculture *255*, 76–81.

Mann, D.G., and Vanormelingen, P. (2013). An inordinate fondness? The number, distributions, and origins of diatom species. J. Eukaryot. Microbiol. *60*, 414–420.

Manuel, R.J., and Kibbler, C.C. (1998). The epidemiology and prevention of invasive aspergillosis. J. Hosp. Infect. *39*, 95–109.

Marques, A., Thanh, T.H., Sorgeloos, P., and Bossier, P. (2006). Use of microalgae and bacteria to enhance protection of gnotobiotic Artemia against different pathogens. Aquaculture *258*, 116–126.

Marrone, V., Piscopo, M., Romano, G., Ianora, A., Palumbo, A., and Costantini, M. (2012). Defensome against toxic diatom aldehydes in the sea urchin *Paracentrotus lividus*. PLOS ONE *7*, e31750.

Martins, B.M., and Locke, J.C. (2015). Microbial individuality: how single-cell heterogeneity enables population level strategies. Curr. Opin. Microbiol. *24*, 104–112.

Matallah-Boutiba, A., Ruiz, N., Sallenave-Namont, C., Grovel, O., Amiard, J.-C., Pouchus, Y.F., and Boutiba, Z. (2012). Screening for toxigenic marine-derived fungi in Algerian mussels and their immediate environment. Aquaculture *342–343*, 75–79.

Mayer, A.M.S., Rodríguez, A.D., Taglialatela-Scafati, O., and Fusetani, N. (2013). Marine Pharmacology in 2009–2011: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action. Mar. Drugs *11*, 2510–2573.

Medlin, L.K. (2011). A Review of the Evolution of the Diatoms from the Origin of the Lineage to Their Populations. In The Diatom World, J. Seckbach, and P. Kociolek, eds. (Dordrecht: Springer Netherlands), pp. 93–118.

Medlin, L.K. (2016). Evolution of the diatoms: major steps in their evolution and a review of the supporting molecular and morphological evidence. Phycologia *55*, 79–103.

Medlin, L.K. (2018). Mini review: Diatom species as seen through a molecular window. Braz. J. Bot. *41*, 457–469.

Mendiola, J.A., Torres, C.F., Toré, A., Martín-Álvarez, P.J., Santoyo, S., Arredondo, B.O., Señoráns, F.J., Cifuentes, A., and Ibáñez, E. (2007). Use of supercritical CO2 to obtain extracts with antimicrobial activity from *Chaetoceros muelleri* microalga. A correlation with their lipidic content. Eur. Food Res. Technol. *224*, 505–510.

Mendiola, J.A., Santoyo, S., Cifuentes, A., Reglero, G., Ibáñez, E., and Señoráns, F.J. (2008). Antimicrobial activity of sub- and supercritical CO2 extracts of the green alga *Dunaliella salina*. J. Food Prot. *71*, 2138–2143.

Miller, M.B., and Bassler, B.L. (2001). Quorum sensing in bacteria. Annu. Rev. Microbiol. *55*, 165–199.

Mimouni, V., Ulmann, L., Tremblin, G., and Robert, J.-M. (2003). Desaturation of linoleic acid in the marine diatom *Haslea ostrearia* simonsen (Bacillariophyceae). Cryptogam. Algol. *24*, 269–276.

Miranda, C.D., and Zemelman, R. (2002). Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture *212*, 31–47.

Mitchell, P.H., and Barney, R.L. (1917). The occurrence in Virginia of green-gilled oysters similar to those of Marennes. (Washington,: Govt. print. off.,).

Mitchell, P.H., and Barney, R.L. (1918). The occurrence in Virginia of green-gilled oysters similar to those of Marennes. Bull. U. S. Bur. Fish. *35*, 135–149.

Mohamed, B., Hajer, A., Susanna, S., Caterina, O., Flavio, M., Hamadi, B., and Aldo, V. (2014). Transcriptomic responses to heat stress and nickel in the mussel *Mytilus galloprovincialis*. Aquat. Toxicol. *148*, 104–112.

Molina-Cárdenas, C.A., and Sánchez-Saavedra, M. del P. (2017). Inhibitory effect of benthic diatom species on three aquaculture pathogenic vibrios. Algal Res. *27*, 131–139.

Molina-Cárdenas, C.A., Sánchez-Saavedra, M. del P., and Lizárraga-Partida, M.L. (2014). Inhibition of pathogenic *Vibrio* by the microalgae *Isochrysis galbana*. J. Appl. Phycol. *26*, 2347–2355.

Molisch, H. (1903). Notiz über eine blaue Diatomee. Berichte Dtsch. Bot. Ges. *21*, 23–26.

Monod, J. (1949). The Growth of Bacterial Cultures. Annu. Rev. Microbiol. *3*, 371–394.

Morga, B., Arzul, I., Chollet, B., and Renault, T. (2009). Infection with the protozoan parasite *Bonamia ostreae* modifies in vitro haemocyte activities of flat oyster *Ostrea edulis*. Fish Shellfish Immunol. *26*, 836–842.

Mori, T., Hirose, H., Hanjavanit, C., and Hatai, K. (2002). Antifungal activities of plant extracts against some aquatic fungi. Biocontrol Sci. *7*, 187–191.

Mouget, J.-L., Tremblin, G., Morant-Manceau, A., Morançais, M., and Robert, J.-M. (1999). Long-term photoacclimation of *Haslea ostrearia* (Bacillariophyta): effect of irradiance on growth rates, pigment content and photosynthesis. Eur. J. Phycol. *34*, 109–115.

Mouget, J.-L., Rosa, P., Vachoux, C., and Tremblin, G. (2005). Enhancement of marennine production by blue light in the diatom *Haslea ostrearia*. J. Appl. Phycol. *17*, 437–445.

Mouget, J.-L., Gastineau, R., Davidovich, O., Gaudin, P., and Davidovich, N.A. (2009). Light is a key factor in triggering sexual reproduction in the pennate diatom *Haslea ostrearia*. FEMS Microbiol. Ecol. *69*, 194–201.

Mousavi, S.., Mirzargar, S.., Ebrahim Zadeh Mousavi, H., Omid Baigi, R., Khosravi, A., Bahonar, A., and Ahmadi, M.. (2009). Evaluation of antifungal activity of new combined essential oils in comparaison with malachite green on hatching rate in rainbow trout (*Oncorhynchus mykiss*) eggs. J. Fish. Aquat. Sci. *4*, 103–110.

Moussa, H.S., Ibrahem, A.B.B., Sayed, A.F.M.E.-, and Mohammed, F.A. (2015). *In vitro* evaluation of anti-microbial activities of marine streptomyces against viral models, bacterial and fungal Strains. Int. J. Virol. *11*, 20–31.

Mudimu, O., Rybalka, N., Bauersachs, T., Born, J., Friedl, T., and Schulz, R. (2014). Biotechnological screening of microalgal and cyanobacterial strains for biogas production and antibacterial and antifungal effects. Metabolites *4*, 373–393.

Muller-Feuga, A. (2000). The role of microalgae in aquaculture: situation and trends. J. Appl. Phycol. *12*, 527–534.

Murakami, M., Makabe, K., Yamaguchi, K., Konosu, S., and Wälchli, M.R. (1988). Goniodomin a, a novel polyether macrolide from the dinoflagellate *Goniodoma pseudogoniaulax*. Tetrahedron Lett. *29*, 1149–1152.

Myrand, B., Tremblay, R., and Sévigny, J.-M. (2009). Impact of suspension culture using mesh sleeves on genetic characteristics of *Mytilus edulis* L. in Canada. Aquaculture *291*, 147–153.

- N -

Naas, K.E., N1ss, T., and Harboe, T. (1992). Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus L.*) in green water. Aquaculture *105*, 143–156.

Nagai, H., Satake, M., and Yasumoto, T. (1990). Antimicrobial activities of polyether compounds of dinoflagellate origins. J. Appl. Phycol. *2*, 305–308.

Nagai, H., Mikami, Y., Yazawa, K., Gonoi, T., and Yasumoto, T. (1993). Biological activities of novel polyether antifungals, gambieric acids A and B from a marine dinoflagellate *Gambierdiscus toxicus*. J. Antibiot. (Tokyo) *46*, 520–522.

Najdenski, H.M., Gigova, L.G., Iliev, I.I., Pilarski, P.S., Lukavský, J., Tsvetkova, I.V., Ninova, M.S., and Kussovski, V.K. (2013). Antibacterial and antifungal activities of selected microalgae and cyanobacteria. Int. J. Food Sci. Technol. *48*, 1533–1540.

Nassiri, Y., Robert, J.-M., Rincé, Y., and Ginsburger-Vogel, T. (1998). The cytoplasmic fine structure of the diatom *Haslea ostrearia* (Bacillariophyceae) in relation to marennine production. Phycologia *37*, 84–91.

Navarro, A., Faria, M., Barata, C., and Piña, B. (2011). Transcriptional response of stress genes to metal exposure in zebra mussel larvae and adults. Environ. Pollut. *159*, 100–107.

Naviner, M., Bergé, J.-P., Durand, P., and Le Bris, H. (1999). Antibacterial activity of the marine diatom *Skeletonema costatum* against aquacultural pathogens. Aquaculture *174*, 15–24.

Negri, A., Oliveri, C., Sforzini, S., Mignione, F., Viarengo, A., and Banni, M. (2013). Transcriptional response of the mussel *Mytilus galloprovincialis* (Lam.) following exposure to heat stress and copper. PLOS ONE *8*, e66802.

Neori, A. (2010). “Green water” microalgae: the leading sector in world aquaculture. J. Appl. Phycol. *23*, 143–149.

Neuville, D., and Daste, P. (1978). Research on the determinative conditions of the production of blue pigment by *Navicula astrearia* (Gaillon) Bory maintained in culture [application to oyster industry]. Rev. Gen. Bot.

Newaj-Fyzul, A., Al-Harbi, A.H., and Austin, B. (2014). Review: Developments in the use of probiotics for disease control in aquaculture. Aquaculture *431*, 1–11.

Newsome, A.G., Culver, C.A., and van Breemen, R.B. (2014). Nature’s palette: the search for natural blue colorants. J. Agric. Food Chem. *62*, 6498–6511.

Noga, E.J. (2011). Fish Disease: Diagnosis and Treatment (John Wiley & Sons).

Norme AFNOR (NF T90-354) (2007). Qualité de l’eau - Détermination de l’Indice Biologique Diatomées (IBD).

Norme AFNOR (NF T90-354) (2016). Qualité de l’eau - Échantillonnage, traitement et analyse de diatomées benthiques en cours d’eau et canaux.

- O -

Ohta, S., Chang, T., Ikegami, N., Kondo, M., and Miyata, H. (1993). Antibiotic substance produced by a newly isolated marine microalga, *Chlorococcum* HS-101. Bull. Environ. Contam. Toxicol. *50*, 171–178.

Ohta, S., Shiomi, Y., Kawashima, A., Aozasa, O., Nakao, T., Nagate, T., Kitamura, K., and Miyata, H. (1995). Antibiotic effect of linolenic acid from *Chlorococcum* strain HS-101 and *Dunaliella primolecta* on methicillin-resistant *Staphylococcus aureus*. J. Appl. Phycol. *7*, 121–127.

Ohta, S., Ono, F., Shiomi, Y., Nakao, T., Aozasa, O., Nagate, T., Kitamura, K., Yamaguchi, S., Nishi, M., and Miyata, H. (1998). Anti-Herpes Simplex Virus substances produced by the marine green alga, *Dunaliella primolecta*. J. Appl. Phycol. *10*, 349–356.

Øie, G., Reitan, K.I., and Olsen, Y. (1994). Comparison of rotifer culture quality with yeast plus oil and algal-based cultivation diets. Aquac. Int. *2*, 225–238.

Okocha, R.C., Olatoye, I.O., and Adedeji, O.B. (2018). Food safety impacts of antimicrobial use and their residues in aquaculture. Public Health Rev. *39*.

Olsen, A.I., Olsen, Y., Attramadal, Y., Christie, K., Birkbeck, T.H., Skjermo, J., and Vadstein, O. (2000). Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. Aquaculture *190*, 11–25.

Ördög, V., Stirk, W.A., Lenobel, R., Bancířová, M., Strnad, M., Staden, J. van, Szigeti, J., and Németh, L. (2004). Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. J. Appl. Phycol. *16*, 309–314.

- P -

Palmer, P.J., Burke, M.J., Palmer, C.J., and Burke, J.B. (2007). Developments in controlled green-water larval culture technologies for estuarine fishes in Queensland, Australia and elsewhere. Aquaculture *272*, 1–21.

Pandiyan, P., Balaraman, D., Thirunavukkarasu, R., George, E.G.J., Subaramaniyan, K., Manikkam, S., and Sadayappan, B. (2013). Probiotics in aquaculture. Drug Invent. Today *5*, 55–59.

Pane, G., Cacciola, G., Giacco, E., Mariottini, G.L., and Coppo, E. (2015). Assessment of the antimicrobial activity of algae extracts on bacteria responsible of external otitis. Mar. Drugs *13*, 6440–6452.

Papandroulakis, N., Divanach, P., Anastasiadis, P., and Kentouri, M. (2001). The pseudo-green water technique for intensive rearing of sea bream (*Sparus aurata*) larvae. Aquac. Int. *9*, 205–216.

Parrish, C.C. (1987). Separation of Aquatic Lipid Classes by Chromarod Thin-Layer Chromatography with Measurement by Iatroscan Flame Ionization Detection. Can. J. Fish. Aquat. Sci. *44*, 722–731.

Pasquier, A. (1818). Essai Médical sur les Huîtres (Paris, France).

Pérez-Sánchez, T., Mora-Sánchez, B., and Balcázar, J.L. (2018). Biological approaches for disease control in aquaculture: advantages, limitations and challenges. Trends Microbiol. *26*, 896–903.

Pesando, D., Gnassia-Barelli, M., and Gueho, E. (1979a). Partial characterization of a specific antibiotic, antifungal substance isolated from the marine diatom *Chaetoceros lauderi* Ralfs. In Marine Algae in Pharmaceutical Science, (Walter de Gruyter. Berlin-New York), pp. 447–459.

Pesando, D., Gnassia-Barelli, M., and Gueho, E. (1979b). Antifungal properties of some marine algae. In Marine Algae in Pharmaceutical Science, (Walter de Gruyter Berlin-New York), pp. 461–472.

Petschow, B.W., Batema, R.P., and Ford, L.L. (1996). Susceptibility of *Helicobacter pylori* to bactericidal properties of medium-chain monoglycerides and free fatty acids. Antimicrob. Agents Chemother. *40*, 302–306.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.

Pfaffl, M.W., Tichopad, A., Prgomet, C., and Neuvians, T.P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett. *26*, 509–515.

Pinsino, A., Matranga, V., Trinchella, F., and Roccheri, M.C. (2010). Sea urchin embryos as an *in vivo* model for the assessment of manganese toxicity: developmental and stress response effects. Ecotoxicology *19*, 555–562.

Pohnert, G. (2005). Diatom/Copepod Interactions in Plankton: The Indirect Chemical Defense of Unicellular Algae. ChemBioChem *6*, 946–959.

Poulet, S.A., Wichard, T., Ledoux, J.B., Lebreton, B., Marchetti, J., Dancie, C., Bonnet, D., Cueff, A., Morin, L.P., Pohnert, G., et al. (2006). Influence of diatoms on copepod reproduction. I. Field and laboratory observations related to *Calanus helgolandicus* egg production.

Pouvreau, J.-B. (2006). Purification et caractérisation du pigment bleu-vert “marennine” synthétisé par la diatomée marine *Haslea ostrearia* (Gaillon/Bory) Simonsen : propriétés physico-chimiques et activités biologiques. PhD Thesis, Université de Nantes.

Pouvreau, J.-B., Morançais, M., Fleury, F., Rosa, P., Thion, L., Cahingt, B., Zal, F., Fleurence, J., and Pondaven, P. (2006a). Preliminary characterisation of the blue-green pigment “marennine” from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. J. Appl. Phycol. *18*, 757–767.

Pouvreau, J.-B., Morançais, M., Massé, G., Rosa, P., Robert, J.-M., Fleurence, J., and Pondaven, P. (2006b). Purification of the blue-green pigment “marennine” from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. J. Appl. Phycol. *18*, 769–781.

Pouvreau, J.-B., Morançais, M., Fleurence, J., and Pondaven, P. (2006c). Method for the quantification of the blue-green pigment “marennine” synthesized by the marine diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen using HPLC gel-filtration and photodiode-array detection. J. Appl. Phycol. *19*, 263–270.

Pouvreau, J.-B., Housson, E., Tallec, L.L., Morançais, M., Rincé, Y., Fleurence, J., and Pondaven, P. (2007). Growth inhibition of several marine diatom species induced by the

shading effect and allelopathic activity of marennine, a blue-green polyphenolic pigment of the diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. J. Exp. Mar. Biol. Ecol. *352*, 212–225.

Pouvreau, J.-B., Michèle Morançais, Taran, F., Rosa, P., Laurent Dufossé, Fabienne Guérard, Pin, S., Fleurence, J., and Pondaven, P. (2008). Antioxidant and free radical scavenging properties of marennine, a blue-green polyphenolic pigment from the diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen responsible for the natural greening of cultured oysters. J. Agric. Food Chem. *56*, 6278–6286.

Pradhan, J., Das, S., and Das, B.K. (2014). Antibacterial activity of freshwater microalgae: A review. Afr. J. Pharm. Pharmacol. *8*, 809–818.

Prasetiya, F.S., Gastineau, R., Poulin, M., Lemieux, C., Turmel, M., Syakti, A.D., Hardivillier, Y., Widowati, I., Risjani, Y., Iskandar, I., et al. (in press). *Haslea nusantara*, a new blue diatom from the Java Sea, Indonesia: Morphology, biometry and molecular characterization. Plant Ecol. Evol.

Prasetiya, F.S., Safitri, I., Widowati, I., Cognie, B., Decottignies, P., Gastineau, R., Morançais, M., Windarto, E., Tremblay, R., and Mouget, J.-L. (2016). Does allelopathy affect co-culturing *Haslea ostrearia* with other microalgae relevant to aquaculture? J. Appl. Phycol. *28*, 2241–2254.

Prasetiya, F.S., Comeau, L.A., Gastineau, R., Decottignies, P., Cognie, B., Morançais, M., Turcotte, F., Mouget, J.-L., and Tremblay, R. (2017). Effect of marennine produced by the blue diatom *Haslea ostrearia* on behavioral, physiological and biochemical traits of juvenile *Mytilus edulis* and *Crassostrea virginica*. Aquaculture *467*, 138–148.

Pratt, R., Daniels, T.C., Eiler, J.J., Gunnison, J.B., Kumler, W.D., Oneto, J.F., Strait, L.A., Spoehr, H.A., Hardin, G.J., Milner, H.W., et al. (1944). Chlorellin, an antibacterial substance from *Chlorella*. Science *99*, 351–352.

Priyadarshani, I., and Biswajit, R. (2012). Commercial and industrial applications of micro algae - A review. J. Algal Biomass Util. *3*, 89–100.

Provasoli, L., Mclaughlin, J.J., and Droop, M.R. (1957). The development of artificial media for marine algae. Arch. Mikrobiol. *25*, 392–428.

- Q -

Quinn, G.P., and Keough, M.J. (2002). Experimental design and data analysis for biologists (Cambridge).

- R -

Radonić, A., Thulke, S., Achenbach, J., Kurth, A., Vreemann, A., König, T., Walter, C., Possinger, K., and Nitsche, A. (2011). Anionic polysaccharides from phototrophic microorganisms exhibit antiviral activities to Vaccinia virus.

Ranson, G. (1927). L'absorption de matières organiques dissoutes par la surface extérieure du corps chez les animaux aquatiques. Ann. Inst. Océan. *4*, 174.

Ranson, G. (1937). Le verdissement des huîtres. Sciences *8*, 13–24.

Raposo, M.F. de J., de Morais, R.M.S.C., and de Morais, A.M.M.B. (2013a). Health applications of bioactive compounds from marine microalgae. Life Sci. *93*, 479–486.

Raposo, M.F. de J., de Morais, R.M.S.C., and Bernardo de Morais, A.M.M. (2013b). Bioactivity and applications of sulphated polysaccharides from marine microalgae. Mar. Drugs *11*, 233–252.

Raposo, M.F. de J., de Morais, A.M.M.B., and de Morais, R.M.S.C. (2014). Influence of sulphate on the composition and antibacterial and antiviral properties of the exopolysaccharide from *Porphyridium cruentum*. Life Sci. *101*, 56–63.

Raposo, M.F. de J., de Morais, A.M.B., and de Morais, R.M.S.C. (2015a). Marine polysaccharides from algae with potential biomedical applications. Mar. Drugs *13*, 2967–3028.

Raposo, M.F. de J., de Morais, A.M.M.B., and de Morais, R.M.S.C. (2015b). Carotenoids from marine microalgae: A valuable natural source for the prevention of chronic diseases. Mar. Drugs *13*, 5128–5155.

Raposo, M.F. de J., de Morais, A.M.M.B., and de Morais, R.M.S.C. (2016). Emergent sources of prebiotics: Seaweeds and microalgae. Mar. Drugs *14*, 27.

Rasch, M., Buch, C., Austin, B., Slierendrecht, W.J., Ekmann, K.S., Larsen, J.L., Johansen, C., Riedel, K., Eberl, L., Givskov, M., et al. (2004). An inhibitor of bacterial quorum sensing reduces mortalities caused by Vibriosis in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Syst. Appl. Microbiol. *27*, 350–359.

Raven, J.A., and Waite, A.M. (2004). The evolution of silicification in diatoms: inescapable sinking and sinking as escape? New Phytol. *162*, 45–61.

Rech, M., Mouget, J.-L., Morant-Manceau, A., Philippe, R., and Tremblin, G. (2005). Long-term acclimation to UV radiation: effects on growth, photosynthesis and carbonic anhydrase activity in marine diatoms. Bot. Mar. *48*, 407–420.

Rech, M., Morant-Manceau, A., and Tremblin, G. (2008). Carbon fixation and carbonic anhydrase activity in Haslea ostrearia (Bacillariophyceae) in relation to growth irradiance. Photosynthetica *46*, 56–62.

Rechter, S., König, T., Auerochs, S., Thulke, S., Walter, H., Dörnenburg, H., Walter, C., and Marschall, M. (2006). Antiviral activity of Arthrospira-derived spirulan-like substances. Antiviral Res. *72*, 197–206.

Regunathan C., and Wesley S.G. (2004). Control of *Vibrio spp.* in shrimp hatcheries using the green algae *Tetraselmis suecica*. Asian Fish. Sci. *17*, 147–158.

Reitan, K.I., Rainuzzo, J.R., Øie, G., and Olsen, Y. (1997). A review of the nutritional effects of algae in marine fish larvae. Aquaculture *155*, 207–221.

Rhoobunjongde, W., Hatai, K., Wada, S., and Kubota, S.S. (1991). *Fusarium moniliforme* (Sheldon) isolated from gills of kuruma prawn *Penaeus japonicus* (Bate) with black gill disease. Nippon Suisan Gakkaishi *57*, 629–635.

Robert, J.M. (1973). La diatomée *Navicula ostrearia* Bory en baie de Bourgneuf. Rev. Trav. Inst. Pêch. Marit. *37*, 363–368.

Robert, J.-M. (1978). Variations biométriques de l'algue *Navicula ostrearia* Bory (Diatomée Pennée) en culture. Bull. - Société Phycol. Fr. *23*, 38–44.

Robert, J.-M. (1983). Fertilité des eaux des claires ostréicoles et verdissement: Utilisation de l'azote par les diatomées dominantes. University of Nantes.

Robert, J.-M., Maestrini, S.Y., and Zanette, Y. (1982). Production des micro-algues des claires ostréicoles en relation avec l'azote organique dissous excrété par les huîtres. Oceanol. Acta Sp 389–395.

Robert, J.-M., Morançais, M., Pradier, E., Mouget, J.-L., and Tremblin, G. (2002). Extraction and quantitative analysis of the blue-green pigment "marennine" synthesized by the diatom *Haslea ostrearia*. J. Appl. Phycol. *14*, 299–305.

Rodríguez-Meizoso, I., Jaime, L., Santoyo, S., Señoráns, F.J., Cifuentes, A., and Ibáñez, E. (2010). Subcritical water extraction and characterization of bioactive compounds from *Haematococcus pluvialis* microalga. J. Pharm. Biomed. Anal. *51*, 456–463.

Romano, G., Miralto, A., and Ianora, A. (2010). Teratogenic effects of diatom metabolites on sea urchin *Paracentrotus lividus* embryos. Mar. Drugs *8*, 950–967.

Romero, J., Feijoo, C.G., and Navarrete, P. (2012). Antibiotics in Aquaculture – Use, Abuse and Alternatives. In Health and Environment in Aquaculture, (Edmir Daniel Carvalho, Gianmarco Silva David and Reinaldo J. Silva), p.

Rossignol, N., Jaouen, P., Robert, J.-M., and Quéméneur, F. (2000). Production of exocellular pigment by the marine diatom *Haslea ostrearia* Simonsen in a photobioreactor equipped with immersed ultrafiltration membranes. Bioresour. Technol. *73*, 197–200.

Round, F.E., Crawford, R.M., and Mann, D.G. (1990). Diatoms: Biology and Morphology of the Genera (Cambridge University Press).

Rowley, A.F., and Pope, E.C. (2012). Vaccines and crustacean aquaculture—A mechanistic exploration. Aquaculture *334–337*, 1–11.

Rowley, A.F., and Powell, A. (2007). Invertebrate immune systems specific, quasi-specific, or nonspecific? J. Immunol. Baltim. Md 1950 *179*, 7209–7214.

Roy, S.S., and Pal, R. (2015). Microalgae in aquaculture: A review with special references to nutritional value and fish dietetics. Proc. Zool. Soc. *68*, 1–8.

Ruck, E.C., and Theriot, E.C. (2011). Origin and evolution of the canal raphe system in diatoms. Protist *162*, 723–737.

- S -

Safonova, E., and Reisser, W. (2005). Growth promoting and inhibiting effects of extracellular substances of soil microalgae and cyanobacteria on *Escherichia coli* and *Micrococcus luteus*. Phycol. Res. *53*, 189–193.

Salvesen, I., Skjermo, J., and Vadstein, O. (1999). Growth of turbot (*Scophthalmus maximus L.*) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water. Aquaculture *175*, 337–350.

Sánchez-Saavedra, M. del P., Licea-Navarro, A., and Bernáldez-Sarabia, J. (2010). Evaluation of the antibacterial activity of different species of phytoplankton. Rev. Biol. Mar. Oceanogr. *45*, 531–536.

Santos, L., and Ramos, F. (2018). Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. Int. J. Antimicrob. Agents *52*, 135–143.

Santoyo, S., Rodríguez-Meizoso, I., Cifuentes, A., Jaime, L., García-Blairsy Reina, G., Señorans, F.J., and Ibáñez, E. (2009). Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from *Haematococcus pluvialis* microalgae. LWT - Food Sci. Technol. *42*, 1213–1218.

Santoyo, S., Plaza, M., Jaime, L., Ibañez, E., Reglero, G., and Señorans, F.J. (2010). Pressurized liquid extraction as an alternative process to obtain antiviral agents from the edible microalga *Chlorella vulgaris*. J. Agric. Food Chem. *58*, 8522–8527.

Santoyo, S., Jaime, L., Plaza, M., Herrero, M., Rodriguez-Meizoso, I., Ibañez, E., and Reglero, G. (2011). Antiviral compounds obtained from microalgae commonly used as carotenoid sources. J. Appl. Phycol. *24*, 731–741.

Sarthou, G., Timmermans, K.R., Blain, S., and Tréguer, P. (2005). Growth physiology and fate of diatoms in the ocean: a review. J. Sea Res. *53*, 25–42.

Sarwar, S., Chakraborti, S., Bera, S., Sheikh, I.A., Hoque, K.M., and Chakrabarti, P. (2016). The antimicrobial activity of ZnO nanoparticles against *Vibrio cholerae*: Variation in response depends on biotype. Nanomedicine Nanotechnol. Biol. Med. *12*, 1499–1509.

Satake, M., Murata, M., Yasumoto, T., Fujita, T., and Naoki, H. (1991). Amphidinol, a polyhydroxy-polyene antifungal agent with an unprecedented structure, from a marine dinoflagellate, *Amphidinium klebsii*. J. Am. Chem. Soc. *113*, 9859–9861.

Sauvageau, C. (1906). A propos de la présence de la diatomée bleue dans la Méditerranée. Trav. Lab. Arcachon *6*, 46–59.

Schaeffer, D.J., and Krylov, V.S. (2000). Anti-HIV activity of extracts and compounds from algae and cyanobacteria. Ecotoxicol. Environ. Saf. *45*, 208–227.

Schubert, H., Tremblin, G., Robert, J.-M., Sagert, S., and Rincé, Y. (1995). In-vivo fluorescence measurement of photosynthesis of *Haslea ostrearia* Simonsen in relation to marennine content. Diatom Res. *10*, 341–349.

Semba Prasetiya, F. (2015). Greening phenomenon in bivalve by marennine produced from *Haslea ostrearia* and its consequences on bivalve's integrated response. Le Mans.

Seychelles, L.H., Audet, C., Tremblay, R., Lemarchand, K., and Pernet, F. (2011). Bacterial colonization of winter flounder *Pseudopleuronectes americanus* fed live feed enriched with three different commercial diets. Aquac. Nutr. *17*, e196–e206.

Sharma, G.M., Michaels, L., and Burkholder, P.R. (1968). Goniodomin, a new antibiotic from a dinoflagellate. J. Antibiot. (Tokyo) *21*, 659–664.

Sharma, V., McKone, H.T., and Markow, P.G. (2011). A global perspective on the history, use, and identification of synthetic food dyes. J. Chem. Educ. *88*, 24–28.

Shimizu, Y. (2003). Microalgal metabolites. Curr. Opin. Microbiol. *6*, 236–243.

Shishido, T.K., Humisto, A., Jokela, J., Liu, L., Wahlsten, M., Tamrakar, A., Fewer, D.P., Permi, P., Andreote, A.P.D., Fiore, M.F., et al. (2015). Antifungal compounds from cyanobacteria. Mar. Drugs *13*, 2124–2140.

Shukla, V., and Bhathena, Z. (2016). Broad Spectrum Anti-Quorum Sensing Activity of Tannin-Rich Crude Extracts of Indian Medicinal Plants. Scientifica *2016*.

Simonsen, R. (1974). The diatom plankton of the Indian Ocean expedition of RV “Meteor.” Hrsg. Von Dtsch. Forschungsgemeinschaft *19*, 46–49.

Singh, S., Kate, B.N., and Banerjee, U.C. (2005). Bioactive compounds from cyanobacteria and microalgae: an overview. Crit. Rev. Biotechnol. *25*, 73–95.

Sivasankar, P., Anix Vivek Santhiya, A., and Kanaga, V. (2015). A review on plants and herbal extracts against viral diseases in aquaculture. J. Med. Plants Stud. *3*, 75–79.

Smetacek, V. (2000). Oceanography: The giant diatom dump. Nature *406*, 574–575.

Smith, V.J. (2016). Immunology of Invertebrates: Cellular. In ELS, (American Cancer Society), pp. 1–13.

Smith, V.J., Desbois, A.P., and Dyrynda, E.A. (2010). Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. Mar. Drugs *8*, 1213–1262.

Smol, J.P., and Stoermer, E.F. (2010). The Diatoms: Applications for the Environmental and Earth Sciences (Cambridge University Press).

Snoeijs, P., and Kasperoviciene, J. (1996). Intercalibration and distribution of diatom species in the Baltic Sea. Balt. Mar. Biol. Publ. *16*, 62.

Soares, A.R., Robaina, M.C.S., Mendes, G.S., Silva, T.S.L., Gestinari, L.M.S., Pamplona, O.S., Yoneshigue-Valentin, Y., Kaiser, C.R., and Romanos, M.T.V. (2012). Antiviral activity of extracts from Brazilian seaweeds against herpes simplex virus. Rev. Bras. Farmacogn. *22*, 714–723.

Solopova, A., Gestel, J. van, Weissing, F.J., Bachmann, H., Teusink, B., Kok, J., and Kuipers, O.P. (2014). Bet-hedging during bacterial diauxic shift. Proc. Natl. Acad. Sci. *111*, 7427–7432.

Sommerset, I., Krossøy, B., Biering, E., and Frost, P. (2005). Vaccines for fish in aquaculture. Expert Rev. Vaccines *4*, 89–101.

Soudant, P., E. Chu, F.-L., and Volety, A. (2013). Host–parasite interactions: Marine bivalve molluscs and protozoan parasites, Perkinsus species. J. Invertebr. Pathol. *114*, 196–216.

Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A. (2006). Commercial applications of microalgae. J. Biosci. Bioeng. *101*, 87–96.

Sprat, T. (1667). History of the Royal Society (Martyn: London, UK).

Sprouffske, K., and Wagner, A. (2016). Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. BMC Bioinformatics *17*.

Srinivasakumar, K.P., and Rajashekhar, M. (2009). *In vitro* studies on bactericidal activity and sensitivity pattern of isolated marine microalgae against selective human bacterial pathogens. Indian J. Sci. Technol. *2*, 16–23.

Steinhardt, R.A., and Epel, D. (1974). Activation of Sea-Urchin Eggs by a Calcium Ionophore. Proc. Natl. Acad. Sci. *71*, 1915–1919.

Stewart, W.D.P., Rowell, P., and Rai, A.N. (1983). Cyanobacteria-eukaryotic plant symbioses. Ann. Inst. Pasteur Microbiol. *134*, 205–228.

Suleria, H.A.R., Osborne, S., Masci, P., and Gobe, G. (2015). Marine-based nutraceuticals: An innovative trend in the food and supplement industries. Mar. Drugs *13*, 6336–6351.

Sun, C.Q., O'Connor, C.J., and Roberton, A.M. (2003). Antibacterial actions of fatty acids and monoglycerides against *Helicobacter pylori*. FEMS Immunol. Med. Microbiol. *36*, 9–17.

Supamattaya, K., Kiriratnikom, S., Boonyaratpalin, M., and Borowitzka, L. (2005). Effect of a *Dunaliella* extract on growth performance, health condition, immune response and disease resistance in black tiger shrimp (*Penaeus monodon*). Aquaculture *248*, 207–216.

- T -

Talero, E., García-Mauriño, S., Ávila-Román, J., Rodríguez-Luna, A., Alcaide, A., and Motilva, V. (2015). Bioactive compounds isolated from microalgae in chronic inflammation and cancer. Mar. Drugs *13*, 6152–6209.

Talyshinsky, M.M., Souprun, Y.Y., and Huleihel, M.M. (2002). Anti-viral activity of red microalgal polysaccharides against retroviruses. Cancer Cell Int. *2*, 8.

Tanguy, A., Boutet, I., Laroche, J., and Moraga, D. (2005). Molecular identification and expression study of differentially regulated genes in the Pacific oyster *Crassostrea gigas* in response to pesticide exposure. FEBS J. *272*, 390–403.

Tanniou, A., Turpin, V., and Lebeau, T. (2012). Comparison of cryopreservation methods for the long term storage of the marine diatom *Haslea ostrearia* (simonsen). Cryobiology *65*, 45–50.

Tardy-Laporte, C., Arnold, A.A., Genard, B., Gastineau, R., Morançais, M., Mouget, J.-L., Tremblay, R., and Marcotte, I. (2013). A 2H solid-state NMR study of the effect of antimicrobial agents on intact *Escherichia coli* without mutating. Biochim. Biophys. Acta BBA - Biomembr. *1828*, 614–622.

Tendencia, E.A., and dela Peña, M. (2003). Investigation of some components of the greenwater system which makes it effective in the initial control of luminous bacteria. Aquaculture *218*, 115–119.

Terekhova, V.E., Aizdaicher, N.A., Buzoleva, L.S., and Somov, G.P. (2009). Influence of extrametabolites of marine microalgae on the reproduction of the bacterium *Listeria monocytogenes*. Russ. J. Mar. Biol. *35*, 355–358.

Tinh, N.T.N., Dierckens, K., Sorgeloos, P., and Bossier, P. (2008). A review of the functionality of probiotics in the larviculture food chain. Mar. Biotechnol. N. Y. N *10*, 1–12.

Tolosa, J., Font, G., Mañes, J., and Ferrer, E. (2014). Natural occurrence of emerging *Fusarium* mycotoxins in feed and fish from aquaculture. J. Agric. Food Chem. *62*, 12462–12470.

Trainer, V.L., Bates, S.S., Lundholm, N., Thessen, A.E., Cochlan, W.P., Adams, N.G., and Trick, C.G. (2012). *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health. Harmful Algae *14*, 271–300.

Trakranrungsie, N. (2011). Plant derived antifungals- trends and potential applications in veterinary medicine: A mini-review (Science against microbial pathogens: communicating current research and technological advances A. Méndez-Vilas (Ed.)).

Travers, M.-A., Boettcher Miller, K., Roque, A., and Friedman, C.S. (2015). Bacterial diseases in marine bivalves. J. Invertebr. Pathol. *131*, 11–31.

Tréguer, P.J., and De La Rocha, C.L. (2013). The World Ocean Silica Cycle. Annu. Rev. Mar. Sci. *5*, 477–501.

Tréguer, P., Bowler, C., Moriceau, B., Dutkiewicz, S., Gehlen, M., Aumont, O., Bittner, L., Dugdale, R., Finkel, Z., Iudicone, D., et al. (2018). Influence of diatom diversity on the ocean biological carbon pump. Nat. Geosci. *11*, 27.

Tremblin, G., and Robert, J.-M. (2001). Carbon Fixation by the Peculiar Marine Diatom *Haslea ostrearia*. Photosynthetica *39*, 215–220.

Tremblin, G., Cannuel, R., Mouget, J.-L., Rech, M., and Robert, J.-M. (2000). Change in light quality due to a blue-green pigment, marennine, released in oyster-ponds: effect on growth and photosynthesis in two diatoms, *Haslea ostrearia* and *Skeletonema costatum*. J. Appl. Phycol. *12*, 557–566.

Turcotte, F., Mouget, J.-L., Genard, B., Lemarchand, K., Deschênes, J.-S., and Tremblay, R. (2016). Prophylactic effect of *Haslea ostrearia* culture supernatant containing the pigment marennine to stabilize bivalve hatchery production. Aquat. Living Resour. *29*, 401.

Turpin, V. (1999). Limiting nutrients of oyster pond seawaters in the Marennes-Oléron region for *Haslea ostrearia*: applications to the mass production of the diatom in mesocosm experiments. Aquat. Living Resour. *12*, 335–342.

Turpin, V., Robert, J.-M., Goulletquer, P., Massé, G., and Rosa, P. (2001). Oyster greening by outdoor mass culture of the diatom *Haslea ostrearia* Simonsen in enriched seawater. Aquac. Res. *32*, 801–809.

- U -

Udomkusonsri, P., Trongvanichnam, K., Limpoka, M., Klangkaew, N., and Kusucharit, N. (2007). *In vitro* efficacy of the antifungal activity of some Thai medicinal-plants on the pathogenic fungus, *Saprolegnia parasitica* H2, in fish. Kasetsart J Nat Sci *41*, 56–61.

Uitz, J., Claustre, H.¤, Gentili, B., and Stramski, D. (2010). Phytoplankton class-specific primary production in the world's oceans: Seasonal and interannual variability from satellite observations. Glob. Biogeochem. Cycles *24*.

US Report of the Secretary of Commerce and Reports of Bureaus (1916). Reports of the Department of Commerce. Report of the Secretary of Commerce and reports of bureaus.

- V -

Valenciennes, A. (1841). Sur les causes de la coloration en vert de certaines huîtres. Comptes Rendus Hebd. Séances Académie Sci. *12*, 345–346.

Van der Meeren, T., Mangor-Jensen, A., and Pickova, J. (2007). The effect of green water and light intensity on survival, growth and lipid composition in Atlantic cod (*Gadus morhua*) during intensive larval rearing. Aquaculture *265*, 206–217.

Van Hai, N. (2015). The use of medicinal plants as immunostimulants in aquaculture: A review. Aquaculture *446*, 88–96.

Vandenberghe, J., Thompson, F.L., Gomez-Gil, B., and Swings, J. (2003). Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems. Aquaculture *219*, 9–20.

Varrella, S., Romano, G., Ianora, A., Bentley, M.G., Ruocco, N., and Costantini, M. (2014). Molecular response to toxic diatom-derived aldehydes in the sea urchin *Paracentrotus lividus*. Mar. Drugs *12*, 2089–2113.

Varrella, S., Romano, G., Costantini, S., Ruocco, N., Ianora, A., Bentley, M.G., and Costantini, M. (2016a). Toxic diatom aldehydes affect defence gene networks in sea urchins. PLoS ONE *11*.

Varrella, S., Romano, G., Ruocco, N., Ianora, A., Bentley, M.G., and Costantini, M. (2016b). First morphological and molecular evidence of the negative impact of diatom-derived hydroxyacids on the sea urchin *Paracentrotus lividus*. Toxicol. Sci. *151*, 419–433.

Venegas, C.A., Nonaka, L., Mushiake, K., Nishizawa, T., and Murog, K. (2000). Quasi-immune response of *Penaeus japonicus* to penaeid rod-shaped DNA virus (PRDV). Dis. Aquat. Organ. *42*, 83–89.

Venkatesan, R., Karthikayen, R., Periyanayagi, R., Sasikala, V., and Balasubramanian, T. (2007). Antibacterial activity of the marine diatom, *Rhizosolenia alata* (Brightwell, 1858) against human pathogens. Res. J. Microbiol. *2*, 98–100.

Vijayakumar, S., and Menakha, M. (2015). Pharmaceutical applications of cyanobacteria—A review. J. Acute Med. *5*, 15–23.

Vine, N.G., Leukes, W.D., and Kaiser, H. (2004). *In vitro* growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. FEMS Microbiol. Lett. *231*, 145–152.

Viso, A.C., Pesando, D., and Baby, C. (1987). Antibacterial and antifungal properties of some marine diatoms in culture. Bot. Mar. *30*, 41–46.

Vo, T.-S., Ngo, D.-H., Ta, Q.V., and Kim, S.-K. (2011). Marine organisms as a therapeutic source against herpes simplex virus infection. Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci. *44*, 11–20.

- W -

Walter, C.S, and Mahesh, R (2000). Antibacterial and antifungal activities of some marine diatoms in culture. Indian J. Mar. Sci. *29*, 238–242.

Wang, D., Lin, Z., Wang, T., Ding, X., and Liu, Y. (2017). An analogous wood barrel theory to explain the occurrence of hormesis: A case study of sulfonamides and erythromycin on *Escherichia coli* growth. PLOS ONE *12*, e0181321.

Wang, R., Zhong, Y., Gu, X., Yuan, J., Saeed, A.F., and Wang, S. (2015a). The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. Front. Microbiol. *6*.

Wang, Y.-T., Xue, Y.-R., and Liu, C.-H. (2015b). A brief review of bioactive metabolites derived from deep-sea fungi. Mar. Drugs *13*, 4594–4616.

Washida, K., Koyama, T., Yamada, K., Kita, M., and Uemura, D. (2006). Karatungiols A and B, two novel antimicrobial polyol compounds, from the symbiotic marine dinoflagellate *Amphidinium sp.* Tetrahedron Lett. *47*, 2521–2525.

Watts, J.E.M., Schreier, H.J., Lanska, L., and Hale, M.S. (2017). The rising tide of antimicrobial resistance in aquaculture: Sources, sinks and solutions. Mar. Drugs *15*, 158.

Wetzel, C.E., Van de Vijver, B., and Ector, L. (2010). *Tursiocola podocnemicola* sp. nov., a new epizoic freshwater diatom species from the Rio Negro in the Brazilian Amazon Basin. Vie Milieu *60*, 177–184.

Wey SB, Mori M, Pfaller MA, Woolson RF, and Wenzel RP (1988). Hospital-acquired candidemia: The attributable mortality and excess length of stay. Arch. Intern. Med. *148*, 2642–2645.

Wichard, T., Poulet, S.A., Boulesteix, A.L., Ledoux, J.B., Lebreton, B., Marchetti, J., Pohnert, G., and Pohnert, G. (2008). Influence of diatoms on copepod reproduction. Ii. Uncorrelated effects of diatom-derived alpha,beta,gamma,delta-unsaturated aldehydes and polyunsaturated fatty acids on *Calanus helgolandicus* in the field.

Wijesekara, I., Pangestuti, R., and Kim, S.-K. (2011). Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. Carbohydr. Polym. *84*, 14–21.

Wraige, E.J., Belt, S.T., Massé, G., Robert, J.-M., and Rowland, S.J. (1998). Variations in distributions of C25 highly branched isoprenoid (HBI) alkenes in the diatom, *Haslea ostrearia*: influence of salinity. Org. Geochem. *28*, 855–859.

Wrolstad, R.E., and Culver, C.A. (2012). Alternatives to those artificial FD&C food colorants. Annu. Rev. Food Sci. Technol. *3*, 59–77.

- X -

Xu, L., Meng, W., Cao, C., Wang, J., Shan, W., and Wang, Q. (2015). Antibacterial and antifungal compounds from marine fungi. Mar. Drugs *13*, 3479–3513.

- Y -

Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., Takriff, M.S., and others (2014). An overview: biomolecules from microalgae for animal feed and aquaculture. J Biol Res *21*, 1–10.

Yanong, R.P.E. (2003). Fungal diseases of fish. Veterinary Clin. North Am. Exot. Anim. Pract. *6*, 377–400.

Yasuhara-Bell, J., and Lu, Y. (2010). Marine compounds and their antiviral activities. Antiviral Res. *86*, 231–240.

Yim, J.H., Kim, S.J., Ahn, S.H., Lee, C.K., Rhie, K.T., and Lee, H.K. (2004). Antiviral effects of sulfated exopolysaccharide from the marine microalga *Gyrodinium impudicum* strain KG03. Mar. Biotechnol. *6*, 17–25.

- Z -

Zheng, C.J., Yoo, J.-S., Lee, T.-G., Cho, H.-Y., Kim, Y.-H., and Kim, W.-G. (2005). Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. FEBS Lett. *579*, 5157–5162.

Zhu, H., He, C.-C., and Chu, Q.-H. (2011). Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*. Lett. Appl. Microbiol. *52*, 269–274.

Zhu, S., Kojima, S., and Homma, M. (2013). Structure, gene regulation and environmental response of flagella in *Vibrio*. Front. Microbiol. *4*.

Zorriehzahra, M.J., Delshad, S.T., Adel, M., Tiwari, R., Karthik, K., Dhama, K., and Lazado, C.C. (2016). Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: A review. Vet. Q. *36*, 228–241

ANNEXES

ANNEXE 1 : Publication

Falaise et al. (2016), *Marine Drugs* [*Texte Complet*]

Falaise, C., François, C., Travers, M.-A., Morga, B., Haure, J., Tremblay, R., Turcotte, F., Pasetto, P., Gastineau, R., Hardivillier, Y., Leignel, V., Mouget, J.-L., 2016. **Antimicrobial compounds from eukaryotic microalgae against human pathogens and diseases in aquaculture.** *Marine Drugs* 14, 159-186.

Review

Antimicrobial Compounds from Eukaryotic Microalgae against Human Pathogens and Diseases in Aquaculture

Charlotte Falaise [1], Cyrille François [2], Marie-Agnès Travers [2], Benjamin Morga [2], Joël Haure [2], Réjean Tremblay [3], François Turcotte [3], Pamela Pasetto [4], Romain Gastineau [1], Yann Hardivillier [1], Vincent Leignel [1] and Jean-Luc Mouget [1,*]

1. FR CNRS 3473 IUML Mer-Molécules-Santé (MMS), Université du Maine, Avenue O. Messiaen, Le Mans 72085, France; Charlotte.Falaise@gmail.com (C.F.); gastineauromain@yahoo.fr (R.G.); Yann.Hardivillier@univ-lemans.fr (Y.H.); Vincent.Leignel@univ-lemans.fr (V.L.)
2. Ifremer, SG2M-LGPMM, Laboratoire de Génétique et de Pathologie des Mollusques Marins, Avenue Mus de Loup, La Tremblade 17390, France; Cyrille.Francois@ifremer.fr (C.F.); Marie.Agnes.Travers@ifremer.fr (M.-A.T.); Benjamin.Morga@ifremer.fr (B.M.); Joel.Haure@ifremer.fr (J.H.)
3. Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, 310 des Ursulines, Rimouski, QC G5L 3A1, Canada; Rejean_Tremblay@uqar.ca (R.T.); Francois.Turcotte@uqar.ca (F.T.)
4. UMR CNRS 6283 Institut des Molécules et Matériaux du Mans (IMMM), Université du Maine, Avenue O. Messiaen, Le Mans 72085, France; Pamela.Pasetto@univ-lemans.fr

* Correspondence: Jean-Luc.Mouget@univ-lemans.fr; Tel.: +33-243-833-242

Academic Editor: Peer B. Jacobson
Received: 12 July 2016; Accepted: 24 August 2016; Published: 2 September 2016

Abstract: The search for novel compounds of marine origin has increased in the last decades for their application in various areas such as pharmaceutical, human or animal nutrition, cosmetics or bioenergy. In this context of blue technology development, microalgae are of particular interest due to their immense biodiversity and their relatively simple growth needs. In this review, we discuss about the promising use of microalgae and microalgal compounds as sources of natural antibiotics against human pathogens but also about their potential to limit microbial infections in aquaculture. An alternative to conventional antibiotics is needed as the microbial resistance to these drugs is increasing in humans and animals. Furthermore, using natural antibiotics for livestock could meet the consumer demand to avoid chemicals in food, would support a sustainable aquaculture and present the advantage of being environmentally friendly. Using natural and renewable microalgal compounds is still in its early days, but considering the important research development and rapid improvement in culture, extraction and purification processes, the valorization of microalgae will surely extend in the future.

Keywords: biological activity; antimicrobial; antibacterial; antifungal; antiviral; biological activity; *Haslea*; microalgae; sustainable aquaculture

1. Introduction

Microalgae are present in almost all ecosystems around the world. They evolved in extreme competitive environments, are largely grazed by highly diverse consumers and exposed to microbial pathogens such as bacteria, viruses and fungi. In order to survive, they had to develop tolerance or defense strategies. The variety of these mechanisms resulted in a high diversity of compounds synthesized from diverse metabolic pathways. It appears that many of these metabolites present very specific chemical structures that are not encountered among terrestrial organisms, and sometimes with a structural complexity that makes often too difficult to reproduce them by hemi-synthesis or complete synthesis [1].

In recent decades, there has been a great trend for research and industrial applications of marine compounds and biotechnology [2–7]. Among the large spectrum of marine organisms, microalgae represent a promising resource for blue technologies, due to their rapid growth and usually simple nutriment requirements. Furthermore, many microalgal species are able to grow in saline water or wastewater. This represents an invaluable advantage, considering that freshwater resources are becoming scarce. Microalgae are usually very versatile and able to acclimate to various and changing environments. They offer the opportunity to discover novel molecules or produce known molecules at a lower cost. Due to a tremendous phenotypic plasticity, the nature and amount of their secondary metabolites can be manipulated through control of the culture conditions. Many valuable compounds can be extracted from microalgae, including pigments, lipids, proteins, polysaccharides, vitamins or minerals [8–11]. If an important research effort in microalgal biotechnology is made to promote the production of biofuels [12–14], the variety of compounds generated by microalgae can serve a broad spectrum of applications such as pharmaceuticals, cosmetics, human and animal nutrition, environmental restoration and protection or bioenergy [15–20]. Several compounds have shown potent biological activities, such as antioxidants, anticoagulants, anti-inflammatory, antimicrobial or antitumoral [8,9,21,22]. The possible use of these compounds as a source of prebiotics, nutraceuticals, chemopreventive agents or antimicrobial drugs was investigated and has demonstrated promising results [11,23–26]. Microalgae are also valuable for their production of a diverse range of pigments such as chlorophyll, phycobiliproteins or carotenoids that can be used as dyes for food industry or cosmetics.

The earliest and most common use of microalgae is for aquaculture. They have been used as food source and feed additive to promote the growth of the larvae or the juveniles of various aquatic animals such as finfish, shellfish or crustaceans and can be used for refining process at adult stages [27]. Microalgae can also be supplied to the zooplankton used to feed the larvae of finfish and crustaceans [28]. They are not only essential as a food source but they also permit to improve the quality of aquaculture stock. For instance, the carotenoid astaxanthin, especially abundant in the green microalga *Haematococcus pluvialis*, can be supplied to give or increase color to the flesh of salmon and trout [29]; the blue pigment marennine, produced by the diatom *Haslea ostrearia*, gives a blue-green color to the gills and labial palps of oysters, increasing their market value [30].

Other examples of the microalgae use in aquaculture are the so-called "green-water" and "pseudo green-water" techniques. The "green-water" technique refers to natural phytoplankton populations in outdoor ponds, while the "pseudo green-water" technique relates to the regular addition of selected microalgae. Both techniques allow providing favorable turbidity conditions and/or continuous nutrition to larvae or to the live food [27,31,32]. They have proven multiple benefits over the "clear water" system, for which a series of external filters allows to maintain the water quality, in terms of survival and growth of several animal species [33–36]. Indeed, "green-water" cultures can help to provide food with high nutritional value with chemical and digestive stimulants, and to improve and stabilize the quality of the culture medium [37,38]. Such culture techniques increase general health and resistance to diseases, thanks not only to a better nutrition [39], but also to the production of antimicrobial compounds by some microalgae. The "green-water" culture, and especially the "pseudo green-water" culture present many advantages as they allow direct supply of nutrients, are easy to manage, environmentally friendly and could lower the use of antibiotics in rearing systems [40].

Considering the remarkable biodiversity of microalgae and the improvement in culture, screening, extraction and purification techniques, it is likely that these microorganisms will represent an important source of new products in the future as part of blue technology. So far, bioactive compounds from cyanobacteria have been more studied than those from eukaryotic microalgae, probably due to their simpler culture methods, and have been the subject of several recent papers [41–44]. One of the major difficulties of microalgae mass culture is the bacterial contamination [45] while the culture media of cyanobacteria species studies are generally more resistant.

The present work is thus an update of previous works [1,2,23], and a complement to a recent review on freshwater microalgae [46]. It aims to present the available information about the biological activities of eukaryotic microalgae, by focusing on their (i) antibacterial; (ii) antifungal and (iii) antiviral properties, with a special interest on the activity against human pathogens and their potential application in aquaculture against various microbial diseases.

2. Antibacterial Activity from Microalgae

2.1. Antibacterial Activity from Microalgae against Human Pathogenic Bacteria

The increasing resistance of pathogenic bacteria against a significant number of antibiotics, with consequences for human health, has been a great concern for the past decades and has forced the efforts to find new antibacterial substances [6,47,48]. Some bacteria may infect and cause serious diseases in humans and some others can also provoke foodborne illness inducing moderate to severe nausea, vomiting and diarrhea. Since the pioneer work of Pratt in 1944, which demonstrated the activity of the green alga *Chlorella* against several Gram-positive (G+) and Gram-negative (G−) bacteria [49], the interest for antibacterial compounds from microalgae has been growing. Numerous studies followed to detect compounds with antibacterial activity in microalgae, either to develop new drugs against bacterial infections, or to develop additives for food preservations.

Large screening programs have thus been conducted to assess the potential antibacterial activity of various microalgal extracts against pathogenic and foodborne bacteria. Numerous microalgal species from distinct taxonomical groups originating from various areas [50–52], mainly from marine environment [53–58], but also from freshwater environment [59,60], or even from the soil [61] were shown to have potent antibacterial activity against both (G+) and (G−) bacteria (Table 1). As screening studies can sometimes include hundreds of different microalgae [51,55,59], Table 1 only presents the microalgae with the highest antibacterial activity or the wider spectrum of activity from these screenings.

Table 1. Antibacterial activity from microalgae against human pathogenic bacteria.

Microalgae Species	Antibacterial Compound/Fraction	(G+) Bacteria Growth Inhibition	(G−) Bacteria Growth Inhibition	References
Green microalgae				
Chlamydomonas reinhardtii	Aqueous or methanolic and exanolic extracts	*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*	*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*	[62]
Chlorella minutissima	Ethanolic extracts	*S. aureus*	*E. coli*, *P. aeruginosa*	[51]
Chlorella pyrenoidosa	Various organic solvent extracts	*B. subtilis*, *S. aureus*	*E. coli*, *P. aeruginosa*	[63]
Chlorella vulgaris	Chlorellin	*B. subtilis*, *S. aureus*, *Streptococcus pyogenes*	*E. coli*, *P. aeruginosa*	[49]
Chlorella vulgaris	Aqueous or methanolic and hexanolic extracts	*B. subtilis*, *S. aureus*, *S. epidermidis*	*E. coli*, *P. aeruginosa*, *S. typhi*	[62]
Chlorococcum HS-101	alpha-linolenic acid	*B. subtilis*, *Bacillus cereus*, *S. aureus*, MRSA	*Enterobacter aerogenes*	[53,56,64]
Chlorococcum humicola	Various organic solvent extracts and purified pigments (carotenoid, chlorophyll)	*B. subtilis*, *S. aureus*	*E. coli*, *P. aeruginosa*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Vibrio cholerae*	[65]
Desmococcus olivaceus	Ethanolic extracts	*S. aureus*	*E. coli*, *P. aeruginosa*	[51]
Dunaliella primolecta	Polyunsatured fatty acids: alpha-linolenic acid	*B. cereus*, *B. subtilis*, *S. aureus*, MRSA	*E. aerogenes*	[53,64]
Dunaliella salina	Indolic derivative, polyunsaturated fatty acids, beta-ionone and neophytadiene	*S. aureus*	*E. coli*, *P. aeruginosa*	[52,66,67]
Dunaliella sp.	Lysed cells	*S. epidermidis*, *Micrococcus luteus*	*Proteus vulgaris*	[68]

Table 1. *Cont.*

Microalgae Species	Antibacterial Compound/Fraction	(G+) Bacteria Growth Inhibition	(G−) Bacteria Growth Inhibition	References
Haematococcus pluvialis	Short-chain fatty acids	*S. aureus*	*E. coli*	[69,70]
Klebsormidium sp.	Pellet	*B. subtilis*	Ne	[50]
Pseudokirchneriella subcapitata	Methanolic extracts	*S. aureus*	*P. aeruginosa*	[52]
Scenedesmus obliquus	Long chain fatty acids	*S. aureus*	*E. coli*, *P. aeruginosa*, *Salmonella* sp.	[71]
Scenedesmus quadricauda	Various organic solvent extracts	*B. subtilis*, *S. aureus*	*E. coli*, *P. aeruginosa*	[63]
Scenedesmus sp.	Ethanolic extracts	*S. aureus*	*E. coli*, *P. aeruginosa*	[51]
Red microalgae				
Porphyridium aerugineum	Phycobiliproteins	*S. aureus*, *S. pyogenes*	Nt	[72]
Porphyridium purpureum	Methanolic extracts	*B. subtilis*	*E. coli*	[50]
Porphyridium sordidum	Pellet	*B. subtilis*	*E. coli*, *Pseudomonas fluorescens*	[50]
Rhodella reticulata	Exopolysaccharides	*S. aureus*, *B. cereus*, *S. pyogenes*	Ne	[72]
Diatoms				
Asterionella glacialis	Whole cell	*S. aureus*, *S. epidermidis*, *M. luteus*, *Sarcina* sp.	*E. coli*	[58]
Attheya longicornis	Methanolic extracts	*S. aureus*, MRSA	Ne	[73]
Chaetoceros muelleri	Unsaturated fatty acid-containing lipidic fractions (triglycerides and docosa-pentaenoic acid (DPA))	*B. subtilis*, *S. aureus*	*E. coli*	[74,75]
Navicula delognei (*Parlibellus delognei*)	transphytol ester, hexadecatetraenoic and octadecatetraenoic acids	*S. aureus*, *S. epidermidis*	*S. typhimurium*, *P. vulgaris*	[76]
Phaeodactylum tricornutum	eicosapentaenoic acid (EPA), palmitoleic and hexadecatrienoic acids (HTA)	*B. cereus*, *Bacillus weihenstephanensis*, *S. aureus*, *S. epidermidis*, MRSA	Ne	[77,78]
Rhizosolenia alata	Various organic solvent extracts	*B. subtilis*, *S. aureus*	*E. coli*, *P. aeruginosa*, *P. vulgaris*, *S. typhi*, *V. cholerae*	[79]
Thalassiothrix frauenfeldii	Non-axenic culture and organic solvent extracts	*B. subtilis*, *S. aureus*	*E. coli*, *P. aeruginosa*, *Salmonella paratyphi*, *S. typhi*, *V. cholerae*	[80]
Skeletonema costatum	Aqueous and organic extracts	*B. subtilis*, *S. aureus*	*P. aeruginosa*	[81]
Skeletonema costatum	Various organic solvents extracts	*S. aureus*, *Staphylococcus peoria*, *S. fecalis*, *S. pyogenes*	Ne	[54]
Haptophytes				
Isochrysis galbana	Chlorophyll a derivatives: pheophytin a and chlorophyllide a	*S. aureus*, *Streptococcus faecalis*, *S. pyogenes*, *Micrococcus* sp.	Nt	[54,82]

Ne = No effect of the microalgal compound against the bacteria tested; Nt = Not tested; MRSA = Methicillin resistant *S. aureus*.

It appeared from these studies that the production of antibiotics is largely dependent on the microalgal species [65]. The presence of antibiotic agents can vary widely between different species from the same class, even if some studies presume that the antibacterial activity is predominantly found among the members of the classes Bacillariophyceae and Chrysophyceae [54,55]. The antibacterial activity can also differ within a same species, with ecotypes adapted to different environments [83]. Indeed, the green microalga *Dunaliella* sp. isolated from highly polluted waters proved to be more active against bacteria than its ecotypes isolated from less polluted waters [68].

2.1.1. Toward Improving Extraction Techniques

Beside the microalgal species, the presence of the antibacterial compounds in the microalgal extracts is also highly dependent on the solvent used during the extraction. As the biological activity is rarely found in aqueous extracts [59,79,80], it seems that compounds with an activity against foodborne and human pathogenic bacteria in microalgae are mostly hydrophobic and can be more readily extracted with organic solvents. Some authors found that antibacterial activity was generally found in methanolic extracts [50,59], while some other studies described a better extraction using acetone [79,80], benzene and ethyl acetate [65] or petroleum ether and hexane [66].

Other techniques have been tested to extract bioactive compounds from microalgae, such as supercritical CO_2, pressurized liquid extraction (PLE) or subcritical water extraction (SWE). These techniques are considered as "greener" than the traditional ones as they do not need large quantities of organic solvents, allow a faster extraction and are more selective toward the compounds of interest [84]. Supercritical CO_2 method allowed obtaining lipid fractions from *Chaetoceros muelleri* with antibacterial activity against *Staphyloccocus aureus* and *Escherichia coli* [75], while a classic extraction with solvents such as hexane, dichloromethane and methanol did not demonstrate any activity against *E. coli* [74]. PLE and SWE permitted to extract antimicrobial agents from *H. pluvialis* in the red phase with good efficiency [66,69,70]. SWE presents the advantage of not requiring the use of toxic solvents, and low temperatures such as 50 °C can allow a good extraction. SWE could therefore represent an interesting green technique to obtain extracts for natural ingredients, and particularly for the food industry.

2.1.2. Diversity of Antibacterial Compounds Extracted from Microalgae

In some studies, the antibacterial compounds present in the organic extracts were characterized. These bioactive compounds can be pigments, such as phycobiliproteins [72] or chlorophyll derivatives [82,85], but they are most of the time free fatty acids. Short chain fatty acids from *H. pluvialis* [69,70] and long chain fatty acids from *Scenedesmus obliquus* [71] present antibacterial activity against *E. coli* and *S. aureus.* The polyunsaturated fatty acids from *Chlorococcum* strain HS-101 and *Dunaliella primolecta* demonstrated antibacterial activity against the methicillin-resistant *S. aureus* (MRSA), a bacterium causing infections that kill thousands of people per year and which can be highly resistant to conventional antibiotics [64]. Desbois et al. also found fatty acids from the diatom *Phaeodactylum tricornutum* with a very potent antibacterial activity against the MRSA and have characterized three different unsaturated fatty acids involved in the antibacterial activity: the polyunsaturated fatty acid eicosapentaenoic acid (EPA), the monounsaturated fatty acid palmitoleic acid (PA) and the relatively unusual polyunsaturated fatty acid hexadecatrienoic acid (HTA) [77,78]. It has also been observed that the fusiform morphotype of this diatom produced greater levels of EPA, PA and HTA compared to its oval morphotype [86].

In natural environments, fatty acids are released when the microalgal cell loses its integrity and they seem to be involved in an "activated" defense mechanism to protect an algal population against grazing predators [87] and when pathogenic bacteria are around the algae [26]. Moreover, it has previously been shown that fatty acids possess bactericidal properties against a diverse range of bacteria [88]. The exact mechanism of the antibacterial activity of the bioactive compounds is not yet fully elucidated, but it seems that bacterial cellular membranes would be the main site of action [89]. There is some evidence of deleterious effects of fatty acids in the bacterial membrane, causing a cell leakage, a reduction of the nutrient intake and a reduction of the cellular respiration [26]. The antibacterial action of fatty acids may also be mediated by the inhibition of bacterial fatty acid synthesis [87].

These mechanisms could explain why (G+) bacteria are more susceptible to microalgae bioactive compounds than (G−) bacteria. In fact, the bacterial growth inhibition is generally lower when microalgae are tested against (G−) bacteria, and in some cases the tested extracts do not present any bactericidal effect. As examples, the phycobiliproteins and exopolysaccharides from the red microalgae *Porphyridium aerugineum* and *Rhodella reticulata* respectively, were active against the

(G+) bacteria *S. aureus* and *Streptoccocus pyogenes* but presented no effect against the (G−) bacteria *E. coli* and *Pseudomonas aeruginosa* [72]. The diatom *P. tricornutum* did not demonstrate antibacterial effect against these two (G−) bacteria either, whereas a good antibacterial activity against the (G+) MRSA was observed [78]. Thus, the difference in sensitivities between bacteria may be due to their complex membrane permeability, making the penetration and the bactericidal action of the compound more difficult.

The potent activity of microalgal compounds, especially free fatty acids, against various bacteria straightens further development in the search for drugs and food preservatives from microalgae. Bacterial resistance to free fatty acids has not been encountered yet [90,91], so their exploitation in medicine deserves to be further investigated [78]. Furthermore, as consumers tend to avoid synthetic additives, microalgae could be good candidates as natural sources against food-borne pathogens.

2.2. Use of Microalgae against Pathogenic Bacteria in Aquaculture

Bacteria are nowadays considered as main causes of infections in intensive aquaculture of finfish and shellfish worldwide [92], occurring as well in hatcheries and nurseries as in rear and grow-out ponds. These infections can lead to serious mass mortalities and imply considerable economic losses. A wide range of bacteria are known to infect farmed and wild species with minor to severe consequences on health and survival. Among the important bacterial diseases in finfish we can cite those caused by *Aeromonas* sp. and *Pseudomonas* sp., inducing hemorrhages in many fresh-water species, or *Vibrio* sp. inducing vibrioses in marine fish species [93]. *Vibrio* are able to infect a wide variety of hosts [94] and are also the most common and harmful shrimp pathogenic bacteria [95], with luminous species such as *Vibrio harveyi* involved in mass mortalities in shrimp hatcheries [96]. *Vibrio* sp. have also been demonstrated to be involved in a large number of massive mortalities in bivalve hatcheries [97–101].

Antibiotics have been largely used in intensive aquaculture, as well for finfish [102,103], shrimp [104–107] or shellfish cultures [108,109]. However, as for humans and terrestrial animals, bacterial resistance in aquaculture is increasing and most antibiotics are less effective [110–112]. The presence of drug residues in tissues of aquatic animals and the risk of transferring resistant bacteria to humans have led to a great concern about the use of antibiotics for public health [113]. A few antibiotics are now licensed in aquaculture due to the establishment of strict regulations by the European Conformity (EC) or the Food and Drug Administration (FDA), such as the regulation (EC) No 470/2009 of the European Parliament and the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin. Various alternative and natural compounds are available today to control aquatic pathogenic bacteria, mainly derived from plants. They present the advantages of decreasing the side effects observed with synthetic antibiotics, being less expensive [114]. In this context, several microalgae species have been investigated for their antibacterial activity in vitro, and in co-culture with pathogenic bacteria, and some studies have also been conducted in vivo with the "green water" technique and using microalgae as food supplements (Table 2).

Table 2. Antibacterial activity from microalgae against diseases in aquaculture.

Microalgae Species	Compound/Fraction Tested	Target Bacteria/Antibacterial Effect	References
In vitro experiments			
Chaetoceros lauderi	Whole cell	*Vibrio anguillarum*, *Aeromonas salmonicida*	[58]
Dunaliella tertiolecta	Aqueous extract	*Vibrio campbellii*	[115]
Euglena viridis	Organic solvent extracts	*Aeromonas hydrophila*, *Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida Vibrio alginolyticus*, *V. anguillarum*, *Vibrio fluvialis*, *Vibrio harveyi*, *Vibrio parahaemolyticus*	[116]
Haslea karadagensis	Purified pigment (intra- and extracellular forms)	*Polaribacter irgensii*, *Pseudoalteromonas elyakowii*, *Vibrio aestuarianus*	[117]

Table 2. *Cont.*

Microalgae Species	Compound/Fraction Tested	Target Bacteria/Antibacterial Effect	References
Haslea ostrearia	Purified marennine (intra- and extracellular forms)	*P. irgensii*, *P. elyakowii*, *V. aestuarianus*	[118]
	Purified marennine (intracellular form)	*V. anguillarum*	[119]
	Purified marennine (extracellular form)	*Vibrio splendidus*-related	[30]
Phaeodactylum tricornutum	Aqueous and organic extracts	*Alcaligenes cupidus*, *Alteromonas communis*, *Alteromonas haloplanktis*, *Vibrio fischeri*, *V. parahaemolyticus*	[81]
	Polyunsaturated free fatty acid	*Vibrio anguillarum*, *M. luteus*, *Photobacterium* sp.	[78]
Skeletonema costatum	Aqueous and Organic extracts	*A. cupidus*, *A. communis*, *Pseudomonas marina*, *V. fischeri*, *V. parahaemolyticus*	[81]
	Organic and purified extracts	*V. anguillarum*, *Vibrio mytili T*, *Vibrio* spp. *S322*, *Vibrio* spp. *VRP*	[120]
	Aqueous extracts	*Vibrio campbellii*	[115]
Stichochrysis immobilis	Microalgal homogenates	*Xanthomonas* sp. *1*, *Flavobacterium* sp. *1*, *Pseudomonas* sp. *Strain 101*	[121]
Tetraselmis suecica	Microalgal supernatant and microalgal homogenates of a commercial spray-dried preparation	*A. hydrophila*, *A. salmonicida*, *Serratia liquefaciens*, *V. alginolyticus*, *V. anguillarum*, *V. parahaemolyticus*, *Vibrio salmonicida*, *Vibrio vulnificus*, *Yersinia ruckeri*	[122,123]
Co-culture experiments			
Chaetoceros calcitrans	Axenic culture	*V. harveyi*	[124]
Chlorella minutissima	Axenic culture	*V. alginolyticus*, *V. anguillarum*, *Vibrio lentus*, *V. parahaemolyticus*, *Vibrio scophthalmi*, *V. splendidus*	[125]
Chlorella sp.	Axenic culture	*V. harveyi*	[40]
Isochrysis galbana	Non-axenic culture	*V. alginolyticus*, *V. campbellii*, *V. harveyi*	[126]
Isochrysis sp.	Axenic culture	*V. alginolyticus*, *V. lentus*, *V. splendidus*, *V. scophthalmi*, *V. parahaemolyticus*, *V. anguillarum*	[125]
Nannochloropsis sp.	Axenic culture	*V. alginolyticus*, *V. anguillarum*, *V. lentus*, *V. parahaemolyticus*, *V. scophthalmi*, *V. splendidus*	[125]
Nitzchia sp.	Axenic culture	*V. harveyi*	[124]
S. costatum	Exometabolites in the culture fluid	*Listeria monocytogenes*	[127]
	Axenic culture	*Pseudomonas* sp., *Vibrio* sp.	[128]
Tetraselmis chui	Axenic culture	*V. alginolyticus*, *V. anguillarum*, *V. lentus*, *V. parahaemolyticus*, *V. scophthalmi*, *V. splendidus*	[125]
In vivo experiments			
C. minutissima	30 min incubation of enriched *Artemia metanauplii*	Decrease of the bacterial load in *Artemia* and diminution of presumptive *Vibrio*	[129]
D. tertiolecta	Daily diet of *Artemia franciscana*	Protection against *V. campbellii* and *V. proteolyticus*	[39]
H. ostrearia	Incubation of *Mytilus edulis* larvae with supernatant containing extracellular pigments	Higher survival and physiological conditions of larvae challenged with *V. splendidus*-related	[130]
Tetraselmis sp.	4 h incubation of *Artemia franciscana*	Diminution of associated bacteria, better bacterial diversity and the flora less dominated by *V. alginolyticus*	[131]
T. suecica	Food supplement for the Atlantic salmon *Solmo salar*	Reduction of *A. hydrophila*, *A. salmonicida*, *Serratia liquefaciens*, *V. anguillarum*, *V. salmonicida*, *Y. ruckeri* infections, reduction of bacterial populations in water tanks and increase of the microbial communities in the digestive tract	[123]
	Food supplement for the broodstock and partial live larvae feed for the white prawn *Fenneropenaeus indicus*	Reduction of *Vibrio* numbers in the water tank, better egg hatching rate and survival rate of the larvae	[132]

2.2.1. Benefits of the "Green-Water" Technique against Bacterial Diseases in Aquaculture

The "green water" technique has demonstrated beneficial effects on health, survival rates and resistance of different organisms [32–37,39]. Addition of the microalga *Isochrysis galbana* allowed a better viability and a faster grow of the turbot *Scophthalmus maximus* larvae as well as a lower proliferation of opportunistic bacteria [133]. The green microalga *Tetraselmis suecica* first showed good antibacterial activity in vitro against several *Vibrio* species [122] and then proved to reduce in vivo the number of various bacterial species in the water tank of the Atlantic salmon *Salmo salar* [123]. Adding *T. suecica* also reduced the number of *Vibrio* species in broodstock gut, eggs and larvae of the white prawn *Fenneropenaeus indicus*, resulting in improved egg hatching and larval survival [132]. A better survival and physiological conditions of the blue mussel *Mytilus edulis* larvae and the scallop *Placopecten magellanicus* larvae were observed when incubated with supernatant containing marennine, the extracellular blue pigment of the diatom *H. ostrearia*, at concentrations as low as 0.1 mg/L [130]. The use of the diatoms *Skeletonema costatum* and *Chaetoceros calcitrans* to feed *Penaeus monodon* larvae allowed a growth inhibition of the luminous bacteria *Vibrio harveyi* [134]. However, this seemed mainly due to the microbial flora associated with the diatoms rather than their metabolic products. Indeed, the effectiveness of the "green water" culture in preventing bacterial infections and outbreaks can also be attributed to the presence of antibacterial factors in the bacterial, fungal and phytoplankton microbiota associated with this culture technique [124].

Yet, several co-culture experiments with axenic microalgae demonstrated the ability of some species to produce and release compounds with potent activity against pathogenic bacteria. Axenic cultures of *Chlorella minutissima*, *Tetraselmis chui*, *Isochrysis* sp. and *Nannochloropsis* sp. limited the growth of various *Vibrio* species [125], and growth of the luminous bacteria *V. harveyi* highly decreased when co-cultured with pure *Chlorella* sp. [40], *C. calcitrans* or *Nitzschia* sp. [124].

Finally, genetically modified microalgae were also used in vivo. In a recent study, better growth, resistance to bacteria and survival rate were observed in the shrimp postlarvae *P. monodon* fed with "fusant" *Chlorella* and *Dunaliella* [135]. The so-called "fusant" microalgae resulted from the generation of a unique cell through somatic hybridization by fusion of the two protoplasts. This fusion technology is especially used to transfer agronomically useful traits to plants [136] and would allow an improvement of valuable metabolites production from these two microalgae. Another genetically modified microalga tested in "green water" systems is the transgenic line of *Nannochloropsis oculata*, able to produce the bovine antimicrobial lactoferricin (LFB) peptide. These LFB-containing transgenic microalgae were developed and tested as food supplement for the medaka fish *Oryzias latipes* infected with *Vibrio parahaemolyticus*, which displayed a significantly better survival rate after 24 h [137].

2.2.2. Microalgae to Improve the Live-Food Quality

Microalgae also show advantages for the live-food quality [39,138], by reducing the number of associated pathogenic bacteria such as *Vibrio* and allowing a lower risk of transmission to fish larvae. Daily addition of *Dunaliella tertiolecta* to feed the brine shrimp *Artemia franciscana*, considered as an essential part of the live food chain for the culture of fish, conferred a full protection against *Vibrio campbellii* and *Vibrio proteolyticus* [39]. A 4 h incubation of *A. franciscana* with the microalgae *Tetraselmis* sp. resulted in a diminution by 75% of associated bacteria, with a better bacterial diversity and the flora less dominated by *Vibrio alginolyticus* [131]. Similar observations were made by Makridis et al. with incubation of *Artemia* with *T. chui* and *C. minutissima* [129]. These authors proposed that the reduction of *Vibrio* cells in *Artemia* cultures could either be due to compounds released by microalgae or to (G+) associated bacteria.

2.2.3. In Vitro Efficiency of Microalgal Compounds against Marine Bacteria

Several in vitro studies demonstrated the ability of various microalgae to produce antibacterial compounds effective against relevant marine pathogenic bacteria. The whole cells of *Chaetoceros*

lauderi [58], supernatant and homogenates of *T. suecica* [122,123] or homogenates from *Stichochrysis immobilis* [121] induced a growth inhibition of various marine bacteria. However, Berland et al. noted that limited attention should be paid to antibacterial activity obtained with broken cells, as in natural conditions substances synthesized have to be released into the water to target another organism [121]. Microalgae producing antibacterial compounds that are not released in the medium cannot indeed be considered gainful in "green water" techniques, but these compounds can be highly useful in the design of novel drugs.

Only a few compounds with activity against marine bacteria have been characterized, such as the polyunsaturated free fatty acid in *P. tricornutum*, identified as eicosapentaenoic acid [78]. An antibacterial activity was also demonstrated in vitro with the blue pigment of the two diatoms *H. ostrearia* and *Haslea karadagensis.* These blue pigments can be observed in the apex of the microalgae (intracellular form) but can also be released in the medium (extracellular form). The purified forms of these pigments [139] have been shown to inhibit the growth of several marine bacteria including *Vibrio* such bacteria belonging to *Vibrio splendidus* clade or *Vibrio aestuarianus* species [30,117–119], involved in oysters mass mortality [140–142].

A series of experiments were conducted to assess the spectrum of activity of purified extracellular marennine, the pigment produced by *H. ostrearia*, against pathogenic *Vibrio* species. The growth of *V. splendidus*-related strain (*Vibrio tasmaniensis* LGP32 (CIP107715)) was slowed down when exposed to a concentration of marennine up to 10 μg per mL and seemed totally inhibited with a concentration of 1 mg per mL (Figure 1).

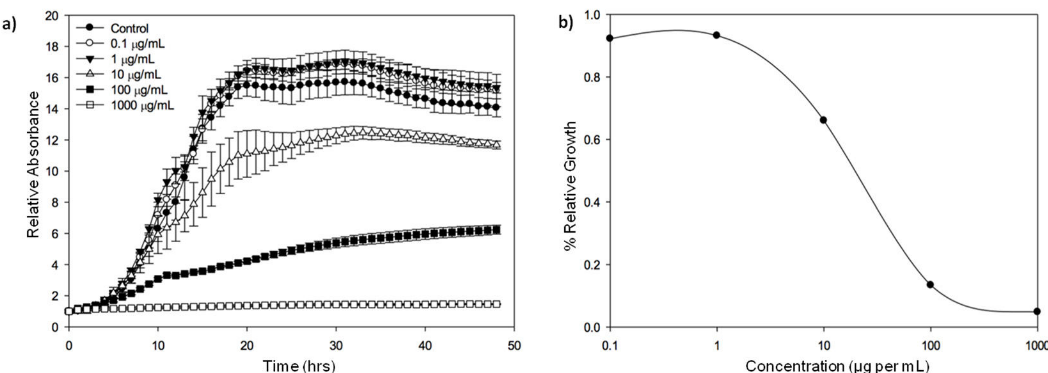


Figure 1. Growth inhibition of *Vibrio tasmaniensis* CIP 107715 by purified marennine, the blue pigment produced by *Haslea ostrearia*. (**a**) *V. tasmaniensis* was grown over night at 25 °C, cells were then washed with sterile water 2 times and adjusted to an OD_{600} = 0.5. Cells were added to wells with marennine in the following concentrations: 0, 0.1, 1, 10, 100 and 1000 μg per mL. Kinetics were run at OD_{600} for 48 h, with measurements taken every 30 min (n = 3); (**b**) Relative values were graphed in order to account for the absorbance differences due to the pigment. The effective concentration reducing bacteria growth rate by 50%, EC_{50}, was estimated at 19.14 μg per mL (Standard error: 6.73) (original results).

In another series of experiments, a comparative approach combining several species and strains was chosen to reflect the diversity of strains within species. The sensitivity of various strains recognized for their virulence, of *V. aestuarianus* [143], *Vibrio coralliilyticus* [144] and *Vibrio tubiashii* [145] to extracellular marennine purified as previously described [139] was assessed by exposing the bacteria to marennine concentration ranging from 1 to 100 μg per mL. For all the three species, after 48 h, the higher the marennine concentration, the higher was the bacterial growth inhibition (data not shown), confirming the antibacterial activity of the pigment produced by the diatom *H. ostrearia*. Moreover, when comparing different ecotypes within a same *Vibrio* species, the sensitivity of the strains exposed to marennine could be significantly different (Figure 2). It can be noted that the strains coming from collections, used to describe the species and often isolated besides mortality events, seemed more sensitive to marennine comparing to virulent strains. Complementary experiments are under way in

order to confirm and precise the biological effect of marennine on these *Vibrio* species and to highlight the variability in sensitivity of different strains within a same species.

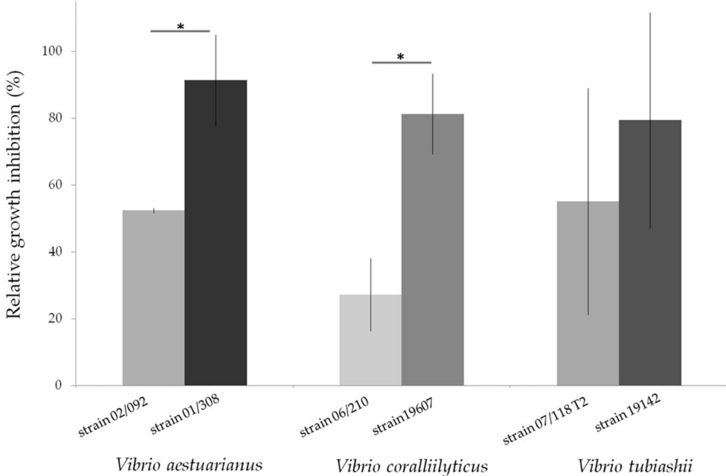


Figure 2. Relative growth inhibition of three *Vibrio* species, *V. aestuarianus*, *V. coralliilyticus*, *V. tubiashii*, after a 48 h exposition to purified extracellular marennine, produced by the diatom *Haslea ostrearia.* Each strain was grown over night in a Mueller-Hinton Broth medium at 22 °C and their concentration was then adjusted to an OD_{600} = 0.1. Bacterial cultures were exposed for 48 h to marennine at a concentration of 100 µg per mL before OD measurement. The relative growth inhibition was assessed in comparison with the growth of the control, not exposed to marennine. Results are means ± SD, for two separate experiments conducted using triplicates. A significant difference of sensitivity (*) between the two *V. aestuarianus* and *V. coralliilyticus* strains was observed (ANOVA statistical test, *p*-value 0.01 and 7×10^{-4} respectively). (original results).

2.2.4. Interactions between Microalgae and Marine Bacteria

The antibacterial mechanisms of action of microalgae are still unclear and the bioactive compounds released by the cells could either be bactericidal or prevent the bacterial multiplication. The very rapid growth inhibition of various *Vibrio* species in co-culture with *Chlorella* sp., *Isochrysis* sp. or *Nannochloropsis* sp. with no recovery after few days [40,125] allows considering a bactericidal action of the extracellular substances produce by some microalgae [126]. Austin et al. also observed a prompt inhibition of several *Vibrio* species by *T. suecica* in vitro and noticed a very rapid decrease in bacterial mobility with an elongation and vacuolisation of the cells in less than 20 min. Though, a reduction of the inhibitory activity was observed after only 5 h. It was suggested that the bioactive substance could have been denatured or adsorbed by some bacterial cells, allowing others to grow [122,123].

In some cases, the antibacterial activity of microalgae can be induced by the presence of bacteria in the vicinity of the microalgae, or can be constitutive and always present in the algal culture medium [61]. The constitutive production of antibacterial exometabolites by some microalgae was highlighted with the growth diminution of *Listeria monocytogenes* in co-culture with the cell-free culture media of *S. costatum* [127].

Microalgae can influence marine bacteria in different ways. They can either inhibit or stimulate bacterial growth, or have no apparent effect, depending on the target bacteria [121,128]. As an example, the diatom *S. costatum* inhibits the growth of *Pseudomonas* and *Vibrio* in co-culture, but enhances the growth of *Flavobacterium* [128]. The production of antibacterial compounds by microalgae such as lipids or fatty acids varies according to the taxonomic group, the growth conditions, the available nutrients and their concentration in the medium, the light intensity, the temperature or the pH. The development stage of the algal culture is also highly significant as it is assumed that various secondary metabolites are produced and released in the medium at different growth phases [1]. Terekhova et al. showed

that only the exometabolites produced by *S. costatum* during the middle steady-state growth phase presented an antibacterial activity against *L. monocytogenes* while compounds released during the exponential growth phase had no effect on these bacteria [127]. Cooper et al. have also demonstrated the direct relation between cell growth phase and antibacterial activity, and showed that *P. tricornutum* presented a better activity against a wide spectrum of marine bacteria during the exponential growth phase compared to the stationary phase, while the reverse relationship was found for *S. costatum* [81].

3. Antifungal Activity from Microalgae

3.1. Antifungal Activity from Microalgae against Human Pathogens

The search for antifungal compounds from microalgae started much later than screening for antibacterial activity. As a matter of fact, fungi have been considered as harmful human pathogens since the 1970s, when mortality induced by fungal infections and the frequency of nosocomial mycoses increased in hospitalized patients. Increase of fungal infections was mainly due to therapies that depress patients' immune system such as the use of intensive and aggressive chemotherapy regimens, the expansion of organ transplant programs and the spread of the AIDS epidemic. [146,147]. The incidence of invasive aspergillosis (induced by *Aspergillus* species), and the number of cases of candidemia (an infection and a disease caused by *Candida* species), have been rising inexorably from that time [148,149]. The growing use of antifungal agents in recent years has led to the development of drug resistance [150,151]. Thus, there is a need for novel drugs and several studies were recently conducted to find fungicide activity from natural marine products against human pathogenic fungi [152–157], including antifungal agents from microalgae (Table 3).

There are fewer screening activities for microalgal fungicides than for bactericides, and most of the studies focus not only on the antifungal activity but also on antibacterial activities [50,51,58,60,63,65,72,80,117,158,159]. As for antibacterial activity, antifungal activity varies widely depending on microalgal species, type of solvent used to extract the compound and the microorganism tested. It does not seem to be a taxonomic trend for the antifungal activity, and the capability to produce antifungal compounds would have evolved independently of phylogenetic relationship in microalgae [50]. However, Pesando et al. noticed a significant activity of the genus *Chaetoceros* [160] and Kellam et al. also indicated that marine microalgae showed more potential in the search for new antifungal agents than freshwater species [161].

Table 3. Antifungal activity from microalgae.

Microalgae Species	Antifungal Compounds/Fraction	Target Microorganims	References
Green microalgae			
Chlorella vulgaris	Microalgal supernatant	Yeast: *Candida kefyr* Mold: *Aspergillus fumigatus*, *Aspergillus niger*	[62]
Chlorococcum humicola	Organic solvent extracts and pigments: beta carotene, Chlorophyll a and Chlorophyll b	Yeast: *C. albicans* Mold: *A. flavus*, *A. niger*	[65]
Heterochlorella luteoviridis	Microalgal supernatant	Yeast: *C. albicans*	[50]
Haematococcus pluvialis	Short-chain fatty acids	Yeast: *C. albicans*	[70]
Scenedesmus quadricauda	Organic solvent extracts	Yeast: *C. albicans*, *S. cerevisiae* Mold: *A. flavus*, *A. niger*, *P. herquei* Other: *A. brassicae*, *F. moniliforme*, *Helminthosporium* sp.	[63]
Red microalgae			
Porphyridium aerugineum	Phycobiliproteins	Yeast: *C. albicans*	[72]
Porphyridium purpureum	Microalgal supernatant	Yeast: *C. albicans*	[50]
Rhodella reticulata	Exopolysaccharides	Yeast: *C. albicans*	[72]

Table 3. *Cont.*

Microalgae Species	Antifungal Compounds/Fraction	Target Microorganims	References
Diatoms			
Chaetoceros lauderi	Polysaccharides	Mold: *A. fumigatus*, *Blastomyces dermatitidis*, *Emmonsia parva*, *Madurella mycetomi*, *Sporothrix schenckii* Dermatophyte: *Epidermophyton floccosum*, *Microsporum audouini*, *Microsporum canis*, *Microsporum ferrugineum*, *Microsporum gypseum*, *Microsporum nanum*, *Microsporum persicolor*, *Trichophyton* spp.	[58,162,163]
Chaetoceros muelleri	Lipidic fractions: triglycerides, docosapentaenoic acid (DPA)	Yeast: *C. albicans*	[75]
Haslea karadagensis	Purified pigment (intra- and extracellular forms)	*Corollospora maritima*, *Lulworthia* sp., *Dendryphiella salina*	[117]
Thalassiothrix frauenfeldii	culture filtrates and organic solvent extracts	Yeast: *C. albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Cryptococcus neoformans* Mold: *A. niger*	[80]
Dinoflagellates			
Amphidinium sp.	Polyols: karatungiols A(1)	Mold: *A. niger*	[164]
Gambierdiscus toxicus	Gambieric acids A and B forms	Mold: *A. fumigatus*, *A. niger*, *Aspergillus oryzae*, *Penicillium citrinum*, *Penicillium chrysogenum*, *Paecilomyces variotii* Dermatophyte: *E. floccosum*, *T. mentagrophytes*	[165]
Goniodoma pseudogonyaulax	Goniodomin A (polyether macrolide)	Yeast: *C. albicans*, *C. neoformans*, *S. cerevisiae* Mold: *Penicillium* sp. Dermatophyte: *T. mentagrophytes*	[166,167]
Prorocentrum lima	Polyethers	Yeast: *Candida rugosa* Mold: *A. niger*, *Penicillium funiculosum*	[159]

As illustrated in Table 3, several antifungal compounds from various microalgae have been characterized. Polysaccharides with high molecular weight were identified in the diatom *C. lauderi*. They presented a large spectrum of activity against dermatophytes, moulds and phyto-fungi, but no activity was detected against the yeasts tested [58,162,163]. Gambieric acids from the dinoflagellate *Gambierdiscus toxicus* also had an antifungal activity against several dermatophytes and moulds but showed no activity against yeasts like *Candida albicans* or *Saccharomyces cerevisiae* [165]. The diatom *Thalassiothrix frauenfeldii* was meanwhile active against yeasts and moulds but not against dermathophytes [80]. Other compounds such as pigments like beta-carotene, chlorophyll-a and chlorophyll-b from *Chlorococcum humicola* [65], or phycobiliproteins from *Porphyridium aerugineum* have also demonstrated antifungal activities [72]. The polyene-polyhydroxy metabolites amphidinols were extracted from the dinoflagellate *Amphidinium klebsii* [168,169]. Polyenes are metabolites known for having a potent antifungal activity as they target the biosynthetic pathway of ergosterols, found in fungi membranes [150]. These few results illustrate that the search for novel antifungal compounds from microalgae has not been greatly developed so far, although an increasing number of fungi display drug resistance phenomenon.

3.2. Potential Use of Microalgae against Fungal Diseases in Aquaculture

An increase in fungal infections has been observed in the last few decades with the modernization and intensification of aquaculture at an industrial scale, resulting in huge losses for aquaculture

industries. Fungal infections in aquaculture may cause severe diseases and mortality events leading to economic losses. They are often considered secondary to other factors or pathogens such as consequences of water quality problems, fish trauma by rough handling or temperature shock, bacterial diseases or parasites [170]. Several fungi are known to induce diseases by developing in the skin and the gills of the infected fish or in eggs, or by producing toxins [171]. Indeed, mycotoxins can provoke many disorders and can accumulate in fish tissues, representing a risk for public health [172].

Chemicals used to treat infected animals are limited and, due to the increasing resistance of fungi against conventional drugs, environmental legislations and consumer's safety, the alternative "herbal formulations" and alternative safe and cheap methods have become of renewed interest [173]. Studies have been conducted in order to find new antimycotics of natural origin such as plant extracts [174–176] or essential oils [177,178], which should have no harmful effect on fish, fish eggs, human health and ecosystems.

So far, very few studies have been conducted to assess the antifungal activity from microalgae against pathogenic fungi in aquaculture systems (Table 3). Gastineau et al. have demonstrated in vitro the antifungal activity of the pigment produced by the diatom *H. karadagensis* against three marine fungal species *Corollospora maritima*, *Dendryphiella salina*, *Lulworthia* sp., which can be involved in the phenomenon of biofouling [117]. Organic extracts of the green microalgae *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* have demonstrated antifungal activity against *Fusarium monofiliform* [63] reported of causing black gill disease in shrimps [179]. This result is of great interest as *Fusarium* sp. were recently shown to produce toxins that accumulate in fish [180]. *Aspergillus fumigatus* is also a fungus susceptible to produce toxins and it thus represents a potential contaminant for seafood, particularly for marine bivalves [181]. It can be inhibited by compounds produced by *C. lauderi* [58], *G. toxicus* [165], and *Chlorella vulgaris* [62].

More generally, fungi from the taxa *Fusarium* and *Aspergillus* are found in many countries in water, sediments and marine invertebrates, and their presence in shellfish farming areas evidences the necessity to pay attention to shellfish contamination by such fungi [182]. Furthermore, reports of fungi causing deleterious effects are frequently related [183,184], which encourage a larger screening of microalgae for the production of novel antifungal compounds.

4. Antiviral Activity from Microalgae

4.1. Antiviral Activity from Microalgae against Pathogenic Human Viruses

Viral pathogens are the leading cause of human diseases and mortality worldwide. Treatments to block the entry of the virus or its replication directly are difficult to design, as they can have adverse effects on the infected host cells [185]. Thus, treatments against diseases caused by viruses are limited, and resistance to these available treatments demonstrate the need for new medicines [186]. Many drugs exhibiting selective inhibition of mammalian originate from synthetic organic chemicals or from natural products, for instance secondary metabolites in plants [185]. Along with the development of "blue" technology and extraction improvement, there is a growing interest in marine-derived antiviral compounds. Thus, thousands of compounds from various marine organisms such as algae, bacteria, fungi, marine invertebrates or sponges have been screened [153,186–188] and some of them have demonstrated antiviral activities and are commercially available.

Potential antiviral activity from algal compounds has been first demonstrated in the 1950s by Gerber et al. who observed that the polysaccharides extracted from *Gelidium cartilagenium* afforded protection for embryonated eggs against influenza B and mumps viruses [189], and the first brood-based studies of seaweeds for their antiviral substances started in the 1970s [190]. Screenings for antiviral compounds from macroalgae are still predominant [191–195], but the interest for antiviral compounds from microalgae and cyanobacteria rapidly increased as they also present relevant antiviral activities and are easier to culture. Cyanobacteria are promising sources of antiviral compounds, and their simple growth needs make them good candidates for the production of antiviral

agents at an industrial scale [44,196]. The sulphated polysaccharide isolated from the cyanobacteria *Spirulina platensis*, named spirulan, has demonstrated potent antiviral activity against the herpes simplex virus type 1 (HSV-1) and also against the human immunodeficiency virus type 1 (HIV-1) [197]. A spirulan-like molecule isolated from *Arthrospira platensis* has also been indicated to possess antiviral activities against these two viruses, with absence of cytotoxic effects [198].

Several studies have been conducted to test microalgae compounds against pathogenic human viruses (Table 4). Antiviral compounds extracted from microalgae are mainly polysaccharides. Their mechanisms of action against viruses are not fully understood but it seems that they can inhibit different stages of the viral infection, such as the adhesion, the penetration or the replication. Polysaccharides have gained interest in the biomedical and pharmaceutical industries as they are easily available in nature and most of them are nontoxic, safe, biodegradable and biocompatible [199]. However, numerous polysaccharides with antiviral activities were not developed for clinical use. The reasons are probably the very high molecular weights of some polysaccharides, preventing them to pass through the different barriers of the body and the incapacity of enzymes to digest these large and complex molecules, leading to their accumulation in the body and to potential cytotoxic effects [200].

Table 4. Antiviral activity from microalgae.

Microalgae Species	Antiviral Compound and Cytotoxicity (µg/mL)	Target Virus	Mechanism of Action and Efficiency (µg/mL)	References
Green microalgae				
Chlorella vulgaris	Polysaccharide-rich fraction CC_{50} > 1600 (Vero cells)	HSV-1	Inhibits attachment, replication	[201]
			IC_{50} = 61	
Dunaliella primolecta	Pheophorbide-like compound Not cytotoxic (Vero cells)	HSV-1	Inhibits adsorption, invasion	[202]
			MIC = 5 (totally inhibit the CPE)	
Dunaliella salina	Short chain fatty acids, β-ionone, neophytadiene, phytol, palmitic and α-linolenic acids CC_{50} = 1711 (Vero cells)	HSV-1	Inhibits infectivity	[203]
			IC_{50} = 85	
Haematococcus pluvialis	Polysaccharide-rich fraction CC_{50} = 1867 (Vero cells)	HSV-1	Inhibits attachment, penetration, replication	[203]
			IC_{50} = 99	
Red microalgae				
Porphyridium cruentum	Sulphated exopolysaccharide Not cytotoxic at 100 (HeL cells)		Inhibits penetration, replication	[204]
		HSV-1	EC_{50} (HSV-1) = 34	
		HSV-2	EC_{50} (HSV-2) = 12	
		Vaccina	EC_{50} (Vaccina) = 12	
Porphyridium purpureum	Exopolysaccharide Not cytotoxic at 500 (HEp-2 cells)	Vaccina	Interaction with free viral particles	[205]
			IC_{50} = 0.65	
Porphyridium sp.	Sulphated polysaccharide Not cytotoxic at 250 (Vero cells) and 2000 (in vivo in rats)	HSV-1	In vitro: inhibits adsorption, replication	[200,206]
			CPE_{50} = 1	
			In vivo: prevents the development of symptoms at 100	
			Inhibits adsorption, replication	
		HSV-2	CPE_{50} (HSV-2) = 5	
		VZV	CPE_{50} (VZV) = 0.7	
Porphyridium sp.	Purified polysaccharide Not cytotoxic at 1000 (NIH/3T3 cells)	MuSV/MuLV	Inhibits the production of retroviruses in the cells	[207]
			RT_{50} reduction = 5	
		MuSV-124	Inhibits cell transformation	
			ffu_{50} protection = 10	

Table 4. *Cont.*

Microalgae Species	Antiviral Compound and Cytotoxicity (μg/mL)	Target Virus	Mechanism of Action and Efficiency (μg/mL)	References
Diatoms				
Haslea karadagensis	Purified pigment: intra- and extracellular forms	HSV-1	Inhibits infection, cell destruction	[117]
	CC_{50} (Int) = 87		EC_{50} (Int) = 62	
	CC_{50}(Ext) > 200 (Vero cells)		EC_{50} (Ext) = 23	
Haslea ostrearia	Purified pigment: intra- and extracellular forms	HSV-1	Inhibits infection, cell destruction	[117]
	CC_{50} (Int) > 200 (Vero cells)		EC_{50} (Int) = 24	
	CC_{50} (Ext) = 107 (Vero cells)		EC_{50} (Ext) = 27	
	Water soluble extract CC_{50} > 200 (Vero and MT-4 cells)	HSV-1	Inhibits replication	[208]
			EC_{50} = 14	
Navicula directa	Sulphated polysaccharide: Naviculan		Inhibits adhesion, penetration	[209]
	CC_{50} (HSV-1) = 3800 (Vero cells)	HSV-1	IC_{50} (HSV-1) = 14	
	CC_{50} (HSV-2) = 3800 (Vero cells)	HSV-2	IC_{50} (HSV-2) = 7.4	
	CC_{50} (IFV-A)= 5400 (MDCK cells)	IFV-A	IC_{50} (IFV-A) = 170	
	CC_{50} (HIV-1) = 4000 (HeLA cells)	HIV-1	IC_{50} (HIV-1) = 53	
Dinoflagellates				
Cochlodinium polykrikoides	Extracellular sulphated polysaccharides: A1 and A2		Inhibits replication and the CPE	[210]
	CC_{50} (HIV-1) > 100 (MT-4 cells)	HIV-1	IC_{50} (HIV-1) = 1.7	
	CC_{50} (IFV-A) > 100 (MDCK cells)	IFV-A	IC_{50} (IFV-A) = 0.45–1	
	CC_{50} (IFV-B) > 100 (MDCK cells)	IFV-B	IC_{50} (IFV-B) = 7.1–8.3	
	CC_{50} (RSV-A) > 100 (Hep-2 cells)	RSV-A	IC_{50} (RSV-A) = 2–3	
	CC_{50} (RSV-B) > 100 (Hep-2 cells)	RSV-B	IC_{50} (RSV-B) = 0.8	
	A1	HSV-1	IC_{50} = 4.5	
	CC_{50} > 100 (HMV-2 cells)			
	A2	PFluV-2	IC_{50} = 0.8	
	CC_{50} > 100 (HMV-2 cells)			
Gyrodinium impudicum	Purified sulphated exopolysaccharide: p-KG03	EMCV	Inhibits the development of the CPE, suppress tumor cell growth EC_{50} = 27	[211]
	CC_{50} = 3.4 (MT-4 cells)			
	CC_{50} = 59.9 (Vero cells)			
	CC_{50} > 1000 (HeLa cells)			
	Not in MDCK cells CC_{50} > 100		Inhibits adsorption	[212]
		IFV-A	EC_{50} (IFV-A) = 0.19–0.48	
		IFV-B	EC_{50} (IFV-B) = 0.26	

EMCV: encephalomyocarditis virus; HIV-1: human immunodeficiency virus type 1; HSV-1: Herpes simplex virus type 1; HSV-2: herpes simplex virus type 2; IFV-A: influenza A virus; IFV-B: influenza B virus; MuLV: murine leukemia virus; MuSV-124: murine sarcoma virus; RSV: respiratory syncytial virus; VZV: varicella zoster virus. CC_{50}: concentration that kills 50% of the infected cells; CPE_{50}: concentration that offers 50% protection against the cytopathic effect; EC_{50}: concentration requires to inhibit 50% of the virus-induced cytopathic effects (CPE); ffu_{50}: concentration that offers 50% protection against the formation of foci of malignant cells; IC_{50}: concentration that inhibits 50% of the virus infection; MIC: minimum inhibitory concentration; RT_{50}: concentration that offers 50% reduction of reverse transcriptase activity.

Some sulphated polysaccharides present a broad antiviral spectrum against enveloped viruses. Naviculan, extracted from the diatom *Navicula directa*, or A1 and A2 extracted from *Cochlodinium polykrikoides* demonstrated to potent antiviral activity against several enveloped viruses such as HIV-1, HSV-1 or influenza virus type A (IFV-A) [210,211]. The sulphated polysaccharide p-KG03 extracted from *Gyrodinium impudicum* did not demonstrate antiviral activity against HSV-1 and HSV-2, but presented a good activity against the encephalomyocarditis RNA virus (EMCV) [211], and against several strains of influenza viruses with efficiency comparable to some existing drugs [212]. Antiviral activities of

microalgae against HSV type 1 and 2 are the most studied [117,118,200–203,206,208–210]. More than one third of the world population is affected by HSV-1 or HSV-2, infections that cause contagious diseases such as oral and genital herpes [187]. The efficiency and the low toxicity of some of the microalgal compounds tested attest their advantageous use as antiviral agents. They could help to control viral diseases occurring in humans, but also in animal species with economic value.

4.2. Potential Use of Microalgae against Viruses in Aquaculture

Aquaculture production undergoes numerous viral diseases, which can affect organism health and survival rates, and can sometimes lead to mass mortality. Viral diseases are spreading and are consequently important limiting factors for the expansion of aquaculture. Many different viruses, from various virus families, are known to infect farmed species, such as finfish, crustaceans or molluscs [213–215]. As a few relevant examples, we can cite the infectious pancreatic necrosis virus (IPNV), isolated from a very wide host range among finfish [215], the yellow head virus (YHV) and the white spot syndrome virus (WSSV) causing important losses in shrimp culture, or the ostreid herpesvirus-1 (OsHV-1) leading to high mortality in marine bivalves [216] and which can be transmitted between different bivalve species [217]. It appears that diseases induced by RNA viruses are the highest cause of ecological and socio-economic impacts in European farmed finfish [218]. Viruses in aquaculture species are either established for decades or are newly emerging because of the intensification of farming practices that facilitates rapid transmission of diseases.

Viral diseases in aquaculture are challenging to manage. They are difficult to treat directly and a few, if any, efficacious treatments are available other than destroying all organisms in infected farms and avoiding their movements to and from infected areas. In some particular cases, vaccination is used in farmed finfish, mainly to treat trout and salmon [218,219]. The vaccination issue did not arise in invertebrates, as it was widely assumed that, unlike vertebrates, they do not possess the capacity to develop long-term acquired immunity. Nevertheless, there are evidences for specific immune memory in some invertebrates [220] such as crustaceans [221], and several studies have demonstrated the antiviral protection of shrimp by "vaccination" [222,223]. A recent work has also demonstrated the presence of an antiviral system in oysters after injection of a synthetic viral analogue (Poly I:C) against OsHV-1. This immune response showed similarity with the vertebrate interferon response pathway [224]. The lack of marine mollusc cell culture not only restricts virus isolation capacities and subsequent characterization work, but also limits investigations on host-virus interaction. However, recent progress has been made with the development of stem cells in the cupped oysters *Crassostrea gigas* [225].

Plant and herbal extracts with activity against viral diseases in aquaculture production have recently been reviewed by Sivasankar et al. [226]. Some extracts have already been successfully tested in vivo, such as the plant extract of *Cyanodon dactylon* against the WSS virus of the shrimp *P. monodon* [227]. Plant extracts, acting as immunostimulants, have the advantage of being easily delivered by oral administration and may be eco-friendly as they are biodegradable.

In contrast, very few studies have been conducted to assess the antiviral activity of microalgae against viruses in aquaculture. Some polysaccharide extracts of various microalgae have been tested against the viral hemorrhagic septicaemia virus (VHSV) [228], a virus of economic importance afflicting over 50 species of fresh water and marine fish including salmonid fish [215]. Endocellular extracts of *Porphyridium cruentum*, *D. tertiolecta*, *Ellipsoidon* sp., *Isochrysis galbana* var. *Tiso* and *Chlorella autotrophica* inhibited the viral infection of VHSV in vitro in epithelioma papulosum cyprinid (EPC) cells. Concentrations lower than 2 µg of extracts per mL of *P. cruentum* and *D. tertiolecta* were sufficient to detect an antiviral activity. Exocellular extracts of these algal species were also able to inhibit the viral infection, except for *I. galbana* var. *Tiso*. This study has also demonstrated that there is no correlation between the content of sulphated polysaccharides of each microalga and its capacity to inhibit the viral replication. Thus, the observed antiviral effects would be due to different polysaccharide molecular species with differences in molecular size.

A higher resistance to the WSSV was observed in the tiger shrimp *P. menodon* reared in a "green water" system using commercially available extracts of *Dunaliella salina* [35]. Culture bath treatments with the microalga *C. minutissima* significantly reduced the mortality of *Epinephelus marginatus* showing signs of Viral Encephalopathy and Retinopathy (VER) [229]. These results indicate that the control of the disease is probably due to the antiviral effect of *C. minutissima* cultures, which thus needs to be further investigated using in vitro testing of water-soluble algal extracts.

Viral diseases are increasingly spreading, causing great economic losses for aquaculture industries. No effective treatment has yet been developed, as vaccination possibility is limited and chemical drugs are gradually avoided because of their toxicity and potential residue accumulation. Plant and herbal extracts have shown potent antiviral activity against several pathogenic viruses in aquaculture. They could be promising candidates for antiviral agents as part of an environmentally friendly and sustainable aquaculture, although their production and delivery processes could be limiting factors. In this context, the use of microalgae as a source of antiviral agents should be further studied, as water-soluble compounds present in algal supernatants could be a valuable alternative.

5. Conclusions and Final Remarks

The diversity of microalgae is immense, with species, genera or even classes being discovered every year. On the estimated millions of existing species, about 30,000 have been described, but only a dozen are cultivated in a large scale for biotechnological applications. The main obstacle for their commercial exploitation remains the production cost, but it should be bypassed by the optimization of mass culturing conditions. Microalgae are promising source of high-value products, and their application as antimicrobials is only in its onset. The development of novel drugs with no microbial resistance and the use of environmentally friendly antibiotics in a context of sustainable aquaculture are needed. The efficiency of various microalgal compounds against human or aquatic pathogens is very encouraging and there is no doubt that their exploitation and application will expand.

Acknowledgments: The authors acknowledge H.T. Badawy for her help with *Vibrio splendidus* experiments, the Region Pays de la Loire and Le Mans Centre for Technology Transfer for facilities. This publication benefited from funding from the European Commission under the Community's Seventh Framework Program BIOVADIA (contract No. FP7-PEOPLE-2010-IRSES-269294, Biodiversity and Valorisation of Blue Diatoms).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Borowitzka, M.A. Microalgae as sources of pharmaceuticals and other biologically active compounds. *J. Appl. Phycol.* **1995**, *7*, 3–15. [CrossRef]
2. Bhatnagar, I.; Kim, S.-K. Immense essence of excellence: Marine microbial bioactive compounds. *Mar. Drugs* **2010**, *8*, 2673–2701. [CrossRef] [PubMed]
3. Dyshlovoy, S.A.; Honecker, F. Marine compounds and cancer: Where do we stand? *Mar. Drugs* **2015**, *13*, 5657–5665. [CrossRef] [PubMed]
4. Jha, R.K.; Zi-rong, X. Biomedical compounds from marine organisms. *Mar. Drugs* **2004**, *2*, 123–146.
5. Kang, H.K.; Seo, C.H.; Park, Y. Marine peptides and their anti-infective activities. *Mar. Drugs* **2015**, *13*, 618–654. [CrossRef] [PubMed]
6. Mayer, A.M.S.; Rodríguez, A.D.; Taglialatela-Scafati, O.; Fusetani, N. Marine Pharmacology in 2009–2011: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action. *Mar. Drugs* **2013**, *11*, 2510–2573. [PubMed]
7. Suleria, H.A.R.; Osborne, S.; Masci, P.; Gobe, G. Marine-based nutraceuticals: An innovative trend in the food and supplement industries. *Mar. Drugs* **2015**, *13*, 6336–6351. [CrossRef] [PubMed]
8. Encarnacao, T.; Pais, A.A.; Campos, M.G.; Burrows, H.D. Cyanobacteria and microalgae: A renewable source of bioactive compounds and other chemicals. *Sci. Prog.* **2015**, *98*, 145–168. [CrossRef] [PubMed]
9. Singh, S.; Kate, B.N.; Banerjee, U.C. Bioactive compounds from cyanobacteria and microalgae: An overview. *Crit. Rev. Biotechnol.* **2005**, *25*, 73–95. [CrossRef] [PubMed]

10. Shimizu, Y. Microalgal metabolites. *Curr. Opin. Microbiol.* **2003**, *6*, 236–243. [CrossRef]
11. Talero, E.; García-Mauriño, S.; Ávila-Román, J.; Rodríguez-Luna, A.; Alcaide, A.; Motilva, V. Bioactive compounds isolated from microalgae in chronic inflammation and cancer. *Mar. Drugs* **2015**, *13*, 6152–6209. [CrossRef] [PubMed]
12. Ahmad, A.L.; Yasin, N.H.M.; Derek, C.J.C.; Lim, J.K. Microalgae as a sustainable energy source for biodiesel production: A review. *Renew. Sustain. Energy Rev.* **2011**, *15*, 584–593. [CrossRef]
13. Ghasemi, Y.; Rasoul-Amini, S.; Naseri, A.T.; Montazeri-Najafabady, N.; Mobasher, M.A.; Dabbagh, F. Microalgae biofuel potentials (Review). *Appl. Biochem. Microbiol.* **2012**, *48*, 126–144. [CrossRef]
14. Halim, R.; Danquah, M.K.; Webley, P.A. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnol. Adv.* **2012**, *30*, 709–732. [CrossRef] [PubMed]
15. Barra, L.; Chandrasekaran, R.; Corato, F.; Brunet, C. The challenge of ecophysiological biodiversity for biotechnological applications of marine microalgae. *Mar. Drugs* **2014**, *12*, 1641–1675. [CrossRef] [PubMed]
16. Cadoret, J.-P.; Garnier, M.; Saint-Jean, B. Microalgae, functional genomics and biotechnology. *Adv. Bot. Res.* **2012**, *64*, 285–341.
17. Priyadarshani, I.; Biswajit, R. Commercial and industrial applications of micro algae—A review. *J. Algal Biomass Utln.* **2012**, *3*, 89–100.
18. De Jesus Raposo, M.F.; de Morais, R.M.S.C.; de Morais, A.M.M.B. Health applications of bioactive compounds from marine microalgae. *Life Sci.* **2013**, *93*, 479–486. [CrossRef] [PubMed]
19. Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A. Commercial applications of microalgae. *J. Biosci. Bioeng.* **2006**, *101*, 87–96. [CrossRef] [PubMed]
20. Yaakob, Z.; Ali, E.; Zainal, A.; Mohamad, M.; Takriff, M.S. An overview: Biomolecules from microalgae for animal feed and aquaculture. *J. Biol. Res.* **2014**, *21*, 1–10. [CrossRef] [PubMed]
21. De Jesus Raposo, M.F.; de Morais, R.M.S.C.; Bernardo de Morais, A.M.M. Bioactivity and applications of sulphated polysaccharides from marine microalgae. *Mar. Drugs* **2013**, *11*, 233–252. [CrossRef] [PubMed]
22. De Jesus Raposo, M.F.; de Morais, A.M.B.; de Morais, R.M.S.C. Marine polysaccharides from algae with potential biomedical applications. *Mar. Drugs* **2015**, *13*, 2967–3028. [CrossRef] [PubMed]
23. Amaro, H.M.; Guedes, A.C.; Malcata, F.X. Chapter: Antimicrobial activities of microalgae: An invited review. In *Science against Microbial Pathogens: Communicating Current Research and Technological Advances*; Formatex Microbiology Book Series; Mendez-Vilas, A., Ed.; Formatex Research Center: Badajoz, Spain, 2011; Volume 2.
24. De Jesus Raposo, M.F.; de Morais, A.M.M.B.; de Morais, R.M.S.C. Emergent sources of prebiotics: Seaweeds and microalgae. *Mar. Drugs* **2016**, *14*, 27. [CrossRef] [PubMed]
25. De Jesus Raposo, M.F.; de Morais, A.M.M.B.; de Morais, R.M.S.C. Carotenoids from marine microalgae: A valuable natural source for the prevention of chronic diseases. *Mar. Drugs* **2015**, *13*, 5128–5155. [CrossRef] [PubMed]
26. Smith, V.J.; Desbois, A.P.; Dyrynda, E.A. Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. *Mar. Drugs* **2010**, *8*, 1213–1262. [CrossRef] [PubMed]
27. Muller-Feuga, A. The role of microalgae in aquaculture: Situation and trends. *J. Appl. Phycol.* **2000**, *12*, 527–534. [CrossRef]
28. Borowitzka, M.A. Microalgae for aquaculture: Opportunities and constraints. *J. Appl. Phycol.* **1997**, *9*, 393–401. [CrossRef]
29. Lorenz, R.T.; Cysewski, G.R. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* **2000**, *18*, 160–167. [CrossRef]
30. Gastineau, R.; Turcotte, F.; Pouvreau, J.-B.; Morançais, M.; Fleurence, J.; Windarto, E.; Prasetiya, F.S.; Arsad, S.; Jaouen, P.; Babin, M.; et al. Marennine, promising blue pigments from a widespread *Haslea* diatom species complex. *Mar. Drugs* **2014**, *12*, 3161–3189. [CrossRef] [PubMed]
31. Neori, A. "Green water" microalgae: The leading sector in world aquaculture. *J. Appl. Phycol.* **2010**, *23*, 143–149. [CrossRef]
32. Papandroulakis, N.; Divanach, P.; Anastasiadis, P.; Kentouri, M. The pseudo-green water technique for intensive rearing of sea bream (*Sparus aurata*) larvae. *Aquac. Int.* **2001**, *9*, 205–216. [CrossRef]
33. Naas, K.E.; N1ss, T.; Harboe, T. Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L.) in green water. *Aquaculture* **1992**, *105*, 143–156. [CrossRef]
34. Reitan, K.I.; Rainuzzo, J.R.; Øie, G.; Olsen, Y. A review of the nutritional effects of algae in marine fish larvae. *Aquaculture* **1997**, *155*, 207–221. [CrossRef]

35. Supamattaya, K.; Kiriratnikom, S.; Boonyaratpalin, M.; Borowitzka, L. Effect of a *Dunaliella* extract on growth performance, health condition, immune response and disease resistance in black tiger shrimp (*Penaeus monodon*). *Aquaculture* **2005**, *248*, 207–216. [CrossRef]
36. Van der Meeren, T.; Mangor-Jensen, A.; Pickova, J. The effect of green water and light intensity on survival, growth and lipid composition in Atlantic cod (*Gadus morhua*) during intensive larval rearing. *Aquaculture* **2007**, *265*, 206–217. [CrossRef]
37. Cahu, C.L.; Zambonino Infante, J.L.; Péres, A.; Quazuguel, P.; Le Gall, M.M. Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: Effect on digestive enzymes. *Aquaculture* **1998**, *161*, 479–489. [CrossRef]
38. Palmer, P.J.; Burke, M.J.; Palmer, C.J.; Burke, J.B. Developments in controlled green-water larval culture technologies for estuarine fishes in Queensland, Australia and elsewhere. *Aquaculture* **2007**, *272*, 1–21. [CrossRef]
39. Marques, A.; Thanh, T.H.; Sorgeloos, P.; Bossier, P. Use of microalgae and bacteria to enhance protection of gnotobiotic Artemia against different pathogens. *Aquaculture* **2006**, *258*, 116–126. [CrossRef]
40. Tendencia, E.A.; dela Peña, M. Investigation of some components of the greenwater system which makes it effective in the initial control of luminous bacteria. *Aquaculture* **2003**, *218*, 115–119. [CrossRef]
41. Burja, A.M.; Banaigs, B.; Abou-Mansour, E.; Grant Burgess, J.; Wright, P.C. Marine cyanobacteria—A prolific source of natural products. *Tetrahedron* **2001**, *57*, 9347–9377. [CrossRef]
42. Costa, M.; Costa-Rodrigues, J.; Fernandes, M.H.; Barros, P.; Vasconcelos, V.; Martins, R. Marine cyanobacteria compounds with anticancer properties: A review on the implication of apoptosis. *Mar. Drugs* **2012**, *10*, 2181–2207. [CrossRef] [PubMed]
43. Gupta, V.; Ratha, S.K.; Sood, A.; Chaudhary, V.; Prasanna, R. New insights into the biodiversity and applications of cyanobacteria (blue-green algae)—Prospects and challenges. *Algal Res.* **2013**, *2*, 79–97. [CrossRef]
44. Vijayakumar, S.; Menakha, M. Pharmaceutical applications of cyanobacteria—A review. *J. Acute Med.* **2015**, *5*, 15–23. [CrossRef]
45. Deschênes, J.-S.; Boudreau, A.; Tremblay, R. Mixotrophic production of microalgae in pilot-scale photobioreactors: Practicability and process considerations. *Algal Res.* **2015**, *10*, 80–86. [CrossRef]
46. Pradhan, J.; Das, S.; Das, B.K. Antibacterial activity of freshwater microalgae: A review. *Afr. J. Pharm. Pharmacol.* **2014**, *8*, 809–818.
47. Jin, L.; Quan, C.; Hou, X.; Fan, S. Potential Pharmacological Resources: Natural Bioactive Compounds from Marine-Derived Fungi. *Mar. Drugs* **2016**, *14*, 76. [CrossRef] [PubMed]
48. Shannon, E.; Abu-Ghannam, N. Antibacterial Derivatives of Marine Algae: An Overview of Pharmacological Mechanisms and Applications. *Mar. Drugs* **2016**, *14*, 81. [CrossRef] [PubMed]
49. Pratt, R.; Daniels, T.C.; Eiler, J.J.; Gunnison, J.B.; Kumler, W.D.; Oneto, J.F.; Strait, L.A.; Spoehr, H.A.; Hardin, G.J.; Milner, H.W.; et al. Chlorellin, an antibacterial substance from *Chlorella*. *Science* **1944**, *99*, 351–352. [CrossRef] [PubMed]
50. Mudimu, O.; Rybalka, N.; Bauersachs, T.; Born, J.; Friedl, T.; Schulz, R. Biotechnological screening of microalgal and cyanobacterial strains for biogas production and antibacterial and antifungal effects. *Metabolites* **2014**, *4*, 373–393. [CrossRef] [PubMed]
51. Ördög, V.; Stirk, W.A.; Lenobel, R.; Bancířová, M.; Strnad, M.; van Staden, J.; Szigeti, J.; Németh, L. Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *J. Appl. Phycol.* **2004**, *16*, 309–314. [CrossRef]
52. Pane, G.; Cacciola, G.; Giacco, E.; Mariottini, G.L.; Coppo, E. Assessment of the antimicrobial activity of algae extracts on bacteria responsible of external otitis. *Mar. Drugs* **2015**, *13*, 6440–6452. [CrossRef] [PubMed]
53. Chang, T.; Ohta, S.; Ikegami, N.; Miyata, H.; Kashimoto, T.; Kondo, M. Antibiotic substances produced by a marine green alga, *Dunaliella primolecta*. *Bioresour. Technol.* **1993**, *44*, 149–153. [CrossRef]
54. Duff, D.C.B.; Bruce, D.L.; Antia, N.J. The Antibacterial Activity of Marine Planktonic Algae. *Can. J. Microbiol.* **1966**, *12*, 877–884. [CrossRef] [PubMed]
55. Kellam, S.J.; Walker, J.M. Antibacterial activity from marine microalgae in laboratory culture. *Br. Phycol. J.* **1989**, *24*, 191–194. [CrossRef]
56. Ohta, S.; Chang, T.; Ikegami, N.; Kondo, M.; Miyata, H. Antibiotic substance produced by a newly isolated marine microalga, *Chlorococcum* HS-101. *Bull. Environ. Contam. Toxicol.* **1993**, *50*, 171–178. [CrossRef] [PubMed]
57. Srinivasakumar, K.P.; Rajashekhar, M. In vitro studies on bactericidal activity and sensitivity pattern of isolated marine microalgae against selective human bacterial pathogens. *Indian J. Sci. Technol.* **2009**, *2*, 16–23.

58. Viso, A.C.; Pesando, D.; Baby, C. Antibacterial and antifungal properties of some marine diatoms in culture. *Bot. Mar.* **1987**, *30*, 41–46. [CrossRef]
59. Cannell, R.J.P.; Owsianka, A.M.; Walker, J.M. Results of a large-scale screening programme to detect antibacterial activity from freshwater algae. *Br. Phycol. J.* **1988**, *23*, 41–44. [CrossRef]
60. Katircioglu, H.; Beyatli, Y.; Aslim, B.; Yüksekdag, Z.; Atici, T. Screening for antimicrobial agent production of some microalgae in freshwater. *Internet J. Microbiol.* **2005**, *2*, 1–5.
61. Safonova, E.; Reisser, W. Growth promoting and inhibiting effects of extracellular substances of soil microalgae and cyanobacteria on *Escherichia coli* and *Micrococcus luteus*. *Phycol. Res.* **2005**, *53*, 189–193. [CrossRef]
62. Ghasemi, Y.; Moradian, A.; Mohagheghzadeh, A.; Shokravi, S.; Morowvat, M.H. Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: Characterization of antimicrobial activity of *Chroococcus dispersus*. *J. Biol. Sci.* **2007**, *7*, 904–910.
63. Abedin, R.; Taha, H. Antibacterial and antifungal activity of cyanobacteria and green microalgae. Evaluation of medium components by Plackett-Burman design for antimicrobial activity of *Spirulina platensis*. *Glob. J. Biotechnol. Biochem.* **2008**, *3*, 22–31.
64. Ohta, S.; Shiomi, Y.; Kawashima, A.; Aozasa, O.; Nakao, T.; Nagate, T.; Kitamura, K.; Miyata, H. Antibiotic effect of linolenic acid from *Chlorococcum* strain HS-101 and *Dunaliella primolecta* on methicillin-resistant *Staphylococcus aureus*. *J. Appl. Phycol.* **1995**, *7*, 121–127. [CrossRef]
65. Bhagavathy, S.; Sumathi, P.; Jancy Sherene Bell, I. Green algae *Chlorococcum humicola*—A new source of bioactive compounds with antimicrobial activity. *Asian Pac. J. Trop. Biomed.* **2011**, *1*, S1–S7. [CrossRef]
66. Herrero, M.; Ibáñez, E.; Cifuentes, A.; Reglero, G.; Santoyo, S. *Dunaliella salina* microalga pressurized liquid extracts as potential antimicrobials. *J. Food Prot.* **2006**, *69*, 2471–2477. [PubMed]
67. Mendiola, J.A.; Santoyo, S.; Cifuentes, A.; Reglero, G.; Ibáñez, E.; Señoráns, F.J. Antimicrobial activity of sub- and supercritical CO2 extracts of the green alga *Dunaliella salina*. *J. Food Prot.* **2008**, *71*, 2138–2143. [PubMed]
68. Lustigman, B. Comparison of antibiotic production from four ecotypes of the marine alga, *Dunaliella*. *Bull. Environ. Contam. Toxicol.* **1988**, *40*, 18–22. [CrossRef] [PubMed]
69. Rodríguez-Meizoso, I.; Jaime, L.; Santoyo, S.; Señoráns, F.J.; Cifuentes, A.; Ibáñez, E. Subcritical water extraction and characterization of bioactive compounds from *Haematococcus pluvialis* microalga. *J. Pharm. Biomed. Anal.* **2010**, *51*, 456–463. [CrossRef] [PubMed]
70. Santoyo, S.; Rodríguez-Meizoso, I.; Cifuentes, A.; Jaime, L.; García-Blairsy Reina, G.; Señorans, F.J.; Ibáñez, E. Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from *Haematococcus pluvialis* microalgae. *LWT—Food Sci. Technol.* **2009**, *42*, 1213–1218. [CrossRef]
71. Guedes, A.C.; Barbosa, C.R.; Amaro, H.M.; Pereira, C.I.; Malcata, F.X. Microalgal and cyanobacterial cell extracts for use as natural antibacterial additives against food pathogens. *Int. J. Food Sci. Technol.* **2011**, *46*, 862–870. [CrossRef]
72. Najdenski, H.M.; Gigova, L.G.; Iliev, I.I.; Pilarski, P.S.; Lukavský, J.; Tsvetkova, I.V.; Ninova, M.S.; Kussovski, V.K. Antibacterial and antifungal activities of selected microalgae and cyanobacteria. *Int. J. Food Sci. Technol.* **2013**, *48*, 1533–1540. [CrossRef]
73. Ingebrigtsen, R.A.; Hansen, E.; Andersen, J.H.; Eilertsen, H.C. Light and temperature effects on bioactivity in diatoms. *J. Appl. Phycol.* **2015**, *28*, 939–950. [CrossRef] [PubMed]
74. Del Pilar Sánchez-Saavedra, M.; Licea-Navarro, A.; Bernáldez-Sarabia, J. Evaluation of the antibacterial activity of different species of phytoplankton. *Rev. Biol. Mar. Oceanogr.* **2010**, *45*, 531–536. [CrossRef]
75. Mendiola, J.A.; Torres, C.F.; Toré, A.; Martín-Álvarez, P.J.; Santoyo, S.; Arredondo, B.O.; Señoráns, F.J.; Cifuentes, A.; Ibáñez, E. Use of supercritical CO2 to obtain extracts with antimicrobial activity from *Chaetoceros muelleri* microalga. A correlation with their lipidic content. *Eur. Food Res. Technol.* **2007**, *224*, 505–510. [CrossRef]
76. Findlay, J.A.; Patil, A.D. Antibacterial constituents of the diatom *Navicula delognei*. *J. Nat. Prod.* **1984**, *47*, 815–818. [CrossRef] [PubMed]
77. Desbois, A.P.; Lebl, T.; Yan, L.; Smith, V. Isolation and structure characterization of two antibacterial free fatty acids from marine diatom, *Phaeodactylum tricornutum*. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 755–764. [CrossRef] [PubMed]
78. Desbois, A.P.; Mearns-Spragg, A.; Smith, V.J. A fatty acid from the diatom *Phaeodactylum tricornutum* is antibacterial against diverse bacteria including multi-resistant *Staphylococcus aureus* (MRSA). *Mar. Biotechnol.* **2008**, *11*, 45–52. [CrossRef] [PubMed]

79. Venkatesan, R.; Karthikayen, R.; Periyanayagi, R.; Sasikala, V.; Balasubramanian, T. Antibacterial activity of the marine diatom, *Rhizosolenia alata* (Brightwell, 1858) against human pathogens. *Res. J. Microbiol.* **2007**, *2*, 98–100.
80. Walter, C.S.; Mahesh, R. Antibacterial and antifungal activities of some marine diatoms in culture. *Indian J. Mar. Sci.* **2000**, *29*, 238–242.
81. Cooper, S.; Battat, A.; Marsot, P.; Sylvestre, M. Production of antibacterial activities by two Bacillariophyceae grown in dialysis culture. *Can. J. Microbiol.* **1983**, *29*, 338–341. [CrossRef]
82. Bruce, D.L.; Duff, D.C.; Antia, N.J. The identification of two antibacterial products of the marine planktonic alga *Isochrysis galbana*. *Microbiology* **1967**, *48*, 293–298. [CrossRef] [PubMed]
83. Pawlik-Skowrońska, B. Resistance, accumulation and allocation of zinc in two ecotypes of the green alga Stigeoclonium tenue Kütz. coming from habitats of different heavy metal concentrations. *Aquat. Bot.* **2003**, *75*, 189–198. [CrossRef]
84. Herrero, M.; Mendiola, J.A.; Plaza, M.; Ibañez, E. Screening for Bioactive Compounds from Algae. In *Advanced Biofuels and Bioproducts*; Lee, J.W., Ed.; Springer: New York, NY, USA, 2013; pp. 833–872.
85. Jørgensen, E.G. Antibiotic substances from cells and culture solutions of unicellular algae with special reference to some chlorophyll derivatives. *Physiol. Plant.* **1962**, *15*, 530–545. [CrossRef]
86. Desbois, A.P.; Walton, M.; Smith, V.J. Differential antibacterial activities of fusiform and oval morphotypes of *Phaeodactylum tricornutum* (Bacillariophyceae). *J. Mar. Biol. Assoc. UK* **2010**, *90*, 769–774. [CrossRef]
87. Jüttner, F. Liberation of 5,8,11,14,17-Eicosapentaenoic Acid and Other Polyunsaturated Fatty Acids from Lipids as a Grazer Defense Reaction in Epilithic Diatom Biofilms. *J. Phycol.* **2001**, *37*, 744–755. [CrossRef]
88. Kabara, J.J.; Swieczkowski, D.M.; Conley, A.J.; Truant, J.P. Fatty acids and derivatives as antimicrobial agents. *Antimicrob. Agents Chemother.* **1972**, *2*, 23–28. [CrossRef] [PubMed]
89. Galbraith, H.; Miller, T.B. Physicochemical effects of long chain fatty acids on bacterial cells and their protoplasts. *J. Appl. Bacteriol.* **1973**, *36*, 647–658. [CrossRef] [PubMed]
90. Sun, C.Q.; O'Connor, C.J.; Roberton, A.M. Antibacterial actions of fatty acids and monoglycerides against *Helicobacter pylori*. *FEMS Immunol. Med. Microbiol.* **2003**, *36*, 9–17. [CrossRef]
91. Petschow, B.W.; Batema, R.P.; Ford, L.L. Susceptibility of *Helicobacter pylori* to bactericidal properties of medium-chain monoglycerides and free fatty acids. *Antimicrob. Agents Chemother.* **1996**, *40*, 302–306. [PubMed]
92. Austin, B. *Infectious Disease in Aquaculture: Prevention and Control*; Woodhead Publishing: Cambridge, UK, 2012.
93. Austin, B.; Austin, D.A. *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*; Springer Science & Business Media: Berlin, Germany, 2007.
94. Vandenberghe, J.; Thompson, F.L.; Gomez-Gil, B.; Swings, J. Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems. *Aquaculture* **2003**, *219*, 9–20. [CrossRef]
95. Aguirre-Guzmán, G.; Mejia Ruíz, H.; Ascencio, F. A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. *Aquac. Res.* **2004**, *35*, 1395–1404. [CrossRef]
96. Austin, B.; Zhang, X.-H. *Vibrio harveyi*: A significant pathogen of marine vertebrates and invertebrates. *Lett. Appl. Microbiol.* **2006**, *43*, 119–124. [CrossRef] [PubMed]
97. Beaz-Hidalgo, R.; Diéguez, A.L.; Cleenwerck, I.; Balboa, S.; Doce, A.; de Vos, P.; Romalde, J.L. *Vibrio celticus* sp. nov., a new *Vibrio* species belonging to the Splendidus clade with pathogenic potential for clams. *Syst. Appl. Microbiol.* **2010**, *33*, 311–315. [CrossRef] [PubMed]
98. Kesarcodi-Watson, A.; Kaspar, H.; Lategan, M.J.; Gibson, L. Two pathogens of Greenshell mussel larvae, *Perna canaliculus*: *Vibrio splendidus* and a *V. coralliilyticus*/neptunius-like isolate. *J. Fish Dis.* **2009**, *32*, 499–507. [CrossRef] [PubMed]
99. Lambert, C.; Nicolas, J.L.; Cilia, V.; Corre, S. *Vibrio pectenicida* sp. nov., a pathogen of scallop (Pecten maximus) larvae. *Int. J. Syst. Bacteriol.* **1998**, *48*, 481–487. [CrossRef] [PubMed]
100. Prado, S.; Romalde, J.L.; Montes, J.; Barja, J.L. Pathogenic bacteria isolated from disease outbreaks in shellfish hatcheries. First description of *Vibrio neptunius* as an oyster pathogen. *Dis. Aquat. Org.* **2005**, *67*, 209–215. [CrossRef] [PubMed]
101. Travers, M.-A.; Boettcher Miller, K.; Roque, A.; Friedman, C.S. Bacterial diseases in marine bivalves. *J. Invertebr. Pathol.* **2015**, *131*, 11–31. [CrossRef] [PubMed]
102. Armstrong, S.M.; Hargrave, B.T.; Haya, K. Antibiotic Use in Finfish Aquaculture: Modes of Action, Environmental Fate, and Microbial Resistance. In *Environmental Effects of Marine Finfish Aquaculture*; Hargrave, B.T., Ed.; Handbook of Environmental Chemistry; Springer: Berlin, Germany, 2005; pp. 341–357.

103. Cabello, F.C. Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. *Environ. Microbiol.* **2006**, *8*, 1137–1144. [CrossRef] [PubMed]
104. Bermdez-Almada, M.C.; Espinosa-Plascenci, A. The Use of Antibiotics in Shrimp Farming. In *Health and Environment in Aquaculture*; Carvalho, E., Ed.; InTech: Rijeka, Croatia, 2012.
105. Gräslund, S.; Bengtsson, B.E. Chemicals and biological products used in south-east Asian shrimp farming, and their potential impact on the environment—A review. *Sci. Total Environ.* **2001**, *280*, 93–131. [CrossRef]
106. Primavera, J.H.; Lavilla-Pitogo, C.R.; Ladja, J.M.; Dela Peña, M.R. A survey of chemical and biological products used in intensive prawn farms in the Philippines. *Mar. Pollut. Bull.* **1993**, *26*, 35–40. [CrossRef]
107. Silva, E.F.; Soares, M.A.; Calazans, N.F.; Vogeley, J.L.; do Valle, B.C.; Soares, R.; Peixoto, S. Effect of probiotic (*Bacillus* spp.) addition during larvae and postlarvae culture of the white shrimp Litopenaeus vannamei. *Aquac. Res.* **2012**, *44*, 13–21. [CrossRef]
108. Holbach, M.; Robert, R.; Boudry, P.; Petton, B.; Archambault, P.; Tremblay, R. Scallop larval survival from erythromycin treated broodstock after conditioning without sediment. *Aquaculture* **2015**, *437*, 312–317. [CrossRef]
109. Prado, S.; Romalde, J.L.; Barja, J.L. Review of probiotics for use in bivalve hatcheries. *Vet. Microbiol.* **2010**, *145*, 187–197. [CrossRef] [PubMed]
110. Albuquerque Costa, R.; Araújo, R.L.; Souza, O.V.; Vieira, R.H.S.D.F. Antibiotic-Resistant *Vibrios* in Farmed Shrimp. *BioMed Res. Int.* **2015**, *2015*, 505914. [CrossRef] [PubMed]
111. Fernández-Alarcón, C.; Miranda, C.D.; Singer, R.S.; López, Y.; Rojas, R.; Bello, H.; Domínguez, M.; González-Rocha, G. Detection of the floR gene in a diversity of florfenicol resistant Gram-negative bacilli from freshwater salmon farms in Chile. *Zoonoses Public Health* **2010**, *57*, 181–188. [CrossRef] [PubMed]
112. Miranda, C.D.; Zemelman, R. Bacterial resistance to oxytetracycline in *Chilean salmon* farming. *Aquaculture* **2002**, *212*, 31–47. [CrossRef]
113. Sapkota, A.; Sapkota, A.R.; Kucharski, M.; Burke, J.; McKenzie, S.; Walker, P.; Lawrence, R. Aquaculture practices and potential human health risks: Current knowledge and future priorities. *Environ. Int.* **2008**, *34*, 1215–1226. [CrossRef] [PubMed]
114. Citarasu, T. Natural antimicrobial compounds for use in aquaculture. In *Infectious Disease in Aquaculture: Prevention and Control*; Austin, B., Ed.; Woodhead Publishing: Cambridge, UK, 2012; pp. 419–457.
115. González-Davis, O.; Ponce-Rivas, E.; Sánchez-Saavedra, M.D.P.; Muñoz-Márquez, M.-E.; Gerwick, W.H. Bioprospection of microalgae and cyanobacteria as biocontrol agents against *Vibrio campbellii* and their use in white shrimp Litopenaeus vannamei culture. *J. World Aquac. Soc.* **2012**, *43*, 387–399. [CrossRef]
116. Das, B.K.; Pradhan, J.; Pattnaik, P.; Samantaray, B.R.; Samal, S.K. Production of antibacterials from the freshwater alga *Euglena viridis* (Ehren). *World J. Microbiol. Biotechnol.* **2005**, *21*, 45–50. [CrossRef]
117. Gastineau, R.; Hardivillier, Y.; Leignel, V.; Tekaya, N.; Morançais, M.; Fleurence, J.; Davidovich, N.; Jacquette, B.; Gaudin, P.; Hellio, C.; et al. Greening effect on oysters and biological activities of the blue pigments produced by the diatom *Haslea karadagensis* (Naviculaceae). *Aquaculture* **2012**, *368*, 61–67. [CrossRef]
118. Gastineau, R.; Pouvreau, J.-B.; Hellio, C.; Morançais, M.; Fleurence, J.; Gaudin, P.; Bourgougnon, N.; Mouget, J.-L. Biological activities of purified marennine, the blue pigment responsible for the greening of oysters. *J. Agric. Food Chem.* **2012**, *60*, 3599–3605. [CrossRef] [PubMed]
119. Pouvreau, J.-B. Purification et Caractérisation du Pigment Bleu-Vert “Marennine” Synthétisé par la Diatomée Marine Haslea ostrearia (Gaillon/Bory) Simonsen: Propriétés Physico-Chimiques et Activités Biologiques. Ph.D. Thesis, Université de Nantes, Nantes, France, 2006.
120. Naviner, M.; Bergé, J.-P.; Durand, P.; Le Bris, H. Antibacterial activity of the marine diatom *Skeletonema costatum* against aquacultural pathogens. *Aquaculture* **1999**, *174*, 15–24. [CrossRef]
121. Berland, B.R.; Bonin, D.J.; Cornu, A.L.; Maestrini, S.Y.; Marino, J.-P. The antibacterial substances of the marine alga *Stichochrysis immobilis* (chrysophyta). *J. Phycol.* **1972**, *8*, 383–392. [CrossRef]
122. Austin, B.; Day, J.G. Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. *Aquaculture* **1990**, *90*, 389–392. [CrossRef]
123. Austin, B.; Baudet, E.; Stobie, M. Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. *J. Fish Dis.* **1992**, *15*, 55–61. [CrossRef]
124. Lio-Po, G.D.; Leaño, E.M.; Peñaranda, M.M.D.; Villa-Franco, A.U.; Sombito, C.D.; Guanzon, N.G., Jr. Anti-luminous *Vibrio* factors associated with the “green water” grow-out culture of the tiger shrimp *Penaeus monodon*. *Aquaculture* **2005**, *250*, 1–7. [CrossRef]

125. Kokou, F.; Makridis, P.; Kentouri, M.; Divanach, P. Antibacterial activity in microalgae cultures. *Aquac. Res.* **2012**, *43*, 1520–1527. [CrossRef]
126. Molina-Cárdenas, C.A.; del Pilar Sánchez-Saavedra, M.; Lizárraga-Partida, M.L. Inhibition of pathogenic *Vibrio* by the microalgae *Isochrysis galbana*. *J. Appl. Phycol.* **2014**, *26*, 2347–2355. [CrossRef]
127. Terekhova, V.E.; Aizdaicher, N.A.; Buzoleva, L.S.; Somov, G.P. Influence of extrametabolites of marine microalgae on the reproduction of the bacterium *Listeria monocytogenes*. *Russ. J. Mar. Biol.* **2009**, *35*, 355–358. [CrossRef]
128. Kogure, K.; Simidu, U.; Taga, N. Effect of *Skeletonema costatum* (Grev.) Cleve on the growth of marine bacteria. *J. Exp. Mar. Biol. Ecol.* **1979**, *36*, 201–215. [CrossRef]
129. Makridis, P.; Costa, R.A.; Dinis, M.T. Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia metanauplii*. *Aquaculture* **2006**, *255*, 76–81. [CrossRef]
130. Turcotte, F. Effet Protecteur d'un Pigment (Marennine) de Diatomée Bleue Contre la Bactérie Pathogène *Vibrio splendidus* chez les Larves de Moule Bleue *Mytilus edulis*: Utilisation Potentielle en Ecloseries de Vivalves. Master's Thesis, Université du Québec à Rimouski, Rimouski, QC, Canada, 2014.
131. Olsen, A.I.; Olsen, Y.; Attramadal, Y.; Christie, K.; Birkbeck, T.H.; Skjermo, J.; Vadstein, O. Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. *Aquaculture* **2000**, *190*, 11–25. [CrossRef]
132. Regunathan, C.; Wesley, S.G. Control of *Vibrio* spp. in shrimp hatcheries using the green algae *Tetraselmis suecica*. *Asian Fish. Sci.* **2004**, *17*, 147–158.
133. Salvesen, I.; Skjermo, J.; Vadstein, O. Growth of turbot (*Scophthalmus maximus* L.) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water. *Aquaculture* **1999**, *175*, 337–350. [CrossRef]
134. Lavilla-Pitogo, C.R.; Albright, L.J.; Paner, M.G. Will microbial manipulation sustain the ecological balance in shrimp (*Penaeus monodon*) hatcheries? In *Advances in Shrimp Biotechnology*, Proceedings of the 5th Asian Fisheries Forum on Special Session on Shrimp Biotechnology, Chiengmai, Thailand, 11–14 November 1998; Flegel, T.W., Ed.; National Center for Genetic Engineering and Biotechnology: Bangkok, Thailand, 1998; pp. 185–192.
135. Kusumaningrum, H.P.; Zainuri, M. Detection of bacteria and fungi associated with *Penaeus monodon* postlarvae mortality. *Procedia Environ. Sci.* **2015**, *23*, 329–337. [CrossRef]
136. Davey, M.R.; Anthony, P.; Power, J.B.; Lowe, K.C. Plant protoplasts: Status and biotechnological perspectives. *Biotechnol. Adv.* **2005**, *23*, 131–171. [CrossRef] [PubMed]
137. Li, S.-S.; Tsai, H.-J. Transgenic microalgae as a non-antibiotic bactericide producer to defend against bacterial pathogen infection in the fish digestive tract. *Fish Shellfish Immunol.* **2009**, *26*, 316–325. [CrossRef] [PubMed]
138. Øie, G.; Reitan, K.I.; Olsen, Y. Comparison of rotifer culture quality with yeast plus oil and algal-based cultivation diets. *Aquac. Int.* **1994**, *2*, 225–238.
139. Pouvreau, J.-B.; Morançais, M.; Massé, G.; Rosa, P.; Robert, J.-M.; Fleurence, J.; Pondaven, P. Purification of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. *J. Appl. Phycol.* **2006**, *18*, 769–781. [CrossRef]
140. Garnier, M.; Labreuche, Y.; Garcia, C.; Robert, M.; Nicolas, J.-L. Evidence for the Involvement of Pathogenic Bacteria in Summer Mortalities of the Pacific Oyster *Crassostrea gigas*. *Microb. Ecol.* **2007**, *53*, 187–196. [CrossRef] [PubMed]
141. Garnier, M.; Labreuche, Y.; Nicolas, J.-L. Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. *Syst. Appl. Microbiol.* **2008**, *31*, 358–365. [PubMed]
142. Gay, M.; Renault, T.; Pons, A.-M.; Le Roux, F. Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: Taxonomy and host alterations. *Dis. Aquat. Org.* **2004**, *62*, 65–74. [CrossRef] [PubMed]
143. Goudenège, D.; Travers, M.A.; Lemire, A.; Petton, B.; Haffner, P.; Labreuche, Y.; Tourbiez, D.; Mangenot, S.; Calteau, A.; Mazel, D.; et al. A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters. *Environ. Microbiol.* **2015**, *17*, 4189–4199. [CrossRef] [PubMed]
144. Genard, B.; Miner, P.; Nicolas, J.-L.; Moraga, D.; Boudry, P.; Pernet, F.; Tremblay, R. Integrative Study of Physiological Changes Associated with Bacterial Infection in Pacific Oyster Larvae. *PLoS ONE* **2013**, *8*, e64534. [CrossRef] [PubMed]

145. Travers, M.-A.; Mersni Achour, R.; Haffner, P.; Tourbiez, D.; Cassone, A.-L.; Morga, B.; Doghri, I.; Garcia, C.; Renault, T.; Fruitier-Arnaudin, I.; et al. First description of French *V. tubiashii* strains pathogenic to mollusk: I. Characterization of isolates and detection during mortality events. *J. Invertebr. Pathol.* **2014**, *123*, 38–48. [CrossRef] [PubMed]
146. Anaissie, E.; Bodey, G.P. Nosocomial fungal infections. Old problems and new challenges. *Infect. Dis. Clin. N. Am.* **1989**, *3*, 867–882.
147. Alangaden, G.J. Nosocomial fungal infections: Epidemiology, infection control, and prevention. *Infect. Dis. Clin. N. Am.* **2011**, *25*, 201–225. [CrossRef] [PubMed]
148. Manuel, R.J.; Kibbler, C.C. The epidemiology and prevention of invasive aspergillosis. *J. Hosp. Infect.* **1998**, *39*, 95–109. [CrossRef]
149. Wey, S.B.; Mori, M.; Pfaller, M.A.; Woolson, R.F.; Wenzel, R.P. Hospital-acquired candidemia: The attributable mortality and excess length of stay. *Arch. Intern. Med.* **1988**, *148*, 2642–2645. [CrossRef] [PubMed]
150. Ghannoum, M.A.; Rice, L.B. Antifungal agents: Mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **1999**, *12*, 501–517. [PubMed]
151. Accoceberry, I.; Noël, T. Antifongiques: Cibles cellulaires et mécanismes de résistance. *Thérapie* **2006**, *61*, 195–199. [CrossRef] [PubMed]
152. El Amraoui, B.; El Amraoui, M.; Cohen, N.; Fassouane, A. Anti-*Candida* and anti-*Cryptococcus* antifungal produced by marine microorganisms. *J. Med. Mycol.* **2014**, *24*, 149–153. [CrossRef] [PubMed]
153. Cheung, R.C.F.; Wong, J.H.; Pan, W.L.; Chan, Y.S.; Yin, C.M.; Dan, X.L.; Wang, H.X.; Fang, E.F.; Lam, S.K.; Ngai, P.H.K.; et al. Antifungal and antiviral products of marine organisms. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 3475–3494. [CrossRef] [PubMed]
154. Hong, J.-H.; Jang, S.; Heo, Y.M.; Min, M.; Lee, H.; Lee, Y.M.; Lee, H.; Kim, J.-J. Investigation of marine-derived fungal diversity and their exploitable biological activities. *Mar. Drugs* **2015**, *13*, 4137–4155. [CrossRef] [PubMed]
155. Shishido, T.K.; Humisto, A.; Jokela, J.; Liu, L.; Wahlsten, M.; Tamrakar, A.; Fewer, D.P.; Permi, P.; Andreote, A.P.D.; Fiore, M.F.; et al. Antifungal compounds from cyanobacteria. *Mar. Drugs* **2015**, *13*, 2124–2140. [CrossRef] [PubMed]
156. Wang, Y.-T.; Xue, Y.-R.; Liu, C.-H. A brief review of bioactive metabolites derived from deep-sea fungi. *Mar. Drugs* **2015**, *13*, 4594–4616. [CrossRef] [PubMed]
157. Xu, L.; Meng, W.; Cao, C.; Wang, J.; Shan, W.; Wang, Q. Antibacterial and antifungal compounds from marine fungi. *Mar. Drugs* **2015**, *13*, 3479–3513. [CrossRef] [PubMed]
158. Gauthier, M.J. Activité antibactérienne d'une Diatomée marine: *Asterionella notata* (Grun). *Rev. Intern. Oceanogr. Med.* **1969**, *25*, 103–165.
159. Nagai, H.; Satake, M.; Yasumoto, T. Antimicrobial activities of polyether compounds of dinoflagellate origins. *J. Appl. Phycol.* **1990**, *2*, 305–308. [CrossRef]
160. Pesando, D.; Gnassia-Barelli, M.; Gueho, E. Antifungal properties of some marine algae. In *Marine Algae in Pharmaceutical Science*; Walter de Gruyter: Berlin, Germany; New York, NY, USA, 1979; pp. 461–472.
161. Kellam, S.J.; Cannell, R.J.P.; Owsianka, A.M.; Walker, J.M. Results of a large-scale screening programme to detect antifungal activity from marine and freshwater microalgae in laboratory culture. *Br. Phycol. J.* **1988**, *23*, 45–47. [CrossRef]
162. Gueho, E.; Pesando, D.; Barelli, M. Propriétés antifongiques d'une diatomeé *Chaetoceros lauderi* ralfs C C. *Mycopathologia* **1977**, *60*, 105–107. [CrossRef] [PubMed]
163. Pesando, D.; Gnassia-Barelli, M.; Gueho, E. Partial characterization of a specific antibiotic, antifungal substance isolated from the marine diatom *Chaetoceros lauderi* Ralfs. In *Marine Algae in Pharmaceutical Science*; Walter de Gruyter: Berlin, Germany; New York, NY, USA, 1979; Volume 1, pp. 447–459.
164. Washida, K.; Koyama, T.; Yamada, K.; Kita, M.; Uemura, D. Karatungiols A and B, two novel antimicrobial polyol compounds, from the symbiotic marine dinoflagellate *Amphidinium* sp. *Tetrahedron Lett.* **2006**, *47*, 2521–2525. [CrossRef]
165. Nagai, H.; Mikami, Y.; Yazawa, K.; Gonoi, T.; Yasumoto, T. Biological activities of novel polyether antifungals, gambieric acids A and B from a marine dinoflagellate *Gambierdiscus toxicus*. *J. Antibiot.* **1993**, *46*, 520–522. [CrossRef] [PubMed]
166. Sharma, G.M.; Michaels, L.; Burkholder, P.R. Goniodomin, a new antibiotic from a dinoflagellate. *J. Antibiot.* **1968**, *21*, 659–664. [CrossRef] [PubMed]

167. Murakami, M.; Makabe, K.; Yamaguchi, K.; Konosu, S.; Wälchli, M.R. Goniodomin a, a novel polyether macrolide from the dinoflagellate *Goniodoma pseudogoniaulax*. *Tetrahedron Lett.* **1988**, *29*, 1149–1152. [CrossRef]
168. Houdai, T.; Matsuoka, S.; Matsumori, N.; Murata, M. Membrane-permeabilizing activities of amphidinol 3, polyene-polyhydroxy antifungal from a marine dinoflagellate. *Biochim. Biophys. Acta* **2004**, *1667*, 91–100. [CrossRef] [PubMed]
169. Satake, M.; Murata, M.; Yasumoto, T.; Fujita, T.; Naoki, H. Amphidinol, a polyhydroxy-polyene antifungal agent with an unprecedented structure, from a marine dinoflagellate, *Amphidinium klebsii*. *J. Am. Chem. Soc.* **1991**, *113*, 9859–9861. [CrossRef]
170. Yanong, R.P.E. Fungal diseases of fish. *Vet. Clin. N. Am. Exot. Anim. Pract.* **2003**, *6*, 377–400. [CrossRef]
171. Noga, E.J. *Fish Disease: Diagnosis and Treatment*; John Wiley & Sons: New York, NY, USA, 2011.
172. Anater, A.; Manyes, L.; Meca, G.; Ferrer, E.; Luciano, F.B.; Pimpão, C.T.; Font, G. Mycotoxins and their consequences in aquaculture: A review. *Aquaculture* **2016**, *451*, 1–10. [CrossRef]
173. Trakranrungsie, N. Plant derived antifungals-trends and potential applications in veterinary medicine: A mini-review. In *Science against Microbial Pathogens: Communicating Current Research and Technological Advances*; Mendez-Vilas, A., Ed.; Formatex Research Center: Badajoz, Spain, 2011.
174. Hussein, M.M.A.; El-Feki, M.A.; Hatai, K.; Yamamoto, A. Inhibitory effects of thymoquinone from *Nigella sativa* on pathogenic *Saprolegnia* in fish. *Biocontrol Sci.* **2002**, *7*, 31–35. [CrossRef]
175. Mori, T.; Hirose, H.; Hanjavanit, C.; Hatai, K. Antifungal activities of plant extracts against some aquatic fungi. *Biocontrol Sci.* **2002**, *7*, 187–191. [CrossRef]
176. Udomkusonsri, P.; Trongvanichnam, K.; Limpoka, M.; Klangkaew, N.; Kusucharit, N. In vitro efficacy of the antifungal activity of some Thai medicinal-plants on the pathogenic fungus, *Saprolegnia parasitica* H2, in fish. *Kasetsart J. Nat. Sci.* **2007**, *41*, 56–61.
177. Hussein, M.M.A.; Wada, S.; Hatai, K.; Yamamoto, A. Antimycotic activity of eugenol against selected water molds. *J. Aquat. Anim. Health* **2000**, *12*, 224–229. [CrossRef]
178. Mousavi, S.M.; Mirzargar, S.S.; Ebrahim Zadeh Mousavi, H.; Omid Baigi, R.; Khosravi, A.; Bahonar, A.; Ahmadi, M. Evaluation of antifungal activity of new combined essential oils in comparaison with malachite green on hatching rate in rainbow trout (*Oncorhynchus mykiss*) eggs. *J. Fish. Aquat. Sci.* **2009**, *4*, 103–110.
179. Rhoobunjongde, W.; Hatai, K.; Wada, S.; Kubota, S.S. *Fusarium moniliforme* (Sheldon) isolated from gills of kuruma prawn *Penaeus japonicus* (Bate) with black gill disease. *Nippon Suisan Gakkaishi* **1991**, *57*, 629–635. [CrossRef]
180. Tolosa, J.; Font, G.; Mañes, J.; Ferrer, E. Natural occurrence of emerging *Fusarium mycotoxins* in feed and fish from aquaculture. *J. Agric. Food Chem.* **2014**, *62*, 12462–12470. [CrossRef] [PubMed]
181. Grovel, O.; Pouchus, Y.F.; Verbist, J.-F. Accumulation of gliotoxin, a cytotoxic mycotoxin from *Aspergillus fumigatus*, in blue mussel (*Mytilus edulis*). *Toxicon* **2003**, *42*, 297–300. [CrossRef]
182. Matallah-Boutiba, A.; Ruiz, N.; Sallenave-Namont, C.; Grovel, O.; Amiard, J.-C.; Pouchus, Y.F.; Boutiba, Z. Screening for toxigenic marine-derived fungi in *Algerian* mussels and their immediate environment. *Aquaculture* **2012**, *342–343*, 75–79. [CrossRef]
183. Karthikeyan, V.; Gopalakrishnan, A. A novel report of phytopathogenic fungi *Gilbertella persicaria* infection on *Penaeus monodon*. *Aquaculture* **2014**, *430*, 224–229. [CrossRef]
184. Karthikeyan, V.; Selvakumar, P.; Gopalakrishnan, A. A novel report of fungal pathogen *Aspergillus awamori* causing black gill infection on *Litopenaeus vannamei* (pacific white shrimp). *Aquaculture* **2015**, *444*, 36–40. [CrossRef]
185. Kitazato, K.; Wang, Y.; Kobayashi, N. Viral infectious disease and natural products with antiviral activity. *Drug Discov. Ther.* **2007**, *1*, 14–22. [PubMed]
186. Yasuhara-Bell, J.; Lu, Y. Marine compounds and their antiviral activities. *Antivir. Res.* **2010**, *86*, 231–240. [CrossRef] [PubMed]
187. Husseiny, S.; Ibrahem, A.B.B.; El Sayed, A.F.M.; Mohammed, F.A. In vitro evaluation of anti-microbial activities of marine streptomyces against viral models, bacterial and fungal Strains. *Int. J. Virol.* **2015**, *11*, 20–31.
188. Vo, T.-S.; Ngo, D.-H.; Ta, Q.V.; Kim, S.-K. Marine organisms as a therapeutic source against herpes simplex virus infection. *Eur. J. Pharm. Sci.* **2011**, *44*, 11–20. [CrossRef]
189. Gerber, P.; Dutcher, J.D.; Adams, E.V.; Sherman, J.H. Protective Effect of Seaweed Extracts for Chicken Embryos Infected with Influenza B or Mumps Virus. *Exp. Biol. Med.* **1958**, *99*, 590–593. [CrossRef]

190. Ehresmann, D.W.; Deig, E.F.; Hatch, M.T.; DiSalvo, L.H.; Vedros, N.A. Antiviral Substances from California Marine Algae1. *J. Phycol.* **1977**, *13*, 37–40. [CrossRef]
191. Bouhlal Rhimou, R.H. Antiviral activity of the extracts of *Rhodophyceae* from Morocco. *Afr. J. Biotechnol.* **2010**, *9*, 7968–7975. [CrossRef]
192. Luescher-Mattli, M. Algae, a possible source for new drugs in the treatment of HIV and other viral diseases. *Curr. Med. Chem.—Anti-Infect. Agents* **2003**, *2*, 219–225. [CrossRef]
193. Schaeffer, D.J.; Krylov, V.S. Anti-HIV activity of extracts and compounds from algae and cyanobacteria. *Ecotoxicol. Environ. Saf.* **2000**, *45*, 208–227. [CrossRef] [PubMed]
194. Soares, A.R.; Robaina, M.C.S.; Mendes, G.S.; Silva, T.S.L.; Gestinari, L.M.S.; Pamplona, O.S.; Yoneshigue-Valentin, Y.; Kaiser, C.R.; Romanos, M.T.V. Antiviral activity of extracts from Brazilian seaweeds against herpes simplex virus. *Rev. Bras. Farmacogn.* **2012**, *22*, 714–723. [CrossRef]
195. Wijesekara, I.; Pangestuti, R.; Kim, S.-K. Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. *Carbohydr. Polym.* **2011**, *84*, 14–21. [CrossRef]
196. Arif, J.M.; Farooqui, A.; Siddiqui, M.H.; Al-Karrawi, M.; Al-Hazmi, A.; Al-Sagair, O.A. Chapter 5: Novel bioactive peptides from cyanobacteria: Functional, biochemical, and biomedical significance. In *Studies in Natural Products Chemistry*; Bioactive Natural Products; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, The Netherlands, 2012; Volume 36, pp. 111–161.
197. Hayashi, T.; Hayashi, K.; Maeda, M.; Kojima, I. Calcium spirulan, an inhibitor of enveloped virus replication, from a blue-green alga *Spirulina platensis*. *J. Nat. Prod.* **1996**, *59*, 83–87. [CrossRef] [PubMed]
198. Rechter, S.; König, T.; Auerochs, S.; Thulke, S.; Walter, H.; Dörnenburg, H.; Walter, C.; Marschall, M. Antiviral activity of Arthrospira-derived spirulan-like substances. *Antivir. Res.* **2006**, *72*, 197–206. [CrossRef] [PubMed]
199. Ahmadi, A.; Zorofchian Moghadamtousi, S.; Abubakar, S.; Zandi, K. Antiviral potential of algae polysaccharides isolated from marine sources: A review. *BioMed Res. Int.* **2015**, *2015*, 825203. [CrossRef] [PubMed]
200. Huleihel, M.; Ishanu, V.; Tal, J.; Arad, S. Antiviral effect of red microalgal polysaccharides on Herpes simplex and Varicella zoster viruses. *J. Appl. Phycol.* **2001**, *13*, 127–134. [CrossRef]
201. Santoyo, S.; Plaza, M.; Jaime, L.; Ibañez, E.; Reglero, G.; Señorans, F.J. Pressurized liquid extraction as an alternative process to obtain antiviral agents from the edible microalga *Chlorella vulgaris*. *J. Agric. Food Chem.* **2010**, *58*, 8522–8527. [CrossRef] [PubMed]
202. Ohta, S.; Ono, F.; Shiomi, Y.; Nakao, T.; Aozasa, O.; Nagate, T.; Kitamura, K.; Yamaguchi, S.; Nishi, M.; Miyata, H. Anti-Herpes Simplex Virus substances produced by the marine green alga, *Dunaliella primolecta*. *J. Appl. Phycol.* **1998**, *10*, 349–356. [CrossRef]
203. Santoyo, S.; Jaime, L.; Plaza, M.; Herrero, M.; Rodriguez-Meizoso, I.; Ibañez, E.; Reglero, G. Antiviral compounds obtained from microalgae commonly used as carotenoid sources. *J. Appl. Phycol.* **2011**, *24*, 731–741. [CrossRef]
204. De Jesus Raposo, M.F.; de Morais, A.M.M.B.; de Morais, R.M.S.C. Influence of sulphate on the composition and antibacterial and antiviral properties of the exopolysaccharide from *Porphyridium cruentum*. *Life Sci.* **2014**, *101*, 56–63. [CrossRef] [PubMed]
205. Radonić, A.; Thulke, S.; Achenbach, J.; Kurth, A.; Vreemann, A.; König, T.; Walter, C.; Possinger, K.; Nitsche, A. Anionic polysaccharides from phototrophic microorganisms exhibit antiviral activities to Vaccinia virus. *J. Antivir. Antiretrovir.* **2010**, *2*, 51–55. [CrossRef]
206. Huleihel, M.; Ishanu, V.; Tal, J.; Arad, S. Activity of *Porphyridium* sp. polysaccharide against Herpes simplex viruses in vitro and in vivo. *J. Biochem. Biophys. Methods* **2002**, *50*, 189–200. [CrossRef]
207. Talyshinsky, M.M.; Souprun, Y.Y.; Huleihel, M.M. Anti-viral activity of red microalgal polysaccharides against retroviruses. *Cancer Cell Int.* **2002**, *2*. [CrossRef]
208. Bergé, J.P.; Bourgougnon, N.; Alban, S.; Pojer, F.; Billaudel, S.; Chermann, J.C.; Robert, J.M.; Franz, G. Antiviral and anticoagulant activities of a water-soluble fraction of the marine diatom *Haslea ostrearia*. *Planta Med.* **1999**, *65*, 604–609. [CrossRef] [PubMed]
209. Lee, J.-B.; Hayashi, K.; Hirata, M.; Kuroda, E.; Suzuki, E.; Kubo, Y.; Hayashi, T. Antiviral sulfated polysaccharide from *Navicula directa*, a diatom collected from deep-sea water in Toyama bay. *Biol. Pharm. Bull.* **2006**, *29*, 2135–2139. [CrossRef] [PubMed]

210. Hasui, M.; Matsuda, M.; Okutani, K.; Shigeta, S. In vitro antiviral activities of sulfated polysaccharides from a marine microalga (*Cochlodinium polykrikoides*) against human immunodeficiency virus and other enveloped viruses. *Int. J. Biol. Macromol.* **1995**, *17*, 293–297. [CrossRef]
211. Yim, J.H.; Kim, S.J.; Ahn, S.H.; Lee, C.K.; Rhie, K.T.; Lee, H.K. Antiviral effects of sulfated exopolysaccharide from the marine microalga *Gyrodinium impudicum* strain KG03. *Mar. Biotechnol.* **2004**, *6*, 17–25. [CrossRef] [PubMed]
212. Kim, M.; Yim, J.H.; Kim, S.-Y.; Kim, H.S.; Lee, W.G.; Kim, S.J.; Kang, P.-S.; Lee, C.-K. In vitro inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03. *Antivir. Res.* **2012**, *93*, 253–259. [CrossRef]
213. Ahne, W. Viral infections of aquatic animals with special reference to Asian aquaculture. *Annu. Rev. Fish Dis.* **1994**, *4*, 375–388. [CrossRef]
214. Arzul, I.; Corbeil, S.; Morga, B.; Renault, T. Viruses infecting marine molluscs. *J. Invertebr. Pathol.* **2016**, under review.
215. Crane, M.; Hyatt, A. Viruses of Fish: An Overview of Significant Pathogens. *Viruses* **2011**, *3*, 2025–2046. [CrossRef] [PubMed]
216. Segarra, A.; Pépin, J.F.; Arzul, I.; Morga, B.; Faury, N.; Renault, T. Detection and description of a particular *Ostreid herpesvirus* 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.* **2010**, *153*, 92–99. [CrossRef] [PubMed]
217. Arzul, I.; Renault, T.; Lipart, C.; Davison, A.J. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *J. Gen. Virol.* **2001**, *82*, 865–870. [CrossRef] [PubMed]
218. Gomez-Casado, E.; Estepa, A.; Coll, J.M. A comparative review on European-farmed finfish RNA viruses and their vaccines. *Vaccine* **2011**, *29*, 2657–2671. [CrossRef] [PubMed]
219. Sommerset, I.; Krossøy, B.; Biering, E.; Frost, P. Vaccines for fish in aquaculture. *Expert Rev. Vaccines* **2005**, *4*, 89–101. [CrossRef] [PubMed]
220. Loker, E.S.; Adema, C.M.; Zhang, S.-M.; Kepler, T.B. Invertebrate immune systems—Not homogeneous, not simple, not well understood. *Immunol. Rev.* **2004**, *198*, 10–24. [CrossRef] [PubMed]
221. Rowley, A.F.; Pope, E.C. Vaccines and crustacean aquaculture—A mechanistic exploration. *Aquaculture* **2012**, *334–337*, 1–11. [CrossRef]
222. Johnson, K.N.; van Hulten, M.C.W.; Barnes, A.C. "Vaccination" of shrimp against viral pathogens: Phenomenology and underlying mechanisms. *Vaccine* **2008**, *26*, 4885–4892. [CrossRef] [PubMed]
223. Venegas, C.A.; Nonaka, L.; Mushiake, K.; Nishizawa, T.; Murog, K. Quasi-immune response of *Penaeus japonicus* to penaeid rod-shaped DNA virus (PRDV). *Dis. Aquat. Org.* **2000**, *42*, 83–89. [CrossRef] [PubMed]
224. Green, T.J.; Montagnani, C. Poly I: C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with *Ostreid herpesvirus* (OsHV-1 μvar). *Fish Shellfish Immunol.* **2013**, *35*, 382–388. [CrossRef] [PubMed]
225. Jemaà, M.; Morin, N.; Cavelier, P.; Cau, J.; Strub, J.M.; Delsert, C. Adult somatic progenitor cells and hematopoiesis in oysters. *J. Exp. Biol.* **2014**, *217*, 3067–3077. [CrossRef] [PubMed]
226. Sivasankar, P.; Anix Vivek Santhiya, A.; Kanaga, V. A review on plants and herbal extracts against viral diseases in aquaculture. *J. Med. Plants Stud.* **2015**, *3*, 75–79.
227. Balasubramanian, G.; Sarathi, M.; Venkatesan, C.; Thomas, J.; Hameed, A.S.S. Studies on the immunomodulatory effect of extract of *Cyanodon dactylon* in shrimp, *Penaeus monodon*, and its efficacy to protect the shrimp from white spot syndrome virus (WSSV). *Fish Shellfish Immunol.* **2008**, *25*, 820–828. [CrossRef] [PubMed]
228. Fabregas, J.; García, D.; Fernandez-Alonso, M.; Rocha, A.I.; Gómez-Puertas, P.; Escribano, J.M.; Otero, A.; Coll, J.M. In vitro inhibition of the replication of haemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV) by extracts from marine microalgae. *Antivir. Res.* **1999**, *44*, 67–73. [CrossRef]
229. Katharios, P.; Papadakis, I.; Prapas, A.; Dermon, C.; Ampatzis, K.; Divanach, P. Mortality control of viral encephalopathy and retinopathy in 0+ grouper *Epinephelus marginatus* after prolonged bath in dense *Chlorella minutissima* culture. *Bull. Eur. Assoc. Fish Pathol.* **2005**, *25*, 28–31.

© 2016 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).

ANNEXE 2 : Publication

Falaise et al. (2019), *Aquatic Toxicology* [*Texte Complet*]

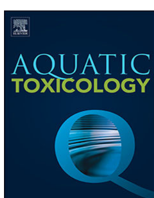
Falaise, C., Cormier, P., Tremblay, R., Audet, C., Deschênes, J.-S., Turcotte, F., François, C., Seger, A., Hallegraeff, G., Lindquist, N., Sirjacobs, D., Gobert, S., Lejeune, P., Demoulin, V., Mouget, J.-L., 2019. **Harmful or harmless: Biological effects of marennine on marine organisms**. *Aquatic Toxicology* 209, 13–25.

Aquatic Toxicology 209 (2019) 13–25

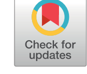
Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Harmful or harmless: Biological effects of marennine on marine organisms



Charlotte Falaise[a], Patrick Cormier[b], Réjean Tremblay[c], Céline Audet[c], Jean-Sébastien Deschênes[c], François Turcotte[c], Cyrille François[d], Andreas Seger[e,f], Gustaaf Hallegraeff[e], Niels Lindquist[g], Damien Sirjacobs[h], Sylvie Gobert[j,i], Pierre Lejeune[j], Vincent Demoulin[h], Jean-Luc Mouget[a,*]

[a] Laboratoire Mer Molécule Santé (EA 2160, FR CNRS 3473 IUML), Le Mans Université, Le Mans, France
[b] Sorbonne Universités (UPMC Paris 06, CNRS, UMR 8227) Biologie Intégrative des Modèles Marins, Station Biologique de Roscoff, Roscoff, France
[c] Institut des sciences de la mer, Université du Québec à Rimouski, Rimouski, Canada
[d] Ifremer, RBE-SG2M-LGPMM, Laboratoire de Génétique et de Pathologie des Mollusques Marins, Station La Tremblade, Avenue Mus Loup, F-17390, La Tremblade, France
[e] Institute for Marine and Antarctic Studies (IMAS), University of Tasmania, Hobart, Australia
[f] South Australian Research and Development Institute (SARDI), Urrbrae, South Australia, Australia
[g] Institute of Marine Sciences, University of North Carolina, Chapel Hill, United States
[h] INBIOS - PHYTOSYSTEMS, Eukaryotic Phylogenomics, Liège University, Liège, Belgium
[i] Université de Liège, MARE, Focus, Océanologie Biologique, Liège, Belgium
[j] Station de Recherche Sous-marines et Océanographiques (STARESO), 20260, Calvi, France

ARTICLE INFO

Keywords:
Diatom
Haslea ostrearia
Marennine
Marine organisms
Natural bioactive compound

ABSTRACT

Marennine is a water-soluble blue-green pigment produced by the marine diatom *Haslea ostrearia*. The diatom and its pigment are well known from oyster farming areas as the source of the greening of oyster gills, a natural process increasing their market value in Western France. Blooms of blue *Haslea* are also present outside oyster ponds and hence marine organisms can be exposed, periodically and locally, to significant amounts of marennine in natural environments. Due to its demonstrated antibacterial activities against marine pathogenic bacteria (*e.g. Vibrio*) and possible prophylactic effects toward bivalve larvae, marennine is of special interest for the aquaculture industry, especially bivalve hatcheries. The present study aimed to provide new insights into the effects of marennine on a large spectrum of marine organisms belonging to different phyla, including species of aquaculture interest and organisms frequently employed in standardised ecotoxicological assays. Different active solutions containing marennine were tested: partially purified Extracellular Marennine (EMn), and concentrated solutions of marennine present in *H. ostrearia* culture supernatant; the Blue Water (BW) and a new process called Concentrated Supernatant (CS). Biological effects were meanwhile demonstrated in invertebrate species for the three marennine-based solutions at the highest concentrations tested (*e.g.*, decrease of fertilization success, delay of embryonic developmental stages or larval mortality). Exposure to low concentrations did not impact larval survival or development and even tended to enhance larval physiological state. Furthermore, no effects of marennine were observed on the fish gill cell line tested. Marennine could be viewed as a Jekyll and Hyde molecule, which possibly affects the earliest stages of development of some organisms but with no direct impacts on adults. Our results emphasize the need to determine dosages that optimize beneficial effects and critical concentrations not to be exceeded before considering the use of marennine in bivalve or fish hatcheries.

1. Introduction

Haslea ostrearia is a cosmopolitan marine pennate diatom that synthesizes and releases a water-soluble blue-green pigment called marennine. This species can bloom erratically in oyster ponds in Western France (Baie de Marennes-Oléron, Baie de Bourgneuf), a phenomenon that has long been known to be responsible for the greening of oyster gills. This phenomenon is of economic interest for the French oyster farming industry as the market value of green oyster is significantly increased (by 20 to 30%) due to changes in the organoleptic properties of the oysters and the scarcity of the product, blooms in ponds being an erratic phenomenon. In the last decade, new

* Corresponding author.
E-mail address: Jean-Luc.Mouget@univ-lemans.fr (J.-L. Mouget).

https://doi.org/10.1016/j.aquatox.2019.01.016
Received 12 November 2018; Received in revised form 16 January 2019; Accepted 17 January 2019
Available online 21 January 2019
0166-445X/ © 2019 Elsevier B.V. All rights reserved.

Fig. 1. Underwater pictures of natural blue *Haslea* blooms forming biofilms observed **A)** in macro-algae *Padina* sp. in the Mediterranean sea, Corsica (France) and **B)** on sediments in the Beaufort Strait, North Carolina (United States).

species of blue *Haslea* have been discovered, some of them producing marennine-like pigments chemically distinct from the originally described marennine molecule (Gastineau et al., 2012, 2016). Blooms of blue *Haslea* are also observed in natural environments, such as in the Mediterranean Sea and East coast of the USA (Fig. 1). Moreover, oysters with green gills have long been observed worldwide, *e.g.*, in Great Britain (Sprat, 1667), Denmark (Petersen, 1916), the USA (Mitchell and Barney, 1917) and Australia (Hallegraeff and Mouget, personal communication). Furthermore, the greening of gills has been reported in other organisms, such as polychaetes, crabs, littorina, mussels (Ranson, 1927), sea-anemones (Gaillon, 1820), scallops and cockles (Gastineau et al., 2018), illustrating that many marine organisms can be exposed to blue *Haslea* populations and marennine-like pigments not only in artificial (oyster) ponds, but also in natural environments.

Although our knowledge of the blue *Haslea* biodiversity has recently increased, little is known about the chemical properties of marennine and marennine-like pigments or their functions for the microalgae. Marennine is thought to be produced *via* a cytoplasmic synthesis pathway (Nassiri et al., 1998) and transiently accumulates at the cell apices (intracellular form of the pigment, IMn). Marennine is excreted from the cells, possibly by exocytosis *via* small vesicles, which collapse and release an extracellular form of marennine (EMn). The two forms of the pigment differ in their UV–vis spectral characteristics and molecular mass (Pouvreau et al., 2006b). Marennine is a complex molecule composed of glycosidic units (Gastineau et al., 2014) attached to one or various aromatic rings (Pouvreau et al., 2006b). A protocol to obtain a purified form of EMn or IMn has been developed (Pouvreau et al., 2006c) and several authors have proposed different methods to estimate marennine concentration in solution, despite incomplete knowledge of its chemical structure (Pouvreau et al., 2006a; Robert et al., 2002).

Various studies have demonstrated that marennine (as purified molecule or raw extract) has multiple biological activities, such as antioxidant (Pouvreau et al., 2008), antiproliferative (Carbonnelle et al., 1998; Gastineau et al., 2012), antiviral (Bergé et al., 1999; Gastineau et al., 2012) and antibacterial (Falaise et al., 2016; Gastineau et al., 2014, 2012). It has also been shown that marennine possesses allelopathic properties, limiting the growth of various microalgae (Pouvreau et al., 2007; Prasetiya et al., 2016). The biological activities of marennine are species- and even strain-dependent in the case of bacteria (Falaise et al., 2016), suggesting that marennine could act on specific molecular targets. Tests conducted with Gram-negative bacteria have demonstrated activity of the marennine-like pigment produced by *Haslea provincialis* (Gastineau et al., 2016) and marennine produced by *H. ostrearia*, on the lipopolysaccharidic cell membrane of *Escherichia coli* (Tardy-Laporte et al., 2013) and of *Vibrio splendidus* (Bouhlel et al., 2018), rendering it more rigid.

In line with these results, particularly those demonstrating the capacity of marennine to limit the proliferation of certain pathogenic marine bacteria (Falaise et al., 2016; Gastineau et al., 2012, 2014), further research has confirmed the protective effect of marennine on the giant scallop (*Placopecten magellanicus*) and blue mussel (*Mytilus edulis*) larvae when challenged with *V. splendidus* (Turcotte et al., 2016). The same study also revealed that a 20 d (day) exposure of mussel larvae to low concentrations of marennine (0.1 μg mL^{-1}) provided a significantly higher survival rate than the control, although exposure to higher concentrations of marennine (1 μg mL^{-1}) resulted in 100% larval mortality. Even brief exposure to marennine has been demonstrated to result in behavioural, physiological and biochemical changes that were still present eight weeks after exposure (Prasetiya et al., 2017).

These results underpin the interest to further study the effects of marennine on early developmental stages of marine organisms, to identify which are possibly sensitive to marennine, as well as defining exposure concentrations and durations that would be beneficial to species of aquaculture interest. The present work presents a broad panel of experiments conducted to provide an overview of the biological effects of marennine solutions on various marine organisms belonging to different phyla. The organisms were selected based on aquaculture interest or their established role in ecotoxicological model assays. Different solutions containing marennine, prepared from *H. ostrearia* culture supernatants, were used in the exposures: the purified EMn (Pouvreau et al., 2006c), the Blue Water (BW; (Turcotte et al., 2016) and a newly patented Concentrated Supernatant (CS; see Materials). Although EMn represents the most purified form of marennine currently available, BW and CS are of particular interest if the use of marennine-based solutions is considered at larger scales than laboratory experiments due to their easier and cheaper method of production. Marennine exposures were conducted on embryos and/or larvae of the mollusc *M. edulis*, the crustacean *Chthamalus bisinuatus*, the chordate *Pseudopleuronectes americanus* and the echinoderms *Sphaerechinus granularis* and *Paracentrotus lividus*. The effects of the blue pigment solutions were also investigated *in vitro* on the fish gill cell line RTgill-W1, on the oyster *Crassostrea gigas* haemocytes, and on prokaryotic models with bacteria of the genus *Vibrio*, providing a broad view of the diversity of marennine effects on marine organisms.

2. Materials and methods

Purified form of extracellular marennine (EMn), Blue Water (BW) and Concentrated Supernatant (CS) were all obtained from *H. ostrearia* culture supernatant (Table 1), but the process to obtain BW and CS is faster and has a better yield in comparison with purified EMn. BW and CS are not purified marennine *per se*, but they allow preparing concentrated solutions of marennine needed to run dose-response experiments between *Haslea* pigment and target organisms. Experiments are presented in the Results section in the following order: 1) Experiments using the BW solution, 2) CS solution, and 3) purified EMn solution.

Table 1
Summary of the marennine based solutions and marine organisms used in the present study.

Marennine based solutions	Production process	UV-visible spectra	Method of quantification	Marine organisms tested and studied effect
Blue Water **(BW)**	Ultrafiltration of *H. ostrearia* culture medium (3-30 kDa)		$\varepsilon_{677} = 12.13\ L\ g^{-1}\ cm^{-1}$ (Pouvreau et al., 2006c)	Mussel *M. edulis,* barnacle *C. bisinuatus* and winter flounder *P. americanus*: Larval survival and/or development Bacterial species of the *Vibrio* genus: Bacterial growth
Concentrated Supernatant **(CS)**	Innovative process being patented		$\varepsilon_{669} = 17.2\ L\ g^{-1}\ cm^{-1}$ (Robert et al., 2002) $\varepsilon_{677} = 12.13\ L\ g^{-1}\ cm^{-1}$ (Pouvreau et al., 2006c)	Bacterial species of the *Vibrio* genus: Bacterial growth Sea urchin *S. granularis*: Fertilization, early embryonic development
Purified Extracellular Marennine **(EMn)**	- Ultrafiltration of *H. ostrearia* culture medium (3-30 kDa) - Anion-Exchange chromatography - Dialysis & freeze-drying (Pouvreau et al., 2006c)	Adapted from Pouvreau et al. (2006)	$\varepsilon_{677} = 12.13\ L\ g^{-1}\ cm^{-1}$ (Pouvreau et al., 2006c) Weighing of the purified EMn dried powder	Preliminary assays with EMn on the sea urchins *S. granularis* and *P. lividus*: Fertilization Oyster *C. gigas* haemocytes: Cytotoxicity Fish gill cell line: Cytotoxicity

2.1. Preparation of the purified extracellular marennine (EMn), Blue Water (BW) and Concentrated Supernatant (CS)

The Blue Water (BW) and purified extracellular marennine (EMn) were produced at the *Station aquicole de Pointe-au-Père* (Québec, Canada) and provided by the *Institut des sciences de la mer de Rimouski-Université du Québec à Rimouski* (ISMER-UQAR; Québec, Canada). The production process of BW and purified EMn were previously described (Turcotte et al., 2016; Pouvreau et al., 2006c). Briefly, *H. ostrearia* strains (NCC 136), isolated from Bourgneuf Bay (France) and provided by NCC (Nantes Culture Collection), were cultured in 100 L photobioreactors until the extracellular marennine concentration reached a maximum of 6 to 8 μg mL^{-1}. Marennine concentration was determined on cell-free culture supernatant (filtered through Sarstedt 0.2 μm syringe filters) using a spectrophotometer (Cary 100 Bio UV–vis, Agilent Technologies) and the Beer-Lambert's equation ($\varepsilon_{677} = 12.13\ L\ g^{-1}\ cm^{-1}$) as proposed by Pouvreau et al. (2006c). The BW was then obtained by concentration of the culture supernatant containing EMn by ultrafiltration (double cut off 3–30 kDa; (Turcotte et al., 2016). To obtain the purified EMn, the BW was further treated by an anion-exchange chromatography process and the fraction collected was dialyzed and freeze-dried (Pouvreau et al., 2006c). BW and purified EMn were stored in the dark at 4 °C and −20 °C respectively. Concentrated Supernatant (CS) was produced in the *Mer Molécule Santé* (MMS) and *Institut des Molécules et Matériaux du Mans* (IMMM) laboratories (Le Mans, France). A strain of *H. ostrearia* (NCC 495) was batch cultured in 500 mL Erlenmeyers flask containing 250 mL of autoclaved sea water prepared from a commercial sea salt mix (Instant Ocean, Aquarium Systems®; pH 7.6 ± 0.2; salinity 32) with an enrichment solution as described in Mouget et al. (2009). Microalgal cultures were maintained in a 16 °C temperature-controlled room at an irradiance of 200 μmol photon $m^{-2}\ s^{-1}$, with illumination provided by cool-white fluorescent tubes in a 14/10 h light/dark cycle. At the beginning of the stationary growth phase the culture supernatant containing EMn was collected by decantation of the microalgal cells and subsequent vacuum filtrations through 15 μm (150 mm Filter paper, Fisher Scientific®) and 1.2 μm (37 mm glass microfiber filters, Whatman®). Filtered culture supernatant was collected in 1 L glass bottles and the EMn was then concentrated using an innovative technique recently patented (patent n°: 1,872,316). The resulting solution was then dialyzed using a 2 kDa dialysis membrane (Spectra/Por®6, Spectrum®). Dialysis tubes were placed in ultra-pure water tanks for 3 d under agitation with the water changed every 24 h. The dialyzed solution was then ultra-filtered (double cut-off 1 kD-30 kDa; Kros Flo® Research IIi TFF System and Kros Flo® Automatic Backpressure valve, Spectrum®) and further concentrated by evaporation with a Rotavapor (Vacuum controller CVC2, Vacuubrand®; Rotary Elevator, Heating Bath Hei-Vap, Base Hei-Vap ML Adv/Pre, Heidolph®). The pH of the resulting CS was neutralized if required to 7.5 ± 0.2 by addition of NaOH 0.1 M. The CS was stored one week in the dark at 4 °C until use. The concentration of marennine in the CS was determined using a spectrophotometer and the Beer-Lambert equation ($\varepsilon_{669} = 17.2\ L\ g^{-1}\ cm^{-1}$) as proposed by (Robert et al., 2002). UV–vis spectra of the different marennine based solutions were conducted using quartz cuvettes with 1 cm path length (UV/Vis Lambda 25 Perkin Elmer spectrophotometer, UV Winlab software).

2.2. Exposure of the mussel Mytilus edulis larvae to BW

Adult mussels were obtained in summer 2017 from a farm in the Magdalen Islands (47° 25′N, 61° 50′W, Quebec, Canada), characterized by pure *M. edulis* populations (Myrand et al., 2009) and conditioned for spawning in the *Station aquicole de Pointe-au-Père* (ISMER-UQAR; Québec, Canada) facilities for one month. Mussels were maintained in 180 L tanks in 1 μm filtered seawater at 20 °C flowing at 1 L min^{-1} and were continuously fed with a mixture of *Pavlova lutherii*, *Isochrysis galbana*, and *Nannochloropsis oculata* (ratio 1:1:1) supplied with a peristaltic pump at constant flow to maintain food load at 0.5 mg L^{-1} as described in Hennebicq et al. (Hennebicq et al., 2013). Before spawning, a dozen individuals were removed from the tank and washed by gently rubbing the shell with a brush and diluted bleach and finally rinsed with filtered, UV sterilized sea water. Spawning was induced by successive thermal shocks from 10 to 25 °C. Fertilized eggs were transferred to 60 L tanks at densities of 10 eggs μL^{-1} and embryos were maintained at 18 °C for 48 h. D-larvae were collected by filtration of the water through a 20 μm mesh screen and transferred to 60 L rearing tanks at densities of 10 D-larvae mL^{-1}. Every 2–3 d, tanks were washed and sterilized and the larvae fed with a mixture of *P. lutherii, I. galbana* and *Chaetoceros gracilis* at a final concentration of 90 cells μL^{-1} (30 cell

μL^{-1} for each component of the diet; Turcotte et al., 2016).

M. edulis embryos were collected prior to the transfer to the embryogenesis tank, and D-larvae prior to the transfer to the rearing tank (48 h post fertilization). Veliger larvae were collected in the rearing tanks (14 d post fertilization) by filtration of the water through a mesh screen. Embryos and larvae were exposed to BW in 12-well flat bottom sterile microplates with each well containing 3 mL of diluted BW and 10–15 embryos or 10–15 larvae per well. Five concentrations of marennine from BW were tested: 0, 0.1, 0.5, 1 and 5 $\mu g\ mL^{-1}$. Marennine concentration from BW was estimated as described in Section 2.1 and Table 1. BW was syringe-filtered through 0.2 µm and diluted in sterile sea water. Microplates were kept in a controlled temperature room at 18 °C without addition of food during the time of the experiment. The mortality of embryos and veliger larvae was assessed using a light microscope (Olympus BX41; W. Carsen Co., Ltd., Don Mills, ON, Canada) coupled to a digital camera (Evolution VF Color, MediaCybernetics, Silver Spring, MD, USA). The percentages of swimming and motionless D-larvae were assessed by observation of the microplates using an inverted microscope (Axiovert 100, Zeiss). Larval size was measured with Image Pro-Express (Media Cybernetics) after addition of formaldehyde to the wells. A minimum of 10 larvae were counted per well with at least 3 wells per concentration tested.

2.3. Exposure of the barnacle *Chthamalus bisinuatus* larvae to BW

Experiments were conducted in June 2015 in the *Center for Marine Biology of Sao Paulo University* (CEBIMar/USP; Sao Paulo, Brazil). Spawners were harvested by collecting rock fragments in the intertidal zone of Calhetas Beach (23°49′ 28″S, 45°25′11″W). The fragments were transferred to the laboratory and placed in seawater pumped from the adjacent bay, as described in Kasten and Flores (Kasten and Flores, 2013). A light source was placed above the tanks containing the breeders to recreate the natural photoperiod. Broodstock was submerged for 30 min every 12.4 h to simulate the natural effect of the tide and stimulate spawning. After a first submersion, the water used to submerge the broodstock was siphoned and filtered to harvest the larvae. The larvae were placed in filtered seawater and isolated using a dissecting microscope to obtain a number of larvae sufficient to run the experiment. Larvae were placed individually in the wells of a 96-well microplate in 2 mL of seawater containing 0, 0.05 or 0.1 $\mu g\ mL^{-1}$ of marennine from BW without food to test only the potential toxicity of BW. The BW was prepared and the concentration estimated as described in Section 2.1 (Table 1). Four larvae were tested at each concentration. The microplate was placed on a stirring plate and no food was provided during the experiment. Larvae were observed once a day and survival recorded over 9 d. The experiment was repeated once with spat from a different production.

2.4. Exposure of the winter flounder *Pseudopleuronectes americanus* larvae to BW

Larvae were reared as described by (Khemis et al., 2003) in 57 L cylindro-conical polyethylene tanks and fed rotifers from d 4 to d 26 (Fraboulet et al., 2010). Rotifers were fed a cocktail of microalgal concentrates (1:1:1 *N. oculata*: *I. galbana*: *P. lutherii*, Instant algae, REED Mariculture) and a SELCO food supplement (1 g M^{-1} rotifers, INVE Aquaculture Nutrition, Gransville, UT, USA). The larvae were kept in green water (addition of *N. oculata* culture directly to basins). From d 2 to d 14, two concentrations of marennine from BW were tested on the larvae, 0.05 and 0.1 $\mu g\ mL^{-1}$, with three tanks per treatment (n = 3). BW preparation, as described in Section 2.1 (Table 1), was added to the tanks in the morning, when water flow was cut to feed the larvae. Water flow was restarted at the end of the day (for a total of about 8 h of exposure) and BW was gradually evacuated from the tanks with the flow of water outlet. A dose of BW was given every two days, for a total of seven treatments per tank for the duration of the experiment. Procedures were the same for the control treatments, without the addition of BW in the control tanks. The effect of BW treatment on bacterial load and larval size was evaluated on d 2, d 6, d 10, and d 14, and the size and physiological condition (assessment of energy reserves) of the larvae were determined at the end of the experiment, *i.e.* at d 14 (12 d of treatment). Standard length was measured on formaldehyde-preserved larval pictures as described in (Hjörleifsson and Klein-MacPhee, 1992) using a dissecting microscope (Olympus SZ61) coupled to a digital camera (Evolution VF; Media Cybernetics) and Image Pro-Plus measurement software 5.0 (Media Cybernetics). Lipids were extracted in a 2:1 mixture of dichloromethane: methanol according to (Folch et al., 1957), the lipid classes (triglyceride [TAG], sterol [ST], acetone mobile polar lipids [AMPL], and phospholipids [PL]) concentrations were quantified by TLC-FID as described by (Parrish, 1987), and chromatograms analyzed using PeakSimple v3.21 software (SRI Inc.). To estimate the potential effect of BW on bacterial load in rearing water, bacterial analyzes were carried out using a flow cytometer on water samples taken from the tanks and frozen with glutaraldehyde according to (Seychelles et al., 2011).

2.5. Exposure of the sea urchins *Sphaerechinus granularis* and *Paracentrotus lividus* to EMn and CS

A first series of assays were conducted in May 2016 with purified EMn on *S. granularis* and *P. lividus*, model animals frequently used for *in vivo* ecotoxicological bioassays (Buttino et al., 2016; Pinsino et al., 2010). Sea urchins were collected in the Brest area (France) and obtained from the *Centre de Ressources Biologiques Marines* (CRBM) at the *Roscoff Biological Station* (Roscoff, France). Other experiments on sea urchins were run in March 2017 using CS rather than purified EMn due to its limited production, and the species *S. granularis* because it is available throughout the year.

Marennine-based solutions were prepared by weighing purified EMn freeze-dried powder or after the estimation of CS concentration as described in Section 2.1 (Table 1). Solutions were diluted in 0.22 µm Millipore-filtered seawater (FSW) and the final solutions were syringe filtered through 0.2 µm. Sea urchin spawning was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in FSW, rinsed twice by centrifugation (2.000 *g*, 2 min) and re-suspended in FSW for a final 2% (v/v) egg solution containing 0.1% (v/v) glycine. Sperm was kept dry at 4 °C until use. Experiments took place in a temperature-controlled room at 16 °C.

Effects of purified EMn and CS were first assessed on fertilization. Eggs were transferred to a 24-well culture plate and incubated during 10 min in marennine-based solutions prior to addition of sperm. Solutions at different concentrations were added in wells containing 1 mL of the egg suspension. Only FSW was added for the control condition. pH strips were used to ensure that the pH did not vary between wells of the different concentrations tested. For fertilization, 50 µL of dry sperm was diluted in 1 mL of FSW shortly before use and 4 µL of diluted sperm added per mL of egg suspension. Observation of the culture plate under phase contrast inverted microscopy allowed the determination of the fertilization rate by counting at least 60 eggs per well (n = 3 wells per concentration tested). For experiments conducted with CS, unfertilized eggs exposed to the highest CS concentrations were rinsed 3 times in FSW using a benchtop centrifuge for 5–10 seconds and exposed to sperm for fertilization as described above. Two hours post fertilization, eggs exposed to CS were fixed on a DNA fluorescent stain (Hoechst fixative: Bisbenzymide 0.1 $\mu g\ mL^{-1}$; methanol 75%, glycerol 25%) and observed under a fluorescence microscope (ApoTome, Zeiss).

The effects of CS were also assessed post fertilization (kinetics of first cleavage and early embryonic development). For fertilization, diluted sperm was added in a 50 mL tube containing 25 mL of eggs suspended in FSW (2% (v/v) egg suspension). When egg batches exhibited greater than 90% fertilization, sperm was removed by centrifugation in

Heraeus Labofuge centrifuge (2.000 *g*, swinging bucket rotor 2 min). Fertilized eggs were transferred to a 24-well plate and CS solutions at 1; 10; 50 and 100 μg mL^{-1} added 10 min post fertilization. The percentage of dividing eggs was determined under phase contrast inverted light microscope; at least 60 eggs per well were counted. Three different sea urchin couples were used to replicate the experiment. For the "washed" condition, embryos from 3 wells per concentration tested were rinsed 6 h post fertilization as described earlier. Culture plates were maintained under constant agitation at 16 °C and embryos were observed 8, 48 and 72 h post fertilization with a Leica DMi8 inverted microscope and pictures acquisition done with LASX software. For each observation time, a 75 μL sample of each well was transferred to a glass slide and pictures of the developmental stages predominantly observed were taken. For post hatching developmental stages, a 1 μL drop of Janus green was added to the samples. Bright Field microscopy technique (BF) was applied for embryos 8 h post fertilization and a Differential Interference Contrast technique (DIC) for embryos 48 h and 72 h post fertilization.

2.6. Exposure of Vibrio species to BW and CS

Experiments were conducted during spring 2018 in *MMS* laboratory (Le Mans, France). BW and CS were prepared as described in Section 2.1 (Table 1), and the concentration of marennine was determined with spectrophotometric measurements using the Beer-Lambert law with the specific extinction coefficient (ε_{677} = 12.13 L g^{-1} cm^{-1}) proposed by (Pouvreau et al., 2006b). CS and BW were diluted with sterile ultrapure water. Salinity and pH were adjusted by addition of NaCl and 0.1 M of NaOH or HCl (pH 7.5 ± 0.2; salinity 32) and the solutions were then syringe-filtered through 0.22 μm (Sarstedt). The three *Vibrio* strains tested, *Vibrio chagasii* (strain 8T3_5), *Vibrio crassostreae* (strain 8T2_1) and *Vibrio* sp. (strain 7G1_11) were previously identified (Bruto et al., 2017) and kindly provided by the *Laboratory of Integrative Biology of Marine Models* (CRBM/Roscoff, France). Bacterial strains were kept at −80 °C in 25% glycerol, inoculated in Mueller Hinton Broth (MHB) + 1% NaCl (pH 7.5 ± 0.2; salinity 32) and incubated overnight at 25 °C. Isolations were done on Petri dishes containing agar prepared with Nutrient Agar (Biokar) + 2.3% NaCl (pH 7.5 ± 0.2; salinity 32). The plates were incubated at 25 °C for one day. The antibacterial activity of BW and CS was assessed according to a method described in the Clinical and Laboratory Standards Institute (CLSI) antimicrobial microdilution guidelines (Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI, 2012). The day prior to the experiment, three different colonies per Petri dish were inoculated in broth media and grown overnight at ambient temperature. Wells of a 96-well sterile microplate with cover and flat bottom were filled with 50 μL of either CS or BW at a final concentration of 10 μg mL^{-1} (or sterile saline water for the controls) and 50 μL of a bacterial inoculate. A negative control was also run with sterile saline water and broth media without bacteria to ensure that no contamination occurred during the experiment. To prepare the bacterial inoculate the optical density (OD) of the broth culture was measured at 630 nm (V-10 Plus Onda Spectrophotometer), the OD was adjusted to 0.1 by dilution in MHB + 1% NaCl and the solution was then further diluted by 1/100 as recommended by the CLSI guidelines (Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI, 2012). Microplates were inserted in a microplate spectrophotometer (xMark Bio-Rad) for a 20 h run at ambient temperature. Growth was managed with microplate Manager 6 Software by taking the OD in each well every 30 min at 600 nm.

2.7. Exposure of fish gill cell lines to EMn

Experiments took place in August 2017 at the Institute for Marine and Antarctic Studies (IMAS; Hobart, Australia). The gill epithelium cell line RTgill-W1 was obtained from the American Type Culture Collection (ATTC; and originally isolated from the Rainbow trout *Oncorhyncus mykiss*). The cell line was maintained and exposures to purified EMn conducted as described in Dorantes-Aranda et al. (2011) in conventional 96-well plates. Gill cells were seeded into a flat-bottom 96-well plate (655180, Greiner) at 2.5×10^5 cells mL^{-1} in L-15 medium (L-1518, Sigma) and allowed to attach for 48 h in the dark. Confluence of cell cultures was verified 12 h before experimental exposure and the L-15 medium replaced by L-15/ex (Schirmer et al., 1997). The concentration of the original purified EMn solution was estimated (540 μg mL^{-1} in 50% methanol) as described in Section 2.1 (Table 1), and the solution was diluted in L-15/ex by factors of 1×10^{-1}, 5×10^{-2}, 1×10^{-2}, 5×10^{-3}, 1×10^{-3}, 5×10^{-4} and 1×10^{-4}, yielding final exposure concentrations of 0–54 μg mL^{-1}. Methanol was added to all dilutions to achieve a constant final MeOH concentration of 5% across all treatments, including the non-toxic control (L-15/ex). Gill cells were exposed to these solutions for 2 h at 20 ± 1 °C in the dark (quadruplicate wells per concentration). After the exposure, wells were rinsed twice with saline phosphate buffer (100 μL per well) and incubated for a further 2 h in the dark with 100 μL of 5% resazurin viability stain in L-15/ex medium. Metabolic reduction of resazurin to resorufin by the gill cells was measured in a microplate reader (Fluostar Omega, BMG Labtech) at excitation and emission wavelengths of 540 and 590 nm, respectively. Results are expressed as percentage viability of the nontoxic control (5% MeOH in L-15/ex).

2.8. Exposure of the oyster Crassostrea gigas haemocytes to EMn

Pacific oysters *C. gigas* were harvested in 2015–2016 in the hatchery of *Laboratoire de Génétique et de Pathologie des Mollusques Marins* (LGPMM; La Tremblade, France) in raceways supplied with a constant flow of seawater enriched with phytoplankton (*Skeletonema costatum, Tetraselmis suecica, I. galbana* and *C. gracilis*). They were maintained in safe conditions, free of known infectious pathogens. The shell of 20 adult oysters was broken with metal clamps and haemolymph withdrawn from the adductor muscle sinus (1 to 1.5 mL of haemolymph per oyster) using 1 mL sterile plastic syringes equipped with a needle (0.90 mm x 25 mm). The haemolymph of the different individuals was pooled, filtered through 60 μm nylon mesh and held on ice to prevent haemocyte aggregation (Auffret and Oubella, 1997). Haemocytes were observed under light microscopy using a Malassez-cell. Haemocytes were then exposed to different concentrations of purified EMn: 0, 1, 50 and 100 μg mL^{-1}. The purified EMn was obtained as described in Section 2.1 (Table 1) and the solutions were prepared by weighing of the EMn dried powder on an analytical balance (Sartorius Entris®) and by dilution on sterile sea water. Purified EMn solutions were added to the haemolymph (1:1 ratio) for a final haemocyte concentration of 2.10^4 cells mL^{-1}. Haemocytes were exposed to EMn during 1, 3 or 6 h and mortality was quantified using 200 μL of cell suspension. Cells were incubated in the dark for 30 min on ice with 50 μL of Propidium Iodide (PI, 1.0 g L^{-1}, Interchim), a fluorescent DNA/RNA-specific dye that only permeates through the membranes of dead cells and stains the nucleic acids. Haemocyte samples were analyzed with flow cytometry using an EPICS XL 4 (Beckman Coulter) and red fluorescence following the protocol of (Morga et al., 2009). Based on size discrimination, only haemocytes were taken into account with 5.000 events counted per sample. Results were depicted as cell cytograms and reported as log scale fluorescence levels. Data were analyzed with *Flowing Software 2*.

2.9. Statistics

Statistical analyses were run using *SigmaPlot 12.3* software for Windows. Differences between treatments were assessed with One-Way or Two-Ways ANOVAs. Normality was tested by the Shapiro-Wilk test and the assumption of homoscedasticity of variance with Fisher's test (F-test) and/or verified visually by the spread of residuals, as suggested by (Quinn and Keough, 2002). Post hoc Tukey's pairwise multiple

comparison tests were used to determine differences between means. Unless specified, alpha value used was 0.05.

3. Results

3.1. Differences in the solutions containing the extracellular marennine

BW and CS presented the same UV–vis spectral characteristics in comparison with purified EMn (Pouvreau et al., 2006b), with one peak around 670 nm in the visible region and two others in the UV region around 250 and 320 nm. Different coefficients of extinction were used to assess the extracellular marennine concentration depending on the preparation and concentration process and on the series of experiments (summarized in Table 1). Despite a difference in calculated concentrations of about 30–40 % resulting from the use of either the apparent extinction coefficient proposed by (Robert et al., 2002) or the one proposed by (Pouvreau et al., 2006a), the solutions tested had comparable concentration ranges and activities as described below.

3.2. Effects of BW on *Mytilus edulis* larvae

After a 48 h exposure of embryos to BW, the percentage of larvae that reached the D-larval stage and the mortality rate were assessed (Fig. 2A). The three lowest concentrations tested did not delay larval development; however, when embryos were exposed to 1 μg mL^{-1} of BW, the percentage of D-larvae was significantly lower (22 ± 1% of D-larvae compared to 52 ± 2% for the control; *p*-value = 0.026). Exposure to 1 μg mL^{-1} significantly increased the mortality rate, with 47 ± 1% of mortality while no mortality was recorded in control condition. Exposure to 1 μg mL^{-1} of BW significantly delayed the development with a D-larvae mean length of 88 ± 7 μm compared to 122 ± 1 μm for the control (*p*-value < 0.001; Fig. 2B). At 5 μg mL^{-1}, none of the embryos survived after a 48 h exposure to BW (Fig. 2A).

In a second series of experiments, D-larvae were exposed to BW in order to assess the effects on larval swimming (Fig. 3A). After 24 h in BW, the percentage of swimming larvae exposed to 1 μg mL^{-1} was significantly lowered (80 ± 9% compared to 100 ± 0% for the control; *p*-value < 0.001). D-larvae exposed to 5 μg mL^{-1} of BW were totally motionless, with the velum highly coloured with marennine (Fig. 3B). After 72 h in BW, the percentage of swimming D-larvae exposed to 1 μg mL^{-1} of BW decreased significantly in comparison with the day one with only 45 ± 2% of swimming larvae (*p*-value 0.012).

When veliger larvae were exposed over a 5 d period to BW (Fig. 4A), a significant increase in mortality was only observed at a concentration of 5 μg mL^{-1}, with 64 ± 7% of dead veliger larvae compared to 7 ± 6% of mortality in control (*p*-value < 0.001). Larval length only differed for treatments of 0.5 and 1 μg mL^{-1} compared to 5 μg mL^{-1} of BW (Fig. 4B; *p*-values 0.036 and 0.030 respectively).

3.3. Effect of BW on the barnacle *Chthamalus bisinuatus* larvae

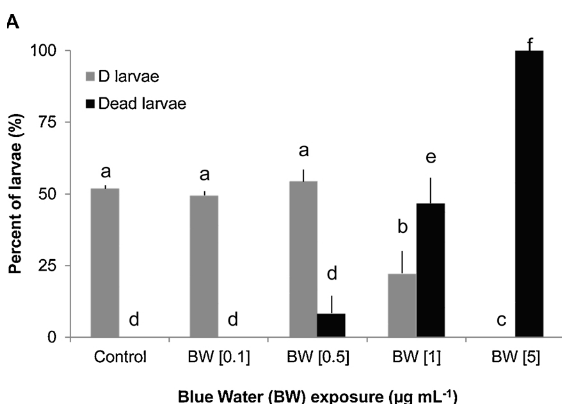
The survival rate of *C. bisinuatus* larvae was measured at two concentrations of BW, 0.05 and 0.1 μg mL^{-1} over a 9 d period, the maximum period to maintain barnacle larvae without food. At both concentrations, exposure to BW had no significant effect on *C. bisinuatus* larval survival rate in comparison with the control (Fig. 5; *p*-value 0.1427).

3.4. Effect of BW on the winter flounder *Pseudopleuronectes americanus* larvae

The addition of BW at 0.05 and 0.1 μg mL^{-1} had no effect on larval growth (*p*-value 0.287). Indeed, the three groups experienced a growth of 0.1 mm d^{-1} from d 2 to d 14 (Fig. 6A). The bacterial load remained comparable in all treatments (*p*-value 0.868), demonstrating that, at such concentration, marennine had no impact on bacterial development in fish rearing tanks (Fig. 6B). Energy reserves in larvae treated with marennine seemed to increase with 4.6 ± 0.7 μg $larva^{-1}$ under 0.1 μg mL^{-1} of BW exposure *vs* 3.9 ± 0.7 μg $larva^{-1}$ for the control, but the difference from the control was not significant due to the high variability among tanks (total lipids, *p*-value 0.1092; triacyglycerol/sterol (TAG/ST) ratio, *p*-value 0.0767). However, the larvae showing the highest lipid content, and the only ones containing TAG, were sampled from the marennine treated tanks (Fig. 6C).

3.5. Effects of CS and purified EMn on *Sphaerechinus granularis* and *Paracentrotus lividus*

A first series of experiments was conducted on the sea urchins *S. granularis* and *P. lividus* to assess if purified EMn had an effect on fertilization and first cleavage of the egg. In both species, fertilization was totally blocked at 50 and 100 μg mL^{-1} and the phenotypes of embryos exposed to 10 μg mL^{-1} were either delayed in comparison with the control or abnormal (*e.g.,* flattened). Four different incubation periods were tested, from 0 to 20 min prior to the addition of purified EMn, but the effects on fertilization were similar. A 10 min incubation of sperm prior to fertilization was also conducted, but the fertilization rate remained unaffected with 100% fertilization observed even when sperm was incubated in EMn at 100 μg mL^{-1} (data not shown). It was checked if purified EMn prevented fertilization or if the absence of fertilization membrane around the eggs was caused by an effect of EMn on the elevation of fertilization membrane steps. To do so, a calcium ionophore (*i.e.*, a chemical that can activate echinoderm eggs by a release of intracellular calcium ions and inducing the membrane elevation



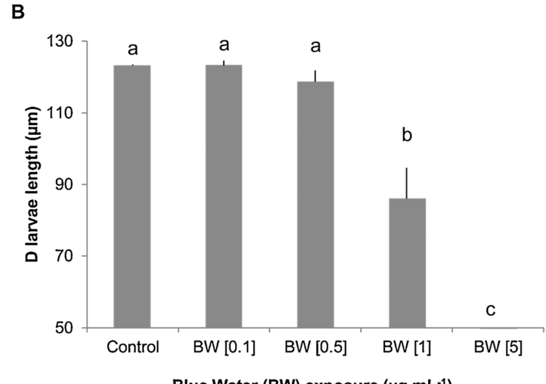


Fig. 2. Exposure of the mussel *Mytilus edulis* embryos to different concentrations (μg mL^{-1}) of Blue Water (BW) over a 48 h period. **A)** Percentage of embryos that reached the D-larval stage (grey bars) and percentage of mortality (black bars). **B)** Length of the larvae that reached the *D* stage (μm). Values are means ± SE (n = 3). Error bars with different lower case letters are significantly different.

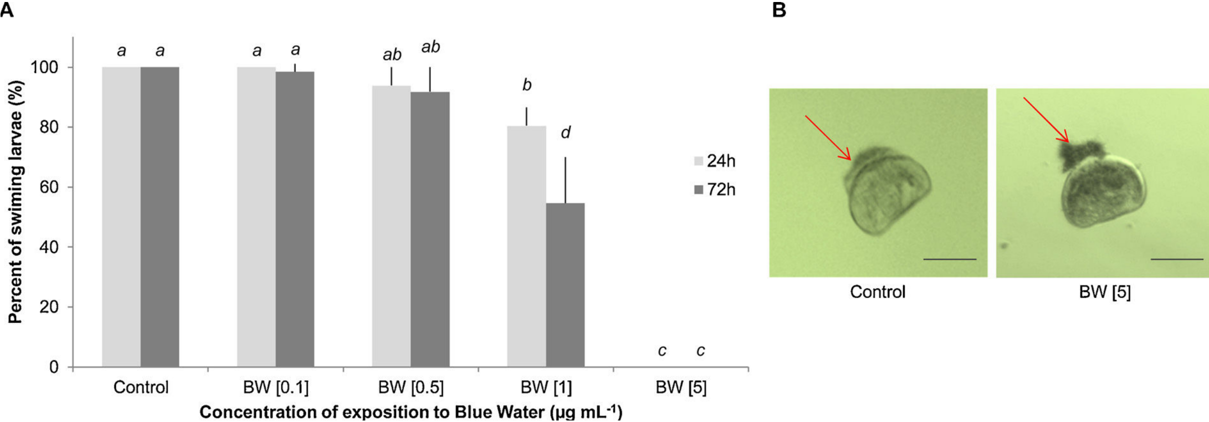


Fig. 3. Exposure of the mussel *Mytilus edulis* D-larvae to different concentrations (µg mL^{-1}) of Blue Water (BW). **A)** Percentage of swimming D-larvae exposed during 24 h (light grey bars) and 72 h (dark grey bars) to BW. Values are means ± SE (n = 6). Error bars with different lower case letters are significantly different. **B)** Observation of D-larvae of the control condition (left picture) and exposed to 5 µg mL^{-1} of BW (right picture) after 24 h under light invert microscopy. D-larvae exposed to 5 µg mL^{-1} of BW were totally motionless with marennine agglutinated on the velum (arrow). Scale bars: 100 µm.

(Steinhardt and Epel, 1974)) was added in EMn incubated eggs. For all concentrations of EMn tested, from 1 to 100 µg mL^{-1}, the calcium ionophore induced the egg activation demonstrating that purified EMn did not interfere with the elevation of the fertilization membrane steps (data not shown).

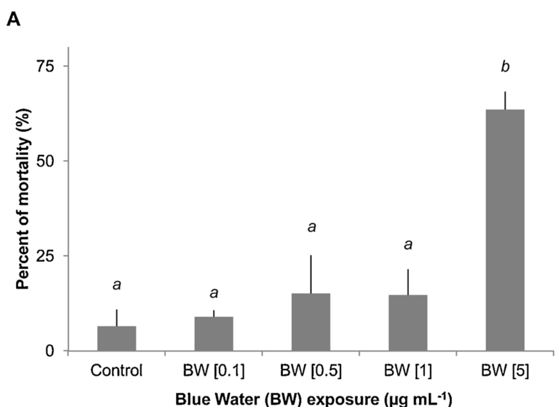
Incubation of *S. granularis* eggs with CS prior to the addition of sperm also exhibited a dose-dependent effect on fertilization (Fig. 7A). Exposure to 10 µg mL^{-1} significantly lowered the fertilization success with 85 ± 8% of fertilization compared to 99 ± 2% for the control condition (*p*-value 0.032). When eggs were incubated in 50 µg mL^{-1}, no fertilization occurred although the sperm was highly active around the eggs (Fig. 7B). When eggs previously exposed to 50 µg mL^{-1} of CS were rinsed and removed in FSW without CS, the fertilization rate was similar to the control condition (*p*-value 0.58) demonstrating that the effect of CS on *S. granularis* eggs and fertilization was reversible (Fig. 7A).

Incubation of newly fertilized *S. granularis* eggs with CS demonstrated a dose dependent effect on the kinetics of first cleavage (Fig. 8A) and on the early embryonic development (Fig. 8B-C). The two lower concentrations tested, 1 and 10 µg mL^{-1}, had no effect on the kinetics of first cleavage: as for the control, divisions started 95 min post fertilization with embryos dividing symmetrically and synchronously, and at 180 min post fertilization all embryos reached at least the 2-cell stage. At higher incubation concentrations, a dose-dependent effect of CS was observed with fewer dividing eggs, asymmetrical and asynchronous divisions. At 180 min post fertilization, less than 85% and 50% of embryos incubated in 50 and 100 µg mL^{-1} of CS, respectively, started to divide and the others quickly degenerated. Similar observations were made for the 3 series of experiments.

Eight hours post fertilization, embryos of the control and those incubated in the presence of CS (1 and 10 µg mL^{-1}) reached the blastula stage (Fig. 8B) while cell division for embryos incubated in 50 and 100 µg mL^{-1} was quickly halted after a few and incoherent divisions or no division at all. Twenty-four hours post fertilization, the gastrula stage was observed for the control embryos and those exposed to 1 µg mL^{-1} while embryos incubated in 10 µg mL^{-1} were still at the blastula stage and did not hatch (data not shown). Forty-eight hours post fertilization, embryos incubated in 1 and 10 µg mL^{-1} exhibited a dose dependent developmental delay with embryos still at the blastula stage and at the early prism stage, respectively, while control embryos reached the late prism stage (Fig. 8C). Three days post fertilization, the early pluteus stage was observed in controls while the early prism stage and hatched blastula stage were observed in those incubated in 1 and 10 µg mL^{-1} of CS, respectively. Washed embryos were able to recover; embryos initially exposed to 1 µg mL^{-1} presented similar developmental stages to the control, 48 and 72 h post fertilization and the development of embryos initially exposed to 10 µg mL^{-1} and rinsed 6 h post fertilization was still slightly delayed in comparison with the control but more advanced than in embryos incubated in the CS.

3.6. Antibacterial effects of BW and CS on Vibrio species

This series of experiments aimed to compare the antibacterial activity of two different solutions of marennine, BW and CS. The bacteria



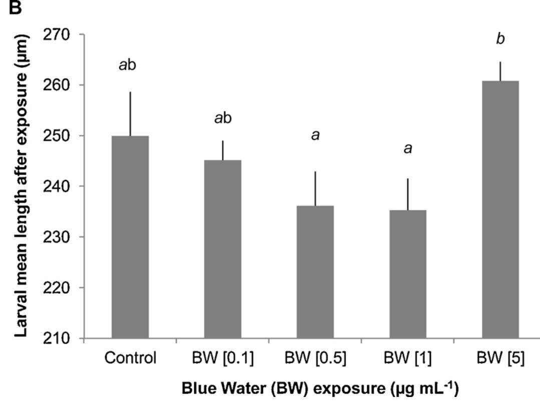


Fig. 4. Mussel *Mytilus edulis* veliger larvae exposed to different concentrations (µg mL^{-1}) of Blue Water (BW) over a 5 d period. **A)** Percentage of veliger larvae mortality and **B)** veliger larvae mean length after BW exposure. Values are means ± SE (n = 3). Error bars with different lower case letters are significantly different.

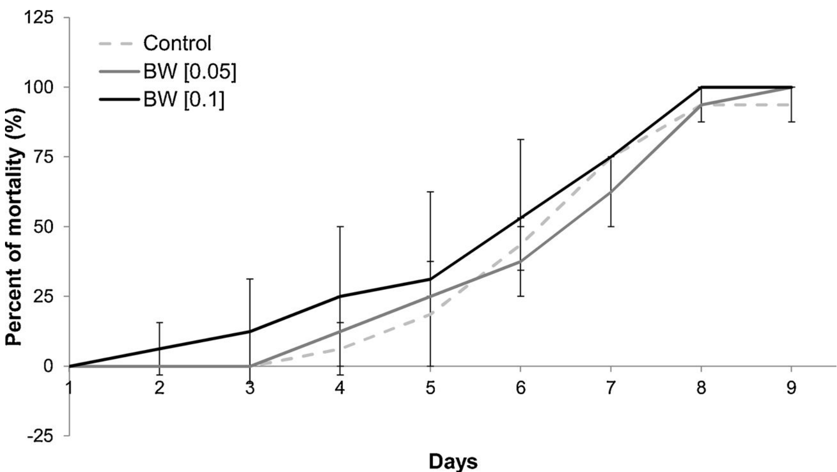
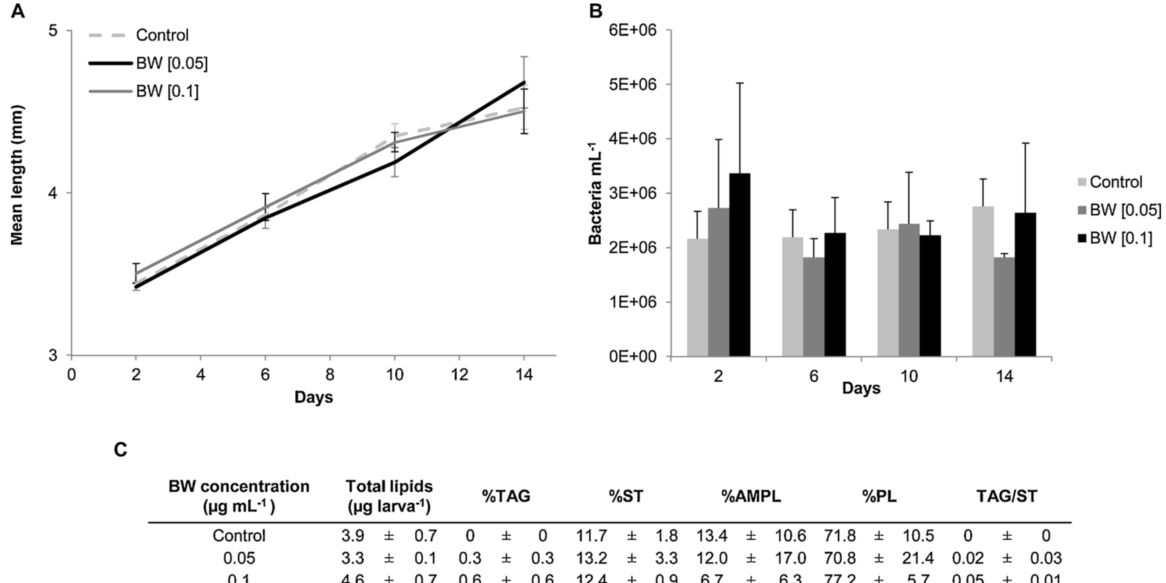


Fig. 5. Percentage of mortality of the barnacle *Chthamalus bisinuatus* larvae exposed to different concentrations (μg mL^{-1}) of Blue Water (BW) over a 9 d period. Results are means ± SE (n = 4).



BW concentration (μg mL^{-1})	Total lipids (μg $larva^{-1}$)	%TAG	%ST	%AMPL	%PL	TAG/ST
Control	3.9 ± 0.7	0 ± 0	11.7 ± 1.8	13.4 ± 10.6	71.8 ± 10.5	0 ± 0
0.05	3.3 ± 0.1	0.3 ± 0.3	13.2 ± 3.3	12.0 ± 17.0	70.8 ± 21.4	0.02 ± 0.03
0.1	4.6 ± 0.7	0.6 ± 0.6	12.4 ± 0.9	6.7 ± 6.3	77.2 ± 5.7	0.05 ± 0.01

Fig. 6. Winter flounder *Pseudopleuronectes americanus* larvae exposed to different concentration (μg mL^{-1}) of Blue Water (BW) over a 14 d period. **A)** Mean lengths (mm) of *P. americanus* larvae. **B)** Bacterial load (bacteria mL^{-1}) in larval tanks and **C)** larval energy reserves: percentages of triglyceride (TAG), sterol (ST), acetone mobile polar lipids (AMPL), and phospholipids (PL) and TAG/ST ratio. Results are means ± SE (n = 3).

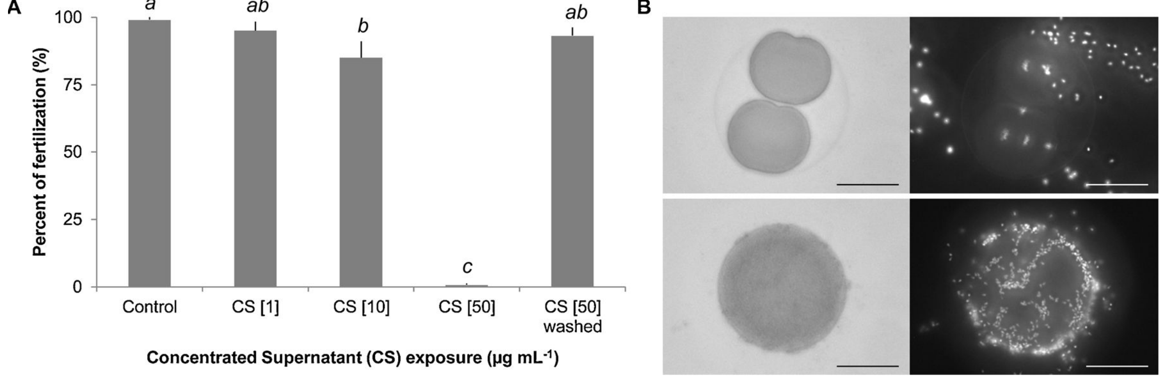


Fig. 7. Sea urchin *Sphaerechinus granularis* eggs exposed to different concentrations (μg mL^{-1}) of *Haslea ostrearia* Concentrated Supernatant (CS) before fertilization. **A)** Fertilization rate and **B)** observation under phase contrast (left panels) and fluorescence (right panels) microscopy (Gx400) of *S. granularis* eggs of the control condition (upper raw) and exposed to CS at 50 μg mL^{-1} (lower raw) 2 h post exposure to CS. Eggs were fixed in a DNA fluorescent stain (Hoechst fixative) to observe cell nuclei of the control dividing egg and spermatozoa agglutinated around the unfertilized egg exposed to 50 μg mL^{-1} of CS. Values in A) are means ± SE (n = 3). Error bars with different lower case letters are significantly different. Scale bars in B): 25 μm.

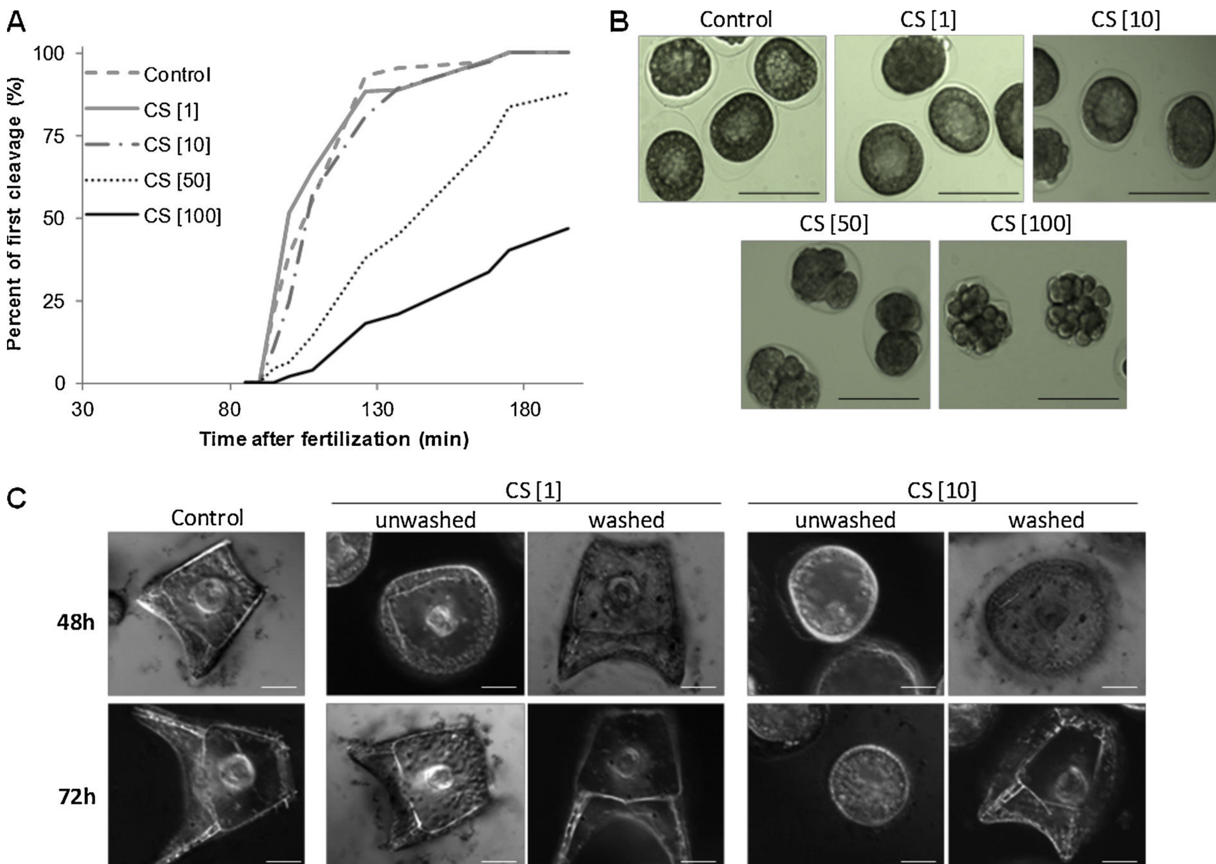


Fig. 8. Exposure of newly fertilized sea urchin *Sphaerechinus granularis* eggs to different concentrations (μg mL^{-1}) of concentrated supernatant (CS) of *Haslea ostrearia* culture. **A)** Kinetic of first cleavage of *S. granularis* eggs exposed to the CS 10 min post fertilization. **B)** Observation of *S. granularis* embryos under phase contrast microscopy 8 h post exposure to CS (Gx200) and **C)** 48 h (top raw) and 72 h (lower raw) post exposure to the CS (Gx400). Embryos of the "unwashed" conditions were still incubated in the CS while embryos of the "washed" condition were rinsed 6 h post exposure to the CS. The kinetics of first cleavage presented in A) is representative of 3 replicate experiments. Views in B) and C) are representative of the observed stages, scale bars: 50 μm.

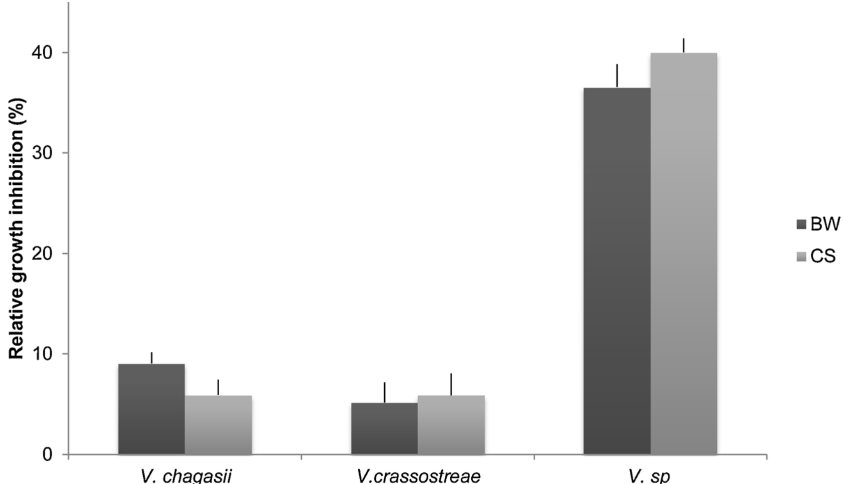


Fig. 9. Relative growth inhibition of the bacteria *Vibrio chagasii, Vibrio crassostreae* and *Vibrio* sp. exposed to 10 μg mL^{-1} of Blue Water (BW) and 10 μg mL^{-1} of Concentrated Supernatant (CS) over a 24 h period. Values are means ± SE (n = 3).

V. chagasii, V. crassostreae and *V.* sp. were exposed to 10 μg mL^{-1} of BW and CS for 20 h and relative growth inhibition in comparison with the control was estimated (Fig. 9). *Vibrio sp* was the most sensitive strain with 37 ± 2% and 40 ± 1% of growth inhibition when exposed to 10 μg mL^{-1} of BW and CS, respectively. The growth of *V. chagasii* exposed to BW and CS also decreased significantly, with percent inhibition as a fraction of the control of 9 ± 1% (p-value < 0.001) and 6 ± 2% (p-value 0.003), respectively. *V. crassostreae* was the least sensitive strain, although growth was also significantly lower than in controls when exposed to BW (5 ± 2% of inhibition, p-value 0.034) and to CS (6 ± 2% of inhibition, p-value 0.020). For each strain tested, growth inhibition induced by BW was statistically similar to the growth inhibition induced by CS (p-values > 0.05) indicating that the two different solutions had similar antibacterial effects at 10 μg mL^{-1}.

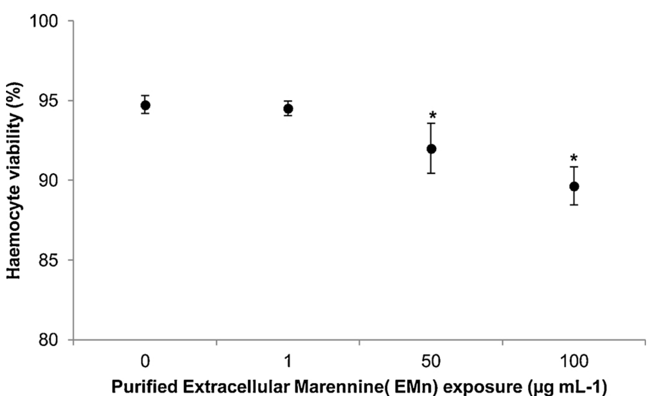


Fig. 10. Percentage of viable oyster *Crassostrea gigas* hemocytes exposed to different concentrations (µg mL^{1-}) of purified Extracellular Marennine (EMn). Values are means and SE (n = 6).

3.7. Effects of EMn on fish gill cell line RTgill-W1

After 2 h exposure to purified EMn, marennine showed no significant effect on fish gill cell viability in the concentration range tested (0–54 µg mL^{-1}).

3.8. Effects of EMn on *Crassostrea gigas* haemocytes

After an exposure of *C. gigas* haemocytes to 1 µg mL^{-1} of purified EMn, the percentage of lysed cells was not significantly different from the control, 6 ± 1% compared to 5 ± 1%, respectively (Fig. 10). The percentage of lysed haemocytes increased in a dose dependent way at 50 (8 ± 1%, *p*-value 0.009) and 100 µg mL^{-1} (10 ± 1%; *p*-value < 0.001). Different durations of exposure were tested (1, 3 and 6 h) without any difference observed in number of lysed cell suggesting that the effects on haemocytes result from the immediate contact with the BW (data not shown).

4. Discussion

This work represents a broad panel of experiments conducted in different laboratories to study the biological effects of marennine on various marine model organisms of aquaculture interest. The results confirm that independently of the mode of preparation, solutions containing marennine can exert biological activities, ranging from growth inhibition of pathogens to the death of bivalve larvae. The effects observed depend on the species, the life cycle stage and concentrations of marennine used. The results are summarized in Table 2 and raise two questions regarding: 1) limitations of the study due to diversity of marennine solutions tested, and 2) interpretation of data in view of importance of these biological activities for the marine environment and potential applications in aquaculture.

4.1. Limitations of the study

This work brings together the results of different experiments, conducted over a three-year period, in different laboratories and using three different marennine-based solutions (purified EMn, BW and CS), as well as different methods to quantify marennine concentrations. Moreover, cultures of *H. ostrearia* were grown on different media, which also may have impacted marennine production and quality. The first series of experiments using purified EMn and CS exposed against *S. granularis* and *P. lividus,* showed similar effects on sea urchin egg fertilization. Furthermore, growth inhibition induced by BW and CS was comparable within the same *Vibrio* species at 10 µg mL^{-1}. We conclude that biological activity was not impaired related to marennine production conditions.

The concentration ranges tested could vary by about 30–40 %, depending on the extinction coefficient used. Various marennine quantification methods have been published so far, using an extinction coefficient determined from raw extract (Robert et al., 2002) or purified marennine (Pouvreau et al., 2006a), or weighing of purified marennine dried powder (Prasetiya et al., 2017; Turcotte et al., 2016 and Gastineau et al., 2012, respectively). As the absolute quality and quantity of the pigment in different solutions could not be clearly assessed without a complete characterization of this pigment, our results provided a range of concentrations showing biological activities against various marine organisms. While the higher concentrations (50 and 100 µg mL^{-1}) provide clues about doses with deleterious effects, the lower concentrations (from 0.01 to 10 µg mL^{-1}) are more ecologically relevant. In natural environments, marennine measurements indicate a range of 1–10 µg mL^{-1} (Turpin et al., 2001), and natural blooms of blue *Haslea* have been recorded worldwide (Fig. 1). *Haslea* blooms are regularly observed in Calvi Bay (Corsica, France) as part of seaweed surveys. These blooms, which mostly occur in spring, result in the development of a blue-green biofilm at the surface of the thallus of seaweeds (*i.e., Padina, Halopteris, Acetabularia*), on short turfs, and to a lower extent on sediments and rocks (STAtion de REcherche Sous-marines et Océanographique - STARESO and Liège University; V. Demoulin, D. Sirjacobs, S. Gobert and P. Lejeune, personal communications). Blue *Haslea* can display very large patches reaching several square meters and be observed at depths ranging from 2 to 8 m. Such extensive blooms are also annually recorded from November through March in central and southern coastal estuaries of North Carolina (USA), in particular along the central coast from lower Core Sound westward across the lower North River estuary and Back Sound to the western portions of

Table 2
Summary of the marennine based solutions effects on the marine organisms tested.

Marennine based solutions	Marine organisms tested	Summary of the observed effects
Blue Water **(BW)**	Mussel *M. edulis*	Higher mortality and developmental delay for embryos from 1 µg mL^{-1} Higher mortality for veliger larvae from 5 µg mL^{-1} Decreased of motility for D-larvae from 1 µg mL^{-1} No observed effects at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
	Barnacle *C. bisinuatus*	No observed effect on larval survival at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
	Winter flounder *P. americanus*	No observed effect on larval and development at 0.1 µg mL^{-1} and 0.5 µg mL^{-1}
Concentrated Supernatant **(CS)**	Bacterial species of the genus *Vibrio*	Growth inhibition at 10 µg mL^{-1} Similar effects observed with BW solutions and CS solutions
	Sea urchin *S. granularis*	Decreased of the fertilization rate from 10 µg mL^{-1} and delay of the embryonic development from 1 µg mL^{-1}
Purified Extracellular Marennine **(EMn)**	Sea urchins *S. granularis* and *P. lividus*	Reversible effects after rinsing the eggs and/or the embryos Similar effects on fertilization observed with CS solutions ad EMn solutions on *S. granularis* and *P. lividus*
	Fish gill cell line Rtgill-W1	No observed effect on cell viability from 0 to 54 µg mL^{-1}
	Oyster C. *gigas* haemocytes	No observed effect up to 1 µg mL^{-1}

Bogue Sound. These blooms of a blue *Haslea* possibly different from *H. ostrearia* do not seem to be associated with the development of biofilms on *Padina* sp. thalli, however they cause a wide-spread greening of oysters, which could underpin emerging green *oyster* industries in the USA (N. Lindquist, personal communications).

Thus it can be inferred that, at the microenvironment scale, high concentrations of marennine could be encountered, and that many marine organisms can periodically and locally face acute marennine exposure. All laboratory experiments to study biological activity of marennine lasted hours or days, and the cumulated effect of long-lasting exposure to low concentrations of marennine has not yet been tested. Greening of oyster gills can be viewed as a cumulative, longer term effect. According to green oyster producers, the greening in oyster ponds can occur in a matter of days, depending on *Haslea* growth and density. In the laboratory, EMn rather than IMn was preferentially responsible for greening, which proved to be time dependent and long-lasting (Gastineau et al., 2018, 2014). However, green oysters have also been observed in natural open environments, in absence of any record of *Haslea* blooms, for instance in oyster leases in Australia (New South Wales and Tasmania). This means that marennine could have biological activities in the long-term, from subacute and chronic exposure, and further work is required to better assess the impact of this pigment in the natural environment.

4.2. The unpredictable "Jekyll and Hyde", good and bad nature of marennine

On the one hand, our results showed that exposure to the tested marennine solutions could lead to adverse effects against many marine animals, depending on the exposure dose. Concentrations from 1 μg mL^{-1} significantly lowered the survival rate of *M. edulis* embryos and locomotion of the D-larvae. Significantly higher mortality rates were observed for veliger larvae exposed to 5 μg mL^{-1} of BW while no mortality was recorded at 1 μg mL^{-1}, which contrasts with results of a previous study where exposure of veliger larvae to 1 μg mL^{-1} of BW led to 100% mortality, but the duration of that experiment was 4 times longer, 20 d *vs* 5 d (Turcotte et al., 2016). Significant dose-response effects of marennine were also observed with the sea urchin model. While high concentrations (50 and 100 μg mL^{-1}) resulted in a total blockage of fertilization and embryonic development, lower concentrations (1 and 10 μg mL^{-1}) of CS induced a developmental delay of *S. granularis* embryos. Previous works also have demonstrated the effects of a water-soluble extract of the diatom *Thalassiosira rotula* on cell division of the sea urchin *P. lividus*, with a blockage of cell division at the higher doses tested and a delay and abnormal development of embryos at lower doses (Buttino et al., 1999). Also, intact diatom cells of six different species could inhibit the egg first cleavage of the sea urchins *Strongylocentrotus droebachiensis* and *Echinus acutus* (Gudimova et al., 2016). Most interestingly, our results indicated that even at high concentrations, the effects of CS on fertilization and embryonic development were reversible after rinsing the eggs or embryos. This confirms previous observations of the effects of water-soluble diatom extracts against various marine invertebrates, including the sea urchin *Psammechinus miliaris* (Caldwell et al., 2002).

The present results could suggest an inability of marennine to bind tightly at the cell surface if considering the reversible effects observed in sea urchins, and to penetrate the cell and exert irreversible damages given the high molecular weight of the molecule (> 10 kDa; Pouvreau et al., 2006b). Marennine could however link to cell surface or membrane receptors and trigger stress responses or even apoptotic signals by inducing changes in cell metabolism. Furthermore, the variability of BW and CS effects observed at both the interspecific and intraspecific levels on the three *Vibrio* species tested, but also those previously shown with *V. aestuarianus*, *V. coralliilyticus* and *V. tubiashii* strains (Falaise et al., 2016) could reflect differences in membrane receptor affinities and in mechanisms of action. Such variability should be considered before application of marennine in aquaculture as an antibacterial agent. However, more data is necessary to confirm these hypotheses.

On the other hand, despite observed deleterious effect at the higher concentrations tested, very low concentrations (0.05 and 0.1 μg mL^{-1}) of BW did not exhibit a negative effect on the barnacle *C. bisinuatus* survival nor on the winter flounder *P. americanus.* Both flounder growth rate and bacterial load in the water remained unaffected throughout the marennine exposure (0.05 and 0.1 μg mL^{-1}), and physiological condition (energetic reserves) improved in response to marennine treatment. These results compare with no observable effect of marennine (0–54 μg L^{-1}) on the viability (metabolic activity) of the rainbow trout gill cell line RTgill-W1. Although the gill cell line exposure was conducted for only 2 h, results provide a strong indication that marennine does not impair fish gill function at the concentration ranges tested here. These results are further supported by (Turcotte et al., 2016) who demonstrated that low exposure to BW (0.1 μg mL^{-1}) enhanced survival and physiological condition of *M. edulis* and *P. magellanicus* larvae. In addition, our results showed that low concentrations of EMn (1 μg mL^{-1}) did not present a cytotoxic effect against haemocytes of adult oysters. Even though cytotoxic effects were evidenced at the highest concentrations (50 and 100 μg mL^{-1}), the point of inflection could not be determined with the concentration range tested. However, it should be emphasized that even after centuries of production of green oysters, no negative impacts on adult oysters, nor green oyster consumers have been reported.

Despite the fact that diatoms are traditionally considered a high quality food source enhancing growth and survival of many marine organisms, they can produce secondary metabolites that function as grazing deterrents (Ianora and Miralto, 2010). Most of these secondary metabolites are polyunsaturated fatty acids (PUFAs) and polyunsaturated aldehydes (PUAs) with a special focus on oxylipins (Caldwell, 2009). Effects of diatom aldehydes on reproductive and fertilization success or embryonic development of various organisms such as crustaceans, echinoderms or arthropods have been widely studied (Caldwell, 2009; Ianora and Miralto, 2010). Aldehyde production is a diatom chemical defense strategy to limit growth of their grazers (Ban et al., 1997; Pohnert, 2005), but other studies reported the absence of a correlation between diatom aldehyde and reproductive biology and early development of marine invertebrates, suggesting the involvement of other unidentified diatom metabolites (Poulet et al., 2006; Wichard et al., 2008). Unlike aldehydes, that are likely to exert their activity following diatom cell destruction and ingestion, the water-soluble marennine may target a larger spectrum of marine organisms and not only diatom grazers.

In conclusion, the present work demonstrates that *H. ostrearia* supernatant containing marennine represents a biologically active water-soluble solution with potential effects on various marine organisms. More studies are required to better estimate the long term impacts of blue *Haslea* blooms in natural environments, facilitated by laboratory studies, investigating chronic exposure to low concentrations of marennine. If the use of *H. ostrearia* supernatant is considered in hatcheries as a preventive or curative anti-infectious agent, concentrated solutions such as BW or CS could be of interest in order to control the delivered marennine doses. It is likely that the adverse effects of marennine only target the early and more succeptable fragile developmental stages such as embryos and larvae, depending on the exposure concentration, whereas adults remain unaffected.

Acknowledgements

We thank the reviewers for their valuable comments that allowed us improving this work. This publication benefited from funding from the research program on the valorization of marine biomass for larval feeding (ISMER/UQAR) (RT), from the RFI Food for Tomorrow (project Maliblue), from the Territorial Collectivity of Corsica and the Rhone-Mediterranean and Corsica Water Agency (STARE-CAPMED - Station of

reference and research on change of local and global anthropogenic pressures on Mediterranean ecosystem drifts), and also from the European Commission under the Horizon 2020 Research and Innovation Program GHaNA (The Genus *Haslea*, New marine resources for blue biotechnology and Aquaculture, grant agreement No [734708/GHANA/H2020-MSCA-RISE- 2016]) (JLM).

References

Auffret, M., Oubella, R., 1997. Hemocyte aggregation in the oyster *Crassostrea gigas*: *In Vitro* measurement and experimental modulation by xenobiotics. Comp. Biochem. Physiol. – Part Physiol. 3, 705–712.

Ban, S., Burns, C., Castel, J., Chaudron, Y., Christou, E., Escribano, R., Umani, S.F., Gasparini, S., Ruiz, F.G., Hoffmeyer, M., Ianora, A., Kang, H., Laabir, M., Lacoste, A., Miralto, A., Ning, X., Poulet, S., Rodriguez, V., Runge, J., Shi, J., Starr, M., Uye, S., Wang, Y., 1997. The paradox of diatom-copepod interactions. Mar. Ecol. Prog. Ser. 157, 287–293. https://doi.org/10.3354/meps157287.

Bergé, J.P., Bourgougnon, N., Alban, S., Pojer, F., Billaudel, S., Chermann, J.C., Robert, J.M., Franz, G., 1999. Antiviral and anticoagulant activities of a water-soluble fraction of the marine diatom *Haslea ostrearia*. Planta Med. 65, 604–609.

Bouhlel, Z., Turcotte, F., Warschawski, D.E., Arnold, A., Lemarchand, K., Deschênes, J.-S., Marcotte, I., Tremblay, R., 2018. Marennine, a potential alternative antibiotic to polymyxine in aquaculture. Presented at the Aquaculture Canada 2018.

Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivesse, S., Alunno-Bruscia, M., Polz, M.F., Le Roux, F., 2017. *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. ISME J. 11, 1043–1052. https://doi.org/10.1038/ismej.2016.162.

Buttino, I., Miralto, A., Ianora, A., Romano, G., Poulet, S.A., 1999. Water-soluble extracts of the diatom *Thalassiosira rotula* induce aberrations in embryonic tubulin organisation of the sea urchin *Paracentrotus lividus*. Mar. Biol. 134, 147–154. https://doi.org/10.1007/s002270050533.

Buttino, I., Hwang, J.-S., Romano, G., Sun, C.-K., Liu, T.-M., Pellegrini, D., Gaion, A., Sartori, D., 2016. Detection of malformations in sea urchin plutei exposed to mercuric chloride using different fluorescent techniques. Ecotoxicol. Environ. Saf., 6th Biannual Ecotoxicology Meeting (BECOME 2014) - Environmental Emergencies: Ecotoxicology as a Management Tool 123, 72–80. https://doi.org/10.1016/j.ecoenv.2015.07.027.

Caldwell, G.S., 2009. The influence of bioactive oxylipins from marine diatoms on invertebrate reproduction and development. Mar. Drugs 7, 367–400. https://doi.org/10.3390/md7030367.

Caldwell, G.S., Olive, P.J.W., Bentley, M.G., 2002. Inhibition of embryonic development and fertilization in broadcast spawning marine invertebrates by water soluble diatom extracts and the diatom toxin 2-trans,4-trans decadienal. Aquat. Toxicol. Amst. Neth. 60, 123–137.

Carbonnelle, D., Pondaven, P., Morancais, M., Masse, G., Bosch, S., Jacquot, C., Briand, G., Robert, J., Roussakis, C., 1998. Antitumor and antiproliferative effects of an aqueous extract from the marine diatom *Haslea ostrearia* (Simonsen) against solid tumors: lung carcinoma (NSCLC-N6), kidney carcinoma (E39) and melanoma (M96) cell lines. Anticancer Res. 19, 621–624.

Clinical and Laboratory Standards Institute (CLSI), 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Document M07-A9. Approved Standard—Ninth Edition. Wayne, PA, USA. .

Falaise, C., François, C., Travers, M.-A., Morga, B., Haure, J., Tremblay, R., Turcotte, F., Pasetto, P., Gastineau, R., Hardivillier, Y., Leignel, V., Mouget, J.-L., 2016. Antimicrobial compounds from eukaryotic microalgae against human pathogens and diseases in aquaculture. Mar. Drugs 14, 159. https://doi.org/10.3390/md14090159.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.

Fraboulet, E., Lambert, Y., Tremblay, R., Audet, C., 2010. Assessment of paternal effect and physiological cost of metamorphosis on growth of young winter flounder *Pseudopleuronectes americanus* juveniles in a cold environment. J. Fish Biol. 76, 930–948. https://doi.org/10.1111/j.1095-8649.2010.02538.x.

Gaillon, B., 1820. Des huîtres vertes, et des causes de cette coloration. J. Phys. Chim. D'histoire Nat. Arts 91, 222–225.

Gastineau, R., Pouvreau, J.-B., Hellio, C., Morançais, M., Fleurence, J., Gaudin, P., Bourgougnon, N., Mouget, J.-L., 2012. Biological activities of purified marennine, the blue pigment responsible for the greening of oysters. J. Agric. Food Chem. 60, 3599–3605. https://doi.org/10.1021/jf205004x.

Gastineau, R., Turcotte, F., Pouvreau, J.-B., Morançais, M., Fleurence, J., Windarto, E., Prasetiya, F.S., Arsad, S., Jaouen, P., Babin, M., Coiffard, L., Couteau, C., Bardeau, J.-F., Jacquette, B., Leignel, V., Hardivillier, Y., Marcotte, I., Bourgougnon, N., Tremblay, R., Deschênes, J.-S., Badawy, H., Pasetto, P., Davidovich, N., Hansen, G., Dittmer, J., Mouget, J.-L., 2014. Marennine, promising blue pigments from a widespread *Haslea* diatom species complex. Mar. Drugs 12, 3161–3189. https://doi.org/10.3390/md12063161.

Gastineau, R., Hansen, G., Davidovich, N.A., Davidovich, O., Bardeau, J.-F., Kaczmarska, I., Ehrman, J.M., Leignel, V., Hardivillier, Y., Jacquette, B., Poulin, M., Morançais, M., Fleurence, J., Mouget, J.-L., 2016. A new blue-pigmented hasleoid diatom, *Haslea provincialis*, from the Mediterranean Sea. Eur. J. Phycol. 51, 156–170. https://doi.org/10.1080/09670262.2015.1110861.

Gastineau, R., Prasetiya, F.S., Falaise, C., Cognie, B., Decottignies, P., Morancais, M., Méléder, V., Davidovich, N.A., Turcotte, F., Tremblay, R., Pasetto, P., Dittmer, J., Bardeau, J.-F., Pouvreau, J.-B., Mouget, J.-L., 2018. Part II: marine molecules for disease treatment / prevention and for biological research, Chap16: marennine-like pigments: Blue diatom of Green oyster cult? In: La Barre, S., Bates, S., Qian, P. (Eds.), Blue Biotechnologies: Production and Uses of Marine Molecules. Wiley VCH, Weinheim, Germany.

Gudimova, E., Eilertsen, H.C., Jørgensen, T.Ø., Hansen, E., 2016. *In vivo* exposure to northern diatoms arrests sea urchin embryonic development. Toxicon 109, 63–69. https://doi.org/10.1016/j.toxicon.2015.11.001.

Hennebicq, R., Fabra, G., Pellerin, C., Marcotte, I., Myrand, B., Tremblay, R., 2013. The effect of spawning of cultured mussels (*Mytilus edulis*) on mechanical properties, chemical and biochemical composition of byssal threads. Aquaculture 410–411, 11–17. https://doi.org/10.1016/j.aquaculture.2013.06.011.

Hjörleifsson, E., Klein-MacPhee, G., 1992. Estimation of live standard length of winter flounder Pleuronectes americanus larvae from formalin-preserved, ethanol-preserved and frozen specimens. Mar. Ecol. Prog. Ser. 82, 13–19.

Ianora, A., Miralto, A., 2010. Toxigenic effects of diatoms on grazers, phytoplankton and other microbes: a review. Ecotoxicol. Lond. Engl. 19, 493–511. https://doi.org/10.1007/s10646-009-0434-y.

Kasten, P., Flores, A.A.V., 2013. Disruption of endogenous tidal rhythms of larval release linked to food supply and heat stress in an intertidal barnacle. Mar. Ecol. Prog. Ser. 472, 185–198. https://doi.org/10.3354/meps10005.

Khemis, I.B., Audet, C., Fournier, R., Noüe, J.D.L., 2003. Early weaning of winter flounder (*Pseudopleuronectes americanus* Walbaum) larvae on a commercial microencapsulated diet. Aquac. Res. 34, 445–452. https://doi.org/10.1046/j.1365-2109.2003.00828.x.

Mitchell, P.H., Barney, R.L., 1917. The Occurrence in Virginia of Green-gilled Oysters Similar to Those of Marennes. Govt. print. off., Washington.

Morga, B., Arzul, I., Chollet, B., Renault, T., 2009. Infection with the protozoan parasite *Bonamia ostreae* modifies in vitro haemocyte activities of flat oyster *Ostrea edulis*. Fish Shellfish Immunol. 26, 836–842. https://doi.org/10.1016/j.fsi.2009.03.018.

Mouget, J.-L., Gastineau, R., Davidovich, O., Gaudin, P., Davidovich, N.A., 2009. Light is a key factor in triggering sexual reproduction in the pennate diatom *Haslea ostrearia*. FEMS Microbiol. Ecol. 69, 194–201. https://doi.org/10.1111/j.1574-6941.2009.00700.x.

Myrand, B., Tremblay, R., Sévigny, J.-M., 2009. Impact of suspension culture using mesh sleeves on genetic characteristics of Mytilus edulis L. in Canada. Aquaculture 291, 147–153. https://doi.org/10.1016/j.aquaculture.2009.03.015.

Nassiri, Y., Robert, J.-M., Rincé, Y., Ginsburger-Vogel, T., 1998. The cytoplasmic fine structure of the diatom *Haslea ostrearia* (Bacillariophyceae) in relation to marennine production. Phycologia 37, 84–91. https://doi.org/10.2216/i0031-8884-37-2-84.1.

Parrish, C.C., 1987. Separation of aquatic lipid classes by chromarod thin-layer chromatography with measurement by Iatroscan flame ionization detection. Can. J. Fish. Aquat. Sci. 44, 722–731. https://doi.org/10.1139/f87-087.

Petersen, C.G., 1916. "Grüne Austern" in Dänemark in 1911–1912. Int. Rev. Gesamten Hydrobiol. Hydrogr. 7, 39–41.

Pinsino, A., Matranga, V., Trinchella, F., Roccheri, M.C., 2010. Sea urchin embryos as an *in vivo* model for the assessment of manganese toxicity: developmental and stress response effects. Ecotoxicology 19, 555–562. https://doi.org/10.1007/s10646-009-0432-0.

Pohnert, G., 2005. Diatom/Copepod interactions in plankton: the indirect chemical defense of unicellular algae. ChemBioChem 6, 946–959. https://doi.org/10.1002/cbic.200400348.

Poulet, S.A., Wichard, T., Ledoux, J.B., Lebreton, B., Marchetti, J., Dancie, C., Bonnet, D., Cueff, A., Morin, L.P., Pohnert, G., Poulet, S.A., Ledoux, J.B., Dancie, C., Morin, L.P., Poulet, S.A., Cueff, C., Pohnert, G., 2006. Influence of Diatoms on Copepod Reproduction. I. Field and Laboratory Observations Related to *Calanus helgolandicus* Egg Production. https://doi.org/10.3354/meps308129.

Pouvreau, J.-B., Morançais, M., Fleurence, J., Pondaven, P., 2006a. Method for the quantification of the blue-green pigment "marennine" synthesized by the marine diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen using HPLC gel-filtration and photodiode-array detection. J. Appl. Phycol. 19, 263–270. https://doi.org/10.1007/s10811-006-9133-8.

Pouvreau, J.-B., Morançais, M., Fleury, F., Rosa, P., Thion, L., Cahingt, B., Zal, F., Fleurence, J., Pondaven, P., 2006b. Preliminary characterisation of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. J. Appl. Phycol. 18, 757–767. https://doi.org/10.1007/s10811-006-9087-x.

Pouvreau, J.-B., Morançais, M., Massé, G., Rosa, P., Robert, J.-M., Fleurence, J., Pondaven, P., 2006c. Purification of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. J. Appl. Phycol. 18, 769–781. https://doi.org/10.1007/s10811-006-9088-9.

Pouvreau, J.-B., Housson, E., Tallec, L.L., Morançais, M., Rincé, Y., Fleurence, J., Pondaven, P., 2007. Growth inhibition of several marine diatom species induced by the shading effect and allelopathic activity of marennine, a blue-green polyphenolic pigment of the diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. J. Exp. Mar. Biol. Ecol. 352, 212–225. https://doi.org/10.1016/j.jembe.2007.07.011.

Pouvreau, J.-B., Morançais, Michèle, Taran, F., Rosa, P., Dufossé, Laurent, Guérard, Fabienne, Pin, S., Fleurence, J., Pondaven, P., 2008. Antioxidant and free radical scavenging properties of Marennine, a blue-green polyphenolic pigment from the diatom *Haslea ostrearia* (Gaillon/Bory) simonsen responsible for the natural greening of cultured oysters. J. Agric. Food Chem. 56, 6278–6286. https://doi.org/10.1021/jf073187n.

Prasetiya, F.S., Safitri, I., Widowati, I., Cognie, B., Decottignies, P., Gastineau, R., Morançais, M., Windarto, E., Tremblay, R., Mouget, J.-L., 2016. Does allelopathy affect co-culturing *Haslea ostrearia* with other microalgae relevant to aquaculture? J. Appl. Phycol. 28, 2241–2254. https://doi.org/10.1007/s10811-015-0779-y.

Prasetiya, F.S., Comeau, L.A., Gastineau, R., Decottignies, P., Cognie, B., Morançais, M., Turcotte, F., Mouget, J.-L., Tremblay, R., 2017. Effect of marennine produced by the

blue diatom *Haslea ostrearia* on behavioral, physiological and biochemical traits of juvenile *Mytilus edulis* and *Crassostrea virginica*. Aquaculture 467, 138–148. https://doi.org/10.1016/j.aquaculture.2016.08.029.
Quinn, G.P., Keough, M.J., 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press, Cambridge.
Ranson, G., 1927. L'absorption de matières organiques dissoutes par la surface extérieure du corps chez les animaux aquatiques. Ann. Inst. Océan. 4, 174.
Robert, J.-M., Morançais, M., Pradier, E., Mouget, J.-L., Tremblin, G., 2002. Extraction and quantitative analysis of the blue-green pigment "marennine" synthesized by the diatom Haslea ostrearia. J. Appl. Phycol. 14, 299–305. https://doi.org/10.1023/A:1021184532581.
Seychelles, L.H., Audet, C., Tremblay, R., Lemarchand, K., Pernet, F., 2011. Bacterial colonization of winter flounder *Pseudopleuronectes americanus* fed live feed enriched with three different commercial diets. Aquac. Nutr. 17, e196–e206. https://doi.org/10.1111/j.1365-2095.2009.00750.x.
Sprat, T., 1667. History of the Royal Society. Martyn, London, UK.
Steinhardt, R.A., Epel, D., 1974. Activation of sea-urchin eggs by a calcium ionophore. Proc. Natl. Acad. Sci. 71, 1915–1919. https://doi.org/10.1073/pnas.71.5.1915.
Tardy-Laporte, C., Arnold, A.A., Genard, B., Gastineau, R., Morançais, M., Mouget, J.-L., Tremblay, R., Marcotte, I., 2013. A 2H solid-state NMR study of the effect of antimicrobial agents on intact *Escherichia coli* without mutating. Biochim. Biophys. Acta BBA - Biomembr. 1828, 614–622. https://doi.org/10.1016/j.bbamem.2012.09.011.
Turcotte, F., Mouget, J.-L., Genard, B., Lemarchand, K., Deschênes, J.-S., Tremblay, R., 2016. Prophylactic effect of *Haslea ostrearia* culture supernatant containing the pigment marennine to stabilize bivalve hatchery production. Aquat. Living Resour. 29, 401. https://doi.org/10.1051/alr/2016032.
Turpin, V., Robert, J.-M., Goulletquer, P., Massé, G., Rosa, P., 2001. Oyster greening by outdoor mass culture of the diatom *Haslea ostrearia* Simonsen in enriched seawater. Aquac. Res. 32, 801–809. https://doi.org/10.1046/j.1365-2109.2001.00615.x.
Wichard, T., Poulet, S.A., Boulesteix, A.L., Ledoux, J.B., Lebreton, B., Marchetti, J., Pohnert, G., Pohnert, G., 2008. Influence of Diatoms on Copepod Reproduction. Ii. Uncorrelated Effects of Diatom-derived alpha,beta,gamma,delta-unsaturated Aldehydes and Polyunsaturated Fatty Acids on *Calanus helgolandicus* in the Field.

ANNEXE 3 : Publication

Falaise et al. (2019), *Marine Drugs* [*Texte Complet*]

Falaise, C., James A., Travers M-A., Zanella M., Badawi M., Mouget, J.-L., 2019. **Complex relationships between the blue pigment marennine and marine bacteria of the genus *Vibrio***. *Marine Drugs,* 17(3), 160-172.

Article

Complex Relationships between the Blue Pigment Marennine and Marine Bacteria of the Genus *Vibrio*

Charlotte Falaise [1], Adèle James [2,3], Marie-Agnès Travers [4], Marie Zanella [1], Myriam Badawi [1] and Jean-Luc Mouget [1,*]

1 Laboratoire Mer Molécule Santé (EA 2160, FR CNRS 3473 IUML), Le Mans Université, 72000 Le Mans, France; charlotte.falaise@gmail.com (C.F.); marie.zanella@univ-lemans.fr (M.Z.); myriam.badawi@univ-lemans.fr (M.B.)
2 Ifremer, Unité Physiologie Fonctionnelle des Organismes Marins, ZI de la Pointe du Diable, 29280 Plouzané, France; adele.james@hotmail.fr
3 Sorbonne Université (UPMC Paris 06, CNRS, UMR 8227) Laboratoire de Biologie Intégrative des Modèles Marins, Station Biologique de Roscoff, 29680 Roscoff, France
4 Ifremer (RBE-SG2M-LGPMM) Laboratoire de Génétique et de Pathologie des Mollusques Marins, Station La Tremblade, Avenue Mus Loup, F-17390 La Tremblade, France; marie.agnes.travers@ifremer.fr
* Correspondence: jean-luc.mouget@univ-lemans.fr; Tel.: +33-243-833-242

Received: 14 February 2019; Accepted: 4 March 2019; Published: 8 March 2019

Abstract: Marennine, the water-soluble blue pigment produced by the marine diatom *Haslea ostrearia*, is known to display antibacterial activities. Previous studies have demonstrated a prophylactic effect of marennine on bivalve larvae challenged with a pathogenic *Vibrio splendidus*, suggesting that the blue *Haslea* is a good candidate for applications in aquaculture as a source of a natural antimicrobial agent. Indeed, the genus *Vibrio* is ubiquitous in aquaculture ecosystems, and regular events of pathogenic invasion cause some of the biggest losses worldwide. To better characterize the effects of marennine on *Vibrios*, a panel of 30 *Vibrio* strains belonging to 10 different species was tested, including bivalve pathogenic species (e.g., *Vibrio crassostreae* and *Vibrio harveyi*). *Vibrio* strains were first exposed to 10 and 25 μg mL^{-1} of Blue Water (BW), a concentrated culture supernatant of *H. ostrearia* containing marennine. This screening evidenced a great diversity in responses, from growth stimulation to a total inhibition, at both the interspecific or intraspecific level. In a second series of experiments, 10 *Vibrio* strains were exposed to BW at concentrations ranging from 5 to 80 μg mL^{-1}. The highest concentrations of BW did not systematically result in the highest growth inhibition as hormetic responses—opposite effects regarding the concentration—were occasionally evidenced. The relationships between marennine and *Vibrio* strains appear more complex than expected and justify further study—in particular, on the mechanisms of action—before considering applications as a natural prophylactic or antibiotic agent in aquaculture.

Keywords: antibacterial activity; diauxie; *Haslea*; hormesis; marennine; *Vibrio*

1. Introduction

The marine diatom *Haslea ostrearia* is characterized by the production of a specific blue-green pigment, named marennine. This water-soluble pigment accumulates at the apices of the cells before its release into the surrounding environment [1]. *H. ostrearia* is a ubiquitous diatom and is of special interest in the Atlantic French coast (e.g., Marennes Bay, Bourgneuf Bay), where blooms in oyster ponds induce the greening of oyster gills that increases the market value of bivalves. Blue diatoms other than *H. ostrearia* have been identified in the last decade, such as *Haslea karadagensis* (Black Sea; [2]), *Haslea provincialis* (Mediterranean Sea; [3]), and more recently, *Haslea nusantara* (Java Sea; [4]). All these species produce blue pigments whose spectral characteristics slightly differ from marennine, and are

named marennine-*like* pigments in the absence of more specific determination (e.g., [2]). Despite an increasing knowledge on blue *Haslea* biodiversity and distribution, questions still remain about these blue pigments and their functions for the algae. Indeed, marennine or marennine-like pigments are highly complex molecules, and their chemical structure remains undetermined. Some glycosidic units attached to one or various aromatic rings have been evidenced, but the exact nature of the chromophore is still unknown yet [5].

Regarding the function of the pigment for the microalga, the significant release of blue pigments by *Haslea* species in seawater (in the range of 1–15 μg mL^{-1} in oyster ponds; [6]), combined with an increasing amount of evidence that marennine interacts with different marine organisms, could advocate for a protective or a competitive role. Indeed, allelopathic effects were demonstrated toward various microalgal species [7,8], as well as antimicrobial effects against several marine bacteria and fungi (reviewed in [9]). More particularly, in vitro experiments have demonstrated antibacterial effects of marennine against various marine bacteria, including strains from the *Vibrio* genus, such as *Vibrio anguillarium* [10], *Vibrio aestuarianus* [11], or *Vibrio splendidus* [12]. The *Vibrio* genus is genetically and metabolically highly diverse, and several species have been described as pathogenic for shellfish [13]. Major pathogens found in hatcheries or in fields belong to *Splendidus*, *Coralliilyticus*, *Harveyi* clades, or to *V. aestuarianus* and *V. tapetis* species. However, it is important to consider the ecological populations, as all strains of a same species do not share colonization and toxicity characteristics, and thus are not pathogenic. Furthermore, different strains of a same *Vibrio* species present distinct sensitivities toward marennine [9]. Hence, bacterial response to marennine exposure can be species- and strain-dependent. This biological activity seems to be intrinsic to blue *Haslea* species, as antibacterial activities were also demonstrated with the marennine-like pigment produced by *H. karadagensis* against *V. aestuarianus* and other species of interest in aquaculture [14].

At the sight of such results, the use of blue *Haslea* and marennine has been considered for aquaculture applications. Marennine biological activities have thus been investigated in vivo, using blue mussel and giant scallop larvae exposed to a concentrated supernatant of *H. ostrearia* culture enriched in extracellular marennine [15]. Low concentrations of this Blue Water (BW) solution significantly increased larval survival when challenged with a pathogenic *V. splendidus* strain [15]. This result is very promising in aquaculture for shellfish and fish larval health, but a better characterization of the interactions between marennine and pathogenic bacteria is needed. Thus, the present work aims to increase further our knowledge about the antimicrobial activity of *H. ostrearia* blue pigment, by assessing the effects of marennine on different species and strains of the genus *Vibrio* that are threatening aquaculture sustainability.

2. Materials and Methods

2.1. Vibrio Strains

Thirty *Vibrio* strains belonging to 10 species were tested for their sensitivity toward Blue Water (BW), a concentrated supernatant of *H. ostrearia* culture containing the extracellular marennine. *Vibrio chagasii* (strain #11, #12, #13), *Vibrio crassostreae* (#51, #52, #53), *Vibrio fortis* (#7, #8, #9), *Vibrio harveyi* (#21, #22, #23), *Vibrio orientalis* (#1, #2, #3), *Vibrio splendidus* (#90, #91, #93), *V. tasmaniensis* (#112, #113, #114), *Vibrio sp.* (isolated from oyster tissues; #36, #37, #38), and *V sp.* (isolated from sea water; #90, #91, #33) strains were provided by the Genomics of *Vibrio* team (Laboratoire de Biologie Intégrative des Modèles Marins (LBI2M), station biologique de Roscoff, France) and were previously described in [16]. *Vibrio aestuarianus* strains (#07/115, #12/016, #03/008) were provided by the Laboratoire de Génétique et de Pathologie des Mollusques Marins (LGPMM) of the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER; La Tremblade, France).

2.2. Vibrio Exposure to Blue Water Solutions

The susceptibility of the *Vibrio* strains to BW was assessed with the method described in the Clinical and Laboratory Standards Institute (CLSI) antimicrobial microdilution guidelines [17]. Bacterial inocula at a defined concentration were exposed to different BW concentrations in a 96-well microplate with a flat bottom and cover (BrandTech™BRAND*plates*™pureGrade™ S 96-well Microplates, Thermo Fisher Scientific, Waltham, MA, USA). Bacterial growth was monitored by Optical Density (OD) measurements with a microplate spectrophotometer (xMark, Bio-Rad, Hercules, CA, USA). Bacterial growth was then recorded using Microplate Manager 6 Software (MPM6, Bio-Rad, Hercules, CA, USA) with OD measurement (at 600 nm, to avoid the absorbance peak of marennine around 677 nm; [5]) of each well every 30 min for 24 h at ambient temperature.

2.2.1. Preparation of Bacterial Inocula

Vibrio strains were kept at −80 °C in 25% glycerol. Broth cultures were prepared with an autoclaved, cation-adjusted, Mueller–Hinton broth media (CaMHB; Biokar, Solabia Group, Pantin, France) by the addition of 1% NaCl (pH 7.5 ± 0.2; salinity = 32) and agar media, prepared with autoclaved, cation-adjusted Nutrient Agar (CaNA; Biokar) by the addition of 2.3% NaCl (final pH 7.5 ± 0.2; salinity = 32). Prior to the antibacterial assays, each *Vibrio* strain was inoculated in CaMHB from the −80 °C sample, incubated overnight at 25 °C under moderate agitation (130 rpm), and isolated on CaNA Petri dishes. After 1 day of incubation at 25 °C, plates containing the isolated colonies were kept at 4 °C for no more than a week. Three different colonies per Petri dish were inoculated in CaMHB (biological replicates, n = 3) and grown overnight at ambient temperature. The next day, the OD (630 nm) of bacteria in the broth culture was measured (V-10 Plus Humeau Spectrophotometer, La-Chapelle-sur-Erdre, France) and the absorbance was adjusted at 0.1 by dilution in CaMHB. To obtain the bacterial inoculum, the solution was further diluted by 1/100 in CaMHB, as recommended by the CLSI guidelines [17]. The bacterial inocula were exposed to BW in the microplates within 15 min after the dilution.

2.2.2. Blue Water (BW) Production

The growth of the 30 *Vibrio* strains exposed to Blue Water (BW) solutions was studied over a 24 h period. BW was prepared from a concentrated supernatant of *H. ostrearia* culture containing the extracellular marennine, and was produced at the Station aquicole de Pointe-au-Père, Institut des Sciences de la Mer à Rimouski-Université du Québec à Rimouski (ISMER-UQAR; Québec, Canada) during the spring of 2017. *H. ostrearia* was cultured in 100 L circular and flat bottom photobioreactors with filtered sea water (temperature: 20 °C; salinity 28) at high irradiance (180 µmol photons m^{-2} s^{-1}), in a 14/10 h light/dark cycle for 3 weeks, until marennine concentration reached around 6–7 µg mL^{-1}, as described in [15]. The supernatant was then collected and concentrated ca. 20 times by ultrafiltration (double cut-off at 3–30 kDa) as described in [18], for a final estimated concentration of ca. 120 µg mL^{-1} (pH 7.7 ± 0.2; salinity = 0), and stored in the dark at 4 °C. The BW concentration was assessed with spectrophotometric measurements (UV/Vis Lambda 25 Perkin Elmer spectrophotometer and UV Winlab Perkin Elmer software (version 6.0.4 2011), Waltham, MA, USA) on a syringe-filtered BW solution (0.2 µm; Sarstedt) and 1 cm path-length quartz cuvettes, using the Beer–Lambert equation (ε_{677} = 12.13 L g^{-1} cm^{-1}) as proposed by [18]. Prior to the antibacterial experiments, the BW stock solution was syringe-filtered on 0.4 µm, and the salinity and the pH were adjusted to be similar to the CaMHB at 32 and 7.5 ± 0.2, respectively, by addition of NaCl and HCl 0.1 M. BW dilutions were prepared with sterile, ultra-pure water plus NaCl (pH 7.5 ± 0.2; salinity = 32). The BW solutions at different concentrations were then syringe-filtered through 0.2 µm and kept at 4 °C.

2.2.3. Antibacterial Essay

In a first series of experiments, the 30 *Vibrio* strains corresponding to 10 different species were screened and exposed to three BW concentrations: 0 μg mL^{-1} (control), 10 μg mL^{-1}, and 25 μg mL^{-1}. In a second series of experiments, 10 *Vibrio* strains presenting different patterns of sensitivity to marennine were exposed to a dilution range of BW: 0 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1}, 25 μg mL^{-1}, 50 μg mL^{-1}, 70 μg mL^{-1}, and 85 μg mL^{-1}. For the screening experiment, the final volume in each well of the microplates was 100 μL, with a final ratio of 1:1 (v/v) bacterial inoculum: BW. In the dilution range experiment, to reach concentrations as high as 70 and 85 μg mL^{-1}, with a BW stock solution of 117 μg mL^{-1}, the final volume in each well was adjusted to 200 μL, with a final ratio of 1:4 (v/v) bacterial inoculum: BW. Microplates were first filled with the BW solutions, and bacterial inocula were then added to each well using a single channel electronic micropipette (Eppendorf Research Pro 50–1000 μL, Eppendorf, Hamburg, Germany). After being completed, microplates were sealed with parafilm and placed in the microplate spectrophotometer for the 24 h run. The experiments were conducted in triplicate, with technical triplicates for each condition. A negative control was also run per microplate for only BW and CaMHB.

2.3. Growth Curve Analyses and Statistics

Bacterial growth kinetics were analyzed using R 3.5.1 software. For the screening experiment, the OD (600 nm) data obtained over the 24 h run were fitted with a bi-phasic logistic growth equation, defined as below:

$$f(x) = \frac{k_1}{1 + e^{-r_1(x-x_1)}} + \frac{k_2}{1 + e^{-r_2(x-x_2)}}.$$

Interpretable metric parameters were then obtained, such as the maximum possible population size in a particular environment for the first phase of growth (k_1 parameter) and for the second phase of growth (k_2 parameter), or the growth rate (r_1 and r_2 parameters). The k_2 parameter was chosen in the screening experiment to study the effects of BW on the *Vibrio* strains.

For the concentration range experiments, the k parameter was also studied, but growth curves were fitted with a simple logistic growth equation, as it was not possible to fit growth curves of a same strain under the different BW exposures with the same bi-phasic logistic growth equation. Growth curves were analyzed with the R package "Growth Curver" [19].

R software was also used for statistical analyses. A Shapiro–Wilkinson test was used to verify data normality, and a Fisher test for the homogeneity of variance. There was no need to perform data transformation. The differences between treatments were assessed with one-way ANOVA, and post-hoc Tukey's pairwise multiple comparison tests were used to determine differences between pairs. Unless specified, data are expressed as mean ± standard error (SE).

3. Results

3.1. Different Patterns of Vibrio Growth Curves Evidenced by the Screening Experiment

The growth curves of the 30 *Vibrio* strains exposed to BW at 0 μg mL^{-1}, 10 μg mL^{-1}, or 25 μg mL^{-1} were recorded for 24 h (Supplementary Figure S1). Typical growth patterns selected from the 30 strains tested are presented in Figure 1. *Vibrio* strains in the CaMHB growth media presented a diauxic growth characterized by two distinct exponential phases [20]. A diauxic growth is typically observed when bacteria grow in a medium containing two different sources of nutrients (e.g., sugars). The diauxic lag phase was particularly marked for the three *V. harveyi* strains tested (Figure 1c) and to a lesser extent to the other tested strains of *V. orientalis*, *V. fortis*, *V. chagasii*, and *V. crassostreae* (Supplementary Figure S1).

Exposure to BW at 10 and 25 μg mL^{-1} affected the growth of 80% of the *Vibrio* strains tested over a 24 h period, and had varying effects on the maximum bacterial population size (Figure 2). An inhibiting dose-dependent effect was observed for 10 strains: *V. orientalis* #3; *V. chagasii* #12 and #13; *V. harveyi* #23; *V. sp.* #36, #37, and #43; *V. crassostreae* #52; *V. tasmaniensis* #114; and *V. aestuarianus* #02/041.

For nine other strains, the inhibiting effect of BW was similar under 10 and 25 μg mL^{-1}: *V. orientalis* #1 and #2, *V. fortis* #7, V. sp. #38, *V. crassostreae* #51 and #52, *V. splendidus* #93, and *V. tasmaniensis* #112 and #113. A growth stimulating effect was observed for *V. fortis* #9 with 36.6 ± 0.9% of stimulation under 10 μg mL^{-1} of BW exposure, and the stimulating effect was reduced to 18.5 ± 2.7% under 25 μg mL^{-1}. The growth stimulation of *V. aestuarianus* #07/115 was higher at 25 μg mL^{-1} than 10 μg mL^{-1}, and was comparable between both concentrations for *V. sp.* #42. For two strains, the growth was modified under BW exposure at 10 μg mL^{-1}, while no effect was observed under 25 μg mL^{-1} exposure: *V. sp.* #41 with a growth inhibition, and *V. splendidus* #90 with a growth stimulation. For six strains, no differences were found for the maximum population size under BW exposure: *V. fortis* #8; *V. splendidus* #91; *V. aestuarianus* #03/008; *V. chagasii* #12 (even if an inhibiting trend was observed, it did not appear to be statistically significant); and *V. harveyi* #21 and #22. Although BW had no effect on the maximum population size of *V. harveyi* #21 and #22, it can be noted that the growth rate of these strains was inhibited with 30.1 ± 1.1% and 37.8 ± 2.3% of inhibition for *V. harveyi* #21, and 38.1 ± 1.6% up to 57.2 ± 1.2% of inhibition for *V. harveyi* #22 under 10 and 25 μg mL^{-1}, respectively (see growth curves in Figure 1c and Supplementary Figure S1).

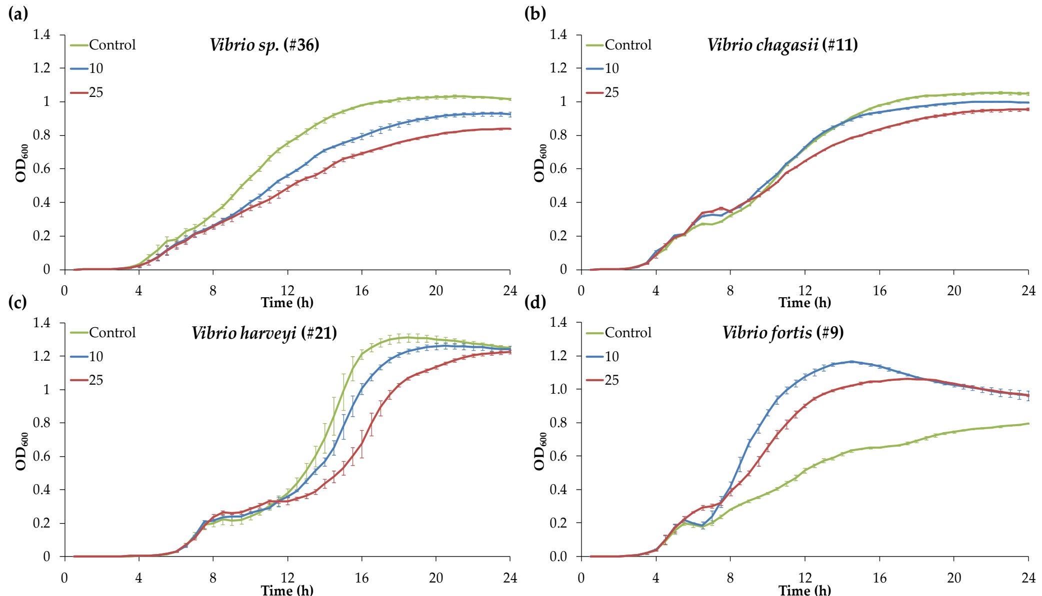
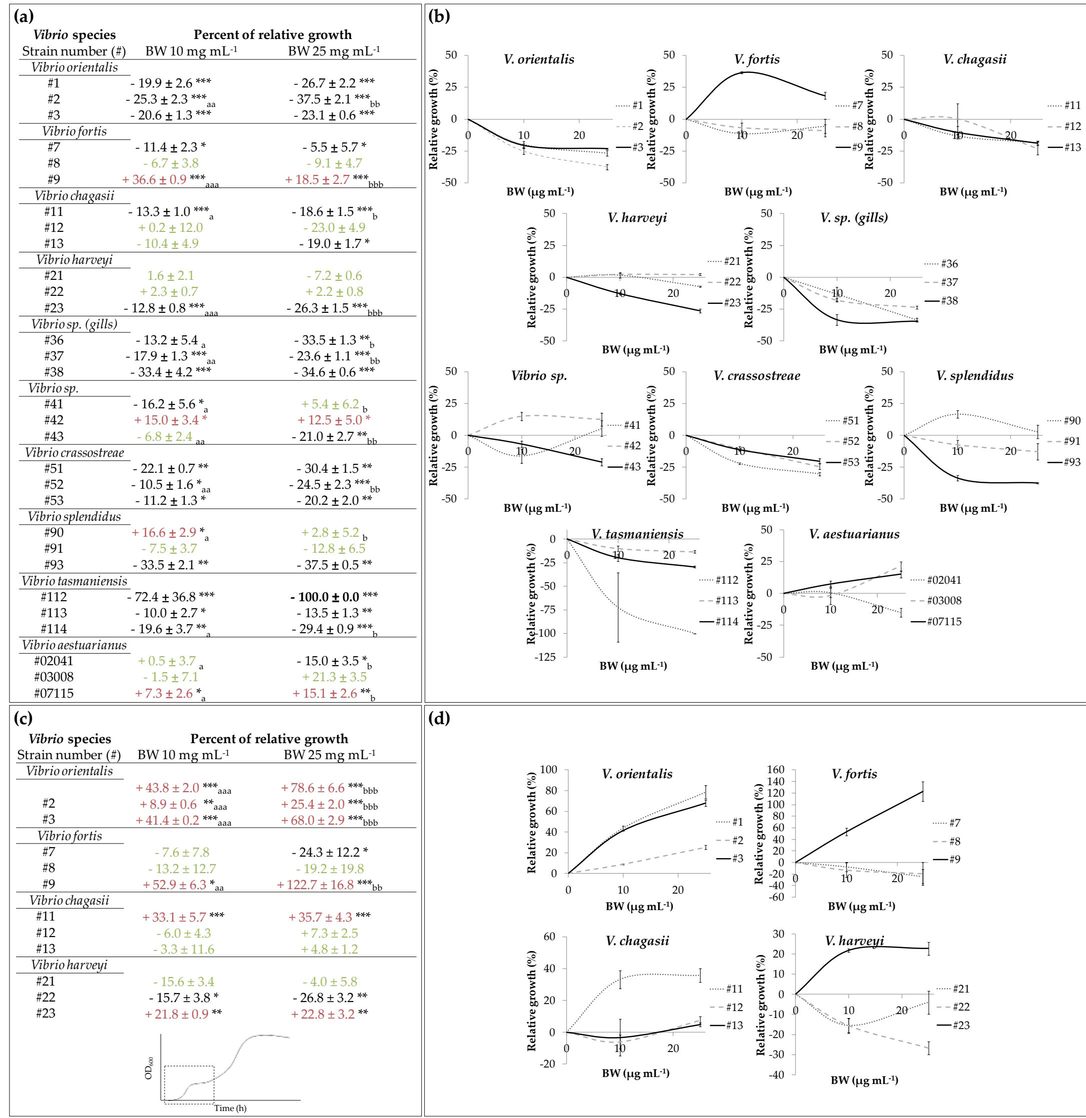


Figure 1. Growth kinetics of four *Vibrio* strains (#) exposed to 0 μg mL^{-1}, 10 μg mL^{-1}, or 25 μg mL^{-1} of Blue Water (BW) over a 24 h period, with growth characteristic features observed for the 30 *Vibrio* strains tested in the screening experiments. BW exposure inhibited the growth of (**a**) *Vibrio sp.* #36, (**b**) *Vibrio chagasii* #11, and (**c**) *Vibrio harveyi* #21, but stimulated the growth of (**d**) *Vibrio fortis* #9. *Vibrios* presented a diauxic growth characterized by two distinct exponential growth phases, with a diauxic lag phase in between. Results are means ± standard error (SE) (n = 3).

Although most *Vibrio* strains exhibited growth inhibition under BW exposure over a 24 h period, growth stimulation was sometimes observed during the first phase of growth. The maximum population size was thus also studied during the first growth phase for some strains that presented a diauxic growth, with a marked diauxic lag phase (i.e., *V. orientalis*, *V. fortis*, *V. chagasii*, and *V. harveyi*), and the percentage of relative growth was calculated (Figure 2c). The first growth phase of *V. orientalis* #1, #2, and #3, as well as *V. fortis* #9 was dose-dependently stimulated under BW exposure. Bacterial growth during the first phase was also stimulated by BW for *V. chagasii* #11 and *V. harveyi* #23, but no differences were found between exposures to 10 and 25 μg mL^{-1}. A growth inhibition was observed during the first growth phase for *V. harveyi* #22 and *V. fortis* #7, while no effect of BW was recorded for *V. fortis* #8, *V. chagasii* #12 and #13, and *V. harveyi* #21 during the first growth phase (Figure 2c,d).



(a)

Vibrio species Strain number (#)	Percent of relative growth BW 10 mg mL-1	 BW 25 mg mL-1
Vibrio orientalis		
#1	- 19.9 ± 2.6 ***	- 26.7 ± 2.2 ***
#2	- 25.3 ± 2.3 ***aa	- 37.5 ± 2.1 ***bb
#3	- 20.6 ± 1.3 ***	- 23.1 ± 0.6 ***
Vibrio fortis		
#7	- 11.4 ± 2.3 *	- 5.5 ± 5.7 *
#8	- 6.7 ± 3.8	- 9.1 ± 4.7
#9	+ 36.6 ± 0.9 ***aaa	+ 18.5 ± 2.7 ***bbb
Vibrio chagasii		
#11	- 13.3 ± 1.0 ***a	- 18.6 ± 1.5 ***b
#12	+ 0.2 ± 12.0	- 23.0 ± 4.9
#13	- 10.4 ± 4.9	- 19.0 ± 1.7 *
Vibrio harveyi		
#21	1.6 ± 2.1	- 7.2 ± 0.6
#22	+ 2.3 ± 0.7	+ 2.2 ± 0.8
#23	- 12.8 ± 0.8 ***aaa	- 26.3 ± 1.5 ***bbb
Vibrio sp. (gills)		
#36	- 13.2 ± 5.4 a	- 33.5 ± 1.3 **b
#37	- 17.9 ± 1.3 ***aa	- 23.6 ± 1.1 ***bb
#38	- 33.4 ± 4.2 ***	- 34.6 ± 0.6 ***
Vibrio sp.		
#41	- 16.2 ± 5.6 *a	+ 5.4 ± 6.2 b
#42	+ 15.0 ± 3.4 *	+ 12.5 ± 5.0 *
#43	- 6.8 ± 2.4 aa	- 21.0 ± 2.7 **bb
Vibrio crassostreae		
#51	- 22.1 ± 0.7 **	- 30.4 ± 1.5 **
#52	- 10.5 ± 1.6 *aa	- 24.5 ± 2.3 ***bb
#53	- 11.2 ± 1.3 *	- 20.2 ± 2.0 **
Vibrio splendidus		
#90	+ 16.6 ± 2.9 *a	+ 2.8 ± 5.2 b
#91	- 7.5 ± 3.7	- 12.8 ± 6.5
#93	- 33.5 ± 2.1 **	- 37.5 ± 0.5 **
Vibrio tasmaniensis		
#112	- 72.4 ± 36.8 ***	**- 100.0 ± 0.0** ***
#113	- 10.0 ± 2.7 *	- 13.5 ± 1.3 **
#114	- 19.6 ± 3.7 **a	- 29.4 ± 0.9 ***b
Vibrio aestuarianus		
#02041	+ 0.5 ± 3.7 a	- 15.0 ± 3.5 *b
#03008	- 1.5 ± 7.1	+ 21.3 ± 3.5
#07115	+ 7.3 ± 2.6 *a	+ 15.1 ± 2.6 **b

(c)

Vibrio species Strain number (#)	Percent of relative growth BW 10 mg mL-1	 BW 25 mg mL-1
Vibrio orientalis		
	+ 43.8 ± 2.0 ***aaa	+ 78.6 ± 6.6 ***bbb
#2	+ 8.9 ± 0.6 **aaa	+ 25.4 ± 2.0 ***bbb
#3	+ 41.4 ± 0.2 ***aaa	+ 68.0 ± 2.9 ***bbb
Vibrio fortis		
#7	- 7.6 ± 7.8	- 24.3 ± 12.2 *
#8	- 13.2 ± 12.7	- 19.2 ± 19.8
#9	+ 52.9 ± 6.3 *aa	+ 122.7 ± 16.8 ***bb
Vibrio chagasii		
#11	+ 33.1 ± 5.7 ***	+ 35.7 ± 4.3 ***
#12	- 6.0 ± 4.3	+ 7.3 ± 2.5
#13	- 3.3 ± 11.6	+ 4.8 ± 1.2
Vibrio harveyi		
#21	- 15.6 ± 3.4	- 4.0 ± 5.8
#22	- 15.7 ± 3.8 *	- 26.8 ± 3.2 **
#23	+ 21.8 ± 0.9 **	+ 22.8 ± 3.2 **

Figure 2. Screening experiment. (**a**) Relative maximum population size (%) of *Vibrio* strains exposed over a 24 h period to concentrations of 10 µg mL^{-1} and 25 µg mL^{-1} of Blue Water (BW). (**b**) Graphical illustrations of the dose response results presented in (**a**). (**c**) Relative maximum population size (%) after the first growth phase (see insert) for some of the *Vibrio* strains presenting a diauxic growth curve and exposed to 10 µg mL^{-1} and 25 mg mL^{-1} of BW. (**d**) Graphical illustrations of the screening experiment results presented in (**c**). Significant growth inhibitions are presented in black, growth stimulations in red, and no observed effects in green. Asterisks (*) indicate a statistical difference with the control, and letters (a,b) indicate significant differences between the two BW concentrations tested.*, a, b; **, aa, bb and ***, aaa, bbb respectively indicate p value < 0.05, p value < 0.01 and p value < 0.001. Values are means ± SE (n = 3).

3.2. Experiment with Blue Water Concentration Range

Different dose response patterns were observed depending on the strain tested, including linear and "U shape" responses (Figure 3). *V. fortis* #8, *V. orientalis* #3, *V. aestuarianus* #02/041, and *V. harveyi* #23 presented a linear dose–response curve, with increasing growth inhibition along with increasing concentration exposure (Figure 3a,b and Supplementary Figure S2). For *V. fortis* #8, a significant inhibitory effect was observed from 5 µg mL^{-1} with 5.1 ± 0.5% growth inhibition (p value < 0.001), up to 64.6 ± 0.7% at 85 µg mL^{-1}, with an IC_{50} (concentration at which the response is inhibited by 50%) at around 50 µg mL^{-1} (Figure 3a). An increasing inhibitory effect was also observed for *V. orientalis* #3

from 5 μg mL^{-1} (p value < 0.001) up to 50 μg mL^{-1}, but there was no difference in growth inhibition between 50 and 85 μg mL^{-1}, with a maximum inhibition level of about 37% (Supplementary Figure S2). A "no observed effect" level (NOEL) was noted for *V. aestuarianus* #02/041 between 0 and 25 μg mL^{-1} (p = 0.351), and the first inhibitory effect was evidenced at 50 μg mL^{-1} with 12 ± 0.2% of inhibition (p = 0.015), while the maximum effect was observed at 85 μg mL^{-1} with 23.4 ± 1.7% of inhibition (Supplementary Figure S2). For *V. harveyi* #23, the diauxic lag phase was particularly marked, and the growth response changed with BW concentration and time. Growth curves were thus analyzed as two independent phases of growth, with phase 1 from 0 to 9 h and phase 2 from 9 to 24 h (Figure 3b). For the first growth phase, a significant growth stimulation was observed starting from 5 μg mL^{-1} (p < 0.001). The second growth phase presented dose-dependent inhibition, with a total blockage of the growth at the highest concentrations tested (i.e., 50, 70, and 85 μg mL^{-1}) and an important increase of the diauxic lag phase for bacteria exposed to 25 μg mL^{-1} at 7 h, versus 2 h for the control.

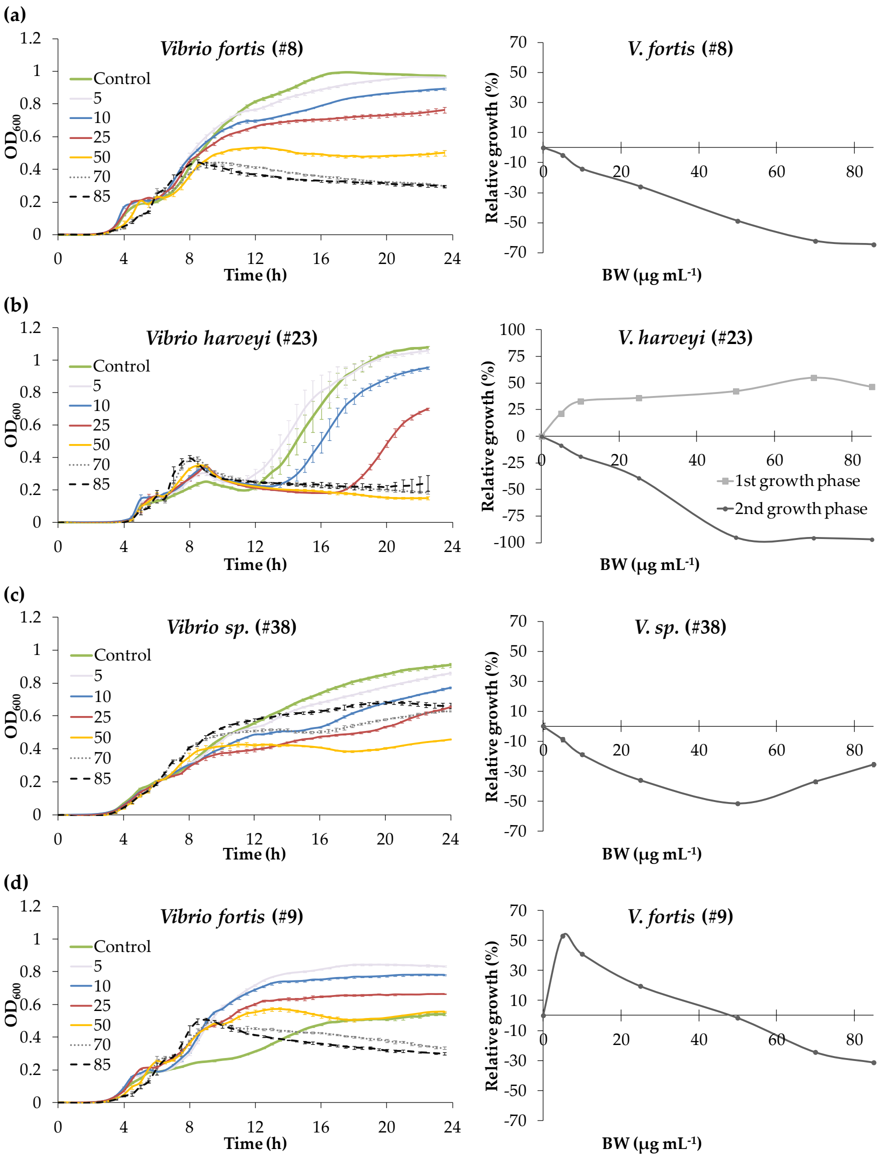


Figure 3. Growth kinetics and dose–response curves of *Vibrio* strains exposed to a concentration range (μg mL^{-1}) of Blue Water (BW) over a 24-h period, presenting linear (**a**,**b**), "U shape" (**c**), or hormetic (**d**) responses. Growth of *V. harveyi* #23 was analyzed in two independent phases, with phase 1 from 0 to 9 h and phase 2 from 9 to 24 h. Values in the growth curves are means ± standard deviation (SD) of (n = 3).

The growth of *V. sp.* 38, *V. chagasii* #13, *V. crassostreae* #53, and *V. splendidus* #90 was also inhibited by the BW concentrations tested, but the dose–response curves expressed in function of the control presented a "U" shape, with the growth inhibitory effect first increasing, then decreasing with increasing BW concentrations (Figure 3c and Supplementary Figure S2). For *V. sp.* #38, the maximum effect was observed at the concentration 50 µg mL^{-1}, with 51.7 ± 0.6% of growth inhibition (Figure 3c). However, the relative growth inhibition decreased to 37.1 ± 1.1% at 70 µg mL^{-1}, and was similar to the inhibition obtained at 25 µg mL^{-1} (p value 0.971). Same observations were made for *V. chagasii* #13 and *V. crassostreae* #53, with the inhibitory effect significantly higher at 50 µg mL^{-1} than at 70 µg mL^{-1} ($p < 0.005$). For *V. splendidus* #90, the percentage of maximum inhibition was 33.3 ± 1.5% at 70 µg mL^{-1}, and the inhibition was significantly lower, at 23.0 ± 0.7% at 85 µg mL^{-1} ($p = 0.004$; Supplementary Figure S2).

The strains *V. tasmaniensis* #114 and *V. fortis* #9 presented a hormetic dose–response curve, with opposite effects depending on the BW concentration. For *V. tasmaniensis* #114 the maximum inhibitory effect was observed between 5 and 10 µg mL^{-1}, with about 23% of inhibition, and decreased until reaching the NOEL between 50 and 70 µg mL^{-1} (Supplementary Figure S2). *V. fortis* #9 presented a growth stimulation at the lower concentrations tested, with a maximum growth stimulation of 53.0 ± 0.1% at 5 µg mL^{-1}, then a decrease with increasing BW concentration. The NOEL was reached at 50 µg mL^{-1}, and the growth inhibition increased dose-dependently, with 31.4 ± 0.6% of inhibition at 85 µg mL^{-1} (Figure 3d).

4. Discussion

The effects of marennine on several *Vibrio* strains with different ecological and virulence properties have been investigated, by monitoring bacterial growth over a 24 h period after addition of BW into the culture medium. The general trend for all the experiments is that BW affected most of the *Vibrio* strains tested (80%), but the results are contrasting. Indeed, BW either inhibited or stimulated the growth of the *Vibrio* strains.

As BW solution (i.e., the concentrated supernatant of *H. ostrearia* containing marennine) was used in the present work, and not a purified form of the pigment, it could be questioned if the observed effects were induced by marennine or by other molecules possibly present in the non-axenic culture medium (e.g., exopolysaccharides (EPS) from *Haslea* or epibiont bacteria). However, previous studies conducted with purified extracellular marennine (EMn) on marine bacteria, including *Vibrio* species, confirmed that the pigment itself did exert an antibacterial activity [9–12]. Moreover, a recent study has demonstrated the biological activities of marennine solutions, including BW and EMn, on the development or survival of various marine organisms, and comparable effects were observed between the different solutions tested [21]. Also, if blue *Haslea* species should be cultured at large scale to ensure the production of marennine solutions for applications as antimicrobials in aquaculture, BW represents the easiest and cheapest production process, compared to the purified pigment.

The observed effect of BW on vibrios was species- but also strain-dependent, as for a same species, the sensitivity of distinct strains could significantly differ. This is in accordance with previous observations of *V. aestuarianus*, *V. coralliilyticus*, and *V. tubiashii* exposed to purified marennine [9]. Moreover, it seemed difficult to perceive a correlation between the effects of BW and the strain ecological characteristics, such as sampling season, distribution (sea water fractions and oyster tissues), or the virulence of the strain toward bivalves (Supplementary Table S1).

However, the present work considerably extends our understanding of the variability of marennine's effect on bacteria of the *Vibrio* genus. The diversity observed in growth responses was especially noteworthy in two species: *V. fortis* on the one hand, with a growth inhibition for strains #7 and #8, and important growth stimulation for strain #9; and on the other hand, *V. tasmaniensis*, with a total inhibition of strain #112, but no effect observed on the strain #113.

In addition, diauxic growth was evidenced for all the *Vibrio* strains tested, a phenomenon commonly observed when bacteria are grown in a medium containing two types of carbon sources

(e.g., sugars), and characterized by two distinct exponential phases, with a diauxic lag phase in between [20]. *Vibrio* diauxic growth is not extensively evidenced in the literature, and was mainly illustrated using *V. cholerae* [22–24] but also *V. alginolyticus* [25]. It is worth noting that complete growth kinetics are rarely recorded for antibacterial assays, as most experiments conducted to screen bioactive compounds against marine *Vibrio* species used the disc diffusion method [26–28], as the ones conducted with marennine and marennine-like pigments on *V. aestuarianus* [11,14]. The effect of purified marennine was previously tested with the microdilution method on another *V. tasmaniensis* strain [9,12] and no obvious diauxic growth was observed then, which could be explained by the use of a different growth media (marine broth media versus CaMHB in the present study), a different time scale, or a different treatment of data.

Bacterial diauxic growth has long been considered as a bacterial phase of enzymatic acclimation to metabolize a different type of sugar; however, there is now growing evidence that this phenomenon could actually correspond to the presence of two bacterial subpopulations in the isogenic culture, with different phenotypic adaptation and growth strategies [29]. Based on this new hypothesis, diauxic growth curves in the present study could indicate the existence of different subpopulations of *Vibrio* strains: Type 1, which can divide quickly by using the most suitable carbon source to sustain its growth; and Type 2, which grows more slowly, but which would be able to metabolize a different carbon source. According to Solopova et al. [29], the Type 1 subpopulation, which cannot switch to an alternative metabolic pathway, will stop dividing, possibly because of the low energy state of cells [29]. This phenotype heterogeneity has been recently demonstrated in *V. cholerae* [24], and would result from the bet-hedging strategy, an evolutionary strategy that allows colonies to cope with fluctuating environments [30–32]. In the present work, although bacterial growth was globally inhibited over the 24 h of the essay, a growth stimulation of the subpopulation Type 1 was sometimes observed under BW exposure (e.g., *V. orientalis*, *V. chagasii*, and *V. harveyi* strains). It can be hypothesized that the subpopulation Type 1 would be able to metabolize part of the sugars constituting the carbon skeleton of the marennine molecule—or, if any, other EPS present in BW solution to sustain its growth—or that the stimulation would result from overcompensation mechanisms by the bacteria under BW exposure, a mechanism that can be observed when cells undergo a disruption in homeostasis [33]. However, other experiments would be needed to confirm these hypotheses. It may also be argued that, when exposed to BW, it is the growth of subpopulation Type 2 that was mainly affected by the BW, as illustrated by the total absence of growth of *V. harveyi* #23 at the highest concentrations tested (from 50 to 80 μg mL^{-1}).

A global observation from the results shows that the dose–response curves of most *Vibrio* strains did not follow a linear or threshold model, with growth inhibition increasing with concentrations. Indeed, some curves presented a “U-shape” response regarding the antibacterial effect, which decreased with increasing BW exposure (e.g., *V. chagasii* #13 or *V. sp* #38). On top of this, opposite effects were observed at low and high concentrations, a phenomenon known as hormesis [34]. Indeed, the lowest BW concentration tested (5 μg mL^{-1}) highly stimulated the growth of *V. fortis* #9, up to 50%, while the growth was significantly inhibited at higher concentrations (from 50 to 80 μg mL^{-1}). Hormetic dose–response relationships have raised a growing awareness in toxicological and ecotoxicological studies, and have been extensively documented over the past two decades in different models, as plants, algae, or fungi [35–37]. The mechanisms of hormesis are not yet clearly understood, and it seems that only a subset of compounds with specific cellular mechanisms would mediate hormetic responses [38,39]. Moreover, hormetic effects are challenging to observe in laboratory conditions, being dependent on various factors, such as endpoint measurements or growth conditions [40]. In the present study, hormetic response of vibrios to BW was observed in the CaMHB medium, a culture medium that was previously reported to promote the observation of hormesis in *Escherichia coli* exposed to antibiotics, while the hormetic effect was not observed in Luria–Bertani (LB) culture media [41].

The mechanisms of action responsible for the antibacterial activity of marennine remain little explored. So far, the *Haslea provincialis* purified blue pigment was demonstrated to interact with the outer membrane of the gram-negative bacteria *E. coli*, rendering it more rigid [42]. Recent studies conducted on *V. cholerae* also demonstrate a disruption of the bacterial membrane integrity and a deformation of the cell architecture by antibacterial agents, such as polyphenols [23] or nanoparticles [43]. For now, it is still unclear how marennine and *Vibrio* interact in vivo, and whether the pigment has a direct effect on *Vibrio* growth or if it could decrease its pathogenicity by fixing on cell membranes, both of which could explain the better survival of bivalve larvae exposed to marennine and challenged with *V. splendidus* [15].

In conclusion, the present work indicates much more complex interactions between marennine and vibrios than a standard linear correlation between dose and effect. Moreover, the antibacterial activity of marennine is dependent on the species, the strain, and possibly the population of bacteria, suggesting that the blue pigment would act on specific targets. Marennine antibacterial mechanisms and low-dose stimulation phenomenon will have to be better understood before considering any application of *Haslea* and marennine as antimicrobials in aquaculture.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/17/3/160/s1, Figure S1: Growth curves of 30 *Vibrio* strains (#) from 10 different species exposed over a 24 h period to 0, 10 or 25 µg mL^{-1} of Blue Water (BW), the concentrated supernatant of *Haslea ostrearia* containing the extracellular marennine. Results are means ± SE (n = 3), Figure S2: Growth kinetics and dose-response curves of *Vibrio* strains exposed to a concentration range (µg mL^{-1}) of Blue Water (BW) over a 24 h period. Values are means ± SD (n = 3), Table S1: Preferential distribution, virulence to oysters and Blue Water (BW) effect over a 24 h period and over the first phase of growth of the 30 *Vibrio* strains tested.

Author Contributions: Conceptualization, C.F. and J.-L.M.; Data curation, C.F.; Formal analysis, C.F. and M.B.; Funding acquisition, J.-L.M.; Methodology, C.F. and M.Z.; Resources, A.J. and M.-A.T.; Software, C.F. and M.B.; Supervision, J.-L.M.; Validation, M.-A.T. and J.-L.M.; Writing—original draft, C.F.; Writing—review & editing, A.J., M.-A.T., M.B., M.Z. and J.-L.M.

Funding: This publication benefited from funding from the Horizon 2020 Research and Innovation Program GHaNA (The Genus *Haslea*, New marine resources for blue biotechnology and Aquaculture) under Grant Agreement No 734708/GHANA/H2020-MSCA-RISE-2016 (JLM).

Acknowledgments: The authors want to thank Frédérique Le Roux from the Biologie Intégrative des Modèles Marins laboratory, Génomique des Vibrios team (Station Biologique de Roscoff, France) for having kindly provided most of the *Vibrio* strains necessary for the present study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nassiri, Y.; Robert, J.-M.; Rincé, Y.; Ginsburger-Vogel, T. The cytoplasmic fine structure of the diatom *Haslea ostrearia* (Bacillariophyceae) in relation to marennine production. *Phycologia* **1998**, *37*, 84–91. [CrossRef]
2. Gastineau, R.; Davidovich, N.A.; Bardeau, J.-F.; Caruso, A.; Leignel, V.; Hardivillier, Y.; Jacquette, B.; Davidovich, O.I.; Rincé, Y.; Gaudin, P.; et al. *Haslea karadagensis* (Bacillariophyta): A second blue diatom, recorded from the Black Sea and producing a novel blue pigment. *Eur. J. Phycol.* **2012**, *47*, 469–479. [CrossRef]
3. Gastineau, R.; Hansen, G.; Davidovich, N.A.; Davidovich, O.; Bardeau, J.-F.; Kaczmarska, I.; Ehrman, J.M.; Leignel, V.; Hardivillier, Y.; Jacquette, B.; et al. A new blue-pigmented hasleoid diatom, *Haslea provincialis*, from the Mediterranean Sea. *Eur. J. Phycol.* **2016**, *51*, 156–170. [CrossRef]
4. Prasetiya, F.S.; Gastineau, R.; Poulin, M.; Lemieux, C.; Turmel, M.; Syakti, A.D.; Hardivillier, Y.; Widowati, I.; Risjani, Y.; Iskandar, I.; et al. *Haslea nusantara*, a new blue diatom from the Java Sea, Indonesia: Morphology, biometry and molecular characterization. *Plant. Ecol. Evol.* **2019**, *151*. (in press).
5. Pouvreau, J.-B.; Morançais, M.; Fleury, F.; Rosa, P.; Thion, L.; Cahingt, B.; Zal, F.; Fleurence, J.; Pondaven, P. Preliminary characterisation of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. *J. Appl. Phycol.* **2006**, *18*, 757–767. [CrossRef]
6. Turpin, V.; Robert, J.-M.; Goulletquer, P.; Massé, G.; Rosa, P. Oyster greening by outdoor mass culture of the diatom *Haslea ostrearia* Simonsen in enriched seawater. *Aquac. Res.* **2001**, *32*, 801–809. [CrossRef]

7. Pouvreau, J.-B.; Housson, E.; Tallec, L.L.; Morançais, M.; Rincé, Y.; Fleurence, J.; Pondaven, P. Growth inhibition of several marine diatom species induced by the shading effect and allelopathic activity of marennine, a blue-green polyphenolic pigment of the diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. *J. Exp. Mar. Biol. Ecol.* **2007**, *352*, 212–225. [CrossRef]
8. Prasetiya, F.S.; Safitri, I.; Widowati, I.; Cognie, B.; Decottignies, P.; Gastineau, R.; Morançais, M.; Windarto, E.; Tremblay, R.; Mouget, J.-L. Does allelopathy affect co-culturing *Haslea ostrearia* with other microalgae relevant to aquaculture? *J. Appl. Phycol.* **2016**, *28*, 2241–2254. [CrossRef]
9. Falaise, C.; François, C.; Travers, M.-A.; Morga, B.; Haure, J.; Tremblay, R.; Turcotte, F.; Pasetto, P.; Gastineau, R.; Hardivillier, Y.; et al. Antimicrobial compounds from eukaryotic microalgae against human pathogens and diseases in aquaculture. *Mar. Drugs* **2016**, *14*, 159. [CrossRef]
10. Pouvreau, J.-B. Purification et Caractérisation du Pigment Bleu-Vert "Marennine" Synthétisé par la Diatomee Marine *Haslea ostrearia* (Gaillon/Bory) Simonsen: Propriétés Physico-Chimiques et Activités Biologiques. Ph.D. Thesis, Université de Nantes, Nantes, France, July 2006.
11. Gastineau, R.; Pouvreau, J.-B.; Hellio, C.; Morançais, M.; Fleurence, J.; Gaudin, P.; Bourgougnon, N.; Mouget, J.-L. Biological activities of purified marennine, the blue pigment responsible for the greening of oysters. *J. Agric. Food Chem.* **2012**, *60*, 3599–3605. [CrossRef]
12. Gastineau, R.; Turcotte, F.; Pouvreau, J.-B.; Morançais, M.; Fleurence, J.; Windarto, E.; Prasetiya, F.S.; Arsad, S.; Jaouen, P.; Babin, M.; et al. Marennine, promising blue pigments from a widespread *Haslea* diatom species complex. *Mar. Drugs* **2014**, *12*, 3161–3189. [CrossRef] [PubMed]
13. Travers, M.-A.; Boettcher Miller, K.; Roque, A.; Friedman, C.S. Bacterial diseases in marine bivalves. *J. Invertebr. Pathol.* **2015**, *131*, 11–31. [CrossRef] [PubMed]
14. Gastineau, R.; Hardivillier, Y.; Leignel, V.; Tekaya, N.; Morançais, M.; Fleurence, J.; Davidovich, N.; Jacquette, B.; Gaudin, P.; Hellio, C.; et al. Greening effect on oysters and biological activities of the blue pigments produced by the diatom *Haslea karadagensis* (Naviculaceae). *Aquaculture* **2012**, *368–369*, 61–67. [CrossRef]
15. Turcotte, F.; Mouget, J.-L.; Genard, B.; Lemarchand, K.; Deschênes, J.-S.; Tremblay, R. Prophylactic effect of *Haslea ostrearia* culture supernatant containing the pigment marennine to stabilize bivalve hatchery production. *Aquat. Living Resour.* **2016**, *29*, 401. [CrossRef]
16. Bruto, M.; James, A.; Petton, B.; Labreuche, Y.; Chenivesse, S.; Alunno-Bruscia, M.; Polz, M.F.; Le Roux, F. *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J.* **2017**, *11*, 1043–1052. [CrossRef] [PubMed]
17. Clinical and Laboratory Standards Institute (CLSI). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Document M07-A9. Approved Standard—Ninth Edition*; Clinical and Laboratory Institute: Wayne, PA, USA, 2012; Volume 32.
18. Pouvreau, J.-B.; Morançais, M.; Massé, G.; Rosa, P.; Robert, J.-M.; Fleurence, J.; Pondaven, P. Purification of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. *J. Appl. Phycol.* **2006**, *18*, 769–781. [CrossRef]
19. Sprouffske, K.; Wagner, A. Growthcurver: An R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinform.* **2016**, *17*, 1–4. [CrossRef]
20. Monod, J. The Growth of Bacterial Cultures. *Annu. Rev. Microbiol.* **1949**, *3*, 371–394. [CrossRef]
21. Falaise, C.; Cormier, P.; Tremblay, R.; Audet, C.; Deschênes, J.-S.; Turcotte, F.; François, C.; Seger, A.; Hallegraeff, G.; Lindquist, N.; et al. Harmful or harmless: Biological effects of marennine on marine organisms. *Aquatic Toxicol.* **2019**, *209*, 13–25. [CrossRef]
22. Bag, J. Glucose inhibition of the transport and phosphoenolpyruvate-dependent phosphorylation of galactose and fructose in *Vibrio cholerae*. *J. Bacteriol.* **1974**, *118*, 764–767.
23. Bhattacharya, D.; Ghosh, D.; Bhattacharya, S.; Sarkar, S.; Karmakar, P.; Koley, H.; Gachhui, R. Antibacterial activity of polyphenolic fraction of Kombucha against *Vibrio cholerae*: Targeting cell membrane. *Lett. Appl. Microbiol.* **2018**, *66*, 145–152. [CrossRef]
24. Chatterjee, T.; Chatterjee, B.K.; Chakrabarti, P. Modelling of growth kinetics of Vibrio cholerae in presence of gold nanoparticles: Effect of size and morphology. *Sci. Rep.* **2017**, *7*, 9671. [CrossRef] [PubMed]
25. Vine, N.G.; Leukes, W.D.; Kaiser, H. In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. *FEMS Microbiol. Lett.* **2004**, *231*, 145–152. [CrossRef]

26. Banerjee, S.; Kim, L.M.; Shariff, M.; Khatoon, H.; Yusoff, F.M. Antibacterial activity of neem (*Azadirachta indica*) leaves on *Vibrio spp.* isolated from cultured shrimp. *Asian J. Anim. Vet. Adv.* **2013**, *8*, 355–361.
27. Cavallo, R.; Acquaviva, M.; Stabili, L.; Cecere, E.; Petrocelli, A.; Narracci, M. Antibacterial activity of marine macroalgae against fish pathogenic *Vibrio* species. *Open Life Sci.* **2013**, *8*, 646–653.
28. Das, B.K.; Pradhan, J.; Pattnaik, P.; Samantaray, B.R.; Samal, S.K. Production of antibacterials from the freshwater alga *Euglena viridis* (Ehren). *World J. Microbiol. Biotechnol.* **2005**, *21*, 45–50. [CrossRef]
29. Solopova, A.; van Gestel, J.; Weissing, F.J.; Bachmann, H.; Teusink, B.; Kok, J.; Kuipers, O.P. Bet-hedging during bacterial diauxic shift. *PNAS* **2014**, *111*, 7427–7432. [CrossRef] [PubMed]
30. Ackermann, M. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat. Rev. Microbiol.* **2015**, *13*, 497–508. [CrossRef]
31. Grimbergen, A.J.; Siebring, J.; Solopova, A.; Kuipers, O.P. Microbial bet-hedging: The power of being different. *Curr. Opin.Microbiol.* **2015**, *25*, 67–72. [CrossRef]
32. Martins, B.M.; Locke, J.C. Microbial individuality: How single-cell heterogeneity enables population level strategies. *Curr. Opin. Microbiol.* **2015**, *24*, 104–112. [CrossRef]
33. Calabrese, E.J. Hormetic mechanisms. *Crit. Rev. Toxicol.* **2013**, *43*, 580–606. [CrossRef] [PubMed]
34. Calabrese, E.J.; Baldwin, L.A. Defining hormesis. *Hum. Exp. Toxicol* **2002**, *21*, 91–97. [CrossRef] [PubMed]
35. Belz, R.G.; Duke, S.O. Herbicides and plant hormesis. *Pest. Manag. Sci.* **2014**, *70*, 698–707. [CrossRef] [PubMed]
36. Calabrese, E.J.; Baldwin, L.A. Hormesis: A Generalizable and Unifying Hypothesis. *Crit. Rev. Toxicol.* **2001**, *31*, 353–424. [CrossRef] [PubMed]
37. Cedergreen, N.; Streibig, J.C.; Kudsk, P.; Mathiassen, S.K.; Duke, S.O. The occurrence of hormesis in plants and algae. *Dose Response* **2006**, *5*, 150–162. [CrossRef] [PubMed]
38. Kendig, E.L.; Le, H.H.; Belcher, S.M. Defining Hormesis: Evaluation of a Complex Concentration Response Phenomenon. *Int. J. Toxicol.* **2010**, *29*, 235–246. [CrossRef]
39. Lushchak, V.I. Dissection of the Hormetic Curve: Analysis of Components and Mechanisms. *Dose Response* **2014**, *12*, 466–479. [CrossRef] [PubMed]
40. Calabrese, E.J.; Baldwin, L.A. A quantitatively-based methodology for the evaluation of chemical hormesis. *Hum. Ecolog. Risk Assess. An. Int. J.* **1997**, *3*, 545–554. [CrossRef]
41. Wang, D.; Lin, Z.; Wang, T.; Ding, X.; Liu, Y. An analogous wood barrel theory to explain the occurrence of hormesis: A case study of sulfonamides and erythromycin on *Escherichia coli* growth. *PLoS ONE* **2017**, *12*, e0181321. [CrossRef]
42. Tardy-Laporte, C.; Arnold, A.A.; Genard, B.; Gastineau, R.; Morançais, M.; Mouget, J.-L.; Tremblay, R.; Marcotte, I. A 2H solid-state NMR study of the effect of antimicrobial agents on intact *Escherichia coli* without mutating. *Biochim. Biophys. Acta (BBA)—Biomembr.* **2013**, *1828*, 614–622. [CrossRef]
43. Sarwar, S.; Chakraborti, S.; Bera, S.; Sheikh, I.A.; Hoque, K.M.; Chakrabarti, P. The antimicrobial activity of ZnO nanoparticles against *Vibrio cholerae*: Variation in response depends on biotype. *Nanomed. Nanotechnol. Biol. Med.* **2016**, *12*, 1499–1509. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

ANNEXE 4 : Publication

Prasetiya et al. (sous presse), *Plant Ecology & Evolution* [*Texte Complet*]

Prasetiya, F.S., Gastineau, R., Poulin, M., Lemieux, C., Turmel, M., Syakti, A.D., Hardivillier, Y., Widowati, I., Risjani, Y., Iskandar, I., Subroto, T., Falaise, C., Mouget, J.-L., Leignel, V., *in press*. ***Haslea nusantara*, a new blue diatom from the Java Sea, Indonesia: Morphology, biometry and molecular characterization**. *Plant Ecology and Evolution*.

Haslea nusantara, a new blue diatom from the Java Sea, Indonesia: Morphology, biometry and molecular characterization

Fiddy Semba PRASETIYA[1,7,8*], Romain GASTINEAU[1], Michel POULIN[2], Claude LEMIEUX[3], Monique TURMEL[3], Agung Dhamar SYAKTI[4], Yann HARDIVILLIER[1], Ita WIDOWATI[5], Yenny RISJANI[6], Iskandar ISKANDAR[7], Toto SUBROTO[8], Charlotte FALAISE[1], Jean-Luc MOUGET[1$] & Vincent LEIGNEL[1$]

[1]FR CNRS 3473 IUML, Mer-Molécules-Santé (MMS), Le Mans Université, Avenue Olivier Messiaen, 72085 Le Mans CEDEX 9, France
[2]Division Recherche et collections, Musée canadien de la nature, C.P. 3443, Succursale D, Ottawa, Ontario, K1P 6P4, Canada
[3]Institut de Biologie Intégrative et des Systèmes, Département de Biochimie, de Microbiologie et de Bio-Informatique, Université Laval, Québec, Canada
[4]Fisheries and Marine Sciences Department, Jenderal Soedirman University, Kampus Perikanan Unsoed Karangwangkal, Jl dr. Suparno, Purwokerto 53123, Indonesia
[5]Faculty of Fisheries and Marine Sciences, Diponegoro University, Jl. Prof. H. Soedharto,SH, Tembalang, Semarang 50275, Indonesia
[6]Faculty of Fisheries and Marine Sciences, University of Brawijaya, Jalan Veteran, Ketawanggede, Malang 65145, Indonesia
[7]Marine Science Department, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran, Jl. Raya Bandung Sumedang KM.21, Jatinangor 45363, Indonesia
[8]Research Center of Biotechnology and Bioinformatics, Jl. Raya Bandung Sumedang KM.21, Jatinangor 45363, Indonesia
($ co-contribution)

*Author for correspondence *: Dr Fiddy Semba PRASETIYA.
Fiddy.Semba_Prasetiya@univ-lemans.fr

Background and aims – The present study aims to describe a new species of pennate blue diatom from the genus *Haslea*, *H. nusantara* sp. nov., collected from Semak Daun Island, the Seribu Archipelago, in Indonesian marine waters.
Methods – Assessment for species identification was conducted using light microscopical (LM), Scanning Electron Microscopical (SEM) and molecular techniques. The morphological characteristics of *H. nusantara* sp. nov. has been described, illustrated and compared to other morphologically similar *Haslea* taxa, while molecular characteristics known worldwide. Additionally, the molecular characteristic (*rbcL* chloroplast gene and *cox1* mitochondrial gene) was also analyzed.
Key results – This new species, named *Haslea nusantara*, is characterized by specific morphological (stria density) and molecular criteria (*rbcL* chloroplast gene and *cox1* mitochondrial gene). It differentiates from other blue *Haslea* species by the presence of a thin central bar, which has been previously reported in non-blue species like *H. pseudostrearia* Massé, Rincé *et* E.J. Cox. The complete mitochondrion (36,288 basepairs, bp) and plastid (120,448 bp) genomes of *H. nusantara* were sequenced and the gene arrangements were compared with other diatom genomes. Phylogeny analyses established using *rbcL* indicated that *H. nusantara* sp. nov. is included in the blue *Haslea* cluster and close to a blue *Haslea* sp. found in Canary Islands (*H. silbo* sp. inedit.).
Conclusions – All investigations carried out in this study show that *H. nusantara* is a new blue-pigmented species, which belongs to the blue *Haslea* clade, with an exceptional geographic distribution in the Southern Hemisphere.

Key words – Indonesia, Bacillariophyceae / blue diatoms / *Haslea* / molecular phylogeny, genomic study

INTRODUCTION

The pennate diatom, *Haslea ostrearia* (Gaillon) Simonsen, has long been known as the only blue diatom ever described, and it is also the type species of the genus *Haslea* Simonsen (Simonsen, 1974; Poulin *et al*., in press, this journal's issue). This microalga produces a water-soluble blue pigment, marennine (Lankester 1886), which accumulates at cell apices and is released into the seawater. This pigment is responsible for the greening of oyster's gills in the Atlantic coast of France (Gaillon 1820). This phenomenon gives a significant economic added value to the French oyster industry, since green oysters, with specific flavor and emerald color, are rarer and more expensive than ordinary oysters. Furthermore, this blue pigment has biological properties like antioxidant (Pouvreau et al. 2008), antibacterial and antiviral (Gastineau et al. 2012a) and allelopathic effect against other diatoms (Pouvreau et al. 2007a, Prasetiya et al. 2016).

For decades, any record worldwide of a blue diatom was assigned to *H. ostrearia*. However, recent works on the blue *Haslea* using scanning electron microscopy (SEM) observation and molecular approaches have enlightened an unsuspected biodiversity of this taxon, with two new species, *H. karadagensis* Davidovich, Gastineau *et* Mouget (Gastineau et al. 2012b) collected in the Black Sea, and *H. provincialis* Gastineau, Hansen *et* Mouget

(Gastineau et al. 2016) in the Mediterranean Sea. Gastineau et al. (2014) also mentioned one undescribed species yet in the Canary Islands (*Haslea silbo* sp. inedit.). Our knowledge about the diversity of non-blue *Haslea* also increased, with *H. sigma* Talgatti, Sar *et* Carvalho Torgan found in salt marshes in Southern Brazil (Talgatti et al. 2014), and seven species more from different collections worldwide (Sterrenburg et al. 2015). To date, the genus *Haslea* encompasses 35 taxa as listed in *AlgaeBase* (Guiry & Guiry 2018) (see http://www.algaebase.org/), but recently, Li et al. (2017) revisited the *Haslea* phylogeny, using SSU rDNA and *rbc*L data, demonstrating that two species must be transferred to the genus *Navicula* Bory, *H. tsukamotoi* Sterrenburg *et* Hinz and *H. avium* Tiffany, Herwig *et* Sterrenburg. Most of *Haslea* species have been encountered in the Northern Hemisphere.

The present study aims to describe a new species of pennate blue diatom from the genus *Haslea*, *H. nusantara* sp. nov., collected from Semak Daun Island, the Seribu Archipelago, in Indonesian marine waters. Morphological, biometry (using SEM), molecular investigations (chloroplast and mitochondrial genomes) were carried out and spectral analysis of the pigment in the culture supernatant was achieved. The specificity of this Indonesian new species was established as compared with other known blue *Haslea* species.

MATERIAL AND METHODS

Diatom sampling, isolation and culture

Specimens of *H. nusantara* were sampled using 50 mL conical centrifuge tubes (Thermo Fisher Scientific, France), on 12 March 2015 by scraping the biofilm covering *Padina* sp. thalli growing in the subtidal zone of Semak Daun Island (5°43'49.27" S, 106°34'20.27" E) in the Seribu Archipelago (“Kepulauan Seribu”), Indonesia (fig. 1). A few days after the sampling, six single cells (IND1 to IND6) were isolated from one tube at Le Mans Université (France), using micropipettes and grown in Petri dishes containing modified artificial seawater (Mouget et al. 2009). The *H. nusantara* cultures were grown in Erlenmeyer flasks in a controlled temperature room at 16 ± 1 °C and an irradiance of 50-100 µmol photons m^{-2} s^{-1}, provided by fluorescent tubes (Philips TLD 36W/965), during a 14:10 h light:dark cycle. Among the six cells initially isolated, only one (IND6) survived and the strain was maintained in culture for several months.

Microscopic observations

The preparation of samples was performed as described in Kaczmarska et al. (2005). Samples were prepared using a Millipore vacuum filtration apparatus with 25 mm diameter and 3 µm pore size polytetrafluoroethylene (PTFE) membrane (General Electric Osmonics, Minnetonka, USA). Samples were washed with distilled water, re-suspended in 5 mL distilled water and cleaned by adding 10 mL of concentrated sulfuric and nitric acid in a water bath at 100 °C for 60 min. Samples were then re-washed using distilled water in the filtration apparatus and re-suspended in 5 mL distilled water. Cleaned samples were then mounted on glass slides using Naphrax and examined using an Olympus BX51 light microscope with a DP72 digital camera, equipped with phase contrast and differential interference contrast (DIC) optics.

For scanning electron microscopic (SEM) examination, cleaned diatom frustules and isolated valves were air-dried onto a circular coverslip, mounted on an aluminum stub prior to coating with platinum or palladium-platinum using a JEOL JUC-5000 sputter coater or JEOL JFC-2300 HR high-resolution fine coater, respectively.

Genome sequencing, annotation and analysis

Total DNA was extracted as described in Doyle & Doyle (1990). An Illumina library of 250 base pairs (bp) DNA inserts was prepared and sequenced on the HiSeq 4000 platform by the Beijing Genomic Institute (Shenzhen). A total of 11 million paired-end reads of 150 bp were assembled using Ray 2.3.0 (Boisvert et al. 2012) and a k-mer value of 45. Contigs of chloroplast and mitochondrial origins were identified by BLAST searches against a local database of organelle genomes and then merged using an overlap-layout consensus approach as implemented in Sequencher 5.4.1 (Gene Codes Corporation, Ann Arbor, USA). Organelle genomes were annotated using a custom set of tools (Turmel et al. 2015) and circular genome maps were drawn using OGDraw (Lohse et al. 2007). Alignments of chloroplast and mitochondrial genomes from diatom taxa belonging to the Naviculales were carried out using the ProgressiveMauve algorithm of Mauve 2.3.1 (Darling et al. 2010)

Phylogenetic trees were built, based on *rbcL* chloroplast gene and *cox1* mitochondrial gene to establish the evolutionary process inside the genus *Haslea*. Our choice focused on *rbcL* (ribulose-bisphosphate carboxylase, large subunit gene) and *cox1* (cytochrome oxidase subunit I) because they are characterized in many *Haslea* species (sequences available in Genbank™). The multiple alignments were carried out using clustalO (https://www.ebi.ac.uk/Tools/msa/clustalo/). The best evolution model was defined using MEGA7 (Kumar et al. 2016). Maximum likelihood (ML) analysis was generated using RaxML (https://embnet.vital-it.ch/raxml-bb, Stamatakis et al. 2008) and MEGA7 with 1000 bootstrap and maximum parsimony (MP) was performed using PAUP (Swofford 1998) and MEGA7 with 1000 bootstrap too.

Pigment characterization with UV-visible spectrophotometry

The marennine-like pigment produced by *H. nusantara* was characterized on cell-free culture supernatant (syringe-filtered on 0.22 µm, Thermo Fisher Scientific) using the Beer-Lambert law. The spectrum was scanned from 200 to 800 nm, in a 5 cm cell using a spectrophotometer (Perkin Elmer, Lambda 25 scan mode). Marennine-like concentration was estimated using the specific extinction coefficient for the extracellular form of marennine (EMn) at the peak wavelength 677 nm ($\varepsilon_{677} = 12.13$ L g^{-1} cm^{-1}), according to Pouvreau et al. (2007b). Spectral characteristics from *H. nusantara* extracellular marennine-like pigment was compared to EMn as control.

RESULTS

Haslea nusantara **Mouget, Gastineau *et* Syakti sp. nov.**

DIAGNOSIS: Cells with two parietal, narrow band-like chloroplasts lying appressed on each side of the valve margin and not reaching the apex. Cell apices characteristically blue

colored. Valves narrow and lanceolate with acute apices. The maximum and minimum length of *H. nusantara* was 91.1 and 54.1 µm, respectively (average, 73.9 ± 1.7 µm, n = 33), while the maximum and minimum width was 8.2 and 5.9 µm (average 6.8 ± 0.1 µm, n = 33). The equation of the fitting curve to describe the relationship between the length to width measurements in *H. nusantara* is:

$$y = 0.023\ x + 5.06,\ R^2 = 0.164,\ n = 33\ (1)\ \text{(Fig 2.)}$$

Axial and central areae are indistinct. Raphe is straight with approximate central endings. Internally, the raphe is bordered on one side by an axial costa, while at center on opposite side, a thin and short central bar can be observed. Striation consists of a transapical pattern (34-37 striae in 10 µm) crossed at right angle by a longitudinal pattern (51-53 striae in 10 µm).

HOLOTYPE **(here designated):** The embedded strain IND6 was acid cleaned and mounted on a glass slide and a SEM stub that have been deposited in the Muséum National d'Histoire Naturelle of Paris, France (PC) under accession numbers PC0576262 and PC0576265, respectively. The cell representative of the type is presented in Figure 3.

*ISOTYPES***:** A slide and a SEM stub with acid cleaned valves of strain IND6 have been deposited at the Bremerhaven Hustedt collection in Bremerhaven, Germany (BRM) under the accession numbers BRM Zu11/18 and BRM R1280, respectively.

*TYPE LOCALITY***:** *Haslea nusantara* was found on a sandy beach in Semak Daun Island, Seribu Archipelago ("Kepulauan Seribu"), Indonesia (5°43'49.27" S, 106°34'20.27" E), associated with *Padina* sp. at depths not exceeding 5 m.

*ETYMOLOGY***:** The species name refers to the Sanskrit word 'nusantara', which is a contemporary Malay term for the Malay Archipelago and Indonesian Archipelago. The word 'nusantara' itself originated from two Sanskrit words, 'nusa' that means 'island', and 'antara' that means 'in between' or 'including' (Evers 2016). Today, Nusantara covers all Indonesian Archipelago or the national territory of Indonesia, where the species *H. nusantara* has been isolated.

Description

Living cells show the typical colored areas associated with blue *Haslea* species, which entirely occupy the space immediately beyond the two parietal chloroplasts lying on both valve margins at both apices (fig. 3). Very few details can be seen regarding the valve features in LM (fig. 3). Our SEM observation showed the full view of the internal valve (fig. 4A). The valve appears as narrow and fusiform or lanceolate with acute apices (fig. 4A): 54.1-91.1 µm in length and 5.9-8.2 µm in width. The ornamentation is hardly seen while a median line holding the raphe system runs from one apex to the other (fig. 4A). In SEM, the external valve surface shows distinct linear, fissure-like openings that run continuously throughout the entire length of the valve from one apex to the other (figs. 4A, B & D). Each of these longitudinal fissures merge with a peripheral stria at the valve margin, which both fuse together at the apex beyond the terminal area (fig. 4D). Most of morphological features

previously observed on the valve of two other species of blue *Haslea* (Gastineau et al. 2012b, 2016) are present in *H. nusantara*, *i.e*., lanceolate valves, acute apices and rectangular frustules in girdle view. The true nature of the striation can be best appreciated internally. It consists of a criss-cross pattern with slightly rectangular areolae forming transapical rows that are crossed at right angle by longitudinal rows. There are 34-37 transapical striae in 10 µm (36.0 ± 1.0 in 10 µm, n = 36) and 51-53 longitudinal striae in 10 µm (52.0 ± 0.2 in 10 µm, n = 33). The internal raphe system is composed of two straight branches, which terminate at center in approximate co-axial endings and at the apex in slightly elongated helictoglossa (fig. 4E). There is a narrow and raised up axial costa on one side of the raphe system running almost continuously but stopping shortly before reaching the apices (fig. 4C). In addition, a short (*ca*. 2 µm long) and narrow central bar is present opposite to the axial costa, which is reported for the first time in a blue *Haslea* (fig. 4C).

The comparative analysis of morphological characteristics between *H. nusantara* and other species of the genus *Haslea* described elsewhere (*H. pseudostrearia* Massé, Rincé *et* E.J. Cox and *H. crucigera* [W. Smith] Simonsen) is presented in Table 3. It shows that, biometrically and morphologically, *H. nusantara* resembles *H. ostrearia*, for instance regarding the mean length and width of the cell, as well as the number of striae in both transapical and longitudinal axis.

Marennine-like pigment

Measurements using UV-visible spectrophotometry revealed that the extracellular form of *H. nusantara* blue pigment demonstrated spectral characteristics similar to marennine produced by *H. ostrearia*, and to marennine-like pigments produced by other blue *Haslea* species, with two peaks, one in the UV and the other in the red part of the spectrum. The maximum spectral absorbance for *H. nusantara* and *H. ostrearia* at pH 7 in the visible part of the spectra was observed at 663 and 666 nm, respectively (fig. 5). It is very similar to the extracellular form of marennine (EMn) produced by *H. ostrearia*.

Structure and organization of organelle genomes

We have sequenced both the mitochondrial and chloroplast genomes of *H. nusantara* and compared them with those of previously examined diatoms. The *H. nusantara* mitochondrial genome was assembled as a circular DNA molecule of 36,288 bp that encodes 36 proteins, 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs) (Table 1 & fig. 6). The accession number of this mitogenome is MH681882. It is among the smallest diatom mitochondrial genomes that have been completely sequenced to date but is in the same size range as other naviculalean genomes (Pogoda et al. 2018). At 29.24 %, its G+C content is similar to those of several other diatom mitochondrial genomes (Pogoda et al. 2018). Comparative analyses of gene order using MAUVE revealed that the *H. nusantara* and *Berkeleya fennica* mitochondrial genomes are colinear, except for a single sequence inversion (fig. 7). In contrast, the gene arrangement of *H. nusantara* is highly scrambled with respect to those of all other naviculaleans.

The 120,448-bp chloroplast genome (accession number: MH681881) of *H. nusantara* features the typical quadripartite architecture observed in many algae (Turmel & Lemieux 2018, Yu et al. 2018); two copies of a large inverted repeat (IR) sequence of 7,241 bp divide

the *H. nusantara* genome into a large (63,119 bp) and a small (42,847 bp) single-copy regions (fig. 8). The circular chloroplast genome encodes 132 proteins, 27 tRNAs and three rRNAs, with the IR including the *ycf89*, *psbY* and *trnP*(ugg) genes in addition to the rRNA operon (fig. 8). Currently available diatom chloroplast genomes all feature the quadripartite architecture (Yu et al. 2018) and tend to be more conserved in size than their mitochondrial counterparts (Tables 1 & 2). MAUVE alignments of the chloroplast genomes of *H. nusantara* and two other naviculaleans showed that the gene order of *H. nusantara* more closely resembles that of *Phaeodactylum tricornutum* Bohlin than that of *Fistulifera solaris* Mayama, Matsumoto, Nemoto *et* Tanaka (fig. 9).

Phylogeny analysis

A phylogeny analysis was carried out to establish the relationship between *H. nusantara* with other *Haslea* species. The best model selected by MEGA7 to *rbcL* fragment (1336 bp) was GTR+G+I (BIC: 7055.021, AIC: 6751.877, lnL: -3336.850, +G parameter: 0.5359, +I: 60.2 % evolutionarily invariable sites). The base frequencies calculated were respectively: f(A)= 0.296, f(T)= 0.321, f(C)=0.177 and f(G)=0.206. The phylogenies (ML/MP), generated using RaxML (ML), PAUP (MP) and MEGA7 (ML/MP) (to compare the typologies generated) showed that *H. nusantara* is included in the blue-pigmented *Haslea* species clade (robust node: 99/92), and it is close to *H. silbo* sp. inedit. (node: 60/73) (fig. 10).

On the other hand, phylogenetic analysis (ML, MP) carried out using RaxML, PAUP and MEGA7 (to compare the results) on mitochondrial *cox1* fragment (696 bp), was based on best model HKY+G (BIC: 6089.902, AIC: 5942.063, lnL: -2948.949, +G parameter: 0.3229). The base frequencies were f(A)= 0.252, f(T)= 0.411, f(C)=0.162 and f(G)=0.176, respectively. The typologies obtained were similar whatever the software used, showing a minor resolution for relationship between the blue *Haslea*, when compared to *rbcL* analysis. *Haslea nusantara* seems genetically closer to *H. ostrearia,* and only another clade including *H. provincialis* and *H. silbo* sp. inedit. appeared (node: 98/100) (fig. 11).

DISCUSSION

Considered as one of the largest archipelago in the tropical region, Indonesia is one of the marine biodiversity hotspots (Roberts et al. 2006). Although some works describe Indonesian diatom flora, most of them only concentrated on freshwater species (Bramburger et al. 2008, 2017; Bramburger & Hamilton 2014) and only a few on marine environment (Sterrenburg et al. 1995; Hendrarto & Nitisuparjo 2011). The diversity of marine diatoms from Indonesia thus remains underexplored. Furthermore, up to now, most of the blue *Haslea* species have been mainly discovered in the Northern Hemisphere, with the exception of the type species *H. ostrearia*, with specimens observed in the Indian Ocean (Simonsen 1974), but for there are neither SEM pictures nor genetic data, and populations of *H. ostrearia* living in the Australian waters (Gastineau et al. 2014). The discovery of a blue *Haslea* species in the Java Sea is thus a novelty.

Characteristics of the new taxon Haslea nusantara

Cells of *H. nusantara* were collected in biofilms associated to *Padina* thalli, and when in culture, they formed aggregates and biofilm on the bottom of the flasks. In light microscopy, as for other blue *Haslea*, *H. nusantara* cells appeared lanceolate and free living, with apices filled with a typical blue pigment. The blue color in *H. nusantara* was similar to those of *H. ostrearia* and *H. provincialis* but different from that of *H. karadagensis*, which is more blue-greyish (Gastineau et al. 2012b). The UV-visible spectrophotometry revealed that at pH 7, the extracellular form of the pigment produced by *H. nusantara* displays a maximum absorbance in the red region with little difference when compared to marennine (663 *vs* 666 nm, respectively). The peak of maximum absorbance in the red region measured in this study for marennine is comparable to those observed in previous works (Pouvreau et al. 2006a, 2006b, 2007b). The spectra being pH-dependent, in absence of more material, however, it cannot be assessed if the differences between pigments of both *Haslea* species are significant. Furthermore, the *H. nusantara* strain having been lost before running further experiments, we were not able to characterize its intracellular form. Therefore, we could not compare this measurement with the intracellular form of marennine (IMn) from *H. ostrearia*.

Observation of cleaned frustule using light microscope revealed that the striation in *H. nusantara* is almost invisible, confirming that this method is of limited use for *Haslea* species identification. In SEM, however, *H. nusantara* is biometrically and morphologically similar to the previously described blue *Haslea* species, for instance *H. ostrearia*, *H. karadagensis* and *H. provincialis,* with lanceolate valves, acute apices in valve view and rectangular frustules in girdle view (Gastineau et al. 2012b, 2016). If the fitting equation for the length to width ratio in *H. nusantara* cells differs from the ones in *H. ostrearia*, *H. karadagensis* and *H. provincialis* (Table 3), the stria density is very similar for both *H. nusantara* and *H. ostrearia*. From these results, it can be inferred that biometrically *H. nusantara* is closely related to *H. ostrearia*, however, using molecular markers, there is no doubt that *H. nusantara* is a new species of blue *Haslea*. Indeed, according to the genetic markers sequenced and the phylogenetic trees, *H. nusantara* is included in the blue-pigmented *Haslea* spp. cluster, and this new species seems distinct from *H. ostrearia* but close to *H. silbo* sp. inedit.

***The Indonesian blue diatom inside the genus* Haslea**

A global consideration of the 35 species of *Haslea* identified so far allows distinguishing different features regarding their morphology, ecology and physiology. For instance, *H. gigantea* Simonsen has been identified as a mesoplanktonic species with a cell length that may reach 500 µm. In contrast to most *Haslea* species that are free benthic organisms, *H. crucigera* (W. Smith) Simonsen is known to be a tube-dwelling species, and *H. wawrikae* (Hustedt) Simonsen is planktonic. The diversity in the frustule morphology can also be viewed within *Haslea* species. For example a pseudostauros (thickening of the transapical central virgae into thick ribs) is present in *H. crucigera*, *H. crucigeroides* (Hustedt) Simonsen and var. *densestriata* Cardinal, Poulin *et* Bérard-Therriault, *H. salstonica* Massé, Rincé *et* E.J. Cox and *H. spicula* (Hickie) Bukhtiyarova (Cardinal et al. 1984; Massé et al. 2001). A molecular phylogeny approach using 16S rDNA in several species suggested the division of the genus *Haslea* into sub-genera, discriminating between taxa with a pseudostauros (*H. crucigera* and *H. salstonica*) on the one hand, the sigmoid *H. nipkowii* (Meister) Poulin *et* Massé (Poulin et al. 2004), the non-blue *H. pseudostrearia* and the blue *H. ostrearia* that do not have a pseudostauros on the other hand, and a third group composed of the planktonic *H. wawrikae* (Hustedt) Simonsen (1974) (Pillet et al. 2011). From the present work, it can be assessed that the new species *H. nusantara* refers to benthic pennate diatoms, with a marennine-like pigment accumulating at cell apices, and a frustule devoid of a pseudostauros but with a short and narrow central bar at the valve center.

Last, the new species *H. nusantara* has been discovered in a geographical region different from the previous ones (*H. karadagensis, H. provincialis* and *H. ostrearia*), which all were from the Northern Hemisphere. This suggests that the diversity of this peculiar taxon is undoubtedly underestimated, and that there are probably more species of blue *Haslea* to discover

ACKNOWLEDGMENTS

This publication benefited from funding from the International research collaboration-scientific publication (Indonesia – France) 'Sustainable valorization of Indonesian phytoplankton in aquaculture: new approaches to control infectious disease' (SALINA, 2015-2017), the Partenariat Hubert Curien (PHC) Nusantara 'Valorisation of algae for sustainable aquaculture and bioactive compounds' (Vasabi) (2016-2017), and also from the Horizon 2020 Research and Innovation Programme GHaNA (The Genus *Haslea*, New marine resources for blue biotechnology and Aquaculture) under Grant Agreement No 734708/GHANA/H2020-MSCA-RISE-2016 (JLM).

REFERENCES

AN S.M., NOH J.H., CHOI D.H., LEE J.H. & YANG E.C., 2016 – Repeat region absent in mitochondrial genome of tube-dwelling diatom *Berkeleya fennica* (Naviculales, Bacillariophyceae). *Mitochondrial DNA Part A* 27: 2137-2138.

AN S.M., KIM S.Y., NOH J.H. & YANG E.C., 2017 – Complete mitochondrial genome of *Skeletonema marinoi* (Mediophyceae, Bacillariophyta), a clonal chain forming diatom in the west coast of Korea. *Mitochondrial DNA Part A* 28: 19-20.
BOISVERT S., RAYMOND F., GODZARIDIS É., LAVIOLETTE F. & CORBEIL J., 2012 – Ray Meta: scalable *de novo* metagenome assembly and profiling. *Genome Biology* 13, R122: 1-13.
BRAMBURGER A. & HAMILTON P.B., 2014 – Effects of a simulated upwelling event on the littoral epilithic diatom community of an ancient tropical lake (Lake Matano, Sulawesi Island, Indonesia). *Hydrobiologia* 739: 133-143.
BRAMBURGER A.J., HAMILTON P.B., HEHANUSSA P.E. & HAFFNER G.D., 2008 – Processes regulating the community composition and relative abundance of taxa in the diatom communities of the Malili Lakes, Sulawesi Island, Indonesia. *Hydrobiologia* 615: 215-224.
CAO M., YUAN X-L. & BI G., 2016 – Complete sequence and analysis of plastid genomes of *Pseudo-nitzschia multiseries* (Bacillariophyta). *Mitochondrial DNA Part A* 27: 2897-2898.
CARDINAL A., POULIN M. & BÉRARD-THERRIAULT L., 1984 – Les diatomées benthiques de substrats durs des eaux marines et saumâtres du Québec. 4. Naviculales, Naviculaceae (à l'exclusion des genres *Navicula*, *Donkinia*, *Gyrosigma* et *Pleurosigma*). *Naturaliste canadien* 111: 369-394.
DARLING A.E., MAU B. & PERNA N. T., 2010 – ProgressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One *5*(6): e11147.
DOYLE J.J. & DOYLE J.L., 1990 – Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.
EVERS H., 2016 - Nusantara: history of a concept. *Journal of Malaysian Branch of the Royal Asiatic Society* 89: 3-14.
FALKOWSKI P.G., KATZ M.E., KNOLL A.H., QUIGG A., RAVEN J.A., SCHOFIELD O. & TAYLOR F.J.R., 2004 – The evolution of modern eukaryotic phytoplankton. *Science* 305: 354-360.
GAGNON J., 2004 – Création d'outils pour l'automatisation d'analyses phylogénétiques de génomes d'organites. M. Sc. Thesis, Université Laval, Quebec City, 211 p.
GAILLON B., 1820 – Des huîtres vertes, et des causes de cette coloration. *Journal de Physique, de Chimie, d'Histoire Naturelle et des Arts* 91: 222-225.
GALACHYANTS Y.P., MOROZOV A.A., MARDANOV A.V., BELETSKY A.V., RAVIN N.V., PETROVA D.P. & LIKHOSHWAY Y.V., 2012 – Complete chloroplast genome sequence of freshwater araphid pennate diatom alga *Synedra acus* from Lake Baikal. *International Journal of Biology* 4: 27-35.
GASTINEAU R., 2011 – Biodiversité, reproduction et phylogénie des diatomées bleues du genre *Haslea* et valorisation de leurs pigments de type marennine. Ph.D. Thesis, Université du Maine, Le Mans, 328 p.
GASTINEAU R., POUVREAU J.B., HELLIO C., MORANÇAIS M., FLEURENCE J., GAUDI, P., BOURGOUGNON N. & MOUGET J.-L., 2012a – Biological activities of purified marennine, the blue pigment responsible for the greening of oysters. *Journal of Agricultural and Food Chemistry* 60: 3599-3605.
GASTINEAU R., DAVIDOVICH N.A., BARDEAU J-F., CARUSO A., LEIGNEL V., HARDIVILLIER Y., JACQUETTE B., DAVIDOVICH O. I., RINCÉ Y., GAUDIN P., COX E.J. & MOUGET J-L., 2012b – *Haslea karadagensis* (Bacillariophyta): a second blue diatom, recorded from the Black Sea and producing a novel blue pigment. *European Journal of Phycology* 47: 469-479.
GASTINEAU R., TURCOTTE F., POUVREAU J.B., MORANÇAIS M., FLEURENCE J., WINDARTO E., PRASETYIA F.S., ARSAD S., JAOUEN P., BABIN M., COIFFARD L., COUTEAU C., BARDEAU J-F., JACQUETTE B., LEIGNEL V., HARDIVILLIER Y., MARCOTTE I., BOURGOUGNON N., TREMBLAY R., DESCHÊNES J-S., BADAWY H., PASETTO P., DAVIDOVICH N., HANSEN G., DITTMER J. & MOUGET J-L., 2014a – Marennine, promising blue pigments from a widespread *Haslea* diatom species complex. *Marine Drugs* 12: 3161-3189.
GASTINEAU R., HARDIVILLIER Y., LEIGNEL V., TEKAYA N., MORANÇAIS M., FLEURENCE J., DAVIDOVICH N., JACQUETTE B., GAUDIN P., HELLIO C., BOURGOUGNON N. & MOUGET J-L., 2014b – Greening effect on oysters and biological activities of the bleu pigments produced by the diatom *Haslea karadagensis* (Naviculaceae). *Aquaculture* 368/369: 61-67.
GASTINEAU R., HANSEN G., DAVIDOVICH N.A., DAVIDOVICH O., BARDEAU J-F., KACZMARSKA I., EHRMAN J.M., LEIGNEL V., HARDIVILLIER Y., JACQUETTE B., POULIN M., MORANÇAIS M., FLEURENCE J., MOUGET J-L. & 2016 – A new blue-pigmented hasleoid diatom, *Haslea provincialis*, from the Mediterranean Sea. *European Journal of Phycology* 51: 156-170.
GUIRY M.D. & GUIRY G.M., 2018 – AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org.
HENDRARTO B. & NITISUPARJO M., 2011 – Biodiversity of benthic diatom and primary productivity of benthic micro-flora in mangrove forest on central Java. *Journal of Coastal Development* 14: 131-140.
KACZMARSKA I., LEGRESLEY M.M., MARTIN J.L. & EHRMAN J., 2005 – Diversity of the diatom genus *Pseudo-nitzschia* Peragallo in the Quoddy region of the Bay of Fundy, Canada. *Harmful Algae* 4: 1-19.

KUMAR S., STECHER G. & TAMURA K., 2016 – MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870-1874.
LANKESTER E.R., 1886 - On green oysters. *Quarterly Journal of Microscopical Science* 26: 1-94.
LI Y., CHEN X., SUN Z. & XU K., 2017 – Taxonomy and molecular phylogeny of three marine benthic species of *Haslea* (Bacillariophyceae), with transfer of two species to *Navicula*. *Diatom Research* 451-463 pp. DOI: 10.1080/0269249X.2017.1401008.
LOHSE M., DRECHSEL O. & BOCK R., 2007 – OrganellarGenomeDRAW (OGDRAW): a tool for the easy generation of high-quality custom graphical maps of plastid and mitochondrial genomes. *Current Genetics* 52: 267-274.
MASSÉ G., RINCÉ Y., COX E.J., ALLARD G., BELT S.T. & ROWLAND S.J., 2001 – *Haslea salstonica* sp. nov. and *Haslea pseudostrearia* sp. nov. (Bacillariophyta), two new epibenthic diatoms from the Kingsbridge Estuary, United Kingdom. *Sciences de la vie/Life Sciences* 324: 617-626.
MOUGET J-L., GASTINEAU R., DAVIDOVICH O., GAUDIN P. & DAVIDOVICH N.A., 2009 – Light is a key factor in triggering sexual reproduction in the pennate diatom *Haslea ostrearia*. *FEMS Microbiology and Ecology* 69: 194-201.
OUDOT-LE SECQ M.P., GRIMWOOD J., SHAPIRO H., ARMBRUST E.V., BOWLER C. & GREEN B.R., 2007 – Chloroplast genomes of the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*: comparison with other plastid genomes of the red lineage. *Molecular Genetics and Genomics* 277: 427-439.
OUDOT-LE SECQ M.P. & GREEN B.R., 2011 – Complex repeat structures and novel features in the mitochondrial genomes of the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*. *Gene* 476: 20-26.
PALINSKA K.A. & SUROSZ W., 2018 – A bleu-pigmented hasleoid diatom, *Haslea* sp., from the Adriatic Sea. *Oceanological and Hydrobiological Studies* 47: 60-66.
PILLET L., VARGAS C. DE, PAWLOWSKI J., 2011 – Molecular Identification of Sequestered Diatom Chloroplasts and Kleptoplastidy in Foraminifera. *Protist* 162: 394-404.
POGODA C.S., KEEPERS K.G., HAMSHER S.E., STEPANEK J.G., KANE N.C. & KOCIOLEK J.P., 2018 – Comparative analysis of the mitochondrial genomes of six newly sequenced diatoms reveals group II introns in the barcoding region of *cox1*. *Mitochondrial DNA Part A* 11: 1-9.
POULIN M., MASSÉ G., BELT S.T., DELAVAULT P., ROUSSEAU F., ROBERT J-M. & ROWLAND S.J., – Morphological, biochemical and molecular evidence for the transfer of *Gyrosigma nipkowii* Meister tot he genus *Haslea* (Bacillariophyta). *European Journal of Phycology* 39: 181-195.
POUVREAU J.B., MORANÇAIS M., MASSÉ G., ROSA P., ROBERT J-M., FLEURENCE J. & PONDAVEN P., 2006a – Purification of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. *Journal of Applied Phycology* 18: 769-781.
POUVREAU J.B., MORANÇAIS M., FLEURY F., ROSA P., THION L., CAHINGT B., ZAL F., FLEURENCE J. & PONDAVEN P., 2006b – Preliminary characterization of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. *Journal of Applied Phycology* 18: 757-767.
POUVREAU J.B., HOUSSON E., LE TALLEC L., MORANÇAIS M., RINCÉ Y., FLEURENCE J. & PONDAVEN P., 2007a – Growth inhibition of several marine diatom species induced by the shading effect and allelopathic activity of marennine, a blue-green polyphenolic pigment of the diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. *Journal of Experimental Marine Biology and Ecology* 352: 212-225.
POUVREAU J.B., MORANÇAIS M., FLEURENCE J. & PONDAVEN P., 2007b – Method for the quantification of the blue-green pigment "marennine" synthesized by the marine diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen using HPLC gel-filtration and photodiode-array detection. *Journal of Applied Phycology* 19: 263-270.
POUVREAU J.B., MORANÇAIS M., TARAN F., ROSA P., DUFOSSÉ L., GUÉRARD F., PIN S., FLEURENCE J. & PONDAVEN P., 2008 – Antioxidant and free radical scavenging properties of marennine, a blue-green polyphenolic pigment from the diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen responsible for the natural greening of cultured oysters. *Journal of Agricultural and Food Chemistry* 56: 6278-6286.
PRASETIYA F.S., SAFITRI I., WIDOWATI I., COGNIE B., DECOTTIGNIES P., GASTINEAU R., MORANÇAIS M., WINDARTO E., TREMBLAY R. & MOUGET J-L., 2016 – Does allelopathy affect co-culturing *Haslea ostrearia* with other microalgae relevant to aquaculture? *Journal of Applied Phycology* 28: 2241-2254.
RAVIN N.V., GALAHYANTS Y.P., MARDANOV A.V., BELETSKY A.V., PETROVA D.P., SHERBAKOVA T.A., ZAKHAROVA Y.R., LIKHOSHWAY Y.V., SKYABIN K.G. & GRACHEV M.A., 2010 – Complete sequence of the mitochondrial genome of a diatom alga *Synedra acus* and comparative analysis of diatom mitochondrial genomes. *Current Genetics* 56: 215-223.

ROBERTS C.M., MITTERMEIER C.G. & SCHUELER F.W., 2006 – Marine biodiversity hotspots and conservation priorities for tropical reefs. *Science* 295: 1280-1284.
RUCK E.C., NAKOV T., JANSEN R.K., THERIOT E.C. & ALVERSON A.J., 2014 – Serial gene losses and foreign DNA underlie size and sequence variation in the plastid genomes of diatoms. *Genome Biology and Evolution* 6: 644-654.
SIMONSEN R., 1974 – The diatom plankton of the India Ocean Expedition of RV Meteor 1964–1965. *'Meteor' Forschungsergebnisse Rehe D* 19: 1-107.
SPRAT T., 1669 – The history of the generation and ordering of green oysters, commonly called Colchester oysters. History of Royal Society of London. 307–319. IN The history of the Royal-Society of London for the improving of natural knowledge by Tho. Sprat. http://name.umdl.umich.edu/A61158.0001.001.
STAMATAKIS A., HOOVER P. & ROUGEMONT J., 2008 – A rapid bootstrap algorithm for the RAxML web-servers. *Systematic Biology* 75: 758-771.
STERRENBURG F.A.S., ERFTEMEIJER P.L.A. & NIENHUIS P.H., 1995 – Diatoms as epiphytes on seagrasses in South Sulawesi (Indonesia) comparison with growth on inert substrata. *Botanica Marina* 38: 1-7.
SWOFFORD D.L., 1998 – PAUP. Phylogenetic analysis using parsimony (and other methods). Version 4. Sinauer Associates. Smithsonian Institution, Sunderland, Massachusetts.
TALGATTI D., SAR E.A. & TORGAN L.C., 2014 – *Haslea sigma* (Naviculaceae, Bacillariophyta) a new sigmoid benthic species from salt marshes of Southern Brazil. *Phytotaxa* 177: 231-238.
TANAKA T., FUKUDA Y., YOSHINO T., MAEDA Y., MUTO M., MATSUMOTO M., MAYAMA S. & MATSUNAGA T., 2011 – High-throughput pyrosequencing of the chloroplast genome of a high neutral-lipid-producing marine pennate diatom, *Fistulifera* sp. strain JPCC DA0580. *Photosynthesis Research* 109: 223-229.
TANG X. & BI G., 2016 – Complete mitochondrial genome of *Fistulifera solaris* (Bacillariophycidae). *Mitochondrial DNA Part A* 27: 4405-4406.
TURMEL M. & LEMIEUX C., 2018 –Evolution of the plastid genome in green algae. *Advances in Botanical Research* 85: 157-193.
TURMEL M., OTIS C. & LEMIEUX C., 2015 – Dynamic evolution of the chloroplast genome in the green algal classes Pedinophyceae and Trebouxiophyceae. *Genome Biololgy and Evolution* 7: 2062-2082.
VILLAIN A., KOJADINOVIC M., PUPPO C., PRIORETTI L., HUBERT P., ZHANG Y., GRÉGORI G., ROULET A., ROQUES C., CLAVERIE J-M., GONTERO B. & BLANC G., 2017 – Complete mitochondrial genome sequence of the freshwater diatom *Asterionella formosa*. *Mitochondrial DNA Part B* 2: 97-98.
YUAN X-L., CAO M. & BI G-Q., 2016 – The complete mitochondrial genome of *Pseudo-nitzschia multiseries* (Bacillariophyta). *Mitochondrial DNA Part A* 27: 2777-2778.
YU M., ASHWORTH M.P., HAJRAH N.H., KHIYAMI M.A., SABIR M.J., ALHEBSHI A.M. & JANSEN R.K., 2018 –Evolution of the plastid genomes in diatoms. *Advances in Botanical Research* 85: 129-155.

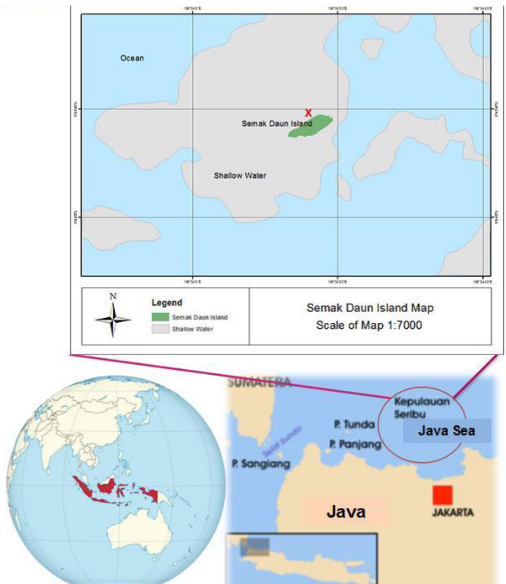


Figure 1 – Sampling location of *Haslea nusantara* sp. nov. near Semak Daun Island (5°43' 49.27" S, 106°34'20.27" E), The Thousand Islands ("Kepulauan Seribu"), Indonesia, during spring 2015. Sampling point is indicated with the cross (x) sign.

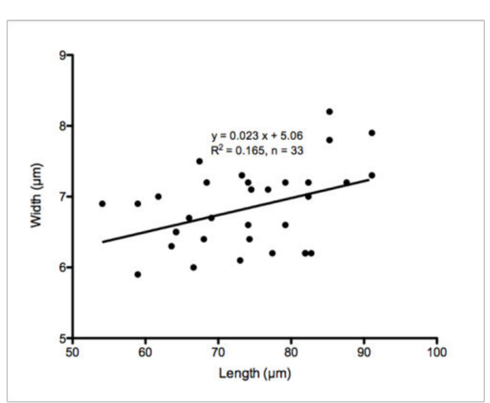


Figure 2 – Distribution of lengths and widths in *Haslea nusantara* from a culture maintained in the laboratory (n = 33). Relationship between mean values of width and length was measured and the obtained equation of the fitting line was: $y = 0.023\,x + 5.06$ (n = 33, $R^2 = 0.165$).

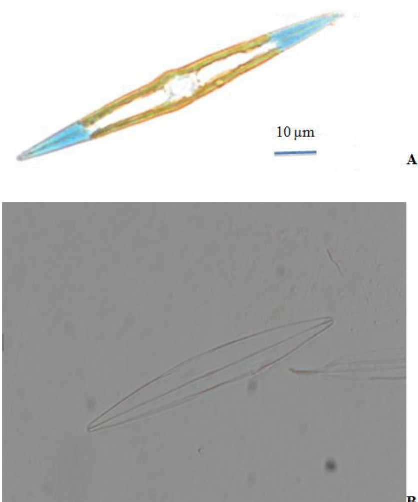


Figure 3 – A, Living cell of *Haslea nusantara* with two parietal chloroplasts and apices filled with blue pigment in light microscopy; Cleaned frustule in the Differential Interference Contrast (DIC) mode. Scale bars A & B: 10 & 5 µm, respectively.

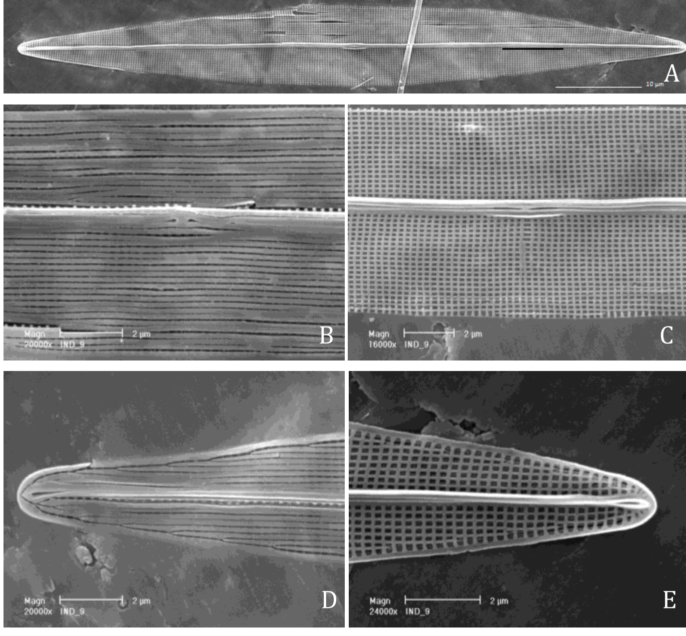


Figure 4 – Holotype material of *Haslea nusantara* from Kepulauan Seribu in scanning electron microscopy: A, Whole valve in internal view; B, External view of the valve center showing approximate raphe endings and continuous longitudinal fissures; C, Internal view of the center with criss-cross stria pattern, axial costa on one side of the raphe and central bar in opposite side; D, External view of apex showing the merging of the two peripheral striae beyond the terminal area; E, Internal view of apex with the helictoglossa.

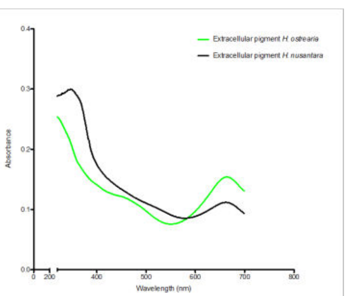


Figure 5 – Absorbance spectrum of *Haslea nusantara* extracellular pigment from 250 to 800 nm at pH 7, compared to that of *H. ostrearia*.

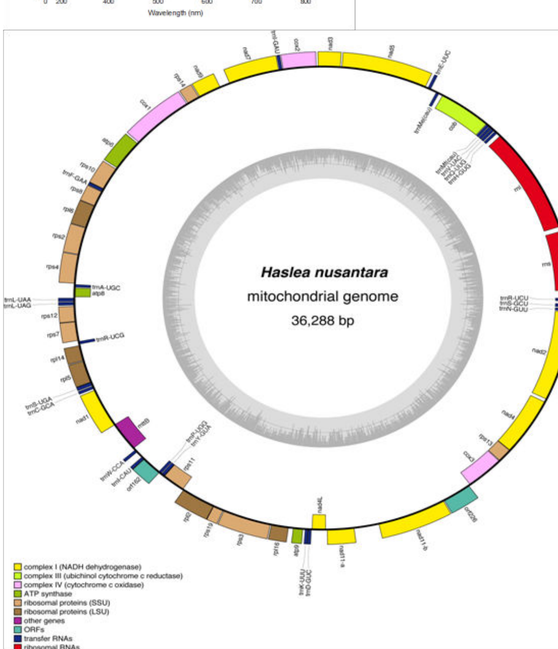


Figure 6 – Gene map of the *Haslea nusantara* mitochondrial genome. Filled boxes represent genes, with colors denoting gene categories as indicated in the bottom left legend. Locally collinear blocks of genome sequences are represented by boxes of identical color and similarly colored blocks are connected by lines. Blocks lying above the center line of the aligned regions are in the same orientation as in the reference (first) genome sequence, while those below this line are in the reverse orientation. Locally collinear blocks of genome sequences are represented by boxes of identical color and similarly colored blocks are connected by lines. Blocks lying above the center line of the aligned regions are in the same orientation as in the reference (first) genome sequence, while those below this line are in the reverse orientation. Genes on the outside of the map are transcribed counterclockwise; those on the inside are transcribed clockwise. The inner ring shows variations in G+C content with the circle inside the G+C content graph marking the 50% threshold (dark gray, G+C; light gray, A+T). bp: base pair.

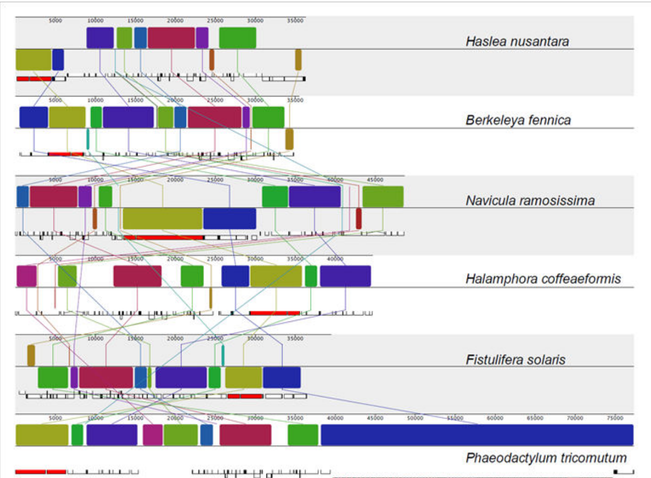


Figure 7 – Extent of mitochondrial genome rearrangements in naviculalean diatoms. The genome alignments were carried out using the Progressive Mauve algorithm of Mauve 2.3.1 (Darling *et al*., 2010).

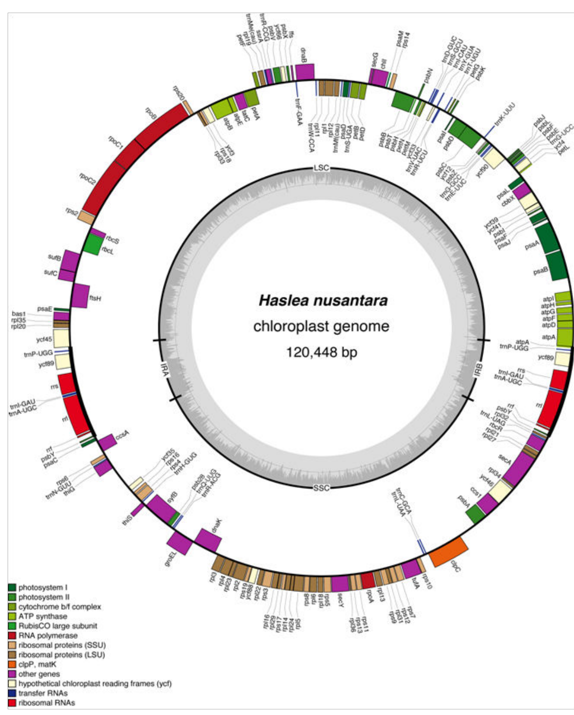


Figure 8 – Gene map of the *Haslea nusantara* chloroplast genome. Filled boxes represent genes, with colors denoting gene categories as indicated in the bottom left legend. Locally collinear blocks of genome sequences are represented by boxes of identical color and similarly colored blocks are connected by lines. Blocks lying above the center line of the aligned regions are in the same orientation as in the reference (first) genome sequence, while those below this line are in the reverse orientation. Genes on the outside of the map are transcribed counterclockwise; those on the inside are transcribed clockwise. The inner ring shows variations in G+C content and the positions of the IR and single-copy regions (SSC and LSC). The circle inside the G+C content graph marks the 50% threshold (dark gray, G+C; light gray, A+T). bp: base pair

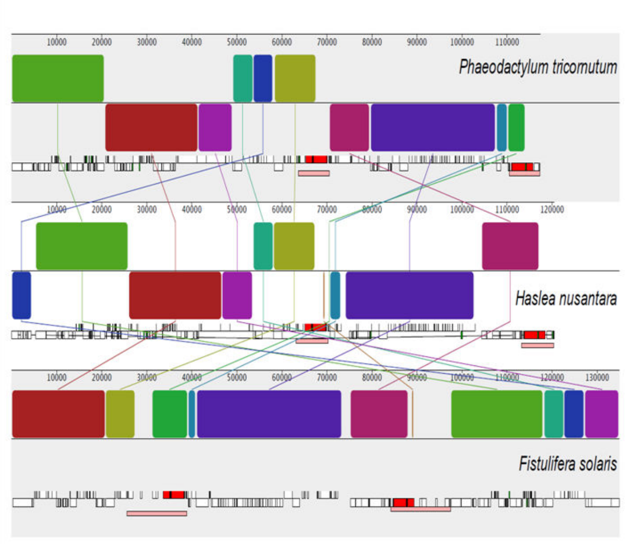


Figure 9 – Extent of chloroplast genome rearrangements in three naviculalean diatoms. The genome alignments were carried out using the Progressive Mauve algorithm of Mauve 2.3.1 (Darling *et al*., 2010).

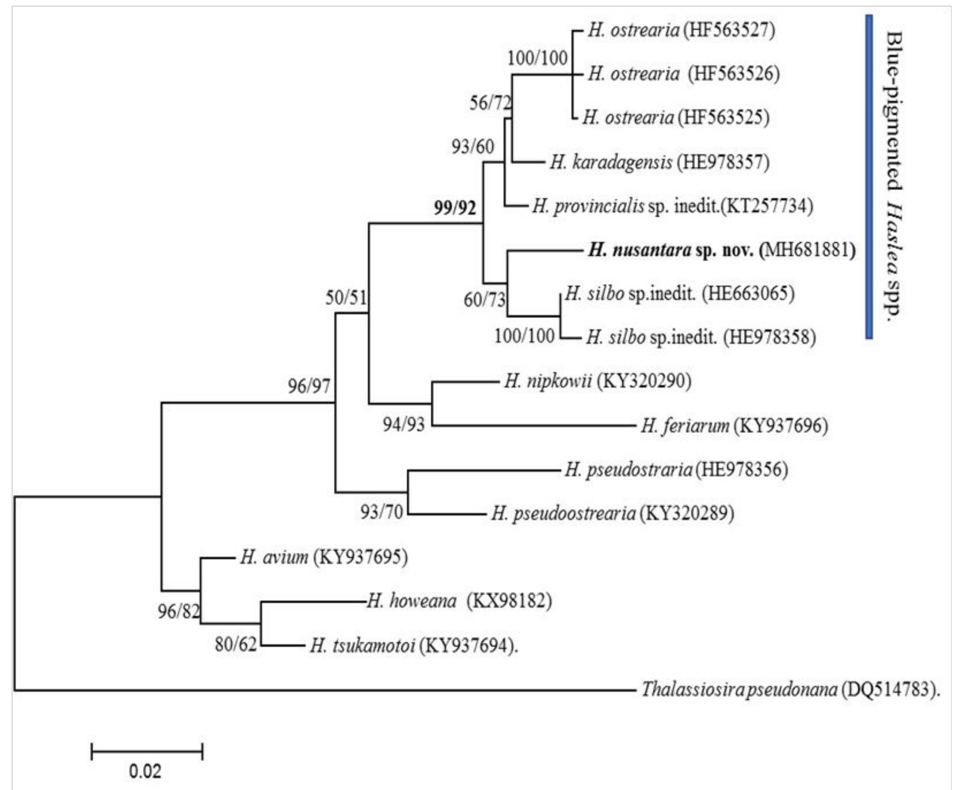


Figure 10 – Maximum likelihood (ML) phylogenetic tree based on *rbcL* (chloroplast gene) from 11 *Haslea* species. The bootstrap values of ML (above branch) and maximum parsimony (below branch) consisted of 1000 replicates.

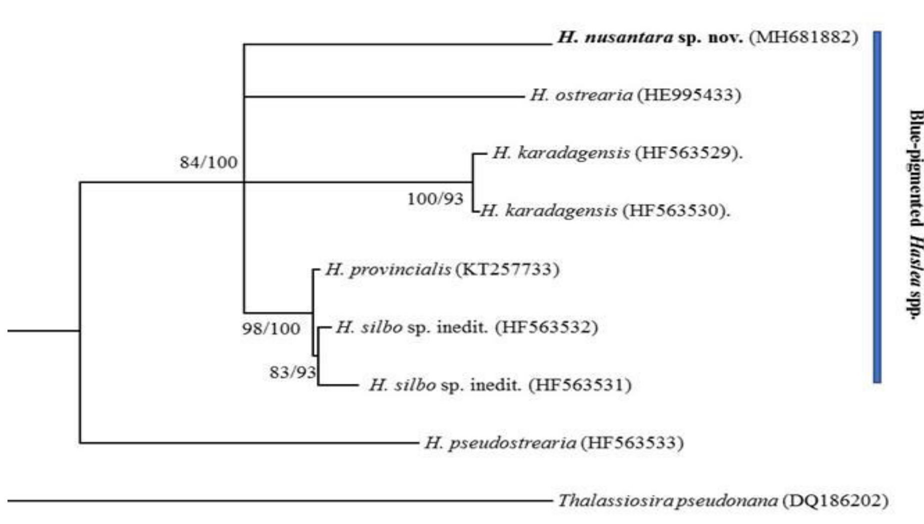


Figure 11 – Maximum likelihood (ML) phylogenetic tree based on *cox1* (mitochondrial gene) from six *Haslea* species. The bootstrap values of ML (above branch) and maximum parsimony (below branch) consisted of 1000 replicates.

Table 1 -General features of diatom mitochondrial genomes.

Order	Species	Accession number	Length of base pairs	GC content (%)	Total genes	Protein-coding genes	tRNA gene	rRNA gene	Reference
Fragilariales	*Asterionella formosa*	KY021079	61,877	26.62	62	35	25	2	Villain et al. 2017
Fragilariales	*Ulnaria acus*	GU002153	46,657	31.77	59	33	24	2	Ravin et al. 2010
Thalassiosirales	*Skeletonema marinoi*	KT874463	38,515	29.73	62	35	25	2	An et al. 2015
Thalassiosirales	*Thalassiosira pseudonana*	NC_007405	43,827	30.11	64	35	27	2	Oudot-Le Secq & Green 2011
Bacillariales	*Pseudo-nitzschia*	KR149143	46,283	31.04	62	37	23	2	Yuan et al. 2016
Naviculales	*Fistulifera solaris*	NC_027978	39,476	28.13	63	33	25	2	Tang & Bi 2015
Naviculales	*Halamphora coffeaeformis*	NC_037727	44,653	32.91	44	38	22	2	Pogoda et al. 2018
Naviculales	*Berkeleya fennica*	KM886611	35,509	29.72	63	36	25	2	An et al. 2014
Naviculales	*Phaeodactylum tricornutum*	NC_016739	77,356	35.01	61	34	24	2	Oudot-Le Secq & Green 2011
Naviculales	***Haslea nusantara***	MH681882	36,288	29.24	61	36	22	2	This study

Table 2 -General features of diatom chloroplast genomes.

Order	Species	Accession number	Length of base pairs	GC content (%)	Inverse Region (IR)	Protein-coding genes	tRNA gene	rRNA gene	Reference
Fragilariales	*Asterionella formosa*	KC509519	121,238	30.67	Yes	132	31	4	Ruck et al. 2014
Fragilariales	*Ulnaria acus*	JQ088178	116,251	30.56	Yes	129	30	4	Galachyants et al. 2012
Thalassiosirales	*Thalassiosira pseudonana*	EF067921	128,814	30.66	Yes	127	27	4	Oudot-Le Secq et al. 2007
Bacillariales	*Pseudo-nitzschia*	KR709240	111,539	31.37	Yes	96	27	3	Cao et al. 2016
Naviculales	*Fistulifera* sp.	AP011960	134,918	32.20	Yes	132	27	3	Tanaka et al. 2011
Naviculales	*Phaeodactylum tricornutum*	EF067920	117,369	32.56	Yes	130	27	3	Oudot-Le Secq et al. 2007
Naviculales	***Haslea nusantara***	MH681881	120,448	31.10	Yes	134	30	4	This study

Table 3 -- Biometric data and morphological features of *Haslea nusantara* from the present study compared to other *Haslea* taxa from the literature (*: from Gastineau *et al*. (2012); **: from Massé *et al*. (2001). Values are given with Standard Error. NA: no data.

	H. nusantara	*H. ostrearia*	*H. provincialis*	*H. karadagensis**	*H. pseudostrearia*	*H. crucigera***
Length (µm)	73.9 ± 1.7 (n = 33)	71.6 ± 5.5 (n = 25)	65.8 ± 0.1 (n = 26)	52.5 ± 0.1 (n = 1148)	55.5 ± 0.2 (n = 25)	95-97
Width (µm)	6.8 ± 0.1 (n = 33)	7.5 ± 1.2 (n = 25)	7.4 ± 0.1 (n = 26)	8.0 ± 0.03 (n = 1148)	8.8 ± 0.1 (n = 25)	11-12
Fitting equation for the length to width ratio	y = 0.02x + 5.06	y = 0.03x + 4.28*	y = 0.02x + 6.33	y = 0.01x + 8.21	NA	NA
Transapical striae in 10 µm	36.0 ± 1.0 (n = 33)	34.8 ± 0.2 (n=30)	32.7 ± 0.2 (n=25)	31.4 ± 0.2 (n=30)	38.6 ± 0.2 (n=25)	17
Longitudinal striae in 10 µm	52.0 ± 2.0 (n = 33)	53.3 ± 0.2 (n=30)	60.2 ± 0.2 (n=25)	57.8 ± 0.3 (n=30)	42.8 ± 0.2 (n=25)	20
Pseudostauros	Not present	Not present	Not present	Not present	Not present	Present
Axial costa	Present	Present	Present	Present	Present	Present
Central bar	Present	Not present	Not present	Not present	Present	Present with pseudostauros
Central raphe endings	Straight	Straight	Straight	Straight	Straight	Deflected
Polar raphe endings	Straight	Straight	Straight	Straight	Straight	Deflected
Colour of pigment at apex	Blue	Blue	Blue	Blue-grey	No pigment	No pigment

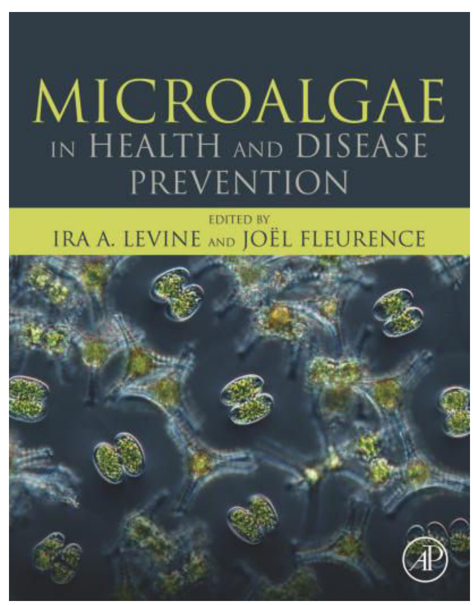
ANNEXE 5 : Chapitres de Livres

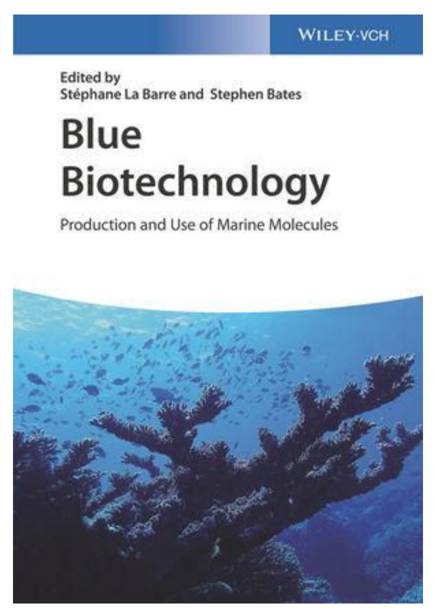
Falaise, Dewi et al. (2018) ; Gastineau et al. (2018) [*Prévisualisations*]

Falaise, C., Dewi, I.C., Hellio, C., Bourgougnon, N., Mouget, J.-L., **2018**. Chapter 12. Anticancer, antiviral, antibacterial and antifungal properties in microalgae, in: *Microalgae in Health and Disease Prevention*. Levine I. and Fleurence J. (Eds.). Elsevier Science, Amsterdam, Netherlands.

et

Gastineau, R., Prasetiya, F.S., Falaise, C., Cognie, B., Decottignies, P., Morancais, M., Méléder, V., Davidovich, N.A., Turcotte, F., Tremblay, R., Pasetto, P., Dittmer, J., Bardeau, J.-F., Pouvreau, J.-B., Mouget, J.-L., **2018**. Chapter 16. Marennine-Like Pigments: Blue Diatom of Green Oyster Cult?, in: *Blue Biotechnology, Production and Use of Marine Molecules*. Stéphane La Barre and Stephen S. Bates (Eds.). Wiley-VCH, Weinheim, Germany.





Chapter 12

Anticancer, Antiviral, Antibacterial, and Antifungal Properties in Microalgae

Intan C. Dewi[1,a], Charlotte Falaise[1,a], Claire Hellio[2], Nathalie Bourgougnon[3], Jean-Luc Mouget[1]

[1]Le Mans Université, Le Mans, France; [2]Universitaire Européen de la Mer (IUEM) - UBO – UEB Technopôle Brest-Iroise, Plouzané, France; [3]Université Bretagne Sud, Vannes, France

Chapter Outline

1. Introduction **235**
2. Anticancer Activity **236**
2.1 Purified Molecules 236
2.1.1 Microalgae 236
2.1.2 Cyanobacteria 237
2.2 Methods for Assessment of Anticancer Activity 239
3. Antiviral Activity **240**
3.1 Screening and Extracts 240
3.2 Purified Molecules 241
3.2.1 Polysaccharides 241
3.2.2 Proteins, Lipids, Pigments 243
3.3 Methods to Assess the Antiviral Activity of a Compound 244
4. Antibacterial Activity **244**
4.1 Antibacterial Activities of Microalgae 245
4.1.1 Green Microalgae 245
4.1.2 Red Microalgae 247
4.1.3 Diatoms 247
4.2 Antibacterial Activities of Cyanobacteria 248
4.3 Methods to Assess the Antibacterial Activity of a Compound 248
4.4 Antibacterial Activity of Microalgae and Cyanobacteria Against Animal Diseases 249
5. Antifungal Activity **249**
5.1 Antifungal Activities From Eukaryotic Microalgae 250
5.1.1 Green and Red Microalgae 250
5.1.2 Diatoms and Dinoflagellates 251
5.2 Antifungal Activities From Cyanobacteria 252
6. Conclusion and Prospects **252**
Acknowledgment **253**
References **253**

1. INTRODUCTION

This chapter mainly focuses on antiproliferative and antimicrobial compounds produced by microalgae (eukaryotic, phototrophic microorganisms) and cyanobacteria, with special emphasis on health and disease prevention. To seek for new bioactive compounds as antiproliferative, antiviral, or antibacterial agents, the pharmaceutical industry has shown a growing interest for a vast marine bioresource, underexplored yet, the aquatic phototrophic microorganisms. Indeed, many strains of microalgae and cyanobacteria are reported to produce intracellular as well as extracellular metabolites with various biological activities, and screening studies have been recurrently carried out with the aim to discover new bioactive metabolites. Illustrating the global interest for microalgae and cyanobacteria as new sources of bioactive agents, several reviews illustrate the increasing number of compounds identified so far (e.g., Ahmadi et al., 2015; Borowitzka, 1995; Encarnacao et al., 2015; Falaise et al., 2016; Raposo et al., 2015; Shimizu, 1996, 2003; Singh et al., 2005). Physicochemical and pharmacological studies have paid special attention to proteins, pigments, terpenoids, and steroids (Abdo et al., 2012; Gastineau et al., 2012a,b; Gustafson et al., 1989; Rechter et al., 2006). In fact, secondary metabolites produced by microalgae and cyanobacteria represent a very chemically diverse reservoir, including nearly every chemical class of natural products, from amino and fatty acids to alkaloids and polyphenolic compounds.

[a] Intan Chandra Dewi and Charlotte Falaise contributed equally to this work.

Microalgae in Health and Disease Prevention. https://doi.org/10.1016/B978-0-12-811405-6.00012-8
Copyright © 2018 Elsevier Inc. All rights reserved.

16

Marennine-Like Pigments: Blue Diatom or Green Oyster Cult?

Romain Gastineau[1], Fiddy S. Prasetiya[1,2,3], Charlotte Falaise[1], Bruno Cognie[4], Priscilla Decottignies[4], Michèle Morançais[4], Vona Méléder[4], Nikolai Davidovich[5], François Turcotte[6], Réjean Tremblay[6], Pamela Pasetto[7], Jens Dittmer[7], Jean-François Bardeau[7], Jean-Bernard Pouvreau[8], and Jean-Luc Mouget[1]

[1] *Le Mans Université, LUNAM, Mer Molecule Santé (MMS), Faculté des Sciences et des Techniques, Mer-Molécules-Santé (MMS) EA 2160, Avenue Olivier Messiaen, 72085 Le Mans Cedex 9, France*
[2] *Universitas Padjadjaran, Marine Science Department, Jl. Raya Bandung Sumedang KM 21, Jatinangor 40600, Indonesia*
[3] *Postgraduate school Universitas Padjadjaran, Program Study of Biotechnology, Research center of Biomolecular and Bioinformatics, Jl. Dipatiukur No. 35 Bandung Gedung Sekolah Pascasarjana, Bandung 40132, Indonesia*
[4] *Université de Nantes, LUNAM, Mer Molecule Santé (MMS), Faculté des Sciences et des Techniques, Mer-Molécules-Santé (MMS) EA 2160, 2 rue de la Houssinière, 44322 Nantes, France*
[5] *Karadag Scientific Station – Nature Reserve of the Russian Academy of Sciences, Laboratory of Algae and Microbiota, p/o Kurortnoe, 98188 Feodosiya, Republic of Crimea, Russia*
[6] *Université du Québec à Rimouski, Institut des Sciences de la Mer à Rimouski, Allée des Ursulines, Québec G5L2Z9, Canada*
[7] *Le Mans Université, Faculté des Sciences et des Techniques, Institut des Molécules et Matériaux du Mans (IMMM), UMR CNRS 6283, Avenue Olivier Messiaen, 72085 Le Mans Cedex 9, France*
[8] *Université de Nantes, Faculté des Sciences et des Techniques, Laboratoire de Biologie et Pathologie Végétales, SFR 4207 QUASAV, 2 rue de la Houssinière, 44322 Nantes, France*

Abstract

This chapter presents new insights into "blue" diatom species and marennine-like pigments in particular, concerning the biodiversity of these microalgae, their pigments, their interactions with bivalves, and their potential use in biotechnology, in particular as natural blue pigments. Historically, the term "blue diatom" refers to the marine diatom *Haslea ostrearia*, known for its specific ability to produce a water-soluble blue pigment, marennine, but it applies now to several new species recently described, all producing blue pigments. Genetically, these species form a well-defined clade inside the genus *Haslea*, and their pigments seem to possess similar spectral characteristics and biological activities, for instance, antimicrobial, especially against some oyster pathogens (e.g., *Vibrio splendidus*). Marennine is the main factor responsible

Blue Biotechnology: Production and Use of Marine Molecules,
First Edition. Edited by Stéphane La Barre and Stephen S. Bates.
© 2018 Wiley-VCH Verlag GmbH & Co. KGaA. Published 2018 by Wiley-VCH Verlag GmbH & Co. KGaA.

for the greening of oyster gills in western France, where oyster farming is a phenomenon that has a noticeable economic impact. The green bivalves are a must for connoisseurs and a way to boost the marketability of regional oyster products. Although the coloration of the gills by marennine has not been correlated with an increase of oyster palatability, the biological activities of marennine could represent a new avenue of valorization of this "old" pigment, especially considering oyster mortality events and the sustainability of molluscan shellfish industry.

16.1 Introduction

Diatoms are silicified brown microalgae of high ecological importance [1, 2], with ever-extending applications in blue biotechnologies (see Chapter 27). They can be used in aquaculture, bioremediation, biofuel production, nanotechnologies, and the production of various high added-value metabolites [3]. The characteristic brown color of diatoms results from their pigment content, chlorophylls *a* and *c*, masked by carotenoids diatoxanthin, diadinoxanthin, and mainly fucoxanthin. In addition to these photosynthetic pigments, some species belonging to the genus *Haslea* synthesize water-soluble blue pigments, known as marennine-like pigments, and are therefore often referred to as "blue diatoms" or "blue *Haslea*" [4–7]. The chemical structure of these pigments is not yet determined, although clues advocate for a polyphenolic [8] or glycosidic nature [6]. Some of these blue pigments, like marennine produced by *Haslea ostrearia* (Figure 16.1), seem to exist under two different forms, one intracellular marennine (IMn) and one extracellular marennine (EMn), with different molecular weights and UV–visible spectral characteristics [8]. Furthermore, their color is pH dependent, reversibly shifting from purple blue in acidic media to green in neutral to alkaline conditions [8–11] (Figure 16.2). *In vivo*, the pigment appears intense blue, especially when accumulated at cell apices, but it can turn the surrounding water green when released from the cells. Furthermore, supernatants of water-soluble extracts of *H. ostrearia* cultures, or purified marennine-like pigments, are known to display several biological activities, for example, antibacterial, antiviral, antifungal, antiproliferative, and antioxidant properties [6, 12–17]. Purified marennine [18] and *H. ostrearia* culture supernatants [19, 20] have also demonstrated allelopathic activities against other species of microalgae. These various activities make blue *Haslea* and their marennine-like pigments a promising model for blue biotechnologies. After a brief presentation of the biodiversity of blue *Haslea* species and their pigments, this chapter will present new insights into the structure and possible applications of marennine-like pigments, mainly as a coloring agent and probiotic in aquaculture or a natural blue colorant in the food industry and cosmetology.

ANNEXE 6 : Essais préliminaires

1) Inactivation d'une suspension du virus OsHV-1 par exposition à la marennine purifiée (EMn) et bio-essai par administration de la suspension traitée à des huîtres creuses

2) Effet de la marennine purifiée (EMn) sur les premières divisions embryonnaires des oursins *Paracentrotus lividus* et *Sphaerechinus granularis :* effets sur la synthèse protéique globale et sur l'activité de CDK1

Les expérimentations présentées ci-après ont été respectivement conduites 1) en Avril 2016 au Laboratoire de Génétique et de Pathologie des Mollusques Marins (RBE/SG2M/LGPMM/Ifremer), Station La Tremblade sous l'encadrement de Cyrille François et 2) en juillet 2016 au Laboratoire de Biologie Intégrative des Modèles Marins, Sorbonne Universités (UPMC Paris 06/CNRS/UMR 8227), Station Biologique de Roscoff, sous l'encadrement de Patrick Cormier.

1) Lors de cet accueil scientifique de 3 semaines au LGPMM, nous avons développé 3 axes de recherches en étudiant les effets de la EMn i) *in vitro* sur la croissance de bactéries du genre *Vibrio* isolées de bivalves ; ii) sur les hémocytes d'huîtres en conduisant des essais de cytotoxicité avec des mesures en cytométrie en flux ; et enfin iii) sur l'inactivation d'une suspension du virus OsHV-1 en menant un bio-essai par administration de la suspension traitée à des huîtres creuses. Les résultats des essais i) et ii) ont pu être valorisés (Falaise et al. (2016), Annexe 1 et Falaise et al. (2019), Annexe 2), de plus les effets observés sur la croissance des souches de *Vibrio* ont pu être davantage étudiés en poursuivant les expérimentations au laboratoire MMS du Mans. Des biais expérimentaux n'ont pas permis d'exploiter les résultats de l'axe de recherche iii), néanmoins la méthodologie employée est présentée dans cette annexe.

2) Lors d'un premier séjour d'une semaine à la station biologique de Roscoff en mai 2016, nous avions réalisé des essais exploratoires pour évaluer si la EMn présentait des effets sur la fécondation et la cinétique de première division embryonnaire chez les oursins *P. lividus* et *S. granularis*. Les résultats préliminaires obtenus ont encouragé la planification d'une seconde série d'expérimentations lors d'un nouvel accueil scientifique de 2 semaines en juillet 2016. Pour ces expérimentations, des tests biochimiques ont été conduits sur *P. lividus* et *S. granularis* pour évaluer si la EMn impactait la synthèse globale de protéines dans l'œuf nouvellement fécondé ou si elle avait un effet sur l'activité du complexe CDK-1 Cycline B lors de la mitose, expliquant ainsi l'absence de division des œufs exposés aux fortes concentrations de pigment. La méthodologie employée pour ces essais et les résultats préliminaires obtenus sont présentés dans cette annexe. Bien que ces tests aient donné des premiers résultats intéressants, il a été décidé, lors d'un 3ème séjour scientifique de 2 semaines en mars 2017, de finaliser les essais concernant les effets de la marennine sur la fécondation et le développement embryonnaire en vue d'une publication. Les expérimentations ont été conduites sur *S. granularis* uniquement car l'espèce *P. lividus* n'était pas disponible en cette période de l'année. Les résultats obtenus lors de ce 3ème séjour scientifique ainsi que certains des résultats préliminaires ont été valorisés dans la publication Falaise et al. (2019), Annexe 2.

HYPOTHESE EXPERIMENTALE : inactivation d'une suspension du virus OsHV-1 par exposition à la marennine purifiée (EMn) et bio-essai par administration de la suspension traitée à des huîtres creuses.

Date et lieu des expérimentations : Avril 2016 au Laboratoire de Génétique et de Pathologie des Mollusques Marins (RBE/SG2M/LGPMM/Ifremer), Station La Tremblade sous l'encadrement de Cyrille François.

Matériel :

- Solution virale d'Ostreid HerpesVirus-1 (OsHV-1) de $6{,}86.10^{8}$ copies mL^{-1}
- Marennine purifiée (EMn) sous forme lyophilisée (poudre)
- Huîtres creuses *Crassostrea gigas* x 215 (lot NSI03-15) :
  - longueur moyenne 49,25 ± 4,0 mm ; masse moyenne : 9,66 ± 1,7 g (n=12)
- Salle d'infection expérimentale (photographie **A**)

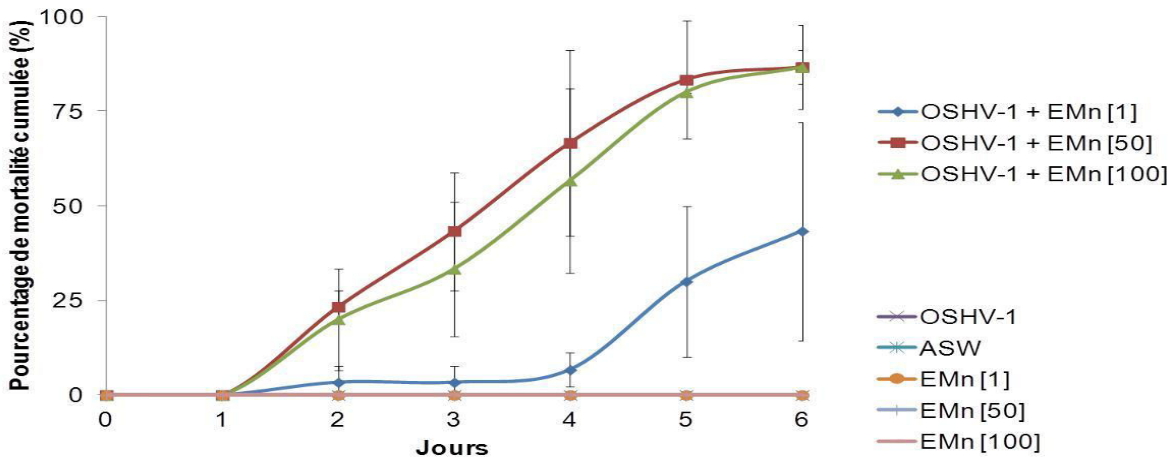
Plan expérimental :

- Dilution de la solution virale à 10^{6} copies mL^{-1} (=dose infectante) dans de l'eau de mer artificielle (ASW)
- Dilution de la EMn dans ASW aux concentrations finales de : 1, 50 et 100 µg mL^{-1}
- Exposition de la solution virale à la EMn aux différentes concentrations « overnight »
- Infection des huîtres
  - Acclimatation des animaux dans la salle d'expérimentation pendant 5 j dans des bacs d'eau de mer naturelle stérile (filtration + traitement UV) à 22°C avec bulleurs
  - Mise à sec des animaux la veille de l'infection
  - Myorelaxation des huîtres dans une solution de chlorure de magnésium (50 g L^{-1}) pendant 1 h
  - Injection intramusculaire des solutions : 1 mL / animal (photographie **B**)
  - Disposition de 10 animaux / bac contenant 3 L d'eau de mer stérile à 22°C et un bulleur
    - 3 bacs de 10 huîtres / modalités ; sauf CTL (+) et CTL (-) 1 bac / modalité (Tableau bilan des modalités **C**)
- Suivis de mortalité pendant 6 j
  - Arrêt du bullage et observation des animaux bâillants
  - Légers choc sur la paroi des bacs pour vérifier que l'animal ne ferme pas ses valves
  - Stimulation à l'aiguille sur le manteau comme test final pour estimer si l'animal est vivant ou non) et retrait des animaux morts des bacs

C

Modalités
CTL négatif (ASW)
CTL positif (OsHV-1 seul)
EMn [1]
EMn [50]
EMn [100]
OsHV-1 + EMn [1]
OsHV-1 + EMn [50]
OsHV-1 + EMn [100]

Résultats :



Suivis de mortalité des huîtres sur 6 jours après injections de solutions contenant de la EMn aux concentrations de 0, 1, 50 ou 100 µg mL^{-1}

L'Ostreid HerpesVirus1 (OsHV-1) est un virus induisant de forts taux de mortalité chez les huîtres *C. gigas* et également chez d'autres bivalves (Arzul and Renault, 2002; Burge et al., 2006; Renault, 2011).

Le contrôle positif (OsHV-1 seul) n'a pas induit de mortalité, alors que la dose infectante administrée aurait dû induire une forte mortalité, ce qui doit se traduire par une erreur expérimentale lors de la préparation de la solution virale à injecter. Un nouveau test d'infection a été alors réalisé quelques jours plus tard pour s'assurer de la pathogénicité de la suspension virale (infection de 10 huîtres) et a résulté en une mortalité totale des huîtres sous 3 jours, confirmant que la solution virale a été mal préparée ou administrée.

Le contrôle de cette expérimentation ayant échoué il est difficile de conclure sur les résultats obtenus. Cependant, il semble que l'injection de EMn seule, même à la plus forte concentration (100 µg mL^{-1}) n'ait pas d'effet sur la survie des huîtres sous 6 jours. Plus surprenant, la solution virale exposée « overnight » à 1 µg mL^{-1} de EMn a induit moins de mortalité que celles exposées à 50 et 100 µg mL^{-1} (ANOVA 2 facteurs dose x jours, *p*-value $< 0{,}01$). En l'absence de contrôle positif valide, l'interprétation des résultats demeure incertaine. Ces tests devraient être reconduits pour confirmer ou infirmer les résultats obtenus. Idéalement, l'infection devrait être effectuée par balnéation plutôt que par injection intramusculaire pour pouvoir tester les effets potentiels de la EMn sur la pathogénicité du virus dans son hôte.

Références

Arzul, I., and Renault, T. (2002). Herpèsvirus et bivalves marins. Virologie *6*, 169–174.
Burge, C.A., Griffin, F.J., and Friedman, C.S. (2006). Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. Dis. Aquat. Organ. *72*, 31–43.
Renault, T. (2011). Les virus infectant les mollusques marins : un exemple d'actualité, les herpèsvirus. Bull. Académie Vét. Fr. *164*, 359–364.

HYPOTHESE EXPERIMENTALE : effet de la marennine purifiée (EMn) sur les premières divisions embryonnaires des oursins *Paracentrotus lividus* et *Sphaerechinus granularis :* effets sur la synthèse protéique globale et sur l'activité de CDK1

Date et lieu des expérimentations : Juillet 2016 au Laboratoire de Biologie Intégrative des Modèles Marins, Sorbonne Universités (UPMC Paris 06/CNRS/UMR 8227), Station Biologique de Roscoff, sous l'encadrement de Patrick Cormier.

Préambule : lors de précédentes expérimentations préliminaires, nous avions remarqué l'effet de la marennine purifiée (EMn) ajoutée 5 minutes après fécondation sur les premières étapes du cycle cellulaire de l'embryon d'oursin Paracentrotus lividus. *Les concentrations de 100 et 50 µg mL^{-1} de EMn avaient eu des effets très nets : observation de divisions asymétriques et asynchrones, puis arrêt total du développement. La concentration 10 µg mL^{-1} avait induit un léger décalage de la cinétique de division cellulaire par rapport au CTL. Il a ensuite été remarqué pour les œufs exposés à 10 µg m^{-1} de EMn un développement anormal des embryons (formes aplatie, absence de spicules). La concentration 1 µg mL^{-1} de EMn n'a pas semblé avoir eu d'effet sur le cycle cellulaire et le développement embryonnaire.*

Plan expérimental :

Deux tests biochimiques sont réalisés à T0 (avant fécondation), T30 (*30 min post fécondation*), T60, T90, T120 et T150 :

1) évaluation de la **synthèse protéique globale** en réponse à la fécondation par la méthode **SUnSET** (Schmidt et al., 2009)
Ajout de puromycine à faible concentration : ce composé mime un acide aminé et se fixe sur le peptide -> un anticorps sera dirigé vers la puromycine ; le marquage à la puromycine est relatif à la quantité de peptides produits.

2) évaluation de **l'activité de CDK1** en dirigeant un anticorps vers le site phosphorylé de la **phosphatase protein 1 (PP1)**
CDK1 (kinase mitotique) est activée par la CyclineB. L'activation de la kinase CDK1 induit la phosphorylation de PP1 : un anticorps sera dirigé vers le site phosphorylé de PP1.
Pour sortir de la métaphase, la CyclineB est inactivée, la quantité de CDK1 diminue.

Principales étapes expérimentales :

-Prélèvement des œufs après fécondation à T0, T30, T60, T90, T120 et T150
-Western Blot
-Exposition aux Ac I et Ac II
-Révélation en ECL (*enhanced chemiluminescence*)
-Traitement des résultats des Western Blot sur Image J

La ponte des oursins et la fécondation sont réalisées selon le protocole décrit dans le Chapitre IV. Partie 2 de ce manuscrit.

Protocole des tests biochimiques réalisés :

Synthèse protéique globale (SuNSET) :

- 200 µL d'œufs à 5%
- 2 µL de puromycine 10mM (diluée dans eau milliQ)
- incubation 10 min
- culot repris dans 100 µL SDS-fix X1 avec bleu de bromophénol (1 mL de bleu dans 10mL de SDS-fix)
- échantillon au congélateur -20℃

SDSFix X 1
pour 30 ml
6ml de SDS 10%
3 ml glycerol
1,875 ml de Tris 1M
1,5 ml β-mercaptoethanol
H20 17,625 *ml*

Activité CDK1 :

- 50 µL d'œufs à 5%
- culot repris dans 25 µL SDS-fix X1 avec bleu de bromophénol
- échantillon au congélateur -20℃

Préparation des gels SDS-PAGE

Couler le gel de séparation à 12 % et laisser polymériser (~2 h) avant de couler le gel de concentration (à laisser polymériser ~20 min)

Gel de séparation

Produits	12% (5mL-une plaque)
LiDS 0,5 %	1 mL
Tris pH 8.8-2,5 M	666 µL
Acrylamide 40 %	1,5 mL
H20	1,83 mL
Persulfate	10 µL
Temed	5 µL

Gel de concentration

Produits	Volume pour 8mL (µL)
H2O	5816
Acry 40%	1024
Tris 1 M pH 6.8	1040
SDS 10%	80
APS 30%	32
Temed	8

Migration des protéines

-Mettre les supports des gels dans cuves de migration contenant du tampon Tris-glycine (TG) SDS 1X

-Déposer 10 µL d'échantillon/puits

-Brancher la cuve au générateur : 100 V dans gel de concentration / 160 V dans gel de séparation

Western Blot

-Placer le gel dans le montage comme suit :

-Mettre le montage dans le support (face noire contre face noire)

-Mettre le système dans une cuve de TG sans SDS 1X + bloc de glace + barreau aimanté

-Mettre la cuve sous agitation dans une chambre froide 4℃ et la brancher au générateur : 100 V constants pendant 1 h.

Résultats du Western Blot – Vérification du transfert avec le rouge Ponceau

-Recouvrir la membrane de nitrocellulose de rouge Ponceau ~1min

-Passer de l'eau distillée sur la membrane

-Rincer la membrane avec du TBS-Tween 1X

Mise en contact des membranes avec l'anticorps primaire

-Saturer la membrane de nitrocellulose en protéines : la recouvrir de **TBST-BSA 1%**

-Mettre sous agitation 45 min

-Exposer les membranes aux Anticorps I (AcI) ; insertion des membranes dans un sachet scellé contenant les AcI :

-SuNSET : anti-puromycine clone 12D10 1/1000

-Activité CDK1 : anti-PP1C-P 1/1000

-Mettre les sachets sous agitation à 4℃ **over night**

Mise en contact des membranes avec l'anticorps secondaire
-Retirer les AcI et rincer les membranes au TBST (3x10min sous agitation)
-Recouvrir la membrane d'AcII (dilution dans TBST-BSA 1%) :
-SuNSET : anti-mouse au 1/5000
-Activité CDK1 : anti-rabbit au 1/4000
-Mettre sous agitation pendant 45 min
-Rincer les membranes au TBST (2x5 min sous agitation)

Révélation – chimioluminescence
-SuNSET : **ECL+** _scanner Typhoon :
-Activité CDK1 : **ECL**_révélation sur films photographiques

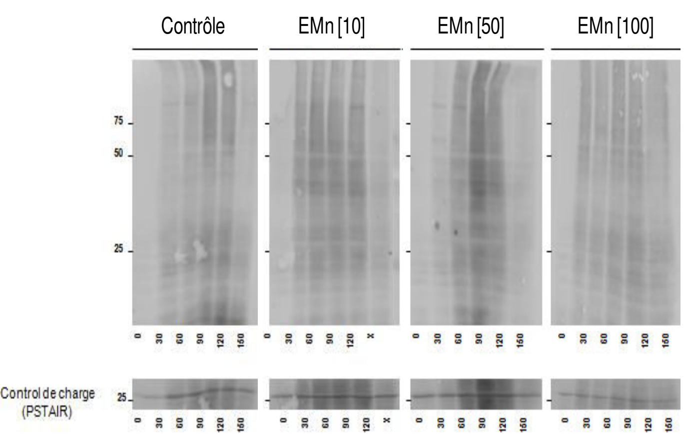
Traitement des données sous image J
Les images scanners des révélations sont analysées sous ImageJ par comparaison des niveaux de gris entre les bandes des différents traitements. Les niveaux de gris de chaque bande sont normalisés avec un contrôle de charge (PSTAIR).

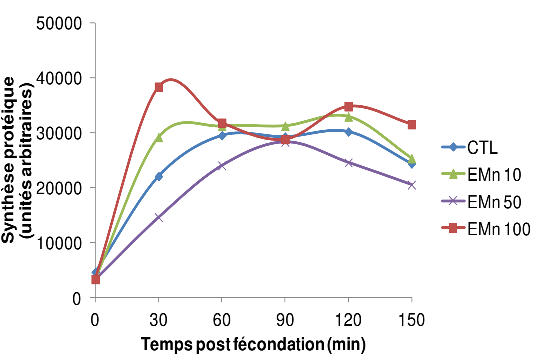
Résultats

- ***Paracentrotus lividus :***

- **Résultats de la synthèse protéique globale**

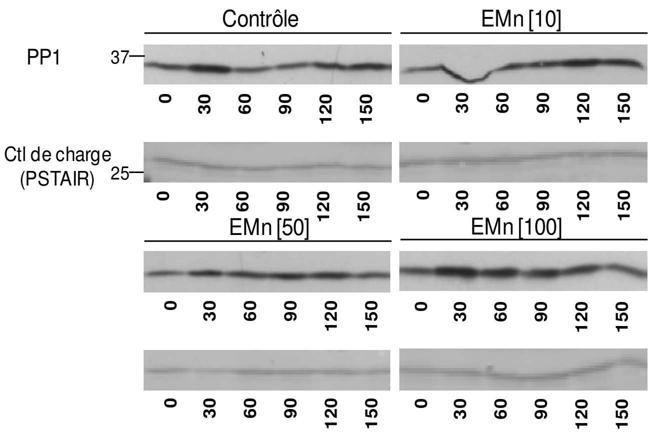
Western Blot et traitement des images sur ImageJ :

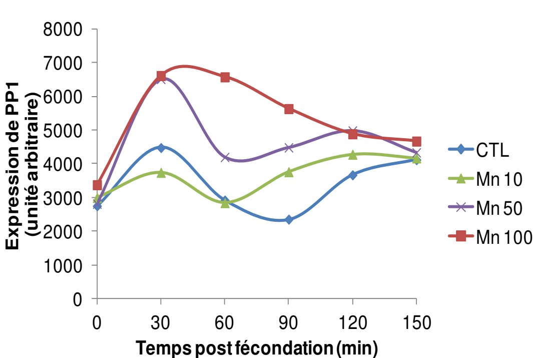




- **Résultats de l'activité de CDK-1**

Western Blot des images sur ImageJ :

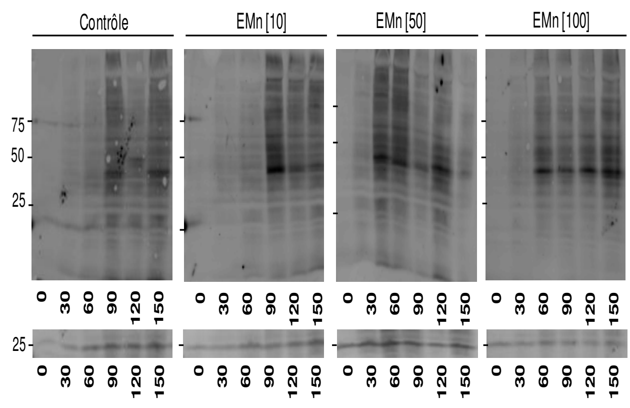


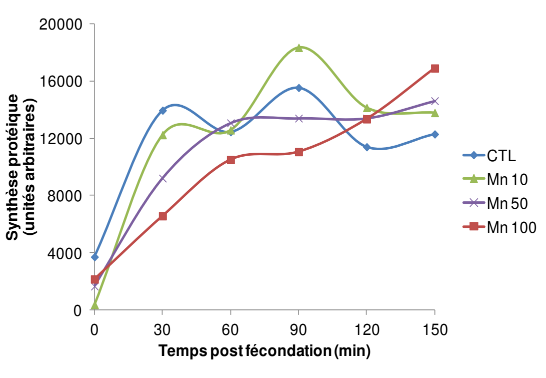


- ***Sphaerechinus granularis :***

- **Résultats de la synthèse protéique globale**

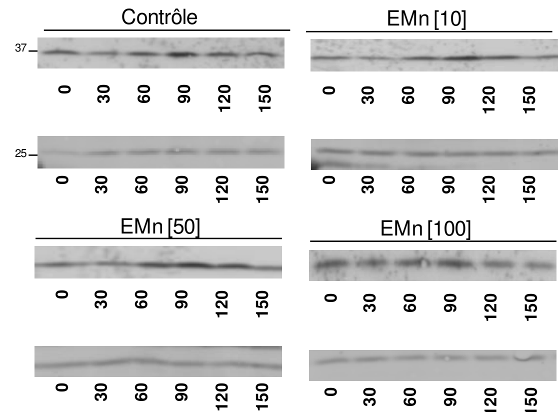
Western Blot et traitement des images sur ImageJ :

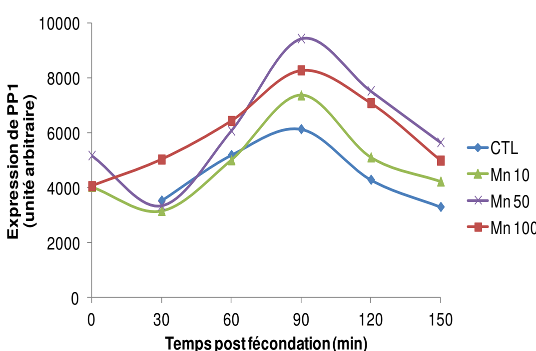




- **Résultats de l'activité de CDK-1**

  Western Blot des images sur ImageJ :





Discussion des résultats

Des œufs d'oursins nouvellement fécondés ont été exposés à de la EMn purifiée. Pour ces tests, préliminaires, un seul réplicat a été effectué par modalité testée.

Chez *P. lividus*, la EMn à 50 et 100 µg mL^{-1} a induit un blocage de la première division mitotique alors que la concentration 10 µg mL^{-1} n'a pas eu d'effet sur la cinétique de $1^{ère}$ division (*données non montrées*). Chez *S. granularis,* contrairement à ce qui était attendu, l'exposition des œufs à la EMn n'a pas eu d'effet sur la cinétique de $1^{ère}$ division, même à 100 µg mL^{-1} (*données non montrées*). Ces résultats sont à l'encontre de tests menés précédemment montrant une sensibilité de *S. granularis* à la EMn à 50 et 100 µg mL^{-1}.

Avec la méthode de purification de la EMn développée par (Pouvreau et al., 2006), la pureté de la EMn obtenue (sous forme de poudre lyophilisée) à l'Université du Québec à Rimouski (UQAR) varie en fonction des fractions sortantes de la colonne de chromatographie par échanges d'ions. Il s'est avéré que la fraction d'EMn utilisée pour ces tests présentait une pureté de seulement 35 % (contre 80 % de pureté pour la fraction utilisée pour les précédents tests), mais ce biais expérimental n'a été déterminé qu'*a posteriori*. Ainsi, la plus forte dose de EMn testée dans les tests présentés ci-dessus ne correspond finalement pas à une concentration réelle de 100 µg mL^{-1} mais à une concentration avoisinant les 35 µg mL^{-1}, ce qui pourrait expliquer l'absence d'effet observé sur la première division mitotique chez *S. granularis*.

Il apparait par ailleurs que l'oursin *P. lividus* est beaucoup plus sensible à la EMn que *S. granularis* puisque les concentrations testées ont induit un blocage des divisions cellulaires chez *P. lividus* mais pas chez *S. granularis.*

Des tests biochimiques ont été conduits pour rendre compte de la synthèse protéique globale ainsi que de l'activité CDK1-CyclineB (en étudiant l'expression de PP1, phosphorylé par CDK1) dans l'œuf d'oursin suite à la fécondation.

Le schéma suivant illustre les événements ayant lieu dans l'œuf suite à la fécondation chez l'oursin :

Chez *P. lividus* les premières divisions mitotiques ont été enregistrées aux alentours de 70 min post fécondations, et celles chez *S. granularis* aux alentours de 100 min post fécondation.

Chez *P. lividus* on observe pour la condition contrôle une augmentation de l'activité CDK1-Cycline B avant la division cellulaire puis une diminution de l'activité témoignant que la cellule sort de la métaphase. En revanche, pour les œufs exposés à la EMn 100 µg mL^{-1} on observe une augmentation de l'activité CDK1-Cycline B telle qu'observée chez le contrôle, mais l'activité ne diminue pas par la suite. Cela suggère que le complexe n'est pas désactivé et que la cellule ne peut pas poursuivre les étapes du cycle mitotique, expliquant ainsi l'absence de division des œufs.

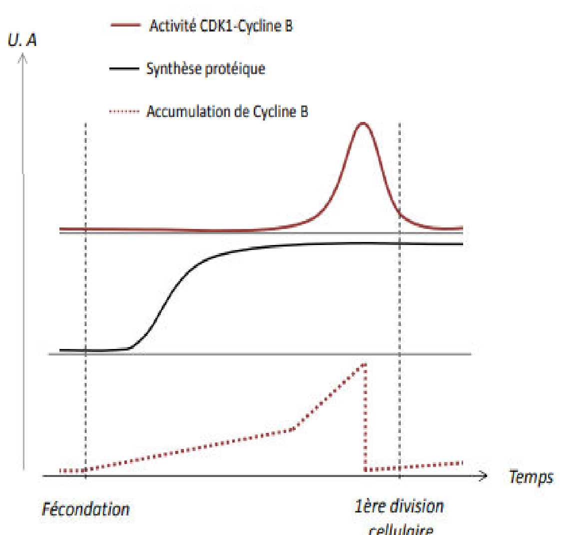


Schéma récapitulatif des évènements ayant lieu à la fécondation chez l'oursin. Adapté de Chasse H. (2016)

Chez *S. granularis* on observe également une augmentation de l'activité CDK1-Cycline B avant la division mitotique mais, bien que le signal de phosphorylation de PP1 chez les œufs exposés à la EMn apparait plus intense comparativement au contrôle sur les résultats du Western Blot, l'activité CDK1-Cycline B diminue par la suite et la division cellulaire se poursuit.

Les résultats concernant la synthèse protéique globale sont difficilement interprétables avec un seul réplicat. Dans les œufs non-fécondés, le taux de synthèse protéique est très faible, et la fécondation provoque une augmentation drastique de la synthèse protéique (Epel, 1967) dont va dépendre l'entrée en mitose (Wagenaar, 1983). Pour *P. lividus* comme pour *S. granularis*, on observe une augmentation de la synthèse protéique avant la mise en place de la première division cellulaire, même chez les œufs de *P. lividus* exposés à la EMn à 100 µg mL^{-1} qui ne se divisent pas par la suite.

Ces résultats préliminaires suggèrent donc un effet anti-mitotique de la EMn en perturbant la désactivation du complexe CDK1-Cycline B et en empêchant les cellules de sortir du stade métaphasique, et ainsi de poursuivre la division cellulaire. Toutefois, ces essais n'ont été conduits qu'une fois avec un seul réplicat et de nouveaux tests seraient nécessaires pour confirmer cette hypothèse.

Références

Chasse, H. (2016). Régulations traductionnelles de l'embryon précoce d'oursin : recrutement des ARNm dans les polysomes à la fécondation. Université Pierre et Marie Curie, Paris VI.

Epel, D. (1967). Protein synthesis in sea urchin eggs: a "late" response to fertilization. Proc. Natl. Acad. Sci. U. S. A. *57*, 899–906.

Pouvreau, J.-B., Morançais, M., Massé, G., Rosa, P., Robert, J.-M., Fleurence, J., and Pondaven, P. (2006). Purification of the blue-green pigment "marennine" from the marine tychopelagic diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen. J. Appl. Phycol. *18*, 769–781.

Schmidt, E.K., Clavarino, G., Ceppi, M., and Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. Nat. Methods *6*, 275–277.

Wagenaar, E.B. (1983). The timing of synthesis of proteins required for mitosis in the cell cycle of the sea urchin embryo. Exp. Cell Res. *144*, 393–403.

Titre : Valorisation des activités biologiques de la diatomée marine *Haslea ostrearia*

Mots clés : aquaculture ; diatomée ; *Haslea* ; marennine ; composé naturel bioactif

Résumé : *Haslea ostrearia* est une diatomée cosmopolite pouvant proliférer en milieu naturel et dont les efflorescences sont particulièrement fréquentes dans les claires à huîtres de la côte Atlantique française. Cette diatomée produit la marennine, un pigment bleu-vert hydrosoluble connu pour induire le verdissement des branchies des huîtres, augmentant ainsi leur valeur marchande. La marennine présente des activités allélopathiques, antioxydantes ou encore antibactériennes *in vitro* et son utilisation en aquaculture a été envisagée suite à la mise en évidence d'effets prophylactiques chez des bivalves. Les objectifs de ce doctorat étaient 1) d'identifier les effets de ce pigment sur la croissance des bactéries du genre *Vibrio,* fréquemment impliquées dans la mortalité massive de bivalves et 2) de s'assurer de son innocuité sur des organismes marins (*e.g.* mollusques, crustacés, échinodermes).

Nos résultats sur la croissance bactérienne ont montré que les relations dose-effet ne présentaient pas systématiquement une tendance linéaire et également que la marennine pouvait totalement inhiber ou bien stimuler la croissance de *Vibrio* en fonction de la souche testée. De plus, des concentrations écologiquement pertinentes ont induit des effets négatifs sur la survie et le développement d'animaux marins. Ces effets indésirables n'ont cependant été observés que chez des stades fragiles de développement tels que les embryons et les larves. Enfin, nos résultats questionnent l'utilisation de *H. ostrearia* en aquaculture et soulignent la nécessité d'établir des doses seuils d'exposition afin de prévenir tout effet indésirable, tout en bénéficiant de la stratégie prophylactique résultant de l'utilisation de la marennine.

Title: Valorization of the biological activities of the diatom *Haslea ostrearia*

Keywords: aquaculture; diatom; *Haslea*; marennine; natural bioactive compound

Abstract: Blooms of the diatom *Haslea ostrearia* are recorded worldwide in marine environments and occur frequently in oyster ponds in the Western French coast. This diatom produces a blue-green water soluble pigment named marennine that is known to turn oyster gills green, which provides a higher market value to the bivalves. Although *H. ostrearia* has been identified centuries ago, little is known about the ecological significance of the blue pigment. Marennine displays allelopathic, antioxidant or antibacterial activities *in vitro* and its use in aquaculture was considered as some prophylactic effects were demonstrated on farmed shellfish. The aims of this thesis were thus 1) to further identify the effects of this pigment on the growth of bacteria from the genus *Vibrio* that are frequently involved in bivalve mass mortality and 2) to ensure its safety on marine organisms (e.g. molluscs, crustaceans, echinoderms).

Our results on bacterial growth demonstrated that the dose-response curves did not systematically present a linear pattern (e.g. "U shape", hormetic responses) and that marennine could either totally inhibit the growth of *Vibrio* or stimulate it depending on the strain tested. Also, ecologically relevant doses could impair the survival and the development of the marine organisms tested. Such noxious effects seemed to only target early and fragile developmental stages as embryos and larvae, while adults appeared unaffected. Finally, our results question the exploitation of *H. ostrearia* in aquaculture and highlight the need to set exposure threshold doses to prevent any adverse effects but to benefit from the prophylactic strategy resulting from the use of marennine.