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Biodiversité, reproduction et phylogénie des diatomées bleues du genre *Haslea* et valorisation de leurs pigments de type marennine

Romain Gastineau

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Ecole Doctorale VENAM

**Thèse de doctorat de l'Université du Maine
Le Mans
France**

Spécialité : Biologie des Organismes

Présentée par
Romain GASTINEAU

Pour obtenir le grade de Docteur de l'Université du Maine

**Biodiversité, reproduction et phylogénie
des diatomées bleues du genre *Haslea* et
valorisation de leurs pigments de type
marennine.**

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Lapidem quem reprobaverunt aedificantes.

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Table des matières

Liste des illustrations	9
Liste des tableaux	11
I. Introduction bibliographique	12
I.1 Généralités sur les diatomées	13
I.2 Utilisation des diatomées	16
I.3 Classification des diatomées	16
I.4 Le genre <i>Haslea</i>	17
I.5 <i>Haslea ostrearia</i>	21
I.5.1 <i>H. ostrearia</i> et le verdissement des huîtres	21
I.5.2 Distribution géographique d' <i>H. ostrearia</i>	23
I.5.3 Dimensions d' <i>H. ostrearia</i>	25
I.5.4 Structure et ultrastructure d' <i>H. ostrearia</i>	26
I.5.5 Ecologie d' <i>H. ostrearia</i>	26
I.5.6 Physiologie d' <i>H. ostrearia</i>	26
I.5.7 La marennine	27
I.6 Objectifs du travail de thèse	28
II. Chapitre 1 : la reproduction sexuée chez <i>H. ostrearia</i>	29
II.1 Introduction générale	30
II.1.1 L'auxosporulation	30
II.1.2 Déroulement général de l'auxosporulation	31
II.1.3 La compatibilité sexuelle	34
II.1.4 Les facteurs induisant la reproduction sexuée	35
II.1.5 L'auxosporulation chez <i>H. ostrearia</i>	36

II.1.6 Les facteurs d'induction de l'auxosporulation chez <i>H. ostrearia</i>	39
II.2 La reproduction sexuée chez <i>H. ostrearia</i> : le facteur concentration	40
II.2.1 Introduction	40
II.2.2 Matériel et méthode	40
II.2.3 Résultats	41
II.2.4 Discussion	42
II.3 La reproduction sexuée chez <i>H. ostrearia</i> : l'impact du pré-conditionnement lumineux	43
II.3.1 Introduction	43
II.3.2 Matériel et méthode	43
II.3.3 Résultats	44
II.3.4 Discussion	44
II.4. La reproduction sexuée chez <i>H. ostrearia</i> : effet de lumières qualitativement différentes	45
II.4.1 Effet de lumières monochromatiques	45
II.4.1 a Matériel et méthode	45
II.4.1 b Résultats	47
II.4.2 Effet de quantités croissantes de radiations dans la zone rouge proche infrarouge	48
II.4.2 a Matériel et méthode	48
II.4.2 b Résultats	49
II.4.3 Discussion	49
III. Chapitre 2 : la diversité biologique chez les diatomées bleues du genre <i>Haslea</i>	52

III.1 Introduction	52
III.2 La baie de Bourgneuf	54
III.3 Marennes-Oléron	55
III.4 Le détroit de Cattégat en Suède	56
III. 5 La Mer Noire et <i>Haslea karadagensis</i>	57
III.5.1 Introduction	57
III.5.2 <i>Haslea karadagensis</i> sp. nov. (Bacillariophyta), a new diatom from the Black Sea producing a novel blue pigment (article)	60
III.5.3 Conclusion et perspectives	89
III.6. L'île de la Gomera et <i>Haslea silbo</i>	90
III.6.1 Introduction	90
III.6.2 <i>Haslea silbo</i> sp. nov. (Bacillariophyta), a new diatom from the Canary Islands producing a marennine-like pigment evidenced by spectrometric methods (article)	93
III.6.3 Conclusion et perspectives	131
III.7 Boulouris et <i>Haslea provincialis</i>	132
III.7.1 Introduction	132
III.7.2 Caractéristiques morphologiques d' <i>H. provincialis</i>	134
III.7.3 Aspects reproductifs	135
III.7.3 Aspects moléculaires	136
III.7.4 Aspects pigmentaires	137
III.7.4.1 Matériel et méthodes	138
III.7.4.2 Résultats	140
III.7.5 Conclusion et perspectives	145

IV. Chapitre 3 : méthodes moléculaires appliquées à l'étude des diatomées bleues	148
IV.1 La phylogénie du genre <i>Haslea</i> et des diatomées bleues	149
IV.1.1 Introduction	149
IV.1.2 Molecular phylogeny of the <i>Haslea</i> spp. (pennate diatoms, Naviculaceae): "To be or not to be blue diatom" Is it the same? (article)	153
IV.2 Héritabilité de l'ADN mitochondrial chez <i>H. ostrearia</i>	185
IV.2.1 Introduction	185
IV.2.2 Inheritance of the mitochondrial DNA in the pennate diatom <i>Haslea ostrearia</i> (Naviculaceae) during auxosporulation suggests a uniparental transmission (article)	187
IV.3 Conclusion et perspectives	215
V. Chapitre 4 : les pigments bleus des diatomées du genre <i>Haslea</i> et leur valorisation	218
V.1 Introduction	219
V.2 Activités antivirales	219
V.2.1 Introduction	219
V.2.2 Matériel et méthodes	220
V.2.3 Discussion et perspectives	220
V.3 Activités antibactériennes	222
V.3.1 Introduction	222
V.3.2 Matériel et méthode	223
V.3.3 Résultats	226
V.3.4 Conclusion et perspectives	226

V.4 Fixation des pigments sur les organes des huîtres	228
V.4.1 Introduction	228
V.4.2 Matériel et méthode	229
V.4.3 Résultats, conclusion et perspectives	230
V.5 Biological activities of purified marennine, the blue pigment responsible for the greening of oysters (article)	231
V.6 The blue-grey pigments of <i>H. karadagensis</i> (Naviculaceae): the Putative utilization to colour the oysters and the Biological properties (article)	261
VI. Conclusion générale	291
VII. Bibliographie	294
VII. Annexes	321

Table des illustrations

Figure 1. Photographie au microscope électronique à balayage du frustule d'une diatomée du genre <i>Achnantes</i> .	14
Figure 2. Schéma des endosymbioses multiples à l'origine des diatomées.	15
Figure 3. Les principales caractéristiques morphologiques du genre <i>Haslea</i> .	18
Figure 4. La variété des diatomées du genre <i>Haslea</i> .	20
Figure 5. Le verdissement des huîtres.	22
Figure 6. Les auteurs du passé.	23
Figure 7. Les phases de l'auxosporulation chez différentes diatomées.	32
Figure 8. Différents types de gamies chez plusieurs espèces de diatomées.	34
Figure 9. L'auxosporulation chez <i>H. ostrearia</i> .	38
Figure 10. La fécondité des croisements entre les clones NCC 136 et NCC 141 en fonction de la concentration cellulaire.	41
Figure 11. La fécondité des croisements entre les clones NCC 234.1 et NCC 148.78 en fonction du préconditionnement lumineux.	44
Figure 12. Spectre de transmittance des trois filtres Lee Filters utilisés.	46
Figure 13. La fécondité des croisements entre les clones NCC 158 et NCC 234.1 en fonction de la qualité lumineuse.	47
Figure 14. Spectre de transmittances des quatres filtres Lee Filters utilisés.	48
Figure 15. La fécondité de croisements entre les clones NCC 158 et NCC 234.1 en fonction de la transmittance dans le rouge des filtres utilisés.	49
Figure 16. <i>H. ostrearia</i> et la Baie de Bourgneuf.	54
Figure 17. <i>H. ostrearia</i> et la Baie Marennes-Oléron.	55

Figure 18. <i>H. ostrearia</i> et le Détroit de Cattéat.	57
Figure 19. <i>H. karadagensis</i> et la réserve de Karadag.	58
Figure 20. <i>H. silbo</i> et l'île de La Gomera.	91
Figure 21. <i>H. provincialis</i> et Boulouris.	133
Figure 22. Les aspects morphologiques d' <i>H. provincialis</i> .	134
Figure 23. Spectre d'absorbance UV-visible de la forme intracellulaire du pigment d' <i>H. provincialis</i> .	140
Figure 24. Spectre d'absorbance UV-visible de la forme extracellulaire du pigment d' <i>H. provincialis</i> .	141
Figure 25. Spectre Raman obtenu in vivo à 514,5 nm sur les apex pigmentés d' <i>H. ostrearia</i> et <i>H. provincialis</i> .	142
Figure 26. Spectre Raman obtenu sur les formes intracellulaire de la marennine et du pigment d' <i>H. provincialis</i> à 514,5 nm.	143
Figure 27. Spectre Raman obtenu sur les formes extracellulaire de la marennine et du pigment d' <i>H. provincialis</i> à 514,5 nm.	144
Figure 28. Le marqueur moléculaire de la cassette ribosomique ITS1-5,8S-ITS2	150
Figure 29. Le marqueur moléculaire <i>rbcL</i> .	151
Figure 30. Le marqueur moléculaire <i>cox1</i> .	152
Figure 31. Les activités antibactériennes et antifongiques des deux formes de la marennine d'après Pouvreau (2006).	222
Figure 32. La méthode de diffusion par disques de papiers sur gel d'agar utilisée au cours de l'étude sur les activités antibactériennes.	224
Figure 33. Le dispositif d'aquariums utilisé pour les expériences de colorations des branchies d'huîtres.	230

Liste des Tableaux

Tableau 1. Les pourcentages d'homologies pour les séquences <i>rbcL</i> des quatre espèces de diatomées bleues étudiées.	136
Tableau 2. Les pourcentages d'homologies pour les séquences <i>cox1</i> des quatre espèces de diatomées bleues étudiées.	137
Tableau 3. Les souches de pathogènes terrestres utilisées au cours de l'étude.	225

I

Introduction bibliographique

I.1 Généralités sur les diatomées

Les diatomées constituent un important groupe de microalgues brunes. Si leur diversité spécifique exacte est inconnue, elle est estimée à potentiellement 200 000 espèces réparties dans une centaine de genres (Mann, 1999). Organismes photosynthétiques possédant un exosquelette siliceux nommé frustule, les diatomées forment un maillon fondamental des cycles du carbone et de la silice en milieu aquatique (Kemp *et al.*, 2006). Les diatomées fixent en effet annuellement un quart du carbone inorganique des océans (Granum *et al.*, 2005), et 20% du carbone fixé par voie photosynthétique à l'échelle de toute la biosphère (Field *et al.*, 1998 ; Smetacek *et al.*, 1999). Ce sont d'importants producteurs primaires des milieux aquatiques (Goldman, 1992), elles sont à la base de nombreux réseaux trophiques, et elles font partie des principaux constituants du phytoplancton.

Le frustule siliceux des diatomées est constitué de deux valves, l'épivalve et l'hypovalve. L'hypovalve, possédant une taille légèrement inférieure, est imbriquée dans l'épivalve. L'ensemble tient au moyen d'un ensemble de ceintures connectives qui forment le cingulum. Les valves siliceuses peuvent être ponctuées par des aréoles. Elles peuvent aussi être ornementées de conduits ou déhiscences permettant l'excrétion dans le milieu extérieur de composés ou la locomotion, comme les fultoportulae et rimoportulae chez les diatomées centriques et certaines araphides, et le raphé chez les diatomées pennées (Round *et al.*, 1990 ; Ruck *et Theriot*, 2011).

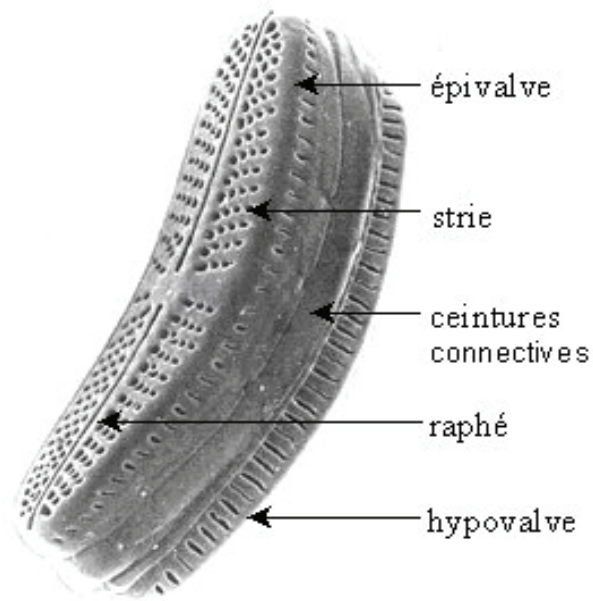


Figure 1. Photographie au microscope électronique à balayage du frustule d'une diatomée du genre *Achnantes*, avec annotations (extrait du site www.pays-de-la-loire.developpement-durable.gouv.fr, propriété du Centre de recherche public Gabriel Lippmann).

Les diatomées sont des organismes ubiquistes, qui peuvent peupler un grand nombre de milieux différents, et établir de nombreux types de relations avec d'autres organismes. Elles peuvent se trouver en eau douce comme en eau salée, dans des eaux tropicales comme dans les polaires, dans les glaces, dans des lacs de haute altitude (Smol et Stoermer, 2010). Certaines sont attachées aux plumes d'oiseaux aquatiques (Croll et Holmes, 1942), à l'épiderme de cétacés (Bennett, 1920), aux écailles de tortues (Wetzel *et al.*, 2010). D'autres existent comme endosymbiontes des foraminifères (Lee *et al.*, 1995), dans des thalles de lichens (Lakatos *et al.*, 2004), à l'intérieur d'éponges (Bavestrello *et al.*, 2000) ou sont au contraire les hôtes de cyanobactéries (Stewart *et al.*, 1983).

Les diatomées sont apparues au cours de l'ère secondaire (Medlin *et al*, 1997 ; Medlin *et al*, 2000 ; Sorhannus, 2007), les espèces fossiles du Crétacé étant les plus abondamment décrites (Round *et al*, 1990). Aussi nommées *Bacillariophyceae*, elles représentent une classe à l'intérieur de l'embranchement des *Ochrophyta*, sous-règne des *Hétérokontophyta* ou Straménopiles. Cette classification prend en compte la composition en pigments photosynthétiques (chlorophylles a et c) ainsi que l'organisation des chloroplastes, résultant chez les *Hétérokontophyta* d'endosymbioses multiples (Armbrust, 2009).

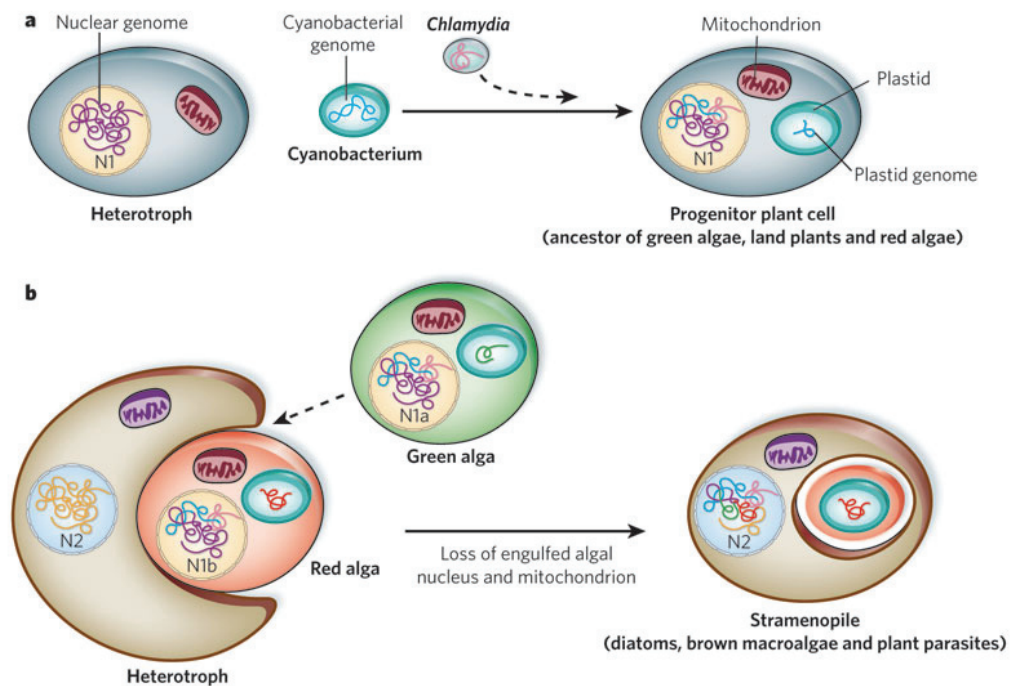


Figure 2. Schéma des endosymbioses multiples à l'origine des diatomées. D'après Armbrust (2009).

I.2 Utilisation des diatomées

Les diatomées sont couramment utilisées en aquaculture, pour le nourrissage du naissain des bivalves en écloserie. Certaines espèces au contenu lipidique riche en acides gras poly-insaturés ω -3 peuvent faire l'objet d'une culture spécifique en vue d'entrer dans la composition de compléments alimentaires (Guihéneuf *et al.*, 2010). L'étude des diatomées fossiles, qui peuvent former une roche nommée diatomite, sert en paléontologie pour la reconstitution des paléoenvironnements (Smol et Stoermer, 2010). Cette diatomite a de nombreuses utilisations industrielles, comme composante de filtres, d'abrasifs, d'absorbants, d'insecticides (Vizinet et de Reviere, 1995 ; Van Den Broeck, 1960). L'indice biologique de diatomées (IBD) a été normalisé par l'AFNOR dès 1996, afin de mesurer la qualité des eaux ainsi que son évolution spatio-temporelle, par l'étude des populations de diatomées. Les frustules des diatomées sont de plus en plus étudiés en vue d'application nanotechnologiques (Gordon *et al.*, 2008). Enfin, les diatomées servent en médecine légale dans le diagnostic des noyades (Ludes et Coste, 1996 ; Jehl, 2004).

I.3 Classification des diatomées

Plusieurs critères ont été utilisés dans la classification des diatomées. Premièrement, la morphologie du test siliceux (Round *et al.*, 1990). Certaines données écologiques, relatives au milieu de vie, ainsi qu'aux températures, pH et salinités supportées (Hustedt, 1939), sont quelquefois invoquées comme discriminantes. D'autres éléments de classification peuvent être la forme des chloroplastes (anciennement chromophores) (Mereschkowsky, 1902-1903) (Cox, 1981) ou de celle des pyrénoides

(Schmid, 2001). Certains auteurs préconisent en outre de prendre en considération les modalités de la reproduction et types de gamies des diatomées (Medlin et Kaczmarska, 2004). Enfin, les classifications précédentes peuvent se voir adjointes à une classification phylogénique sur base moléculaire, ou mises en comparaison avec cette dernière.

L'étude des caractéristiques morphologiques via la microscopie optique a conduit dans un premier temps à la classification des diatomées en deux groupes, centriques et pennées, qui se différenciaient par leur forme générale ainsi que par les axes de symétrie des frustules. En accord avec la classification sur critères morphologiques, les diatomées sont actuellement classées habituellement en trois grands groupes : centriques, pennées araphides et pennées raphides. Les diatomées centriques se reconnaissent à la symétrie centrale de leur frustule. Les diatomées pennées présentent quant à elles une symétrie bilatérale. Les pennées raphides se distinguent des pennées araphides par la présence sur au moins une de leurs valves d'un raphé. (Round *et al.*, 1990 ; Ruck et Theriot, 2011).

I.4 Le genre *Haslea*

Parmi les nombreux genres de diatomées pennées raphides appartenant à la classe des *Naviculaceae* se trouve le genre *Haslea*, plus particulièrement étudié dans ce manuscrit. C'est Simonsen (1974) qui transféra dans le genre *Haslea* plusieurs diatomées autrefois rangées dans les genres *Navicula*, *Amphipleura*, *Pleurosigma*, *Schizonema* et *Stauroneis*, suite à ses observations réalisées en microscopie électronique à balayage.

Le genre *Haslea* se définit d'abord par sa forme pennée. Le raphé est droit, présent sur les deux valves, et ses terminaisons sont peu accentuées. Les chloroplastes sont généralement au nombre de deux, en forme de longues bandes disposées pariétalement. En microscopie électronique à balayage, le frustule apparaît comme étant constitué de bandes longitudinales parallèles aux raphés. Des rangées d'aréoles en quadrilatères sont régulièrement creusées dans la valve, lui donnant un aspect grillagé. Le nom du genre fut donné en l'honneur de la biologiste norvégienne Grethe Rytter Hasle (née en 1920).

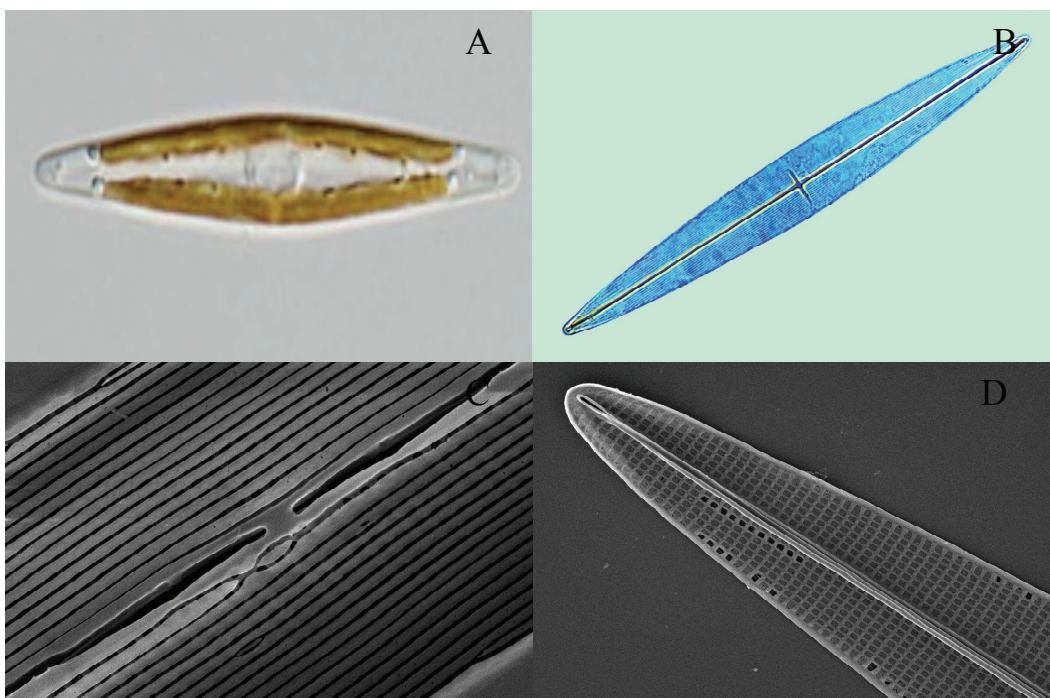


Figure 3. Les principales caractéristiques morphologiques du genre *Haslea*. A : les deux chloroplastes pariétaux (espèce *H. pseudostrearia*, courtoisie de Gert Hansen). B : forme pennée d'un frustule nettoyé par digestion chimique, vue en microscopie optique (espèce *H. balearica*, propriété du site www.diatomloir.eu). C : bandes de silices

longitudinales en vue externe d'un frustule d'*H. ostrearia*. D : aréoles quadrangulaires alignées en vue interne d'un frustule d'*H. ostrearia*.

Dans la diagnose latine du genre (Simonsen, 1974), reproduite ci-dessous, c'est l'espèce *H. ostrearia* qui est choisie comme type générique.

Novum genus familiae Naviculacearum. Frustula membrana tenue, habitu fusiforme, apicibus acutis lateribus convexis, raro in media parte parallelis. Raphe recta, cum poris centralibus parvis, approximatis, poris apicalibus paulo evolutis. Areae axiales centralesque inconspicuae. Structura membranae valvae seriebus areolarum transapicalibus parallelibus apicalibusque rectis composita. Areolae in parte exteriori membrana tenuissima tectae, cum singula rima in unaquaque areola. Multae species planctonicae. Generotypus *Haslea ostrearia* (GAILLON) nov. comb.

Les espèces appartenant au genre *Haslea* les plus couramment citées dans la littérature sont les suivantes : *Haslea britannica*, *Haslea crucigera*, *Haslea crucigeroides*, *Haslea crucigeroides* var. *densestriata*, *Haslea crystallina*, *Haslea frauenfeldii*, *Haslea gigantea* var. *tenuis*, *Haslea gigantea*, *Haslea gretharum*, *Haslea howeana*, *Haslea hyalinissima*, *Haslea indica*, *Haslea kjellmanii* var. *subconstricta*, *Haslea kjellmanii*, *Haslea nautica*, *Haslea nipkowii*, *Haslea ostrearia*, *Haslea pseudostrearia*, *Haslea salstonica*, *Haslea spicula*, *Haslea subagnita*, *Haslea sulcata*, *Haslea trompii*, *Haslea vitrea*, *Haslea wawrikan*.

Les diatomées du genre *Haslea*, bien qu'apparentées par leur test siliceux, peuvent présenter d'importantes différences sur les plans morphologiques, écologiques

et physiologiques. Ainsi, *H. wawriake* possède des apex fortement amincis, ainsi que la particularité d'être une diatomée planctonique (au contraire de la majorité des autres espèces du genre). *H. gigantea* possède une taille atteignant les 500 μm , taille la qualifiant comme constituant du mésoplancton (Simonsen, 1974). *H. crucigera*, *H. salstonica*, et *H. spicula* possèdent un sur-épaississement très marqué de leurs côtes centrales, nommé pseudo-stauros, qui est visible aussi bien en microscopie optique qu'électronique (Round *et al.*, 1990) (Massé *et al.*, 2001). La forme sigmoïdale d'*H. nipkowii* l'a par le passé fait classer dans le genre *Gyrosigma*. *H. ostrearia* a quant à elle ses apex remplis d'un pigment bleu nommé marennine.

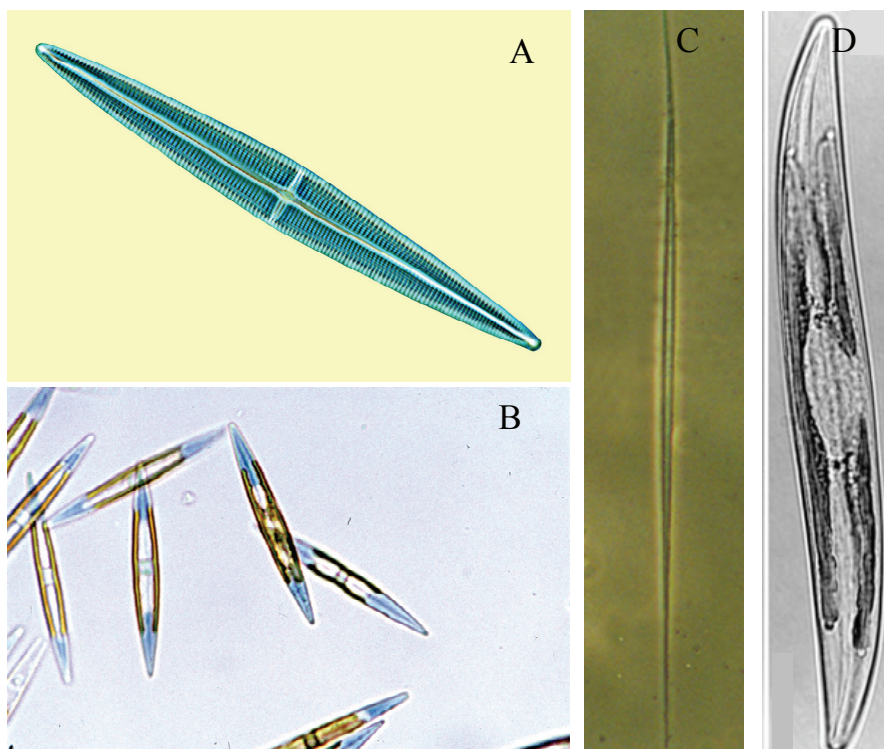


Figure 4. La variété des diatomées du genre *Haslea*. A : *H. crucigera* et son pseudo-stauros central (propriété : diatomloir). B : *H. ostrearia* et ses apex remplis de marennine. C : La diatomée planctonique *H. wawriake* et ses apex effilés (propriété du site <http://planktonnet.awi.de>). *H. nipkowii* et sa forme sigmoïdale (Poulin et al., 2004).

1.5 Haslea ostrearia

1.5.1 *H. ostrearia* et le verdissement des huîtres

H. ostrearia s'est donc toujours démarquée du genre *Haslea* ainsi que des autres espèces de diatomées par sa capacité à produire un pigment bleu surnuméraire, la marennine. A la base des études sur *H. ostrearia* se trouve le phénomène de verdissement des huîtres, qui correspond à la fixation de marennine sur les branchies des bivalves. Cette coloration des branchies apparaît conséquemment à la libération dans le milieu de grandes quantités de marennine, lorsque *H. ostrearia* devient dominante dans les claires ostréicoles où les huîtres sont mises en affinage. Les huîtres verdies ont un prix de vente plus élevé que les huîtres fines de claires n'ayant pas subi ce verdissement. Le phénomène de verdissement est un élément important dans l'activité ostréicole de l'ouest de la France, à la fois économiquement et patrimoniallement (Grelon, 1978).



Figure 5. Le verdissement des huîtres. A : une huître « fine de claire verte ». B et C : la place des huîtres vertes dans la gastronomie et le terroir français.

Ce phénomène de verdissement a été décrit dès 1669 par Thomas Sprat, archevêque de Rochester, dans son histoire de la société royale de Londres (Sprat, 1669), suite à l'observation d'huîtres provenant de Colchester. A l'aide de son microscope, Benjamin Gaillon (1820), botaniste normand, observera un siècle et demi plus tard la pellicule verte s'étant formée sur des huîtres de claires. A cette occasion, il verra se déplacer des organismes de forme linéaire qu'il qualifiera du nom d'animalcule. Souhaitant approfondir, Gaillon ira observer des échantillons épilithiques des thalles verdâtres formés par les agglutinations d'*H. ostrearia* dans les claires. Il y retrouvera les mêmes organismes, décrira leurs apex pigmentés ainsi que leurs déplacements. Leur trouvant des similarités avec le « vibrion triponctué », Gaillon donnera à sa découverte le nom de « vibrion huître », *Vibrio ostrearius*. Plus tard, Bory de Saint-Vincent donnera à cette espèce le nom de *Navicula ostrearia*, tout en lui concédant une nature végétale, en désaccord sur ce point avec Gaillon (Bory, 1824).

Cette dénomination sera conservée jusqu'à ce que Simonsen (1974) transfère *N. ostrearia* dans le genre *Haslea*, en raison des différences morphologiques importantes observées par microscopie électronique à balayage, différences communes au genre *Haslea* (pour une étude complète sur l'histoire du verdissement des huîtres, se reporter à Briée, 2010).



Figure 6. Les auteurs du passé. A : Thomas Sprat (1635-1713). B : Benjamin Gaillon (1782-1839). C : Jean-Baptiste Bory de Saint-Vincent (1778-1846).

I.5.2 Distribution géographique d'*H. ostrearia*

H. ostrearia a été décrite en de nombreux endroits dans le monde, parfois sous son ancien nom de *N. ostrearia*, et souvent avec les techniques qui précèdent l'apparition de la microscopie électronique à balayage, sur la seule foi d'observations en microscopie optique.

Dans l'hémisphère nord, *H. ostrearia* est présente de façon récurrente sur la côte atlantique française dans les baies de Bourgneuf et de Marennes-Oléron. Elle a aussi été observée en mer Méditerranée à côté de Banyuls (Sauvageau, 1906), en Sardaigne (Solazzi et Tolomio, 1976 ; Tolomio, 1978) et en Sicile dans le port de Palerme (Barone, 1979). De même a-t-elle été décrite dans l'Adriatique (Molisch, 1903), dans l'embouchure du canal de Suez (Madkour *et al.*, 2007) ainsi qu'en Mer Noire, dans la partie bordant le Nord Caucase (Proschkina-Lavrenko, 1964). *H. ostrearia* est présente dans les mers du nord de l'Europe, en Grande-Bretagne (Sprat, 1669 ; Hustedt et Aleem, 1951), en Belgique (M'harzi *et al.*, 1998), en Norvège (Hendey, 1964), en Suède (Wulff *et al.*, 2000 ; Cleve-Euler 1951-1955 ; Aleem, 1973 ; Kuylenstierna, 1990.), au Danemark (Petersen, 1916), dans la Mer Baltique (Snoeijs et Kasperovičienė, 1996). Sur la côte Est des États-Unis, elle a été observée en Caroline du Nord ainsi qu'à Rhode Island dans la baie de Narragansett (Kennett et Hargraves, 1991), en Virginie (Mitchell et Barney, 1918), dans le New Jersey (Sullivan, 1977) et potentiellement en Floride (communication personnelle de Julian Sprung). Elle aurait été vue en Amérique Latine, sur les côtes du Honduras (Grunow, 1877). Dans le Pacifique Nord, elle aurait été observée aux îles San Juan (Hardy, 1973), ainsi que de l'autre côté de l'océan, au Japon (Ranson, 1937).

Dans l'hémisphère sud, *H. ostrearia* a été inventoriée dans l'Océan Indien (Simonsen, 1974), ainsi qu'en Australie (Ricard, 1987) et Tasmanie (Volkman *et al.*, 1994)

I.5.3 Dimensions d'*H. ostrearia*

Plusieurs auteurs ont effectué des biométries d'*H. ostrearia*, en milieu naturel comme en culture in-vitro. Robert (1973, 1978) mesure en milieu naturel des longueurs allant de 60 à 120 μm et des largeurs de 6 à 12 μm . En culture, les longueurs extrêmes ont été mesurées par Neuville et Daste (1975) comme étant 36 μm et 140 μm , 36 μm représentant la taille résultant de divisions successives (Neuville et Daste, 1972a) en dessous de laquelle le maintien en culture de la diatomée s'est révélé impossible, 140 μm étant la longueur maximale des cellules initiales obtenues par reproduction sexuée. Davidovich et al. (2009) ont maintenu des cultures jusqu'à la taille minimale de 17 μm , mesurant des cellules initiales d'une taille maximale de 138 μm .

I.5.4 Structure et ultrastructure d'*H. ostrearia*

Les chloroplastes, au nombre de deux, sont pariétaux en vue valvaire. La structure des différents organites a été étudiée par différents auteurs (Neuville *et al.*, 1975 ; Geneves *et al.*, 1976 ; Nassiri *et al.*, 1998). Ce dernier auteur a mis en évidence une particularité propre à *H. ostrearia*, à savoir la modification de l'organisation des thylakoïdes lors de l'accumulation de marennine dans les apex. Il a aussi démontré la présence de vésicules impliquées dans le cycle de production du pigment.

I.5.5 Ecologie d'*H. ostrearia*

H. ostrearia est une espèce benthique, bien que certains auteurs la considèrent comme étant tychopélagique (Robert, 1983), donc potentiellement planctonique lors de phases courtes. *H. ostrearia* est souvent associée à des substrats variés, et peut être épiphytique et épilithique. Elle est euryhaline (Neuville et Daste, 1978 ; Wraige *et al.*, 1998). Elle possède les mécanismes de défense lui permettant de supporter les doses de rayonnement ultraviolet qu'elle reçoit lorsqu'elle est dans les claires ostréicoles à faible hauteur de colonne d'eau (Rech, 2004 ; Fouqueray, 2007). Elle résiste à de fortes intensités lumineuses, telles qu'elles peuvent être reçues par des diatomées benthiques dans des milieux peu profonds à faible turbidité (Mouget *et al.*, 1999). Dans les claires, où elle peut être périodiquement l'espèce de diatomée dominante (Robert, 1983), elle relargue dans le milieu de la marennine, qui a un effet allélopathique sur d'autres diatomées (Pouvreau *et al.*, 2007), ce qui pourrait lui donner un avantage écologique sur d'autres espèces.

I.5.6 Physiologie d'*H. ostrearia*

Comme toute diatomée, *H. ostrearia* est un organisme photosynthétique (Moreau, 1970) (Neuville et Daste, 1978). Elle est capable aussi de mixotrophie, c'est-à-dire qu'elle possède une certaine capacité hétérotrophique (Neuville et Daste, 1978) (Rech, 2004), pour le carbone comme pour l'azote. *H. ostrearia* possède les deux voies d'assimilation du carbone minéral des diatomées que sont les voies celle de la ribulose-biphosphate-carboxylase (RuBPC) associée au cycle de Calvin-Benson et celle de la phosphoénolpyruvatecarboxykinase (PEPCK) (Tremblin et Robert, 2001). Il y aurait

aussi présence d'une voie alternative d'incorporation du carbone de type C₄-like, qui se retrouve chez d'autres diatomées (Rech, 2004). Une activité anhydrase carbonique externe variant en fonction de l'intensité de l'éclairement a aussi été mise en évidence. Plusieurs auteurs ont mesuré les compositions glucidiques, protéiques et lipidiques d'*H. ostrearia* (Robert, 1983 ; Groth-Nard, 1994). Il est apparu qu'*H. ostrearia* possède à la fois une voie de synthèse des acides gras poly-insaturés ω -3 (Mimouni *et al.*, 2003) et qu'elle synthétise aussi des dérivés isoprénoïques, dont certains à 25 carbones nommés Haslènes en référence au genre *Haslea* (Belt *et al.*, 1996 ; Wraige *et al.*, 1998 ; Wraige *et al.*, 1999 ; Allard *et al.*, 2001 ; Belt *et al.*, 2006). *H. ostrearia* possède les pigments photosynthétiques classiques des diatomées, chlorophylle a et chlorophylle c, ainsi que de la diatoxanthine, diadinoxanthine et fucoxanthine. La répartition quantitative de ceux-ci a été étudiée et elle se modifie en fonction de l'éclairement appliqué (Mouget *et al.*, 1999). Le comportement photosynthétique d'*H. ostrearia* varie au cours de l'accumulation de marennine (Tremblin et Robert, 1996). Cette diatomée est aussi capable d'adaptation chromatique en fonction des longueurs d'ondes des éclairagements incidents (Mouget *et al.*, 2004). Certaines longueurs d'ondes stimuleraient d'ailleurs la production de marennine (Mouget *et al.*, 2005).

I.5.7 La marennine

Les mécanismes qui prévalent à la synthèse de marennine, de même que la structure de cette dernière, sont à ce jour inconnus. Plusieurs hypothèses quant à la nature chimique de la marennine ont été suggérées, au nombre desquelles un de co-produit de la dégradation de la chlorophylle ou d'un composé caroténoïque (Bachrach, 1935), une chromoprotéine (Bocat, 1907 ; Robert et Hallet, 1981), éventuellement du

type des phycobiliprotéines présentes chez les cyanobactéries (Ryder, 1884), ou bien un anthocyane (Neuville et Daste, 1972b). Plus récemment, Pouvreau (2007) proposa de classer la marennine parmi les composés polyphénoliques.

Si la nature exacte de la marennine est inconnue, plusieurs de ces activités ont déjà été mises en évidence, au premier rang desquelles sa capacité à verdir les branchies des huîtres (Lankester, 1886 ; Dyer, 1877 ; Puysegur 1880 ; Sauvageau 1907) mais aussi à colorer d'autres organismes aquatiques (Gaillon, 1820 ; Ranson, 1927). Comme indiqué précédemment, la marennine peut inhiber la croissance d'autres espèces de diatomées (Pouvreau *et al.*, 2007). Elle aurait aussi un pouvoir antioxydant (Pouvreau *et al.*, 2008), un effet photoprotecteur (Pouvreau, 2006) ainsi que d'autres activités biologiques qui seront détaillées et précisées plus loin dans ce manuscrit.

I.6 Objectifs du travail de thèse

Le travail de thèse suivant s'inscrit dans la continuité des travaux consacrés à *H. ostrearia* déjà évoqués. Il s'articulera en plusieurs paragraphes, chacun présentant un des aspects de la thèse. Le premier chapitre se focalisera sur l'étude des facteurs contrôlant la reproduction sexuée d'*H. ostrearia*. Le deuxième chapitre est consacré à la mise évidence de la biodiversité des diatomées bleues du genre *Haslea*, ainsi qu'à la description de nouvelles espèces. Les méthodes moléculaires, qui servent tant à la fois à établir une phylogénie des diatomées bleues du genre *Haslea* qu'à préciser le mode de transmission de l'ADN mitochondrial au cours de l'auxosporulation, sont présentées au troisième chapitre. Le quatrième chapitre est dédié à l'étude des activités biologiques des pigments bleus et leur valorisation potentielle.

II

Chapitre 1

La reproduction sexuée chez *H. ostrearia*

II.1 Introduction générale

II.1.1 L'auxosporulation

Le but premier de ce travail de thèse fut de préciser les modalités de la reproduction sexuée chez *H. ostrearia* ainsi que les facteurs contrôlant le déclenchement de l'auxosporulation.

La reproduction sexuée, chez les diatomées, est nécessaire à la restitution de la taille maximale des populations. Chez les diatomées, la division mitotique a en effet comme particularité d'entraîner une réduction de la taille moyenne des populations de ces organismes unicellulaires que sont les diatomées au cours du temps. La mitose chez les diatomées se fait par séparation des deux valves du frustule de la cellule mère, chaque cellule fille héritant d'une valve de la cellule mère et régénérant la valve manquante. Cette nouvelle valve sera toujours la valve inférieure ou interne, d'une taille très légèrement inférieure à la valve supérieure ou externe. De la répétition de ce mécanisme, au fil des nombreuses divisions qui suivront la formation d'une cellule initiale, résultera la diminution progressive de la taille moyenne des cellules d'une culture. Ce phénomène, connu sous le nom de loi de MacDonald-Pfitzer (MacDonald, 1869 ; Pfitzer, 1869), est responsable de l'impossibilité de maintenir la culture d'un même clone de diatomée sur le long terme. Passé un seuil de taille, des difformités apparaissent dans les cultures. En dessous d'une certaine taille critique, il n'est plus possible, pour une cellule, d'entrer en mitose. La culture deviendra donc caduque, et l'on assistera à la disparition définitive du clone, par mort des cellules qui en étaient issues (Geitler, 1932). Lorsqu'une taille critique, évaluée à 45-55% de la taille

maximale (Geitler, 1932) (Davidovich, 2001) est atteinte, les cellules peuvent entrer, si les conditions le permettent, dans une phase reproduction sexuée, nommée auxosporulation chez les diatomées.

II.1.2 Déroulement général de l'auxosporulation

La gamétogénèse se fait par transformation d'une cellule végétative en cellules spécialisées, à savoir les gamètes, suite à un épisode méiotique, suivi ou non d'une ou plusieurs mitoses. Ces gamètes, issus de deux clones sexuellement compatibles, fusionnent pour donner un zygote. Celui-ci va devenir une auxospore, cellule spécialisée qui, en s'allongeant, permettra la restitution de la taille maximale dans l'espèce considérée. L'auxospore se développe par la mise en place progressive de dépôts de silice qui formeront le frustule de la cellule initiale formée. Lorsque cette élongation est terminée, la cellule initiale va briser le périzonium qui l'entourait. Une fois libérée, elle pourra connaître des phases de multiplication végétative.

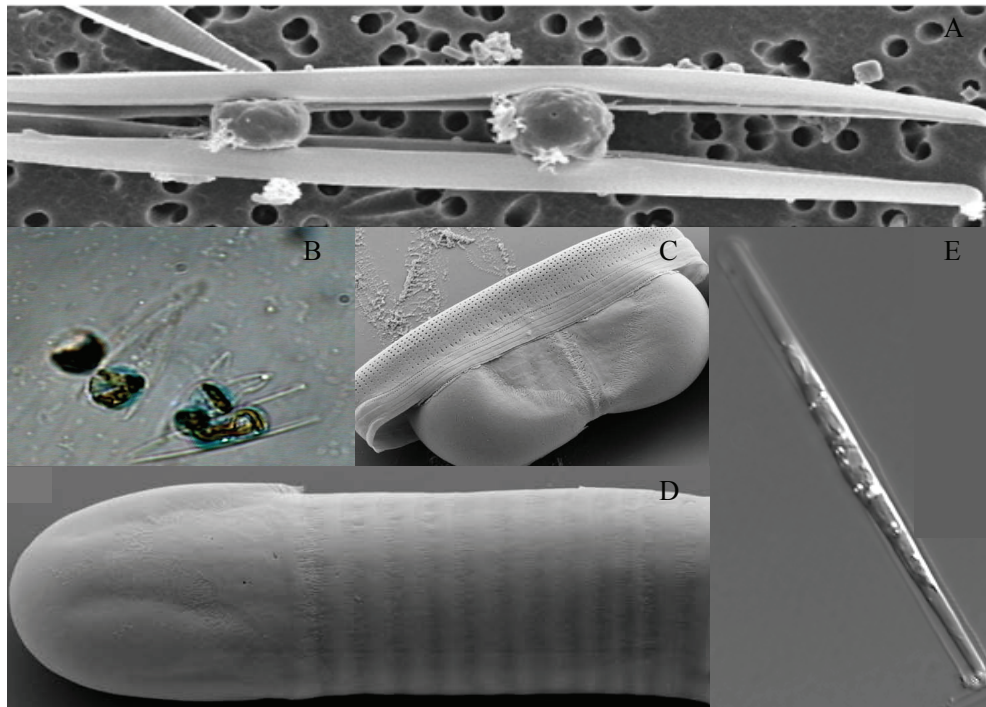


Figure 7. Les phases de l'auxosporulation chez différentes diatomées. A : Gamétogénèse chez *Pseudo-nitzschia multistriata* d'après (D'alelio *et al.*, 2009). B : Fécondation et formation de zygotes chez *H. ostrearia*. C : Auxospore avant élongation chez *Neidium* sp. (d'après Mann et Pouličkova, 2009). D : Auxospore en cours d'élongation chez *Neidium* sp. (d'après Mann et Pouličkova, 2009). E : Cellule initiale entièrement formée encore contenue dans son périzonium chez *Pseudo-nitzschia delicatissima* (d'après Amato *et al.*, 2005).

En fonction des espèces considérées, l'auxosporulation peut se faire selon différents modes de gamétogénèse et de fécondation. Chez les diatomées centriques, elle prend la forme d'une oogamie, une cellule se transformant en gamète femelle sphérique et immobile, le contenu cellulaire d'une autre cellule donnant quant à lui un grand nombre de gamètes mâles flagellés (Jensen *et al.*, 2003) (Chepurnov *et al.*, 2004) (Fig. 8-A, 8-B, 8-C).

Chez les diatomées pennées araphides, une cellule produit des gamètes « femelles » sphéroïdes immobiles, généralement attachés à l'une des valves du frustule ouvert. Les cellules provenant d'un clone « mâle » se transforment en gamètes sphéroïdes non-flagellés, mais capables de locomotion. Ces gamètes sont dans l'immense majorité des cas au nombre de deux. Leurs mécanismes de déplacement sont mal connus (Von Stosch, 1958 ; Davidovich *et al.*, 2010 ; Chepurnov *et al.*, 2004) (Fig. 8-D).

Chez les diatomées pennées dotées d'un raphé, à l'exception notable de l'espèce *Nitzschia longissima* (Chepurnov *et al.*, 2004 ; Davidovich *et al.*, 2006), la gamétogénèse produit le plus souvent des gamètes isomorphes. Ceux-ci sont produits après contact et appariement des cellules parentales. Ces gamètes, bien qu'identiques morphologiquement, peuvent varier dans leur comportement, certains se déplaçant pour entraîner la fécondation, d'autres restant immobiles à l'intérieur du frustule ouvert (Fig. 8-E).

Certaines espèces, par exemple du genre *Achnantes* (Sabbe *et al.*, 2004) ou du genre *Eunotia* (Vanormelingen *et al.*, 2008), sont capables d'une reproduction sexuée uniparentale.

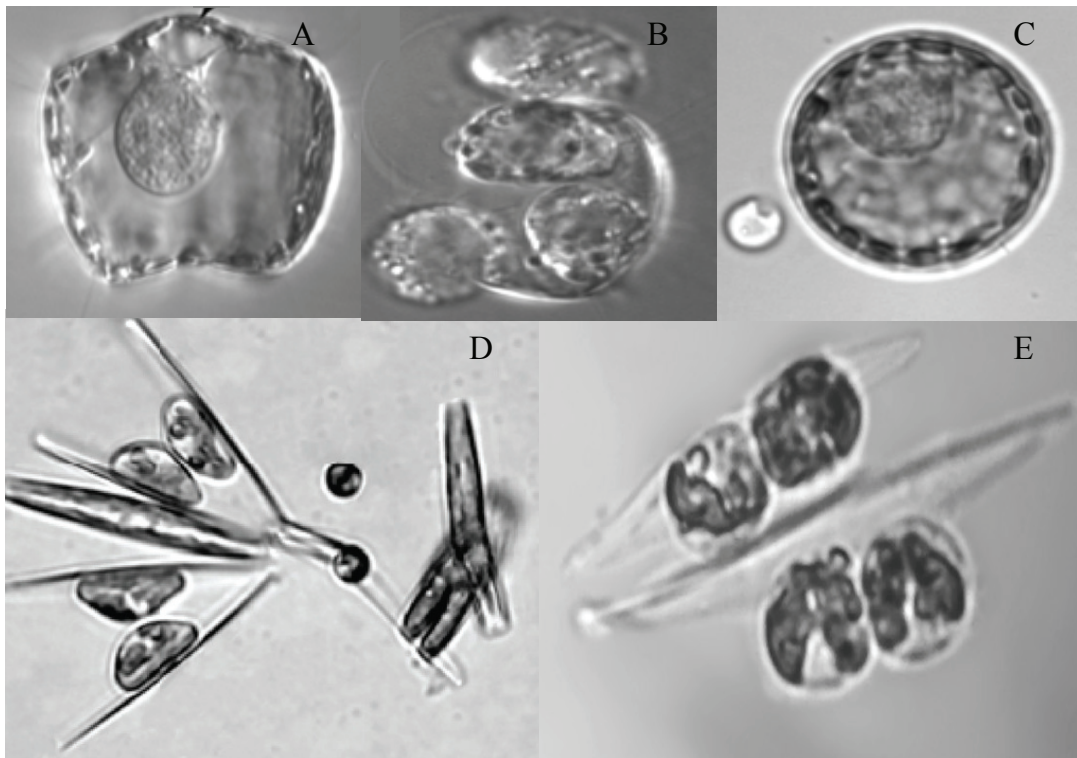


Figure 8. Différents types de gamies chez plusieurs espèces de diatomées. A-B-C : une oogamie chez *Thalassiosira punctigera* avec A : ovogénèse, B : spermatogénèse et C : fécondation (d'après Chepurnov *et al.*, 2006). D : une aplanogamie anisogame chez *Tabularia fasciculata* , gamètes mâles sphériques, gamètes femelles en forme de cloches (d'après Davidovich *et al.*, 2010). E : une aplanogamie isogame chez *H. ostrearia* (d'après Davidovich *et al.*, 2009).

II.1.3 La compatibilité sexuelle

Longtemps les diatomées ont été considérées comme uniquement aptes à une reproduction intra-clonale dite homothallique, du fait d'une monoécie qui serait dominante chez la plupart des espèces. Ceci est le point de vue de Drebes (1977). Des études ultérieures, compilées par Chepurnov *et al.* (2004), ont montré que beaucoup de diatomées présentent un caractère dioïque et sont capables de reproduction inter-clonale ou hétérothallisme. Cet hétérothallisme peut être le seul mode de reproduction connu

chez les espèces considérées. Roschchin et Chepurnov (1999) considèrent désormais que l'hétérothallisme serait majoritaire chez les diatomées pennées. Les termes homothallisme et hétérothallisme sont repris de la mycologie. Ils sont utilisés pour l'étude de la reproduction des oomycètes, qui appartiennent à la même lignée évolutive (hétérokontes) que les diatomées.

II.1.4 Les facteurs induisant la reproduction sexuée

Les facteurs prédominants pour le déclenchement de l'auxosporulation sont avant tout des facteurs internes. Au premier chef il y a la compatibilité, que ce soit celle des types sexuels mis en contact, ou *a fortiori* la proximité génétique, lorsque les individus appartiennent à deux populations dont l'identité spécifique n'est pas encore assurée. En deuxième lieu, l'atteinte d'un seuil critique de taille par les populations clonales considérées est l'autre facteur clef. Certains auteurs font de la réduction de taille un élément dont l'avantage évolutif serait de servir de principal régulateur de l'activité sexuelle, et ce mécanisme de contrôle de la reproduction est considéré comme ayant eu une incidence sur la haute diversité spécifique des diatomées et sur leur abondance (Lewis, 1983).

Chez les diatomées centriques, un grand nombre de facteurs abiotiques sont connus pour induire la reproduction sexuée. Ceux-ci ont été passés en revue par Drebes (1977) puis par Chepurnov *et al.* (2004). Au nombre de ces facteurs, on peut citer les changements de régimes lumineux, en intensité comme en durée de la photopériode. La lumière monochromatique pourrait aussi jouer un rôle. Des modifications importantes dans la salinité, le pH ou la composition en nutriments du milieu de culture

déclenchaient aussi la reproduction. Au niveau des facteurs biotiques, la présence de bactéries spécifiques pourrait être un élément nécessaire à l'auxosporulation.

Pour les diatomées pennées, ce sont les interactions entre les cellules qui semblent être prédominantes dans le déclenchement de la reproduction, que ces interactions se fassent à distance par la voie supposée de phéromones chez les diatomées pennées araphides, ou alors par contact direct lors de l'appariement des cellules parentales chez les diatomées pennées dotées d'un raphé. Des études ont démontré qu'il existe des régimes lumineux favorisant la reproduction sexuée chez les diatomées pennées (Chepurnov *et al.*, 2004). Ces conditions optimales tiennent tout à la fois compte de la photopériode ainsi que de l'intensité de l'éclairement appliqué.

II.1.5 L'auxosporulation chez *H. ostrearia*

Les premières observations d'auxosporulation chez *H. ostrearia* furent consignées dans deux articles par Neuville et Daste (1975 et 1979). Dans ces articles, les auteurs décrivent tout d'abord les stades de reproduction sexuée observés dans des cultures monoclonales dérivées d'échantillons provenant de la Baie de Marennes-Oléron. Dans leur premier article, ces auteurs observent l'apparition de formes sphéroïdes dans leurs cultures, formes qui se développèrent par la suite pour donner des auxospores puis des cellules initiales. Dans leur deuxième article, prenant les dispositions pour observer la reconstitution d'un tel événement, les auteurs purent observer et photographier tous les stades de la reproduction sexuée d'*H. ostrearia*. Ils purent de la même façon évaluer la taille critique d'induction de la reproduction à 65 μm . Les auteurs insistèrent sur la complète identité entre les conditions de culture ayant

eu cours lors des deux épisodes d'auxosporulation. Les algues étaient maintenues sous une illumination de 2000 lux, ce qui correspondrait à une valeur approximative de 27-28 $\mu\text{moles photons m}^{-2} \text{s}^{-1}$, pour une photopériode de 6h/18h lumière/obscurité.

Dans une publication plus récente, Davidovich *et al.* (2009) ont mis en évidence la reproduction hétérothallique de souches d'*H. ostrearia* issues de la Baie de Bourgneuf. L'analyse descriptive du mécanisme reproductif conduit à classer ce dernier dans la catégorie définie par Geitler (1973) comme Ib2a. Le type de gamie est une aplanogamie isogame. L'appariement des cellules sexuellement compatibles se fait sans production d'un mucilage. S'ensuit une gamétogénèse d'où résulte la formation de deux gamètes sphéroïdes par cellule parentale. Après ouverture du frustule, les gamètes sont libérés. Ceux-ci vont fusionner par plasmogamie pour donner un zygote sphéroïde. Il arrive que les zygotes avortent, ou bien que les gamètes ne parviennent pas à fusionner. Il est à noter que chez *H. ostrearia*, gamètes et zygotes sont emplis de marennine, si les cellules parentales en contenaient. Quelques heures après la plasmogamie commence l'expansion de l'auxospore. Au début de cette expansion, deux nucléii résiduels peuvent toujours être observés. Lorsque l'auxospore est pleinement développée, il y a formation de l'épithèque, valve supérieure du frustule, suivie de l'hypothèque. Quand la cellule initiale est intégralement formée, elle peut se libérer de son périzonium.

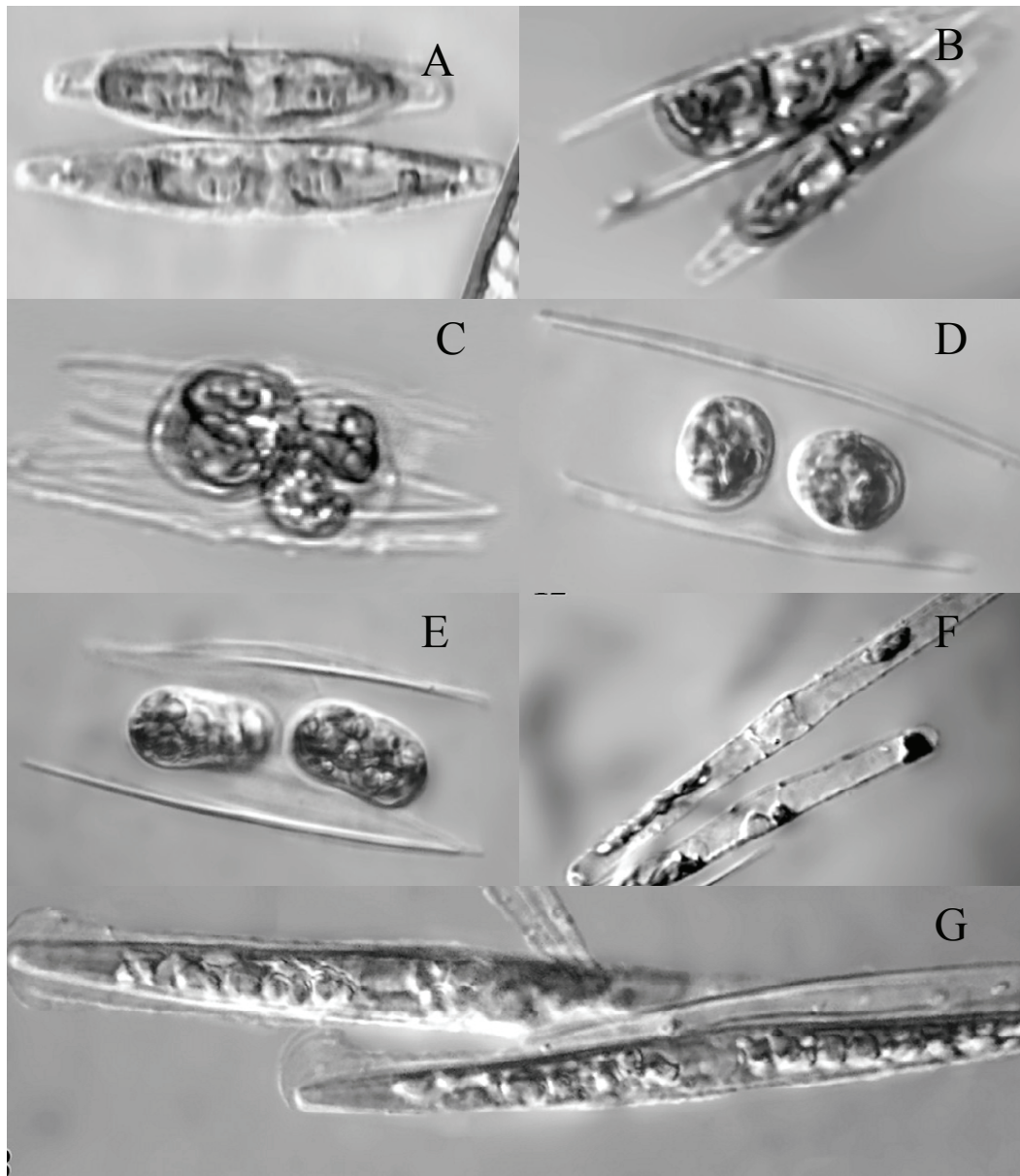


Figure 9. L'auxosporulation chez *H. ostrearia* (d'après Davidovich *et al.*, 2009). A : appariement des cellules parentales. B : gamétogénèse. C : fécondation par plasmogamie. D : formation de zygotes. E : transformation des zygotes en auxospores. F : élongation des auxospores. G : fin d'élongation des auxospores, cellules initiales encore contenues dans le périzonium.

II.1.6 Les facteurs d'induction de l'auxosporulation chez *H. ostrearia*

Suite à la description de reproduction hétérothallique chez *H. ostrearia*, des expériences ont été menées afin de découvrir des facteurs stimulant celle-ci, en particulier au niveau lumineux, et ceci à plusieurs fins. Au point de vue écophysiologique, afin de connaître les conditions les plus favorables à la reproduction, et les mettre en rapport avec les conditions environnementales, en particulier celles prévalant dans les claires ostréicoles. Au point de vue de la biologie reproductive descriptive, pour avoir le maximum de cellules entrant en auxosporulation afin de pouvoir observer tous les stades de cette dernière et réaliser une étude biométrique des cellules initiales. Enfin, comme il apparaîtra plus tard dans l'étude, de façon à rendre plus rigoureuse et sélective l'étude de l'inter-compatibilité, ou l'absence de celle-ci, lors de croisements entre *H. ostrearia* et différentes souches d'algues pourvues de pigments bleus.

L'importance du facteur lumineux a fait l'objet d'une étude préliminaire, étude reprise dans Mouget et al. (2009). Il y apparaît que l'auxosporulation chez *H. ostrearia* est stimulée par de courtes photopériodes et de faibles irradiances appliquées aux couples compatibles de diatomées. La lumière continue semble quant à elle inhiber complètement le processus d'auxosporulation.

Dans le cadre de cette partie, d'autres facteurs que ceux déjà mis en évidence par Mouget et al. (2009) ont été étudiés.

II.2 La reproduction sexuée chez *H. ostrearia* : le facteur concentration

II.2.1 Introduction

Une observation préliminaire faite par Mouget et al. (2009) a conduit à l'observation que certaines concentrations cellulaires utilisées lors des expériences de croisement semblaient être plus favorables à l'induction de la reproduction. Le travail suivant a donc consisté à mettre en évidence l'existence d'une concentration cellulaire optimale pour l'obtention d'un taux de fécondité élevé.

II.2.2 Matériel et méthode

Deux clones sexuellement compatibles, NCC 141 (40 μ m) et NCC 136 (42 μ m), tous deux isolés à partir d'échantillons prélevés en Baie de Bourgneuf, ont été croisés à des concentrations initiales allant de 500 cellules mL⁻¹ à 12000 cellules mL⁻¹ (concentration finale). Les cultures mères ont été maintenues en phase exponentielle par culture en mode semi continu, avec remplacement régulier du milieu de culture après homogénéisation par un dispositif constitué d'un barreau aimanté avec un agitateur magnétique. Le milieu utilisé fut de l'eau de mer artificielle (artificial sea water, ASW) modifiée (Harrison *et al.*, 1980 ; de Brouwer *et al.*, 2002 ; Mouget *et al.*, 2009), dont la composition est donnée en annexe. Les cultures mères ont été placées dans des caissons en bois aux parois recouvertes d'adhésif blanc réfléchissant, illuminées par des tubes fluorescents de type Philips TLD 36W/965. L'irradiance mesurée avec un quantamètre Li-Cor LI-189 couplé avec une sonde 2II Li-Cor Q21284 était de 60 mmol photons m⁻² s⁻¹. La photopériode, fixée par un minuteur, était de 14h/10h lumière/obscurité. Les

algues furent déposées dans des plaques de culture cellulaire de 24 puits, chacune des 6 concentrations utilisées étant réitérée dans 4 puits, dont le volume final était de 2 mL. Les plaques de culture eurent leurs bords entourés de parafilm afin d'éviter toute dessiccation. Elles furent ensuite déposées dans un caisson identique au précédent, avec un dispositif d'éclairage dispensant un éclairage de $28 \text{ mmol photons m}^{-2} \text{ s}^{-1}$ répartis selon 6h/18h lumière/obscurité. Après une semaine, une évaluation de la fécondité est effectuée. Elle se fait en inspectant avec un microscope inversé Nikon TS100 au grossissement x 40 quarante champs optiques par concentrations initiales testées. Le nombre de formes sexuées (gamétocystes, zygotes, auxospores et cellules initiales) est évalué, et ramené au nombre total de cellules observées dans le champ optique. Ce résultat est exprimé sous forme d'un pourcentage, calculé sur la base de trois réplicats d'expériences.

II.2.3 Résultats

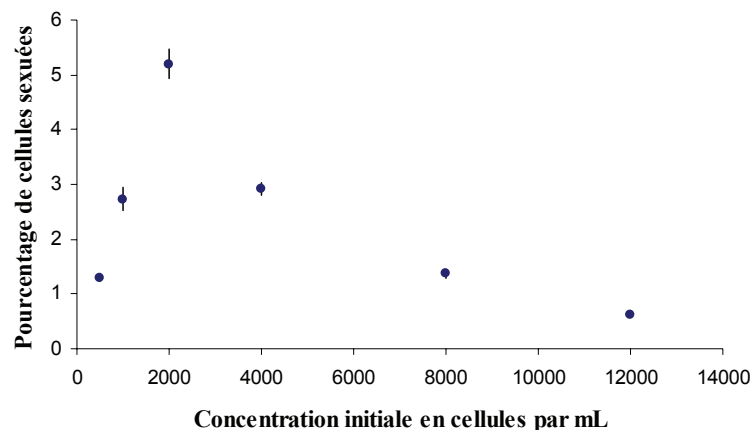


Figure 10. La fécondité des croisements entre les clones NCC 141 et NCC 136 en fonction de la concentration cellulaire (moyenne \pm erreur standard, n=3).

La figure 10 montre un optimum de fécondité supérieur à 5% de cellules sexuées dans les champs optiques observés qui est atteint avec une concentration initiale en cellules de 2000 cellules mL⁻¹.

II.2.4 Discussion

En dessous de la concentration cellulaire optimale mesurée, il est possible que la probabilité de rencontre entre les diatomées soit trop faible pour que se fasse l'appariement entre cellules parentales compatibles. Au dessus de cette concentration, le tapis algal se faisant plus dense, il est possible qu'il y ait dérangement mécanique s'opérant sur les cellules en voies d'appariement, empêchant l'auxosporulation de se faire. Une autre hypothèse a été avancée, faisant intervenir des phéromones. Chez les macroalgues brunes, l'existence de phéromones est un phénomène bien documenté, mis en évidence depuis le milieu du 19^{ème} siècle (Thuret, 1854 ; de Reviers, 2002). Différentes phéromones, du type de celles présentes chez les macroalgues brunes, ont déjà été identifiées chez des diatomées centriques (Derenbach et Pesando, 1986 ; Pohnert et Boland, 1996 ; Hombeck et Boland, 1998). Ces phéromones pourraient être en quantité insuffisante en dessous de l'optimum précité, tandis qu'au dessus, elles pourraient être en quantité saturante, et donc inhibitrice, perturbant un éventuel chimiotactisme des cellules parentales, ou saturant leurs récepteurs.

II.3. La reproduction sexuée chez *H. ostrearia* : l'impact du pré-conditionnement lumineux

II.3.1 Introduction

Dans leur article, Mouget *et al.* (2009) ont étudié l'effet sur la fécondité de différents traitements lumineux appliqués lors de la reproduction. Toutefois, une seule condition lumineuse fut utilisée pour la croissance cellulaire des cultures mères. Le but des expériences suivantes fut d'étudier l'effet de divers préconditionnements lumineux sur la fécondité des croisements.

II.3.2 Matériel et méthodes

Trois lots d'un même couple de clones compatibles, NCC 234.1 et NCC 148.78, sont cultivés dans des erlenmeyers de 250 mL avec 150 mL de milieu ASW. Ces cultures sont maintenues en phase exponentielle par renouvellement régulier du milieu de culture et prélèvement d'une partie de la biomasse. Chaque lot de couple de clones est soumis à un traitement lumineux de photopériode 14h/10h lumière/obscurité, et avec trois intensités lumineuses différentes : 25, 100¹ et 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Les couples de même préconditionnement sont mis en contact dans une plaque unique de culture cellulaire de 24 puits. Cette plaque est placée dans un caisson en bois aux parois recouvertes d'adhésif blanc, avec un dispositif lumineux dispensant un éclairage de 28 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ répartis 6h/18h lumière/obscurité. Après une semaine, les signes d'auxosporulation sont recherchés, et une estimation de la fécondité est réalisée de la

même manière que décrit précédemment. Ces expériences sont réitérées sur cinq réplicats. Une moyenne des taux de fécondité est réalisée.

II.3.3 Résultats

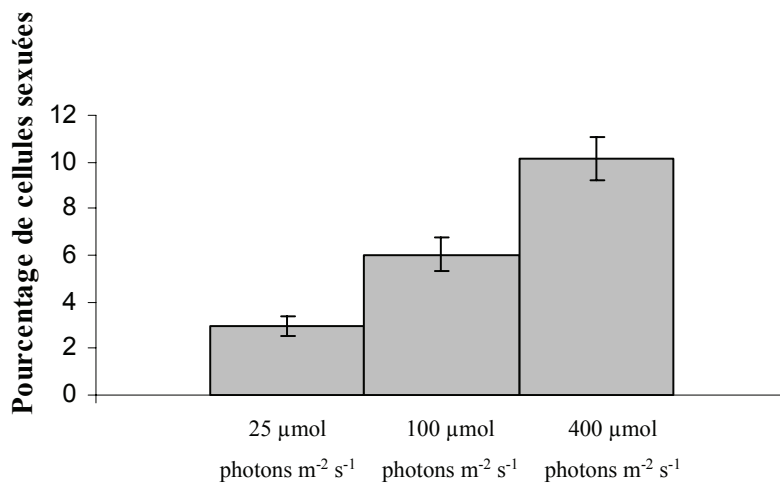


Figure 11. Le taux de fécondité des croisements entre les clones NCC 234.1 et NCC 148.78 en fonction du préconditionnement lumineux (moyenne \pm erreur standard, n=5).

La figure 11 montre que le taux de fécondité des croisements est d'autant plus important que les algues ont été conditionnées à un fort niveau d'éclairement.

II.3.4 Discussion

Dans la publication citée précédemment, Derenbach et Pesando (1986) utilisent un choc lumineux pour induire la reproduction sexuée chez les diatomées centriques *Lithodesmium undulatum* et *Skeletonema costatum*, aboutissant à la la sécrétion

d'ectocarpène, une phéromone présente chez les macroalgues brunes. Peut-être existe-t-il un mécanisme chez les diatomées permettant la synthèse massive de phéromones lors de conditions lumineuses stressantes, telle un changement brutal de luminosité. Auquel cas, selon les résultats ici obtenus, l'importance de cette synthèse serait proportionnelle à l'ampleur de la variation de lumière occasionnée. Toutefois, chez *H. ostrearia*, les mécanismes reproductifs diffèrent par trop de ceux des diatomées centriques évoquées pour que ces dernières soient prises comme modèle. En effet, Derenbach et Pesando (1986) stipulent bien que le traitement lumineux a eu pour effet le déclenchement de l'ovogénèse. Aucune gamétogénèse n'a jamais été observée dans des cultures monoclonales d'*H. ostrearia* utilisées comme témoins dans diverses expérimentations sur l'effet du préconditionnement lumineux.

II.4. La reproduction sexuée chez *H. ostrearia* : effet de lumières qualitativement différentes

Le rôle de la qualité lumineuse dans le déclenchement de l'auxosporulation a été peu étudié. Les expériences suivantes ont eu pour but de rechercher un éventuel impact de lumières de longueur d'onde différentes sur le déclenchement de l'auxosporulation chez *H. ostrearia*.

II.4.1 Effet de lumières monochromatiques

II.4.1 a Matériel et méthode

Le couple compatible formé des clones NCC 158 et NCC 234 a été cultivé en Erlenmeyers de 250 mL avec 150 mL de milieu ASW. Les cultures ont été déposées dans les caissons en bois avec adhésif blanc sur les parois. Les conditions lumineuses de croissance des cultures étaient une lumière d'une intensité de $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ distribuée 14h/10h lumière/obscurité. Les cultures sont maintenues en phase exponentielle par renouvellement du milieu après homogénéisation. Les clones sont ensuite mis en contact dans des plaques de culture cellulaire de 24 puits à une concentration globale de $2000 \text{ cellules mL}^{-1}$, pour un volume final de 2 mL. Huit puits dans la partie centrale des plaques sont ainsiensemencés. Quatre plaques de ce type sont réalisées. Trois de ces plaques sont recouvertes par des filtres de marque Lee Filters dont la transmittance, mesurée avec un spectrophotomètre Spekol 1100, est donnée ci après.

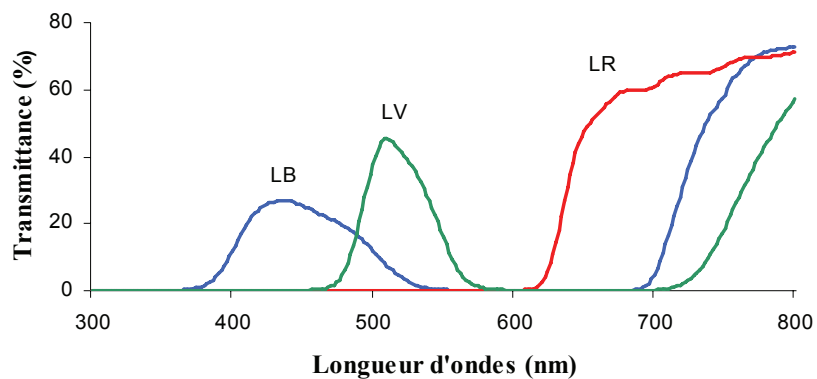


Figure 12. Spectre des transmittances des trois filtres Lee Filters utilisés au cours des expériences.

Les plaques sont déposées dans deux caissons différents, un dont le dispositif lumineux permet d'obtenir une illumination de $28 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ distribuée 6h/18h lumière/obscurité, l'autre ayant un dispositif lumineux avec une source de

lumière plus intense, permettant d'obtenir cette même intensité lumineuse de 28 mmol photons $m^{-2} s^{-1}$ tout en tenant compte de l'absorption occasionnée par les filtres. La photopériode utilisée est la même que dans l'autre caisson. La plaque de culture cellulaire non recouverte de filtre sert de témoin et est placée dans le premier caisson. Les trois autres sont placées dans le deuxième caisson. Les plaques y sont déposées au début de la phase obscure et laissées une semaine avant estimation de la fécondité suivant la méthode décrite précédemment. Les expériences sont réalisées en triplicat.

II.4.1 b Résultats

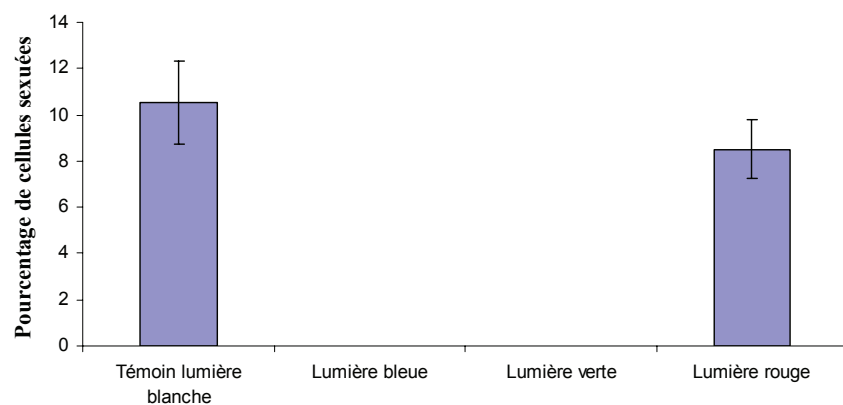


Figure 13. Le taux de fécondité des croisements entre les clones NCC 158 et NCC 234 en fonction du préconditionnement lumineux (moyenne \pm erreur Standard, $n=3$).

Le graphique (figure 13) montre qu'il n'y a pas eu d'auxoporation quand les lumières monochromatiques utilisées furent bleues ou vertes. En lumière rouge, un taux de fécondité approchant celui observé avec le témoin en lumière blanche est obtenu.

II.4.2 Effet de quantités croissantes de radiations dans la zone rouge proche infrarouge

II.4.2 a Matériel et méthode

Le même protocole que celui utilisé précédemment a été reconduit avec une série de filtres ayant une transmittance dans la zone du rouge clair qui va croissante. Les transmittances de ces filtres sont données ci-après.

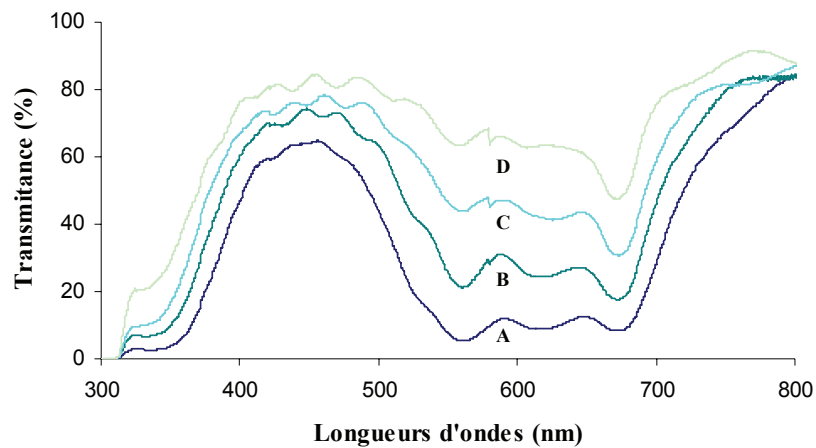


Figure 14. Spectre de transmittance des filtres Lee Filters utilisés.

Un témoin est réalisé en utilisant une cinquième plaque de culture avec expériences de croisement en lumière blanche. Les taux de fécondité sont mesurés après une semaine sous le dispositif lumineux. Ces expériences sont réalisées en trois réplicats. Les résultats sont exprimés en termes de fécondité relative. La fécondité de chacune des conditions lumineuses avec filtres est ramenée en un pourcentage calculé par rapport à la fécondité des expériences témoins.

II.4.2 b Résultats

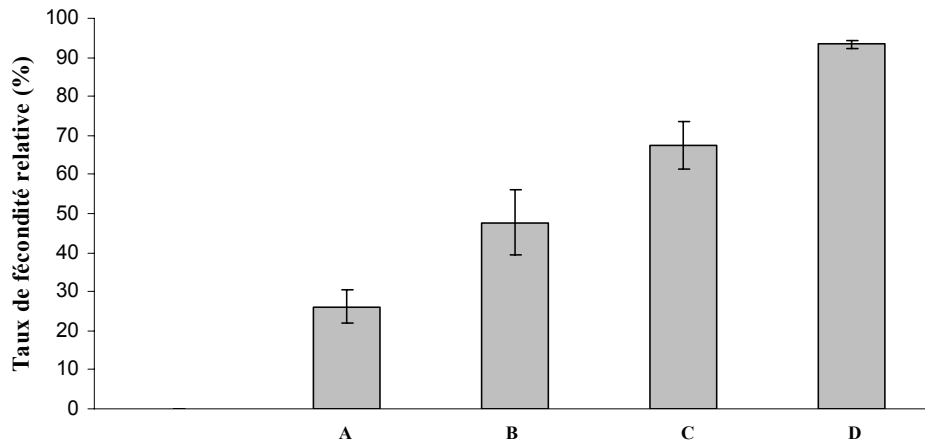


Figure 15. La fécondité relative des croisements entre les clones NCC 158 et NCC 234 en fonction de la concentration cellulaire (moyenne \pm erreur standard, $n=3$).

Le graphique ci-dessus (figure 15) montre l'augmentation du taux de fécondité relative en fonction de l'augmentation de la transmittance des filtres utilisés dans la zone du rouge proche.

II.4.3 Discussion

Il ressort des séries d'expérimentation précédentes que la qualité lumineuse a un effet sur la reproduction sexuée d'*H. ostrearia*. La présence de radiations de longueurs d'onde appartenant au domaine du rouge clair semble favoriser l'auxosporulation. Dans cette série d'expériences, l'absence de ces radiations a toujours inhibé l'apparition de formes sexuées. Le taux de fécondité relative augmente quand la quantité de radiations dans le domaine du rouge proche augmente. Une des explications possible de ces

résultats pourrait être l'intervention d'un photorécepteur, comme par exemple un phytochrome. L'effet de la lumière monochromatique sur la reproduction des diatomées a été peu étudié. Dans une publication en allemand, Baatz (1941) a décrit un effet potentiellement inhibiteur de lumières monochromatiques bleues, vertes et rouges, sur l'apparition de grandes cellules dans les cultures de *Chaetoceros didymus* et ce en comparaison d'un traitement en lumière blanche. Ces cellules de grande taille pourraient être assimilées à des cellules initiales ou bien des auxospores. Un effet d'induction de l'auxosporulation de type phytochrome-like aurait été décrit par Ren et al. (2005) chez la diatomée centrique *Stephanopyxis palmeriana*. Il existe une littérature plus abondante sur les effets de lumière monochromatiques sur d'autres phénomènes biologiques chez les diatomées. Pour *H. ostrearia*, les publications déjà citées (Mouget et al., 2004 ; Mouget et al., 2005) ont mis en évidence des effets sur la croissance ainsi que la physiologie de certaines lumières monochromatiques. L'effet inhibiteur sur la croissance de *Ditylum brightwelli* de flashes de lumière sombre avait été évoqué dès 1973 par Lipps (1973). Le mouvement de certaines espèces de diatomées benthiques peut être stimulé par certaines longueurs d'ondes du visible (McLachlan et al., 2009). Certains gènes chez *Thalassiosira weissflogii* sont régulés par l'alternance rouge proche / rouge lointain (Leblanc et al., 1999), ce qui a suggéré aux auteurs l'action d'un système de type phytochrome. Chez *Thalassiosira pseudonana*, des gènes codant potentiellement pour les parties protéiques de phytochromes ont été découverts (Armbrust et al., 2004).

Quant au volet reproductif, l'implication de photorécepteurs a été supposée ou bien mise en évidence chez nombre d'organismes différents. Les lumières monochromatiques entraînent la libération de gamètes chez la macro-algue brune

Silvetia compressa (Pearson *et al.*, 2004), tandis que chez l'ascomycète *Aspergillus nidulans*, le développement sexuel est inhibé par l'action de la lumière rouge sur un phytochrome (Blumenstein *et al.*, 2005).

Les différents phytochromes de certains végétaux peuvent agir de façon synergique ou antagonistes avec d'autres photorécepteurs pour contrôler un grand nombre de réactions ou phénomènes biologiques différents (Casal, 2000). Il est connu, chez les plantes supérieures, que la régulation des rythmes circadiens se fait à l'aide des phytochromes et des cryptochromes (Somers *et al.*, 1998). Les phytochromes jouent un grand rôle dans la perception de la durée des phases lumineuses entraînant la floraison (Weller *et al.*, 1997) ainsi dans les transports ayant lieu entre cytoplasme et noyau (Yamamoto et Deng, 1999). Plusieurs de ces mécanismes pourraient être mis en rapport avec les hypothèses formulées suite aux résultats précédemment acquis. La régulation photopériodique de l'auxosporulation pourrait avoir comme médiateurs un ensemble de photorécepteurs comme des phytochromes. Des photorécepteurs pourraient aussi permettre l'acheminement vers le noyau de différents composés ayant un rôle dans la régulation du cycle cellulaire, entre autre, un signal déclenchant l'entrée en méiose et donc la gamétogénèse.

Toutes les hypothèses présentées au cours de ce chapitre sont à investiguer aux niveaux physiologiques, biochimiques et moléculaires. Néanmoins, les résultats ici acquis serviront plus tard au cours de la thèse, soit dans les vérifications d'interfécondité entre isolats d'*H. ostrearia* et/ou nouvelles espèces, ou bien lors des expériences sur la transmission de l'ADN mitochondrial.

III

Chapitre 2

La diversité biologique

chez les diatomées bleues du genre *Haslea*

III.1 Introduction

La revue bibliographique de la dispersion géographique d'*H. ostrearia* laisse l'impression d'une espèce largement ubiquiste. Ceci doit cependant être nuancé au regard d'au moins un critère, à savoir le niveau technologique des moyens d'identification utilisés ou disponibles lors des observations. L'unique recours au microscope optique rend possible l'enregistrement sous un seul nom d'espèces qui auraient pu différer par l'ornementation de leur frustule, visible dans toute sa complexité uniquement au moyen d'un microscope électronique à balayage. La littérature n'a pas non plus laissé apparaître qu'il y ait eu des études systématiques des pigments bleus de toutes les algues enregistrées comme *N. ostrearia* ou *H. ostrearia*. Enfin, aucun test d'interfécondité ne semble avoir été effectué entre souches d'*H. ostrearia* ou présumées telles d'origines géographiques diverses.

La prise de contact lors de colloques ou sur des forums spécialisés, les commandes à diverses banques de culture algale, des campagnes d'échantillonnage, ont permis la constitution d'une collection de diatomées bleues du genre *Haslea*. Celle-ci contient trois populations différentes d'*H. ostrearia*, provenant de la Baie de Bourgneuf (France), de la Baie de Marennes-Oléron (France) et du Détroit de Cattégat (Suède). En Crimée a été découverte l'espèce *Haslea karadagensis*, qui fait l'objet d'un chapitre sous forme d'article. Aux îles Canaries ont été isolées *Haslea silbo* (présentée elle aussi sous forme d'article) ainsi qu'une autre diatomée (provisoirement nommée *Haslea acoran* et dont la description est encore en cours). Sur la côte française de la Mer Méditerranée, précisément sur les plages de Boulouris, *Haslea provincialis* a été

découverte. C'est la description de cette biodiversité insoupçonnée, ainsi que des lieux de prélèvement de ces diatomées, qui constitue le présent chapitre.

III.2 La Baie de Bourgneuf

L'ancienne « baie de Bretagne » forme un arc sur la façade atlantique de la Vendée. C'est une zone qui est partiellement abritée du milieu océanique par la presqu'île de Noirmoutiers. Dans cette ancienne région de saliculture, l'activité ostréicole est fortement implantée depuis 1956 (<http://museepaysderetz.free.fr/ostreiculture.html>). Les cultures monoclonales d'*H. ostrearia* conservées à l'algothèque du laboratoire MMS Nantes proviennent en majorité d'échantillons prélevés en ce lieu. C'est à partir de ces spécimens qu'ont été menées les expériences sur la reproduction sexuée d'*H. ostrearia* (Davidovich *et al.*, 2009 ; Mouget *et al.*, 2009). La caractérisation de la marennine (Pouvreau, 2006) fut aussi faite sur du pigment extrait de ces cultures.



Figure 16. A : Vue aérienne de la Baie de Bourgneuf. B : L'activité ostréicole à Bourgneuf, claires pour l'affinage.

III.3 La Baie de Marennes-Oléron

Le bassin ostréicole de Marennes-Oléron, en Charente-Maritime, est constitué d'anciens marais salants utilisés comme claires, c'est-à-dire comme bassins d'affinage des huîtres. L'activité ostréicole et d'affinage y est des plus anciennes et attestée dès le moyen-âge, alors que la région était connue comme gisement d'huîtres depuis la fin de l'antiquité (consigné dans les correspondances d'Ausone, IV^{ème} siècle après Jésus-Christ). Ces bassins ont fourni en huîtres vertes la table du roi Louis XIV, où elles étaient connues comme « marennes vertes » (Grelon, 1978), et bénéficièrent d'un article dans l'Encyclopédie (Diderot et d'Alembert, 1778). Ces claires constituent donc d'excellents lieux de prélèvement dans le but d'en dériver des cultures d'*H. ostrearia*. Plusieurs souches monoclonales d'*H. ostrearia* en furent isolées à partir de la fin de l'année 2010.



Figure 17. A : vue aérienne de la Baie de Marennes-Oléron. B : Les bassins. C : L'activité ostréicole à Marennes-Oléron, une récolte de fines de claires vertes.

III.4 Le détroit de Cattégat en Suède

Le détroit de Cattégat est situé entre le Danemark et la Suède. Il constitue le point de séparation entre la mer du Nord et la mer Baltique. C'est un milieu fortement anthropisé, qui a fortement souffert de la pollution, notamment de l'eutrophisation due aux engrais agricoles (Håkanson, 2009). C'est aussi un milieu possédant un fort gradient de salinité, à la fois suivant la proximité par rapport à la mer du Nord ou la mer Baltique, mais aussi en fonction de la profondeur dans la colonne d'eau (Håkanson, 2009). *H. ostrearia* a été repérée en Mer du Nord en 1955 par Cleve-Euler (Cleve-Euler, 1955). Des huîtres verdies ont été trouvées au Danemark (Petersen, 1916), bien que l'auteur eût minimisé la participation des populations d'*H. ostrearia* observées dans ce phénomène. *H. ostrearia* est aussi présente en Mer Baltique (Snoeijs et Kasperovičienė, 1996) dans la côte ouest de la Suède, près de Göteborg (Aleem, 1973) ainsi que dans l'estuaire de l'Älv, rivière qui se jette dans le détroit de Cattégat (Kuylenstierna, 1990). Des clones d'*H. ostrearia* prélevés sur ce site ont été utilisés pour des études physiologiques par le Dr. Angela Wulff (Wulff *et al.*, 2000), qui est à l'origine des prélèvements effectués de nouveau dans cette zone dans le courant de l'été 2009. Ces échantillons phytoplanctoniques ont montré contenir des diatomées aux apex pigmentés. La microscopie électronique a permis de montrer l'appartenance de ces diatomées à l'espèce *H. ostrearia*, ce qui sera corroboré par la biologie moléculaire comme décrit dans un chapitre ultérieur. Plusieurs des cultures monoclonales dérivées de ces échantillonnages serviront lors des expériences sur la transmission d'un gène mitochondrial au cours de l'auxosporulation.

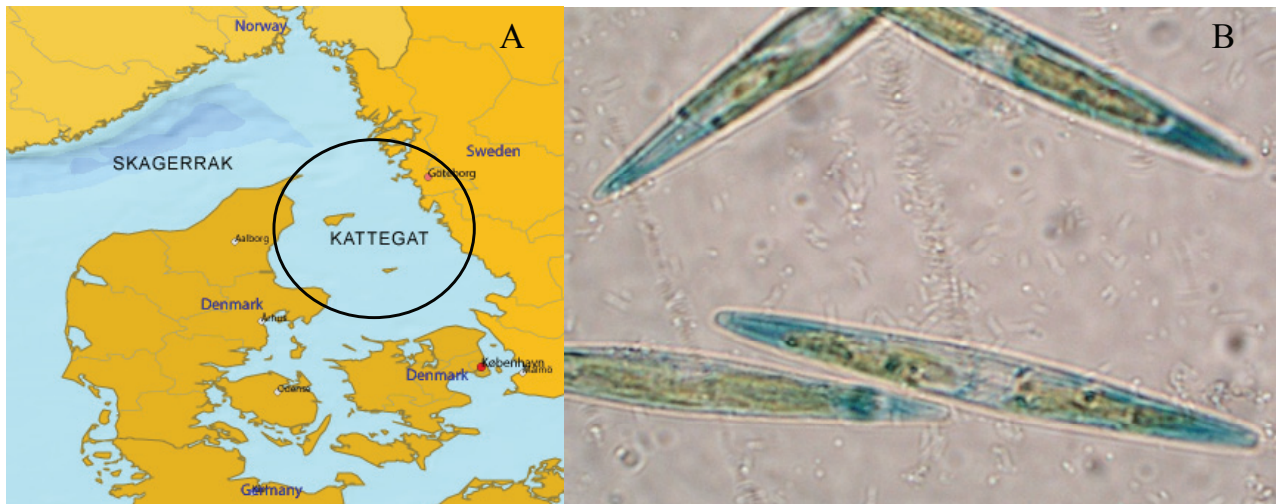


Figure 18. A : carte du Déroit de Cattégat (entouré en noir). B : photo au microscope optique d'*H. ostrearia* en provenance du Cattégat.

III. 5 La Mer Noire et *Haslea karadagensis*

III.5.1 Introduction

La réserve naturelle de Karadag est sise sur la côte sud de la Crimée, république autonome rattachée à l'Ukraine. Elle est constituée d'un massif montagneux, le Karadag, signifiant en Tatar « montagne noire », qui se jette abruptement dans la mer. Ce massif a pour origine un volcan inactif du jurassique culminant à 575 mètres. La nature géologique particulière de ce sol a permis le développement d'une flore unique en Crimée. Le site de Karadag est classé comme réserve naturelle et protégé depuis 1979.

Une première nouvelle espèce de diatomée bleue a été isolée en avril 2008 sur les côtes de la station biologique de Karadag, en Crimée, à partir de biofilms

épilithiques formés sur des galets par des diatomées aux apex bleu-gris sombres. Au cours d'une première campagne de prélèvement en mai 2008, un certain nombre de clones de cette algue ont été isolés puis mis en culture, en association avec le Dr. Nicolai A. Davidovich.

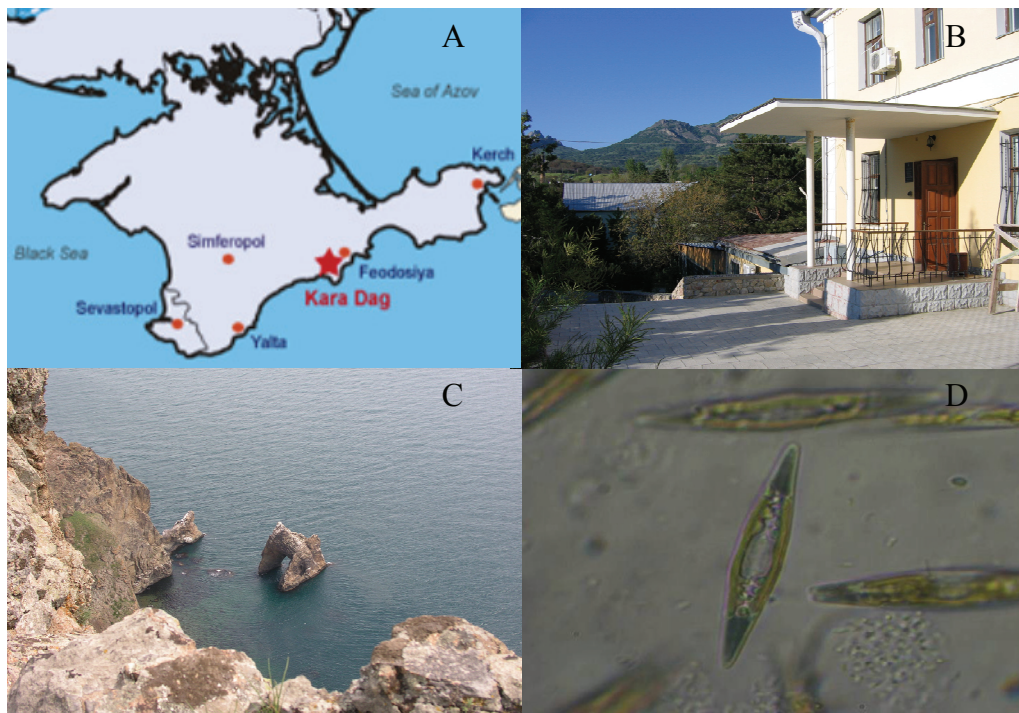


Figure 19. A : Localisation de la réserve de Karadag. B : La station biologique. C : Le site naturel de Karadag. D : La diatomée *H. karadagensis*.

Comme stipulé dans la revue de littérature, une diatomée avait déjà été identifiée comme *H. ostrearia* en Mer Noire par Proshkina-Lavrenko (1964). Les caractéristiques de cette diatomée ont été étudiées à l'aide de plusieurs techniques et méthodes. Tout d'abord, par une approche morphologique, qui a montré que cette diatomée différait de celle consignée par Proshkina-Lavrenko et ne se rattachait pas à l'espèce *H. ostrearia*. A cette même occasion, une biométrie des populations naturelles de cette diatomée a été

réalisée, montrant des extrema de taille différents de ceux observés chez *H. ostrearia*. Enfin, l'utilisation de la spectrométrie Raman a montré que le pigment contenu dans les apex de cette diatomée a un signal Raman différent de celui de la marennine d'*H. ostrearia in vivo*. Ceci a conduit à la description d'une nouvelle espèce de diatomée, *H. karadagensis*. Cette diatomée nouvellement décrite fut la première distincte d'*H. ostrearia* à présenter des apex bleutés.

Ces travaux ont donné lieu à une publication, envoyée à la revue European Journal of Phycology, qui est présentée dans les pages suivantes.

III.5.2 *Haslea karadagensis* sp. nov. (Bacillariophyta), a new diatom
from the Black Sea producing a novel blue pigment

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Running title: *Haslea karadagensis*, a new species of blue diatom

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Abstract

A new species of raphid pennate diatom producing a blue pigment, *Haslea karadagensis* sp. nov., was recently isolated from the Crimean coast of the Black Sea. This organism is very similar to the well-known *Haslea ostrearia*, the first ‘blue’ diatom ever described, which produces marennine, the pigment involved in the greening of oysters. The Ukrainian diatom, *H. karadagensis* sp. nov., differs slightly from *H. ostrearia* in the structure of its frustule, and the two organisms are unable to interbreed. UV-visible spectrophotometry and *in vivo* confocal micro-Raman spectroscopy were used to compare the pigment of *H. karadagensis* sp. nov. with marennine. Both pigments showed absorption bands in the UV and red regions, but positions of the maxima differ between pigments. Significant differences were observed by micro-Raman spectroscopy in the 1000-1700 cm^{-1} wavenumber range, revealing that the pigments are different molecules. *Haslea karadagensis* sp. nov. is the first report of a new ‘blue’ diatom producing a novel blue pigment.

Key words: Bacillariophyta, Black Sea, *Haslea*, marennine-like pigment, micro-Raman spectroscopy, new species

Introduction

The raphid pennate marine diatom, *Haslea ostrearia* (Gaillon) Simonsen, is a tychopelagic/benthic organism, known for its ability to produce a water-soluble blue pigment, commonly called marennine, which accumulates predominantly in the apical regions of the cells. In oyster ponds of western France, where *H. ostrearia* can regularly become dominant, marennine is released into the seawater and is responsible for the 'greening' of oysters. This phenomenon, which has a marked economic impact locally, has long been known (Sprat, 1669). Similarly, the involvement of a microscopic alga was long recognised; *H. ostrearia* was mentioned in the study of green oysters, first described as *Vibrio ostrearius* (Gaillon, 1820; Dyer, 1877), then as *Navicula ostrearia*, and finally transferred to the genus *Haslea* (Simonsen, 1974). The name 'marennine' originates from the Marennes-Oléron region (Lankester, 1886), an area long dedicated to bivalve production in south-western France.

Over the past two centuries, diatoms with blue apices have been observed on all French coasts, from the Channel to the Mediterranean Sea, and ascribed to *H. ostrearia* (reviewed by Neuville & Daste, 1978). They have also been reported from the western Mediterranean Sea: in France near Banyuls (Sauvageau, 1906) and Boulouris (Gert Hansen, Scandinavian Culture Collection of Algae & Protozoa, Copenhagen); in Italy, in Sardinia (Solazzi & Tolomio, 1976; Tolomio, 1978) and in Sicily, in the harbour of Palermo (Barone, 1979) and the north-western coast (Rossella Barone, pers. comm.). 'Blue' diatoms have been reported from the Adriatic Sea (Molisch, 1903), from the Suez canal (Madkour *et al.*, 2007), and from the Black Sea, in north Caucasus (Proschkina-Lavrenko, 1963).

Diatoms with blue tips, recorded as *H. ostrearia*, have also been reported from almost all seas and oceans, both northern and southern hemispheres, either from direct observation or by a greening effect of bivalves. In north-western Europe, this includes the coasts of Great Britain (Sprat, 1669; Hustedt & Aleem, 1951), Belgium (M'harzi *et al.*, 1998), Norway (Hendey, 1964), Sweden (Wulff *et al.*, 2000), Denmark (Limfjord, Petersen, 1916), and in the Baltic Sea (Snoeijs and Kasperovičienė, 1996). In northern Atlantic Ocean, they have been found in the Canary Islands (SCCAP, Gert Hansen), and different places along the North American coast: North Carolina (Beaufort) and Rhode Island (Naragansett Bay) (Kennett and Hargraves, 1991; Gary Wickfors, pers. comm.); Virginia (Chesapeake Bay, Mitchell & Barney, 1918); New Jersey (Sullivan, 1977); Florida (Biscayne Bay, Julian Sprung, pers. com.); and possibly in Honduras (Grunow, 1877). In the northern Pacific Ocean, *H. ostrearia* has been reported from the San Juan Islands, between Washington state and British Columbia (Hardy, 1973), and in Japan (Ranson, 1937). In the southern hemisphere, it was observed in the Indian Ocean (Simonsen, 1974), and reported from northern (Ricard, 1987), eastern (Tasmania, Volkman *et al.*, 1994) and western Australia (Leschenault Estuary, Wasela Hosja, pers. com.). This suggested that the only microalga known to produce a blue pigment, such as marennine, had a worldwide distribution.

In spring 2008, samples taken from the rocky shores of the Karadag Natural Reserve in Crimea (Ukraine), contained diatoms with dark bluish apices. Some of these cells were isolated and different monoclonal strains were cultured under controlled conditions. The protocol used for the induction of the sexual reproduction in *H. ostrearia* (Davidovich *et al.*, 2009; Mouget *et al.*, 2009) was successfully applied to this diatom and auxospores and F1 hybrids were obtained (Davidovich *et al.*, in prep). However, the Ukrainian diatom and *H. ostrearia* have not interbred, thus indicating that

these diatoms could be two different species, but similar enough to belong to the same genus.

The discovery of this Ukrainian diatom raised concerns about the identity of other ‘blue’ diatoms. Most references to *H. ostrearia* occurrences relied on species determinations using light microscopy only. Images from scanning electron microscope were published by Simonsen (1974) to study the frustule of *H. ostrearia*, and to transfer it from *Navicula* to *Haslea*. It is unlikely that all the diatoms recorded worldwide as *Navicula/Haslea ostrearia* have been studied with SEM. Moreover, to the best of our knowledge, no publication has ever dealt with a chemical comparison of the blue pigments of any of the other strains, and the uniqueness of marennine, as the only blue pigment produced by diatoms, has never been questioned.

This paper will first focus on the identification and description of this new diatom, using biometry of the natural populations, light and electronic microscopy for cell and frustule morphology. In addition, UV-visible spectrophotometry, and especially *in vivo* confocal micro-Raman spectroscopy, were used to investigate similarities and differences between the pigments of these two diatoms. As previously reported, Raman spectroscopy can be used to provide a qualitative and quantitative analysis of numerous organic and mineral compounds (Spiro, 1974; Jolivet *et al.*, 2008; Trinkler *et al.*, 2010), and to discriminate between pigments in organisms, such as mollusca (Hedegaard *et al.*, 2006) or corals (Fan and Yang, 2008; Nowak *et al.*, 2008). It has also been used to measure *in vivo* pigment spectra of chlorophylls and carotenoids in the Prymnesiophyceae, Chlorophyceae, Bacillariophyceae and Cyanobacteria (Wu *et al.*, 1998), and to quantify *in vivo* the amount of domoic acid synthesized by the diatom *Pseudonitzschia multiseriata*, for comparison with HPLC results (Wu *et al.*, 2000). We will demonstrate that micro-Raman spectroscopy is a powerful tool for revealing

differences between uncommon biomolecules, such as blue algal pigments. Compared to infra-red spectroscopy, the technique of vibrational spectroscopy has a crucial advantage for samples and biomaterials immersed in water because of its low sensitivity to water content. Furthermore, Raman analyses do not require purified pigment, which in the case of marennine would require large biomass and time-consuming harvesting and purification processes (Pouvreau *et al.*, 2006).

Materials and methods

Algae isolation and culturing

Ukrainian diatoms with blue tips were isolated from epilithic samples collected from the rocky shores of the Black Sea, Crimea, near the Karadag Biological Station (46° 53' 40" N / 35° 12' 05" E), in March 2008. Isolates established from single cells were re-isolated at least five times before being considered monoclonal. For this study, 9 clones (according to the nomenclature of Lakeman *et al.*, 2009), NCC 237 to NCC 244 (Nantes Culture Collection) were derived from natural populations. We used *H. ostrearia* strains NCC 158 and 234, from sexual reproduction in the laboratory, and from the Baie of Bourgneuf in France (46° 59' 19" N / 2° 14' 14" W), respectively. Cultures were grown in Petri dishes or Erlenmeyer flasks containing artificial sea water (Perkins *et al.*, 2006), under controlled conditions (15 ± 1 °C, irradiance of 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 14h/10h L/D cycle).

Light microscopy and biometry

For light microscopy (LM), cells were observed at x40, using either an Olympus CH-2 microscope (Olympus, France) or Biolar PI microscope (PZO, Poland) equipped

with a JVC TK-C1381 camera or PowerShot A95 digital camera accordingly. Biometric measurements were made using the ImageJ software ([http://rsb/info/nih/gov/ij](http://rsb.info.nih.gov/ij)) and a micrometric slide as a scale.

Scanning Electron Microscopy

Cleaned siliceous frustules were prepared for scanning electron microscopy (SEM) by digesting cells in a mixture of sulphuric and oxalic acids in potassium permanganate (Hendey, 1974). Cells were then dried onto aluminium stubs and coated with a thin film of platinum using a JUC5000 sputter coater. SEM observations were then performed with a JEOL 7600F operating at 7 keV incident energy.

UV-visible spectrophotometry

Coloured supernatants of *H. ostrearia* and *H. karadagensis* cultures were simultaneously collected in late exponential growth phase, at the beginning of their photoperiod. Supernatants were first filtered onto 0.45 μm filters, then onto 0.22 μm filters before measurement. The pH values were usually constant (8.0 ± 0.2) and no buffer was required for adjustment. A quartz cell (Hellma) was used for spectrum measurement. Absorbance spectra ranging from 200 nm to 800 nm were recorded using a Thermo electron Helios gamma UV-visible spectrophotometer.

Micro-Raman spectroscopy

For *in vivo* confocal micro-Raman measurements, living algae were placed in Petri dishes. Coloured apices of cells in exponential phase of growth, coloured protoplasts in old cultures, gametes obtained from crossing experiments and cytoplasm were studied using a wavelength of 514.5 nm from a coherent Argon-Krypton ion laser

(Coherent Innova). The Raman spectra were recorded in the back-scattering configuration on a T64000 (Horiba, Jobin Yvon) spectrometer coupled to a liquid-N₂-cooled CCD detector. All measurements were performed at room temperature using a Olympus microscope equipped with a 100x objective to focus the incident laser beam onto the sample and to collect the scattered light. The laser intensity was reduced to less than 0.5 mW at the sample to minimize possible sample deterioration. In order to obtain distinguishable peaks, and Raman spectra with good signal-to-noise ratios, a series of 30 measurements were systematically averaged, with an integration time varying between 10 and 60 s (depending on the samples). The Raman spectra were recorded within the 250-2200 cm⁻¹ wavenumber region and were shifted in the figures (without any baseline correction) for clarity.

Results

Haslea karadagensis sp. nov. Davidovich, Gastineau and Mouget

Latin diagnosis

Valvae lanceolatae hyalinae, poli acuti. Valvae longitudo 28.9-84.0 µm, latitude circiter 4.5-11.2 µm. Raphe directa centrali. Striae non visae per microscopicum luce ; striae transapicales circiter 32 in 10 µm, striae longitudinales circiter 57 in 10 µm.

A Haslea ostrearia simile, sed differt conferto striis transapicalibus et longitudinalibus.

Frustules narrowly rectangular in girdle view. Valves delicate, lanceolate with acute apices, 28.9-84.0 µm long and 4.5-11.2 µm wide. Two band-like plastids, each with several small pyrenoids, lie appressed to the girdle. At each apex, the cell contents appear dark blue in colour throughout the cell cycle. Raphe system central, only faintly

visible in LM. With SEM, the external raphe fissure is seen to be straight, with only very slightly unilaterally deflected, slightly expanded central and apical endings. The external valve surface is covered by longitudinal siliceous strips, parallel to the raphe, merging with a peripheral slit as they abut the valve margin (particularly well seen near the valve apices). Internally, the raphe fissure opens laterally in a slightly thickened rib, except at the apices where it ends in an elongated helictoglossa. A narrow accessory rib runs closely adjacent to the raphe sternum, ending a few micrometres from the apices, where the raphe fissure opening is more lateral and hidden.

The transapical striae are parallel, perpendicular to the raphe throughout the valve, 31.6 ± 0.3 in $10 \mu\text{m}$ (mean \pm SE, $n=31$), comprising rectangular areolae whose longer axes are oriented longitudinally, parallel to the raphe, 57.0 ± 0.5 in $10 \mu\text{m}$ (mean \pm SE, $n=30$). Hymenes were not found, but it is possible that these were removed by the cleaning process. The girdle bands are simple split rings, about $1 \mu\text{m}$ wide throughout, without pores.

Holotype

BM xxxx, National History Museum, London.

Type locality

Type material was collected from the sublittoral zone near the Biological Station ($44^\circ 54' 40'' \text{ N} / 35^\circ 12' 05'' \text{ E}$) of Karadag Natural Reserve, Crimea (Ukraine). Biofilms were collected from stones at a depth of 0.2-0.4 m by N. A. Davidovich, O. I. Davidovich, R. Gastineau, and J-L. Mouget (April and May, 2008). Cells with characteristic blue tips were recorded in the samples taken nearby Karadag Biostation

every spring during the period of observation, starting from 2006. The species was also found in Feodosiya Bay (45° 01' 25" N / 35° 24' 23" E).

Etymology

H. karadagensis sp. nov is named after Karadag, meaning 'black mountain' in the Tatar language, referring to the Jurassic mountain above the shores of this Crimean locality.

Microscopy and biometry measurements

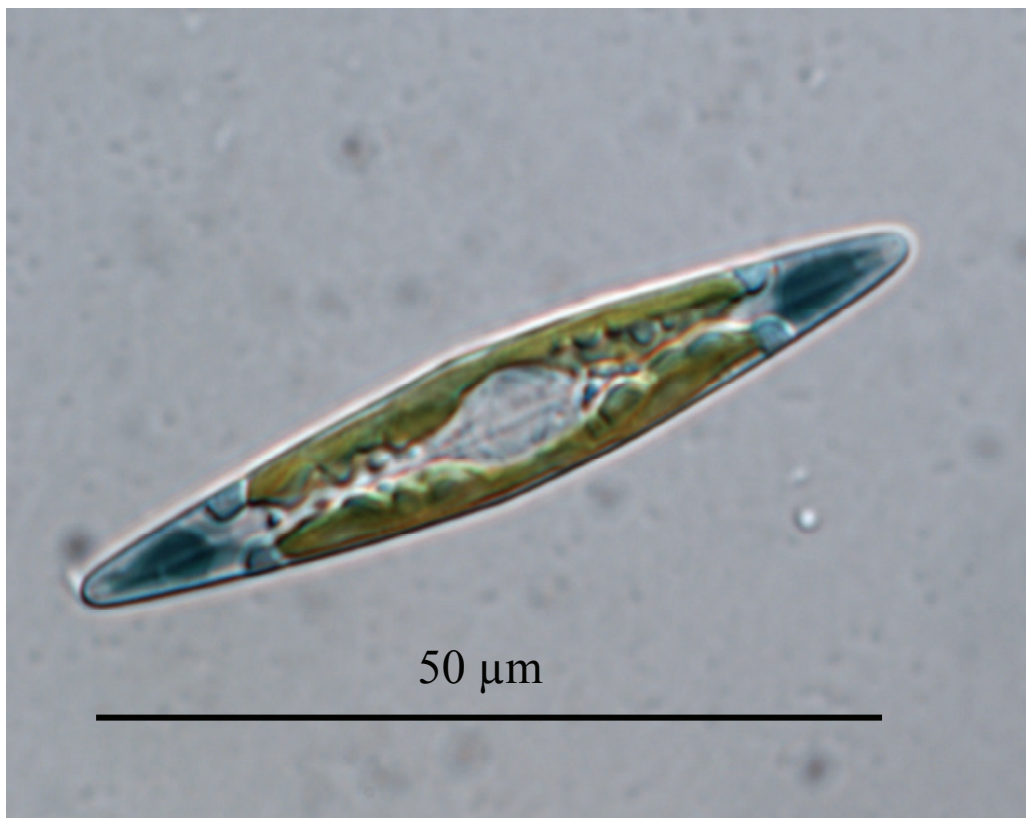
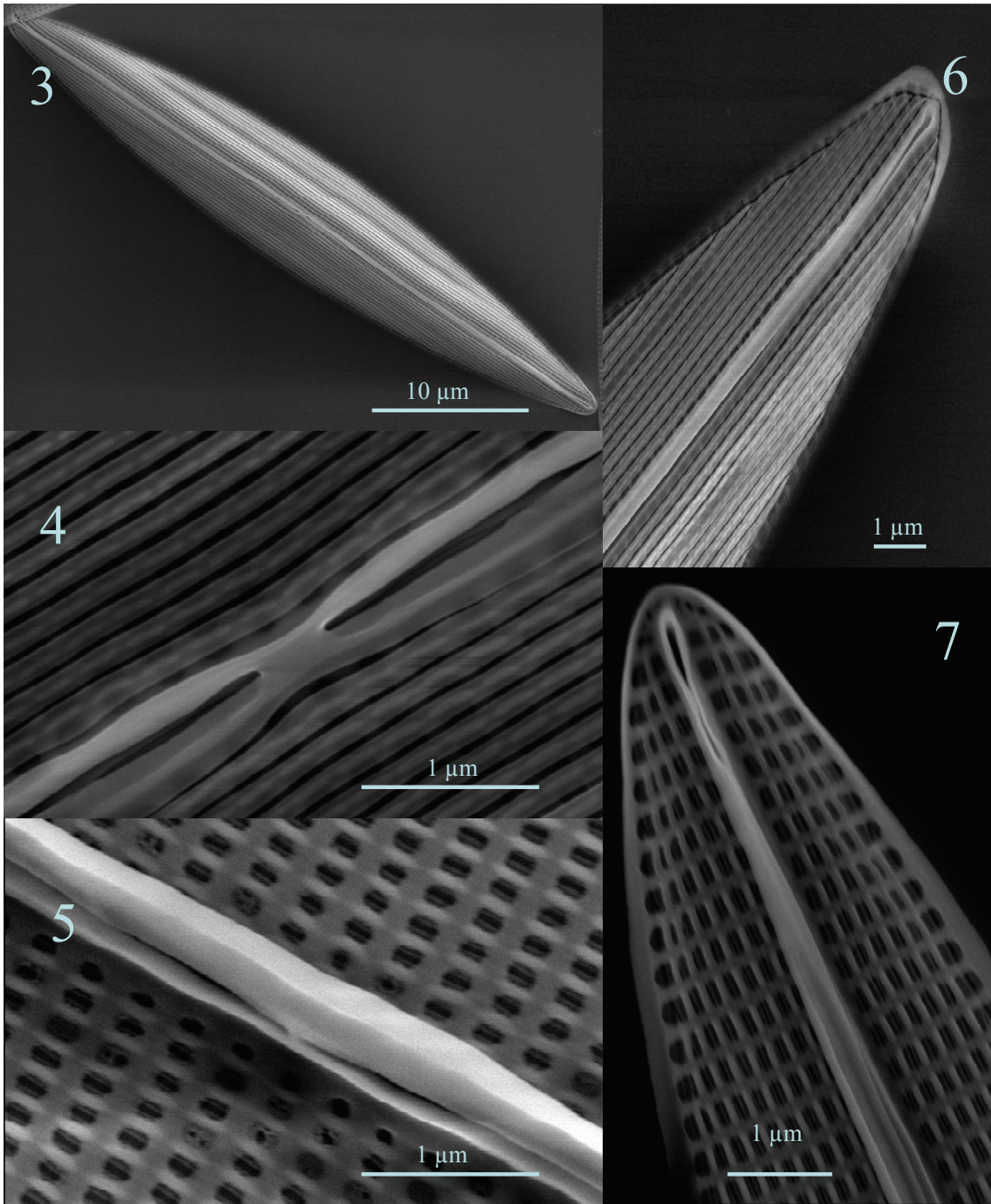


Fig. 1 Live cell of *Haslea karadagensis* sp. nov. under light microscope



Fig. 2 Live cell of *Haslea ostrearia* under light microscope

Figs 1, 2 show live cells of the ‘blue’ diatoms, *H. karadagensis* and *H. ostrearia*, respectively. Apart from the blue apices which are darker in the Ukrainian clones, the two diatoms are very similar. Both have lanceolate valves with two band-like plastids, and the blue colouration is located at the apices. Cells are solitary and free living. The transapical striation of the valve is not resolved with LM.



Figs 3 – 7. Scanning electron micrographs of *Haslea karadagensis* sp. nov. Fig. 3. External view of an entire valve Fig. 4. External view of valve at centre, showing the longitudinal slits and the straight proximal raphe fissures. Fig. 5. Internal view of valve at centre showing the rectangular areolae and the straight proximal raphe ends. Fig. 6.

External view of apex showing the straight distal ending and the longitudinal slits. Fig. 7. Internal view of apex showing the straight helictoglossa and the rectangular areolae.

Under SEM, the external valve surface is covered with parallel, longitudinal strips (Figs 3, 4) overlying the more or less rectangular areolae (Fig. 5), which are probably occluded by hymenes (largely eroded). The proximal raphe fissure endings are very slightly deflected, slightly expanded externally (Fig. 4) and internally (Fig. 5). Externally the polar raphe endings are again very slightly deflected and slightly expanded (Fig. 6), internally they end in an elongate helictoglossa (Fig. 7). Internally, the accessory rib lies very close to the raphe sternum, terminating a few micrometres from the poles where the internal fissure becomes more lateral and hidden (Fig. 7).

Valve morphometrics differ between *H. karadagensis* and *H. ostrearia* (Table 1). The transapical striae are typically 31.6 ± 0.3 (n=31) in *H. karadagensis* sp. nov versus 34.7 ± 0.2 (n=33) in $10 \mu\text{m}$ in *H. ostrearia*; the longitudinal striae are 57.0 ± 0.5 (n=30) and 53.4 ± 0.4 (n=27) in $10 \mu\text{m}$, respectively.

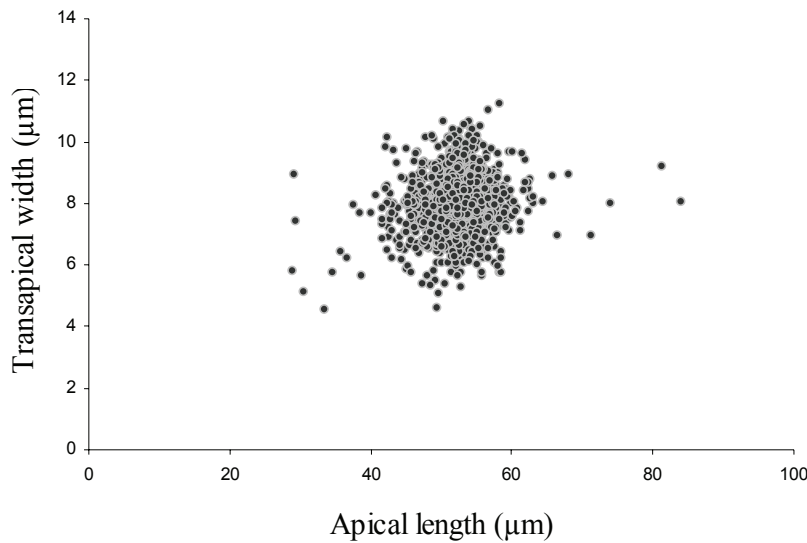


Fig. 8. Distribution of lengths and widths in natural populations of *H. karadagensis* sp. nov. n = 1148 cells from the 10/04/2008 to the 09/05/2008

In situ biometry (Fig. 8) on natural populations of *H. karadagensis* gave an average length of 52.50 ± 0.14 µm and an average width of 8.00 ± 0.03 µm (values are means \pm S.E., n = 1148). The longest cell was 84.0 µm long, and 8.0 µm wide; the smallest 28.9 µm long by 5.8 µm wide. The widest was 11.2 µm for a length of 58.4 µm, the narrowest 4.5 µm for a length of 33.6 µm. *In culture*, of 448 cells measured, the largest cell resulting from sexual reproduction was 96.9 µm long, the smallest at the other end of the life cycle, was 22.0 µm long.

Reproductive behaviour

Possible reproductive compatibility of the two diatoms producing bluish pigments was also studied, by testing heterothallic reproduction (Davidovich *et al.*, 2009). Within each species, crossing experiments between compatible clones under optimal conditions (see Mouget *et al.*, 2009) always resulted in auxospore formation.

Although nine clones of *H. karadagensis* isolated from the Black Sea can mate within the group (Davidovich et al., unpublished), clones of *H. karadagensis* never bred with *H. ostrearia* clones of either sexual type, and no sexual stages (gametes, zygotes, auxospores, initial cells) were ever observed in mixtures of the two organisms. This suggests pre-zygotic isolation between the two populations, which can be interpreted as discrete species. Modalities of sexual reproduction in *H. karadagensis* are under investigation, and more information about auxospore formation, initial cell size, and cardinal points of the life cycle will be described elsewhere (Davidovich et al., in prep).

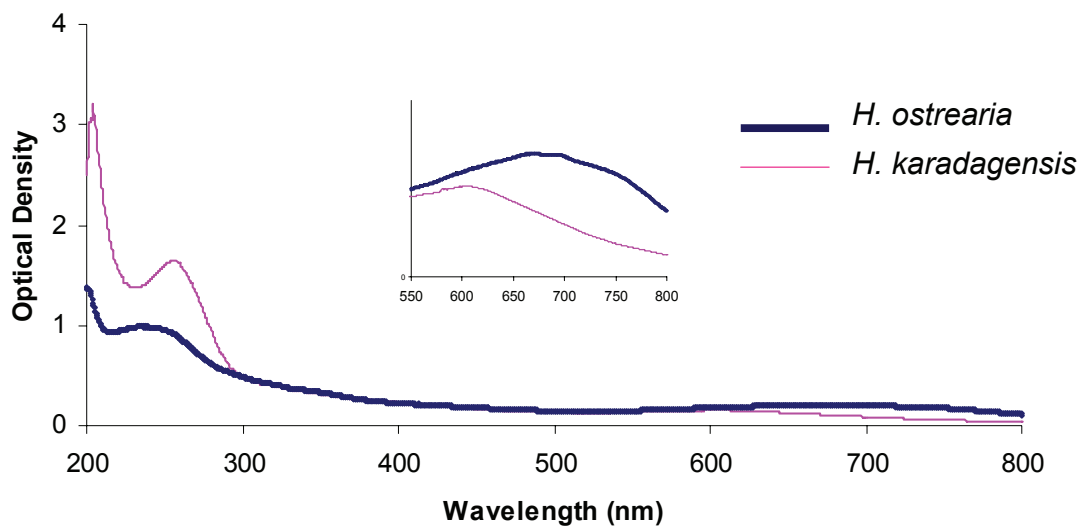


Fig. 9. Absorbance spectra, from 200 to 800 nm, of the pigments released into the medium by *H. ostrearia* and *H. karadagensis* sp. nov. Inset: absorbance spectra, from 550 to 750 nm, of the pigments released into the medium by *H. ostrearia* and by *H. karadagensis* sp. nov.

UV-visible spectrophotometry

UV-visible spectrophotometry showed that the absorption spectra of pigments released into the medium by *H. ostrearia* and *H. karadagensis* are similar, exhibiting a main peak in the UV region, and a secondary peak in the red part of the spectrum. However, there are noticeable differences, as the marennine UV peak is at 247 nm, whereas it is at 260 nm for the pigment from *H. karadagensis* (Fig. 9). Moreover, the visible absorbance peak of marennine occurs at 670 nm, whereas that for the marennine-like pigment of *H. karadagensis* is at 605 nm (inset Fig. 9). Pigment concentrations were not calculated because no reliable method was available.

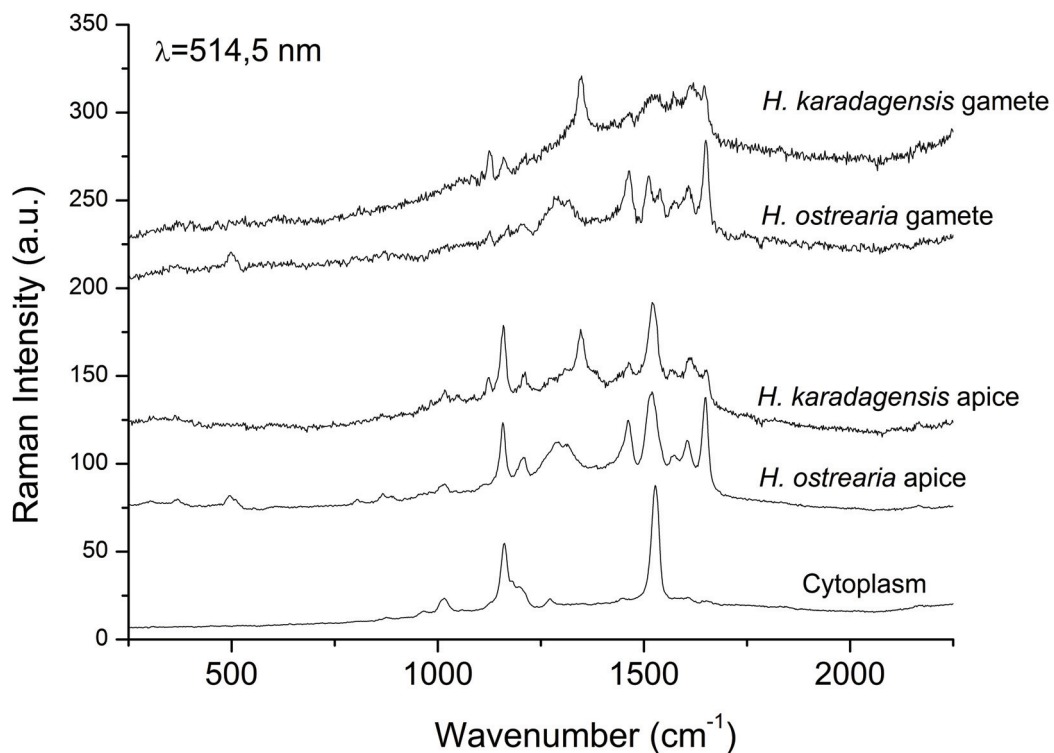


Fig. 10. Raman spectra at 514,5 nm. Haslea-Ukr refers to the pigment from *H. karadagensis* sp. nov. P-Ukr is for the pigment contained in protoplasts obtained from *H. karadagensis* sp. nov. Haslea-Fr refers to the pigment of *H. ostrearia*. P-Fr is for the

pigment contained in the protoplasts obtained from *H. ostrearia*. Cyto is for the cytoplasm.

Micro-Raman spectroscopy

To provide molecular vibrational information on pigments we investigated the living algae in an aqueous environment by confocal Raman spectroscopy. Raman spectra recorded at the centre of the cells, devoid of blue pigment, were identical for both species. Two strong peaks, at 1162 cm^{-1} and 1527 cm^{-1} (Fig. 10), are directly attributed to C-C and C=C stretching vibrations, as already observed in diatoms and other organisms (Saito et al., 1983; Wood *et al.*, 2005; Hedegaard *et al.*, 2006; Heraud *et al.*, 2007). Raman spectra of pigments in the apical regions of vegetative cells of *H. ostrearia* and *H. karadagensis*, and in gametes, are also shown in Fig. 10, and a summary of the main vibrational wavenumbers are listed in Table 2. Two strong peaks, at $1158\text{-}1159\text{ cm}^{-1}$ and $1520\text{-}1522\text{ cm}^{-1}$, are observed in the Raman spectra of both diatoms. The positions of these peaks are similar to those observed for the cytoplasm in both species, suggesting partial light scattering from the cytoplasm during measurement. Raman spectra of *H. ostrearia* apices and gametes showed predominant bands at 1650 and 1462 cm^{-1} , with a broad band at about 1300 cm^{-1} and other weaker bands (Table 2). Raman spectra of *H. karadagensis* apices and gametes (Fig. 10) show an intense band at about 1347 cm^{-1} , two well-resolved bands of medium intensity at 1160 and $1122\text{-}1125\text{ cm}^{-1}$, and several less intense bands.

Discussion

Morphology and biometry support recognition of a new 'blue' diatom

Based on LM and SEM images, the new species, *H. karadagensis*, is morphologically similar to *H. ostrearia* (Figs 1-7, Table 1), but the valve ornamentation of *H. karadagensis* differs from *H. ostrearia* (Peragallo & Peragallo, 1908; Hustedt & Aleen, 1951; Massé et al., 2001), warranting their separation at the species level. Both diatoms produce a peculiar bluish pigment which accumulates at the cell apices, supporting a close taxonomic relationship, within the same genus. Another diatom, *H. pseudostrearia* (Massé et al., 2001) is also very similar in its frustule ultrastructure to *H. ostrearia*, but lacks any blue pigment. Like *H. ostrearia*, *H. karadagensis* and *H. pseudostrearia* lack a pseudo-stauros, or thickened central virgae, which are found in some other diatoms in the genus, such as *H. crucigera* (Round et al., 1990) or *H. salstonica* (Massé et al., 2001). On the other hand (Massé et al. 2001), all these species possess two chloroplasts, one along each side of the cell with numerous pyrenoids, features observed in other species of *Haslea* (Poulin et al., 2004; Cox and Williams, 2006).

The comparison of biometric data from natural populations strengthens the recognition of two species of diatoms in the same genus that are able to produce blue pigments. All the morphological data (Table 1) show higher average lengths for *H. ostrearia* than *H. karadagensis*. Similarly, the lack of reproductive compatibility between the two taxa supports their recognition at the species level.

Raman spectroscopy to discriminate diatom blue pigments and species

In order to discriminate the two species of 'blue' diatoms, their pigment signatures were first recorded *in vitro* by spectrophotometry (external form of the pigments), and *in vivo* by micro-Raman spectroscopy (internal form of the pigments). It has been shown from previous studies (Pouvreau *et al.*, 2006) that the supernatant obtained from cultures of *H. ostrearia* is composed of the external form of marennine with a molecular weight of 9893 ± 1 Da, actively released into the medium, plus some internal form with a molecular weight of 10751 ± 1 Da, released after cell death. Because these authors (Pouvreau *et al.*, 2006) also demonstrated that pH has a bathochromic effect on the absorption spectrum of marennine, we excluded possible artefacts by controlling the pH of the supernatant before measurement. At pH 8.0, both absorption peaks, in UV and visible regions, differed between filtered supernatants from the two diatom cultures. The position of the absorbance peak in the visible region of *H. karadagensis* pigment differs from that of marennine at the same pH. This allows us to hypothesize that these pigments comprise different molecules, belonging to the same chemical family whose structure remains unknown.

To extend our study of *H. ostrearia* and *H. karadagensis*, we performed the first Raman spectroscopic investigations on their blue pigments. The spectral data reveal a high degree of similarity in the 1000-1700 cm^{-1} wave-number range, since most of the vibrational bands are present in both spectra. Nevertheless, it is worth noting that some features allow the two pigments to be easily distinguished. Indeed, it appears that the most intense Raman spectrum bands of *H. ostrearia* are not those observed for *H. karadagensis*. Although the chemical structures of the pigments have not yet been determined, preliminary molecular information can be deduced from Raman spectra.

The 1650 cm^{-1} band could be assigned to the C=C stretching mode of a phenyl ring, and the 1462 cm^{-1} band to a C=O or C=N stretching mode (Johnson *et al.*, 1996; Varsanyi, 1974). Furthermore, the Raman spectra for cell apices and gametes of *H. karadagensis* only, show a strong characteristic band at 1347-1348 cm^{-1} . Full assignment of the observed bands is difficult but our data reveal that the composition and/or the conformational structure of both pigments are different. We have shown that Raman spectroscopy is appropriate for distinguishing between pigments that are probably a complex polycyclic carbide structure containing oxygen and nitrogen (Pouveau *et al.*, 2006). Further work is in progress to clarify the attribution of these Raman-active modes.

In conclusion, this work demonstrates that the new diatom, *H. karadagensis*, isolated from the Black Sea, may be considered the second bluish pigment-producing diatom species after *H. ostrearia*, which has long been considered the only organism able to produce marennine. This raises some other questions. Firstly, was the diatom with bluish apices noticed by Proshkina-Lavrenko (1963) in the Black Sea *H. ostrearia* or *H. karadagensis*? The transapical stria density was 33-36 in 10 μm , and lengths ranged from 50 μm to 81 μm , with a width of 4.5 μm to 7 μm in the diatom observed by Proshkina-Lavrenko. Based on these data, the diatom seen by Proshkina-Lavrenko could be *H. ostrearia*. If so, at least two species of 'blue' diatoms, *H. ostrearia* and *H. karadagensis*, co-exist in the Black Sea. Secondly, the diatom described by Hendey (1964) as *H. ostrearia* had a different transapical stria density to that commonly attributed to *H. ostrearia*, but closer to *H. karadagensis*. Lastly, every record of a diatom with blue tips in seas and oceans, as *H. ostrearia*, without in depth morphological investigations, should be reconsidered, bearing in mind modern

approaches to species determination and for the study of diatoms with blue apices. With this in mind, various other strains of 'blue' diatoms have been obtained from different parts of the world, and are currently under investigation in our laboratory.

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III.5.3 Conclusion et perspectives

Suite à la description de cette nouvelle espèce, des expériences ont été menées en vue d'étudier le phénomène d'auxosporulation chez cette dernière. Il a d'abord été confirmé qu'il n'existe aucune compatibilité sexuelle entre *H. ostrearia* et *H. karadagensis*. La découverte de couples de clones compatibles au sein de l'espèce *H. karadagensis* a permis de caractériser ses modalités reproductives, similaires à celles d'*H. ostrearia* selon la classification de Geitler (1973). Ces résultats font l'objet d'une publication (Davidovich et al., soumis) à la rédaction de laquelle l'auteur du présent manuscrit est associé. *H. karadagensis* sera la quatrième diatomée du genre *Haslea* à voir décrits ses mécanismes reproductifs, après *H. crucigera* (Von Karsten, 1897), *H. subagnita* (Chepurnov, 1993) et *H. ostrearia* (Neuville et Daste, 1975 ; Neuville et Daste, 1979 ; Davidovich *et al.*, 2009). La connaissance et la maîtrise du phénomène reproductif d'*H. karadagensis* seront d'un grand secours dans la continuation du travail de thèse, que cela soit pour vérifier son éventuelle aptitude à la reproduction avec les espèces ultérieurement découvertes, ou afin d'obtenir de nouveaux clones issus de cellules initiales de grande taille. Ces clones seront mis en culture, et survivront à la mort de ceux isolés et ayant été utilisés dans la publication. Ce sont ces clones de première génération qui serviront tout à la fois dans la partie phylogénie de cette étude comme pour la production de pigment.

III.6. L'île de la Gomera et *Haslea silbo*

III.6.1 Introduction

Les îles Canaries, communautés autonomes espagnoles, forment un archipel volcanique de l'océan Atlantique, distinct de la plaque continentale africaine. Cet ensemble d'îles occupe une superficie de 7447 km². L'archipel se trouve à une distance du continent, en l'occurrence du Sahara occidental, d'approximativement 150 km. Au sein des îles Canaries, l'île de la Gomera occupe l'une des places les plus modestes avec ses 372 km². Elle est peu peuplée, et abrite un parc naturel protégé (Garajonay).

Sur les rivages de cette île, des prélèvements contenant des diatomées aux apex colorés en bleu ont été faits en février 2009. Des cultures monoclonales en ont été dérivées au début du mois de mars 2009 par le docteur Gert Hansen, responsable de la « Scandinavian Culture Collection of Algae and Protozoa » (SCCAP) à Copenhague.

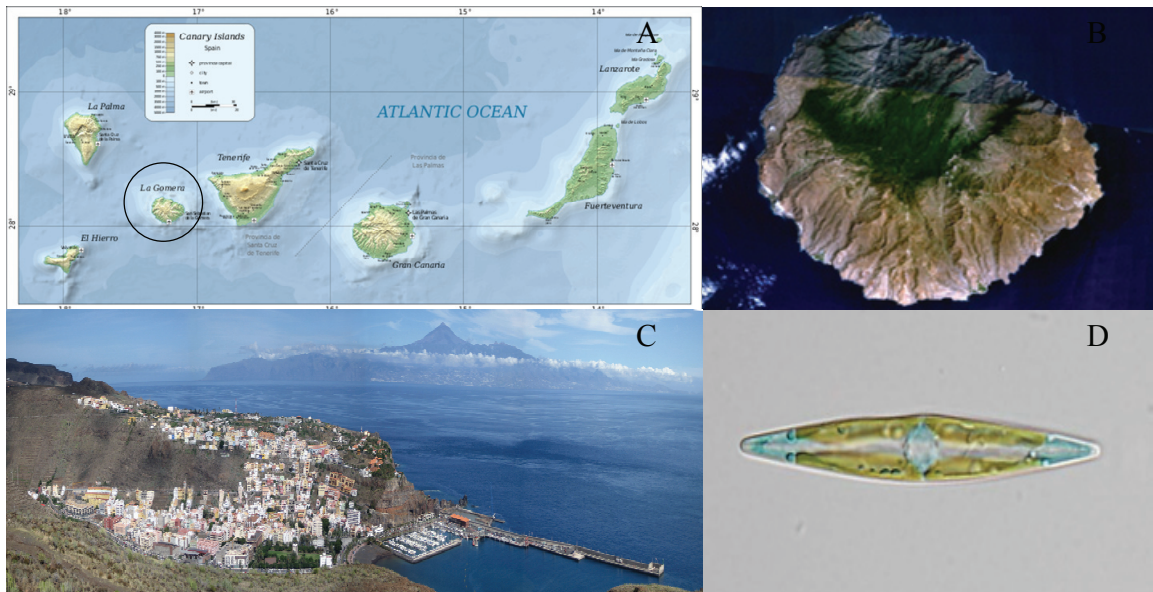


Figure 20. A : carte de l'archipel des Canaries, entourée en noir, l'île de la Gomera. B : vue satellite de l'île de la Gomera. C : vue panoramique de la ville de San Sebastián. D : la diatomée *H. silbo*.

Au laboratoire MMS Le Mans, nous avons pu, en partenariat avec le Dr. Hansen, démontrer que cette diatomée appartenait à une espèce encore non décrite. La méthodologie utilisée lors de la description d'*H. karadagensis* a été de nouveau suivie, et augmentée de nouvelles techniques. Pour cette diatomée, une extraction/purification de pigment a été réalisée en collaboration avec le laboratoire MMS Nantes, et une première approche moléculaire a été mise en place. Morphologiquement, cette diatomée s'est révélée être différente d'*H. ostrearia* pour ce qui est de la densité d'aréoles observables sur son frustule. La forme de ces aréoles est aussi apparue comme plus carrée que celles d'*H. ostrearia*. S'il ne fut pas possible d'obtenir des formes de reproduction par croisements des sept clones de provenance canarienne entre eux, il n'a été davantage possible d'observer une quelconque inter-fécondité entre cette diatomée et *H. ostrearia* ou *H. karadagensis*. L'approche moléculaire, basée sur le séquençage de

la cassette ribosomale ITS1-5.8S-ITS2, a montré que l'homologie entre *H. ostrearia* et cette diatomée était de 60% pour le fragment considéré, homologie comparable à celle existant entre *H. ostrearia* et *H. karadagensis* pour le même marqueur moléculaire. La purification du pigment de la diatomée canarienne aura permis de comparer ses caractéristiques physico-chimiques avec celles de la marennine. Il ressort que les deux pigments sont très proches. Toutefois, là où la marennine présente une forme extracellulaire dont les signatures en spectrométrie UV-visible et Raman diffèrent clairement de celles de la forme intracellulaire, les deux extraits pigmentaires obtenus avec la diatomée canarienne présentent les mêmes signatures, similaires à celles de la marennine interne. Il semblerait qu'il n'y ait aucune excrétion active du pigment, couplée à un clivage de la molécule initiale. Pouvreau (2006) a montré que le poids moléculaire de la marennine externe est inférieur, et a suggéré la possibilité d'une lyse d'une partie de la molécule. Chez la diatomée canarienne, le pigment ne serait relâché dans le milieu qu'à l'occasion de la mort cellulaire ou de division mitotiques, ou bien l'algue posséderait bien des voies d'excrétion active, mais ne faisant pas intervenir un quelconque clivage de la molécule.

Ces résultats ont conduit à la rédaction d'une publication, à destination de la revue *Journal of Phycology*, et décrivant cette diatomée comme une espèce nouvelle du genre *Haslea*, *Haslea silbo* sp. nov. Le nom d'espèce *silbo* fut attribué en référence à une particularité culturelle et linguistique de l'île de la Gomera. Cette publication est présentée ci-après.

III.6.2 *Haslea silbo* sp. nov. (Bacillariophyta), a new diatom from the Canary Islands producing a marennine-like pigment evidenced by spectrometric methods

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ABSTRACT

Haslea silbo sp. nov. is a new species of diatom, originated from the Canary Islands, and singularized by its ability to produce a blue pigment. Despite sharing this peculiarity with the well-known diatom *Haslea ostrearia*, the one that produces the blue pigment called “marennine”, *H. silbo* sp. nov. mainly differs from it under many features. Biometrics and morphological analyses showed the differences in frustule’s ornamentation density and shape. No sexual inter compatibility has ever been observed when crossing strains of both species. Genetic divergence, considering the ribosomal box ITS1-5.8S-ITS2 sequences, has found to be about 40%. *In vitro* Raman spectrometry and UV-visible spectrophotometry done with purified blue pigments showed the strong similarities between them. However, whereas *H. ostrearia* is able to produce an external form of the pigment different from the internal one, this do not seems to be the case with *H. silbo* sp. nov. Indeed, the pigment released in the external medium by *H. silbo* sp. nov. has physical properties identical to those of *H. silbo* sp. nov.’s internal pigment and *H. ostrearia*’s internal marennine, but clearly different from *H. ostrearia*’s external marennine, as demonstrated by *in vitro* Raman spectrometry.

KEYWORDS: algal pigment, blue diatoms, Canary Islands, novel species, marennine, Raman spectrometry,

INTRODUCTION

The marine pennate diatom *Haslea ostrearia* (Gaillon) Simonsen is tagged by the specific blue pigment called marennine it has the capacity to produce. This organism was observed on oyster shells and first described as *Vibrio ostrearius* by Gaillon (1820), who hypothesized the microalga and its pigment played a central role in the greening of oysters. This phenomenon has long been known, as it was previously noticed by Sprat (1669), in the area of Colchester (U.K.), and later experimentally demonstrated by Dyer (1877) and Lankester (1886). Indeed, in the laboratory, marennine produced by *H. ostrearia* during growth is released and accumulates in the culture medium. In oyster ponds, when *H. ostrearia* outcompetes other microalgae, marennine is released in the seawater and filtered by the bivalves, the gills of which turn green. This phenomenon has a noticeable local economical impact (added value to the oysters which benefit from a red label, the so-called ‘vertes de claire’ or ‘fines de claire verte’, claire referring to the oyster pond), especially in the Marennes-Oléron Bay, an area dedicated to bivalve production in south-western France.

Such a diatom with blue apices is easily noticed when present in a phytoplanktonic sample, as well as the greening of bivalves, and both observations have been recorded many times, elsewhere in northern and southern seas and oceans (for a recent review, Gastineau et al, submitted). According to the literature *H. ostrearia* has a cosmopolitan distribution, and its presence was deduced either directly from observation of diatoms with bluish apices, or indirectly from a greening effect of bivalves. A careful identification, if any, of blue diatoms at the species level did not rely on detailed studies of morphology and morphometrics using Scanning Electron Microscopy (SEM) technique, rather on observations with light microscope, despite this

equipment has proven to be insufficient to observe clearly the ornamentations of the frustules, which may be very informative and used for specific classification, even in absence of data on the sexual behaviour (Mann, 1999). For example of a possible fuzzy identification, a blue diatom once collected in Honduras was referred to as *Navicula fusiformis* var. *ostrearia* (Grünow, 1877). However, morphological data showed similarities in frustule striations with *H. ostrearia*, identified as *Navicula ostrearia* at that time, which argued for a same species, as proposed by different authors (Sauvageau, 1906, Ranson, 1951, Hendey, 1964), although none of these works allowed to discard the hypothesis these two blue diatoms were two different species.

Our knowledge about the biodiversity of blue diatoms recently increased with the description of a new species of *Haslea* which produces a marennine-like pigment, collected in the Black Sea, and named *Haslea karadagensis* (Gastineau et al., submitted). This diatom is morphologically similar to *H. ostrearia*, but has revealed to be completely isolated in its reproductive ability. Moreover, the pigment produced by *H. karadagensis* has been shown to significantly differ from marennine regarding its spectroscopic characteristics. Confocal Raman micro-spectrometry, which is a non-contact and non-destructive optical technique, has already proven to be one of the most powerful and versatile tool for detecting and distinguishing quantitative differences in chemical composition in various materials including organic and inorganic compounds (Brame et al. 1976, Lin-Vien et al. 1991, Jolivet et al. 2008, Trinkler et al. 2010). Raman spectroscopy has also been shown to be a useful in situ method for rapid discrimination of pigments in organisms like mollusca (Hedegaard et al., 2006), corals (Fan et al. 2008, Nowak et al. 2008) and algae (Brahma et al. 1983, Wu et al. 1998, Gastineau et al., submitted). Recently, this technique allowed us to characterize *in vivo*

the two blue pigments and to distinguish clearly marennine produced by *H. ostrearia* from the pigment produced by *H. karadagensis*.

Diatoms with blue apices were obtained from natural phytoplanktonic samples collected in Canary Islands. Preliminary observations demonstrated that the Canarian blue diatom displayed differences in the frustule morphology in comparison with both *H. ostrearia* and *H. karadagensis*, and that its pigment presented *in-vivo* close similarities with marennine, as evidenced by spectrophotometry. The aim of the present work was thus to describe the new species of blue diatom originating from the Canary Islands, hereafter named *H. silbo* sp. nov., using both a morphological approach based on the characteristics of the frustule, a molecular investigation analyzing the variability of the ribosomal DNA (ITS1-5.8S-ITS2 region) and a biological approach resulting from interbreeding experiments with the two other blue diatom species, *H. ostrearia* and *H. karadagensis*. The present work will also emphasize the information retrieved from the blue pigments, extracted and purified according to Pouvreau et al. (2006), used as possible taxonomic markers, despite the fact their chemical structure is still unknown

MATERIALS AND METHODS

Algae isolation and culture

Phytoplanktonic samples were collected in La Gomera (Canary Islands, Spain) (28° 6' xx" N / 17° 6' yy" W), in the second half of February 2009. Clones of *Haslea silbo* sp. nov. were isolated on the beginning of March 2009 (SCCAP, Copenhagen). The *H. ostrearia* clone NCC 345 was obtained from phytoplanktonic samples collected

in the Bay of Marennes-Oléron on the 22nd September, 2010 in the oyster-ponds of M. Bonnain (46°12'12"N/1°11'43"W), and isolated early October 2010. The *H. ostrearia* clone NCC 148.75 was obtained in laboratory following interbreeding of strains originating from the Bay of Bourgneuf (46°59'51"N/2°01'50"W). The strain of *H. crucigera* NCC 185 was isolated from “l'Etier du Pouliguen”. The strain of *H. karadagensis* NCC 313 is an offspring obtained after mating experiments conducted in January 2009 on a couple of clones (NCC237.1 and 241.1) derived from natural populations (sampling point at 46°53'33"N/2°07'59"W). All cultures were grown in 250 mL Erlenmeyer flasks containing 150 mL of an artificial sea water (ASW) modified according to MOUGET *et al.* (2009), under controlled conditions (15±1 °C, irradiance of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 14/10 L/D cycle). Illumination was provided by fluorescent tubes Philips TLD 36W/965 and light level measured with a Li-Cor LI-189 quantum meter coupled with a 2II Li-Cor Q21284 quantum sensor.

Induction of auxosporulation and reproductive behaviour

The study of the interbreeding ability of the Canarian blue diatom followed the method described in Mouget *et al.* (2009). One couple of sexually compatible clones of *H. ostrearia* (NCC 158.4 and NCC 148.78) and three strains of *Haslea silbo* sp. nov (SCCAP 1283, SCCAP 1286 and SCCAP 1287) were grown as described above. Cultures were maintained in exponential phase of growth by periodic dilution with fresh medium. Cell density of cultures was estimated using a Nageotte counting cell. Mixtures of parental cultures were made in sterile polystyrene Petri dishes, at a concentration of 2000 cellules mL⁻¹, a concentration which is optimal for the triggering of auxosporulation in *H. ostrearia* (Gastineau, unpublished results). Plates were

surrounded by parafilm against desiccation. Mixtures were made according to the crossing chess presented in Table 3, and were placed in a closed box whose lightening equipment produced an irradiance of $28 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ under a 6h/18h L/D cycle. Experiments were run in triplicates.

Microscopy

For Scanning Electron Microscopy (SEM), living material has been digested in a mixture of sulphuric and oxalic acids in potassium permanganate (HENDEY, 1964). Cells were dried onto aluminium and coated with platinum with a JUC5000, then pictures were take with a JEOL 7600F.

Biometrics

SEM pictures were taken on the *H. ostrearia* NCC 148 clone and the *H. silbo* sp. nov SCCAP 1289 clone, whose sizes were respectively $79.6 \pm 0.5 \mu\text{m}$ and 72.7 ± 0.3 (mean \pm S.E., n = 5) μm . Measurements were made using the ImageJ software ([http://rsb/info/nih/gov/ij](http://rsb.info.nih.gov/ij)). In complement with the striation of the frustule, the geometry of the apices of the two species was compared as follows. Calculation of apical angles was made by creating isosceles triangles, with the upper point originating at the end of a cell apex, as described in Figure 1. A series of 7 angles corresponding to different distances from the cell apex were used to compare the geometry of the apices of *H. ostrearia* and *H. silbo* sp. nov.

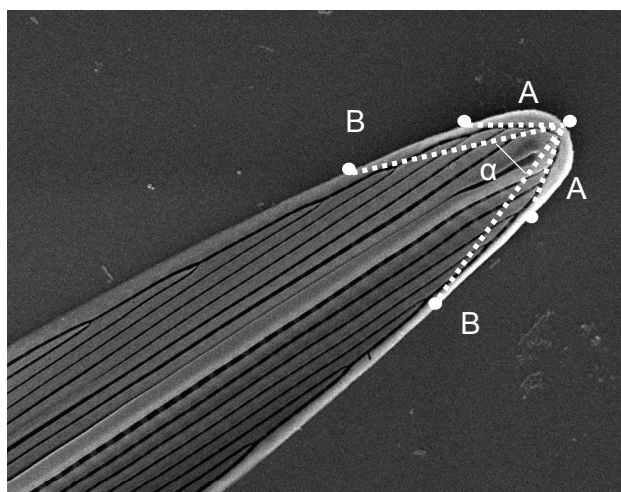


Fig. 1. Method used for the calculation of the angles formed at the apices

Pigment extraction and purification

Cultures were harvested weekly after homogenization. Three-quarters of the flasks were usually collected, containing both coloured supernatant and cell suspension. Cells were separated from the supernatant by gentle centrifugation (10 min, 900 g, slow acceleration/deceleration, 4°C), using a Sigma 3K15 and a rotor n°11133 (Bioblock Scientific). An amount of 24 g fresh weight of biomass and 20 L of blue supernatant from *Haslea silbo* sp. nov. were thus collected. Pigments were purified using Pouvreau et al. (2006b) protocole. Cells were grounded in liquid nitrogen, by mortar and pestle, in 250 mmol L⁻¹ NH₄HCO₃ buffer, pH 8. The crude extract obtained was then centrifuged, and the supernatant collected. The 20 L of coloured supernatant were filtered on a 0.45 µm filter. Then, two ultrafiltrations were performed. Cartridges fitted with regenerated cellulose spiral membranes (Prep/Scale-TFF cartridge 0.23 m², Millipore) were used

with first a 30 kDa cut-off membrane and then a 3kDa cut-off membrane. At the end of the process, the retentate was collected. Pigment was then separated by anion-exchange chromatography, using an UV-visible detector to identify the fraction, according to its optic properties. The fraction collected was dialysed and freeze-dried. Purified pigments were stored in the dark at -20°C. The same method systematically applied to algal pasta and supernatant from various cultures of *H. ostrearia* originated from the Bay of Bourgneuf (France) grown in the Nantes Culture Collection.

Raman spectrometry

For reliable *in-vivo* analyses using micro-Raman spectrometry, living diatoms were placed in Petri dishes a few hours before measurements. A 514.5 nm wavelength radiation from a coherent spectrum argon/krypton ion laser was selected to provide a good signal/noise ratio. The laser beam was focused onto the coloured apices of diatoms (at a power less than 0.1mW at the specimen) lying into the Petri dishes using a microscope equipped with a $\times 100$ objective (average spot diameter 0.8 μm , spatial resolution about 1–2 μm). The Raman spectra were recorded in the back-scattering configuration on a confocal T64000 (Horiba, Jobin Yvon) spectrometer coupled to a liquid-N₂-cooled CCD detector. In order to obtain distinguishable peaks and reliable data, *in vivo* Raman spectra on a series of about 10 diatoms were systematically collected and averaged with an integration time varying between 6s and 10 s (depending on the samples).

For *in-vitro* Raman spectrometry, 200-500 μg of freeze-dried pigments of both intracellular and extracellular forms of each species were dissolved in 0.25 M Na_2HPO_4 / NaH_2PO_4 buffer, pH 7, then the mixture was placed on a glass slide and dried at room temperature. The laser beam was focused at the pellets on the slides, where pigments were at high concentration. Raman spectra were collected at different sample points on purified blue pigments and averaged with an integration time varying between 200s and 600s and an excitation power approximately less than 0.3 mW at the sample. All Raman spectra were recorded within the wavenumber region $250\text{-}2250\text{ cm}^{-1}$ and were shifted in the figures (without any baseline correction) for clarity.

UV-visible spectrophotometry

Purified pigments were dissolved in distilled water, at a concentration of 10 mg mL^{-1} . A series of flasks containing 100 mL of 0.25 M Na_2HPO_4 / NaH_2PO_4 buffer, were prepared, with pH ranging from 2 to 12. Buffers were filtered on 0.22 μm membrane using syringe. A mixture of 50 μL of buffer and 50 μL of pigment solution was made in Hellma quartz cell. Mixture was shake using parafilm. Measurements were done using a Thermo electron Helios gamma UV-visible spectrophotometer. Blank was made with buffer solution. The spectra were recorded on computer, ranging from 220 nm to 800 nm.

Molecular biology

Monoclonal cultures of the clones SCCAP 1283 (*H. silbo* sp. nov), NCC 345 (from Marennes-Oléron), NCC 148.75 (Bourgneuf), *H. karadagensis* strain NCC 313 and *H. crucigera* NCC 185 were harvested by gentle centrifugation, using the same equipment and process as described above for biomass collection. Algal biomass was grounded by mortar and pestle after freezing in liquid nitrogen. DNA was extracted using a Qiagen DNeasy Plant mini Kit. The ribosomal box ITS1-5.8S-ITS2 was amplified using universal primers ITS-1 and ITS-4 (White et al. 1990). PCR mediated amplification of gDNA (100ng) was performed with 0.2 μ M of primers ITS 1: 5'-TCCGTAGGTGAACCTGCGG-3' (Forward) and ITS 2: 5'-TCCTCCGCTTATTGATATGC-3' (Reverse), and finally 2.5 U of GoTaq® Flexi DNA Polymerase (Promega, France). For a final volume of 25 μ L, mixtures were done with 1X green GoTaq® flexi buffer, 2.5 mM of MgCl₂ 25 mM, and 200 μ M of PCR nucleotide mix (Promega, France). Thirty six cycles of XX s denaturation at 94 °C, XX min annealing at XX °C and XX min extension at 72 °C were performed. PCR products were separated by agarose gel electrophoresis. UV revelation was made using a CN-1000 Darkroom (Vilber Lourmat). Bands of interests were eluted with a Wizard® SV Gel and PCR Clean-up System by Promega (France). DNA was integrated into pGEM® T-Easy Vector (Promega, France). Competent *Escherichia coli* cells NEB-5-alpha (New-England Biolabs) were transformed with the recombined vector. Positive colonies were identified by white/blue selection. The sequencing reactions were carried out by Beckmann Coulter Genomics (United-Kingdom, <http://www.cogenicsonline.com>) using SP6 and T7-promoters primers. Homology of the sequences with already-known diatoms'ITS was requested with BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>),

alignment was made using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and visualised using Genedoc software (<http://www.nrbsc.org/gfx/genedoc/>).

RESULTS

Haslea silbo sp. nov. *Gastineau, Hansen and Mouget*

Latin diagnosis

Valvae lanceolatae hyalinae, poli acuti. Valvae longitudo 72.7 μm , latitude circiter 4.5-10.4 μm . Raphe directa centrali. Striae non visae per microscopicum luce ; striae transapicales circiter 33.0 in 10 μm , striae longitudinales circiter 33.6 in 10 μm .

A Haslea ostrearia simile, sed differt conferto striis transapicalibus et longitudinalibus.

Frustules narrowly rectangular. Valves lanceolate with sub acute apices, 72.7 μm long and 10.4 μm wide. Two band-like plastids are lying appressed on each side of the cell (Figure 2). Apical area at each pole of every cell appears in a blue colour that remains all along the cell cycle. Raphe system central, only weakly discernable in LM. The raphe is straight with straight central and apical endings, no extension of the raphe sternum forming central or apical nodules. Raphe fissure on a siliceous rib as wide as two of the longitudinal strips. Central and apical endings slightly expanded. Internally, raphe fissure opens laterally except at the apices where it ends in helictoglossa. Externally the valves look covered by longitudinal strips, separated by slits parallel to the raphe, merging with a peripheral slit near the apices. Internally, the areolae are squared poroids not occluded by hymens. Transapical striation 33.0 in 10 μm , transversal striation 33.6 in 10 μm .

Holotype

A sample of the corresponding clonal culture is deposited at the National History Museum, London (UBM xxxx), and at the University of Copenhagen, Denmark, strain number SCCAP xx.

Type locality

The material was collected in La Gomera, Canary Islands, on February 2009, and clones were isolated in Copenhagen, early March 2009.

Etymology

The species name *silbo* comes from the name of a whistled language ‘spoken’ by the Guanches, native inhabitants of Berber origin of the Canary Islands, and still in use today in La Gomera.

Microscopy and biometry measurements



Fig. 2. Light microscopy view of *H. silbo* sp. nov. from the Scandinavian Culture Collection for Algae and Protozoa.

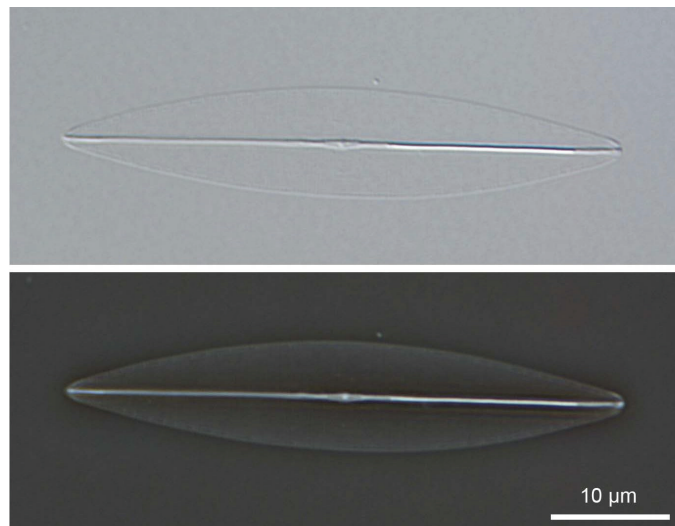


Fig. 3. Light microscope views of an empty frustule of *H. silbo* sp. nov.

Cells are naviculoid and lanceolate, apices appear sub-acute. They do not form any mucilage, or colony. Cells are solitary and free living, and they contain two band-

like plastids and blue apices (Figure 2). In comparison with *H. ostrearia*, cells of *H. silbo* sp. nov. look more ‘stocky’. The ratio of length to width measured on a same cell was 7.0 ± 0.2 (mean \pm S.E., n=5), as compared to 11.7 ± 0.4 (mean \pm S.E., n=5) in *H. ostrearia*, for clones exhibiting a similar cell length ($79.6 \pm 0.5 \mu\text{m}$, mean \pm S.E., n=5). LM does not allow to resolve the transapical striation of the frustule (Figure 3).

In SEM, the external valve is covered with parallel and longitudinal slits (Figure 4A). Proximal raphe fissure is straight (Figure 4A). Areolae are square shaped (Figures 4C to 4E). Helictoglossa is straight in external (Figure 4B) and internal (Figures 4D and 4E) sides of the frustule. Average length was $72.7 \pm 0.3 \mu\text{m}$ (mean \pm S.E., n = 5) and average width was $10.4 \pm 0.2 \mu\text{m}$ (mean \pm S.E., n = 5). Typically the transapical and transversal striae are 33.0 ± 0.3 and 33.6 ± 0.7 in $10 \mu\text{m}$, respectively (mean \pm S.E., n = 10). There may be an accessory rib, as suggested by Figures 4C and 4E.

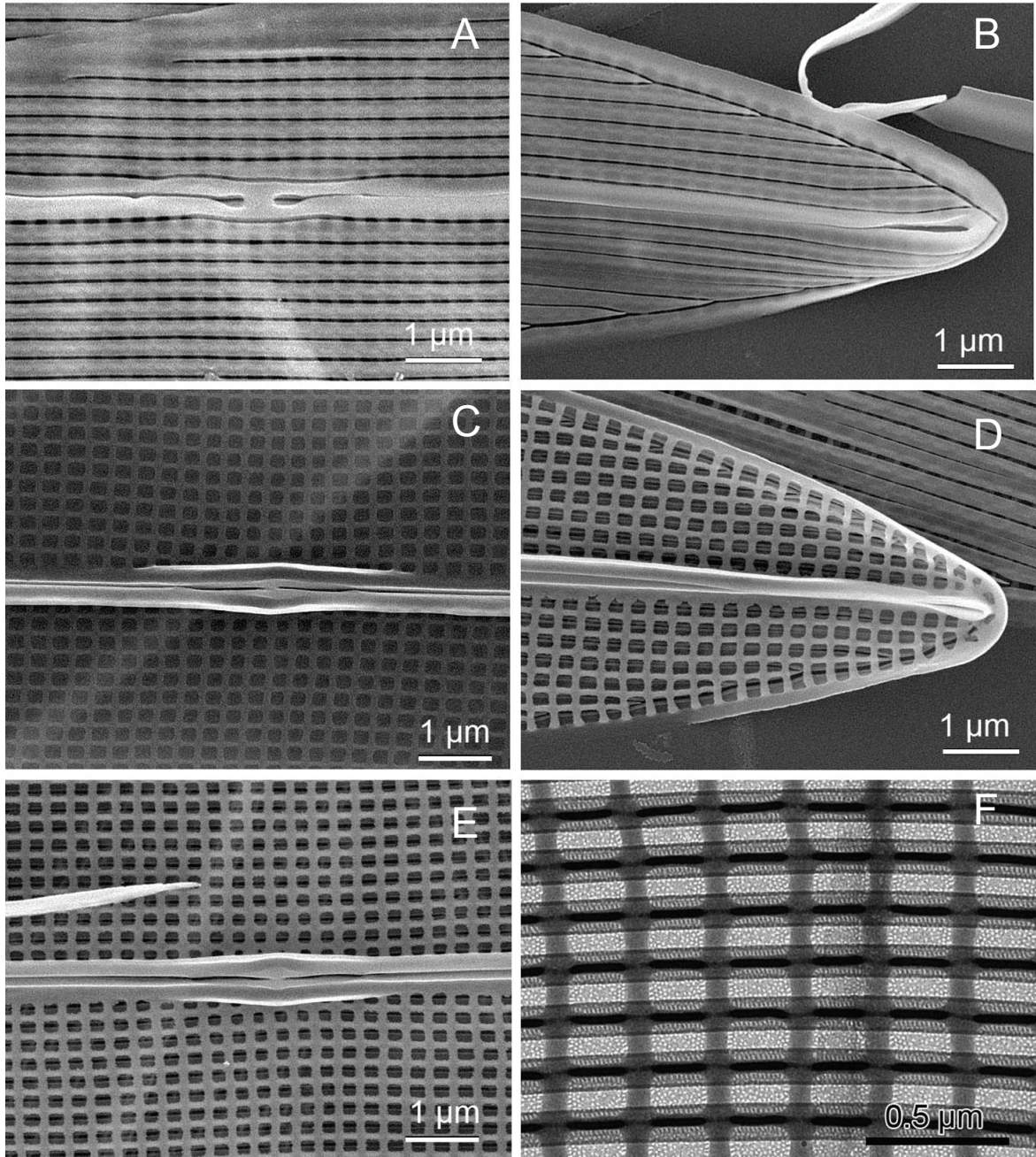


Fig. 4. Scanning electron microscopy pictures of *H. silbo* sp. nov.: (A) central raphe ending in external view and longitudinal slits; (B) straight apical raphe ending in external; (C) and (E) central ending of the raphe in internal view with the square areolae and the eventual accessory rib; (D) apices in internal view with the straight apical ending of the raphe; (F) high magnification of the square areolae. Scale bar 1 µm (A to E), 0.5 µm (F).

Different angles of the apex of *H. silbo* sp. nov. were measured as described in M&M, and compared with corresponding values in *H. ostrearia* (Table 1). Whatever the rank of the measurement, the apical angle is always less acute in *H. silbo* sp nov., indicating a larger apex in this species, in comparison with *H. ostrearia*. The average dimensions of areolae in both species are compared in Table 2. In *H. silbo* sp. nov, areolae surfaces are twice bigger than those of *H. ostrearia*, and the ratio of transapical to transversal length is closer to one, illustrating their square shape.

Distance from the apex (μm)	<i>Haslea ostrearia</i>	<i>Haslea silbo</i> sp. nov.
1	70.2°	84.3°
2.5	52.3°	56.9°
5	33.9°	40.5°
10	23.7°	30.3°
15	18.7°	25.2°
20	15.6°	23.2°
25	15.0°	21.5°

Table 1. Comparison between the angles (in degrees) of the isosceles triangles formed at the apices of *H. ostrearia* and *H. silbo* sp. nov. (see Figure 1 for explanation).

	Ratio between transapical and transversal length	Average surface of areolae (nm ²)
<i>H. ostrearia</i>	1.22 ± 0.02	21745 ± 326
<i>H. silbo</i> sp. nov.	1.03 ± 0.02	44150 ± 145

Table 2. Comparison of the ratio of transapical to transversal length for the areolae and of the average surface of areolae in nm² in *H. ostrearia* and *H. silbo* sp. nov. (results are mean ± S.E., n = 25).

Reproduction behaviour

No interbreeding ever occurred between *H. ostrearia* and *H. silbo* sp. nov strains (Table 3), nor between *H. silbo* sp. nov. and *H. karadagensis* strains (not shown). None of the stages of the sexual reproduction, even the early ones (gametangia pairing, meiosis), was ever observed. Neither did happen auxosporulation after crossing experiment between the three *H. silbo* sp. nov strains maintained in culture. In contrast, mating experiments between the two compatible strains of *H. ostrearia* clones were successful, as described in Davidovich et al. (2009).

	NCC	NCC	NCC	SCCAP	SCCAP	SCCAP
	158.4	148.78	313	1283	1286	1287
NCC 158.4	-	+	-	-	-	-
NCC 148.78		-	-	-	-	-
NCC 313			-	-	-	-
SCCAP 1283				-	-	-
SCCAP 1286					-	-
SCCAP 1287						-

Table 3. Results of the crossing chess obtained with different clones of *H. ostrearia* and *H. silbo* sp. nov. The sign + indicates that auxosporulation occurred. The sign – indicates that no sexual stage was observed.

UV-visible spectrophotometry

For all forms of pigments, an isobestic point was observed in the visible region of the spectrum, close to 640 nm (Figures 5-8). For the extracellular form of marennine, another isobestic point was usually observed in the 490 nm region. For all pigments, the peaks of maximum absorption's positions changed with the pH, moving to the higher

wavelength as the pH increases. The different isobestic points and the peaks of maximum absorption wavelengths as a function of the pH are presented in Tables 4 and 5, respectively. Peak wavelengths increased with the pH, and were highly similar, except for the intracellular form of the pigment of *H. silbo* sp. nov. at the lowest pH. In the range of seawater pH values (7.5-8.5), the peak was almost constant (674 nm) for both pigments, intracellular and extracellular forms.

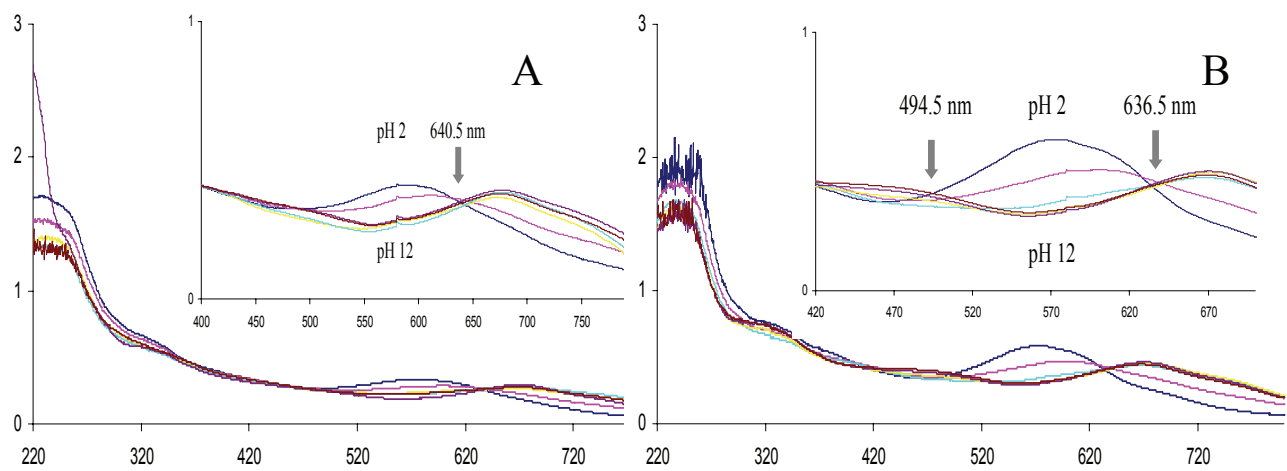


Fig. 5. UV-visible spectrum of the purified intracellular (A) and extracellular (B) forms of marennine from *H. ostrearia* depending on the pH. Inset: isobestic points in the visible region.

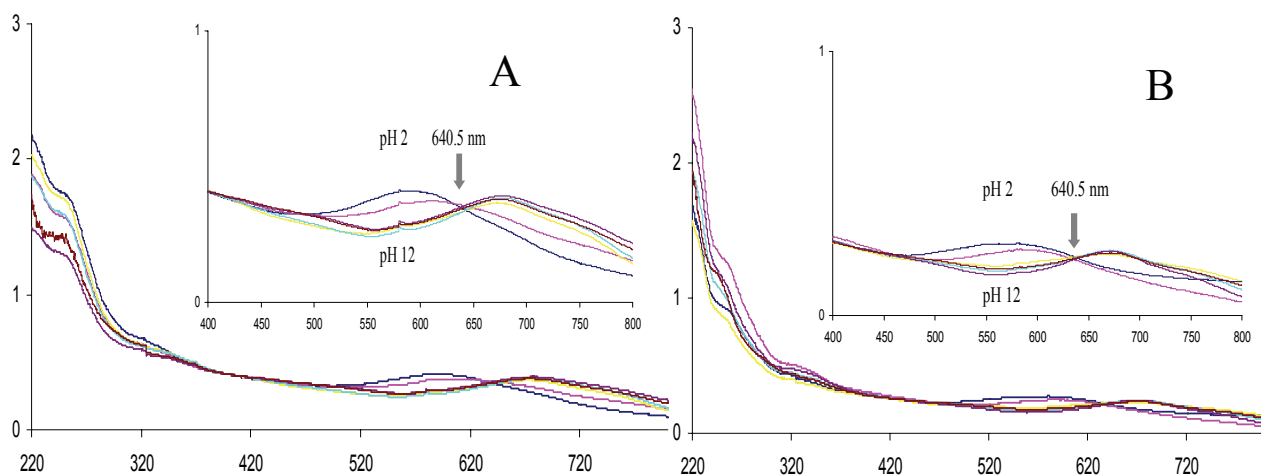


Fig. 6. UV-visible spectra of the purified intracellular (A) and extracellular (B) forms of the pigment from *H. silbo* sp. nov. depending on the pH. Inset: isobestic point in the visible region.

Chemical species	Internal marennine	External marennine	Internal <i>H. silbo</i> sp. nov. pigment	External <i>H. silbo</i> sp. nov. pigment	Internal marennine*	External marennine*
Positions of isobestic points	641 nm	494 nm 636 nm	641 nm	641 nm	650 nm	490 nm 635 nm

Table 4. Wavelengths of the isobestic points corresponding to the different purified pigments observed in UV-visible spectrophotometry.* Values from Pouvreau et al. (2006)

	pH 2	pH 4	pH 6	pH 8	pH 10	pH 12
Internal marennine	577	604	667	673	674	675
External marennine	574	604	672	674	674	674
Internal pigment of <i>H. silbo</i> sp. nov.	593	616	673	674	675	675
External pigment of <i>H. silbo</i> sp. nov.	572	590	670	674	674	675

Table 5. Changes with pH of the wavelengths (nm) corresponding to the peaks of the different pigments, observed in the visible region of the spectrum.

Raman spectrometry

Figure 9 shows *in vivo* Raman spectra of the blue pigments accumulated in the apex of *H. ostrearia* and *H. silbo* diatoms in addition of Raman spectrum of the cytoplasm. As already observed in diatoms (Gastineau, submitted), the characteristic Raman peaks of pigments are found in the region 1000–1700 cm^{-1} . The two predominant peaks, which appeared in each spectrum around at 1160 and 1525 cm^{-1} , are directly attributed to C-C and C=C stretching vibrations modes of the chloroplast. Indeed, as diatoms can move laterally in Petri dish during measurements it is common to collect light scattering from the cytoplasm. The spectral data of *H. ostrearia* and *H. silbo* reveal a high degree of similarity and 7 distinctive bands are clearly evidenced. Raman spectrum of *H. ostrearia* shows two intense and well-resolved bands at 1648 and 1462 cm^{-1} , two well-resolved band of medium intensity at 1606 and 1205 cm^{-1} , a poorly resolved Raman bands centred near 1300 cm^{-1} consisting of at least two broad bands at about 1317 and 1286 cm^{-1} and a distinct weak-intensity band at 1570 cm^{-1} . Similarly, *in vivo* Raman spectrum of *H. silbo* shows two intense and well-resolved bands at 1652 and 1465 cm^{-1} , two well-resolved band of medium intensity at 1609 and 1204 cm^{-1} , two broad bands at about 1320 and 1291 cm^{-1} , a distinct weak-intensity band at 1570 cm^{-1} and several less intense bands in the 250-600 cm^{-1} wavenumber range. A summary of the main vibrational wavenumbers are listed in Table 7.

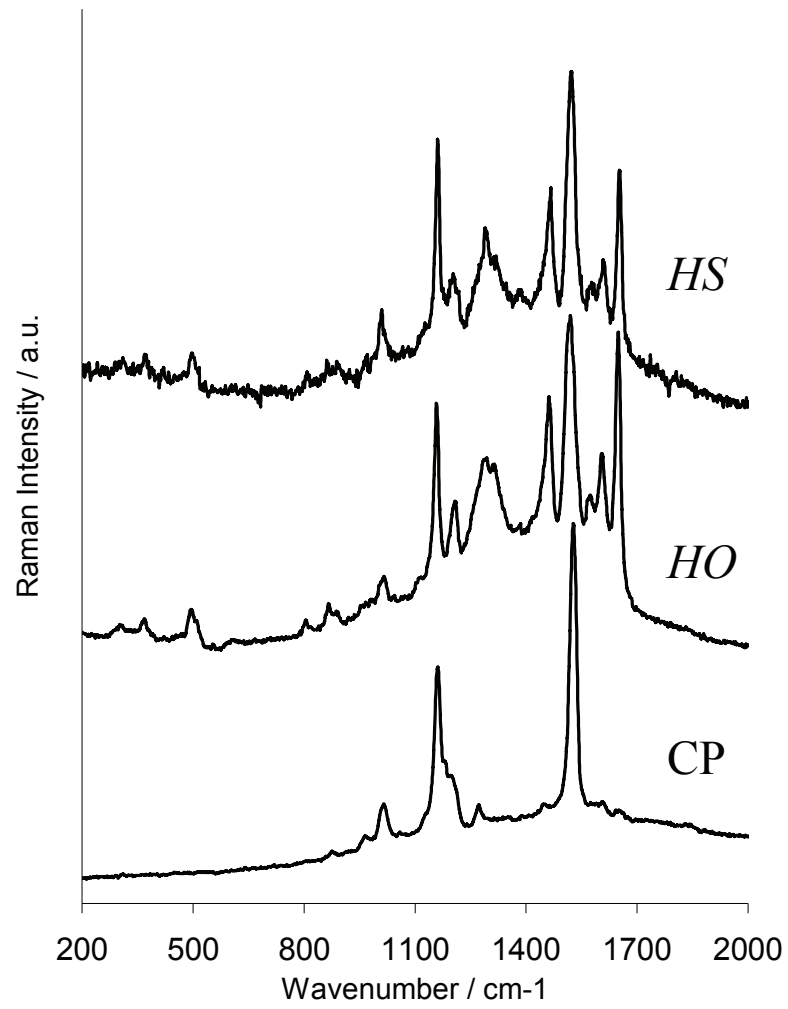


Fig. 8. Comparison of the in vivo Raman spectra recorded on blue pigments observed at the apices of *H. ostrearia* (HO) and *H. silbo* sp. nov. (HS). CP is for the chloroplast.

Cytoplasm	<i>H. ostrearia</i>	<i>H. silbo</i>
1525 (vs)	1648 (s)	1652 (s)
	1606 (m)	1609 (m)
	1570 (w)	1570 (w)
1160 (vs)	1462 (s)	1465 (s)
	1317 (m)	1320 (w)
	1286 (m)	1291 (m)
	1205 (m)	1204 (w)

Table 7: Summary of main^a vibrational bands (wavenumber cm^{-1}) observed by Raman spectroscopy for *H. ostrearia* apices and *H. silbo* apices. The most intense bands are shown in bold.^a vs : very strong, s : strong, m: medium, w : weak

Figure 10 shows Raman spectra of both intracellular and extracellular forms of purified blue pigments of *H. silbo* and *H. ostrearia*. Unlike the *in vivo* results described above, the Raman spectra of the purified pigments are poorly resolved in the 1000-1700 cm^{-1} wavenumber range. Although Raman spectra are superimposed on a relatively intense photoluminescence, broad bands can still be identified (Table 8). Raman investigations of the intracellular and extracellular forms of the purified pigments show similar spectra for *H. silbo* while the spectra for *H. ostrearia* are significantly different with a very intense and broad feature centred at 1470 cm^{-1} wavenumber for the extracellular form of the pigment of *H. ostrearia*.

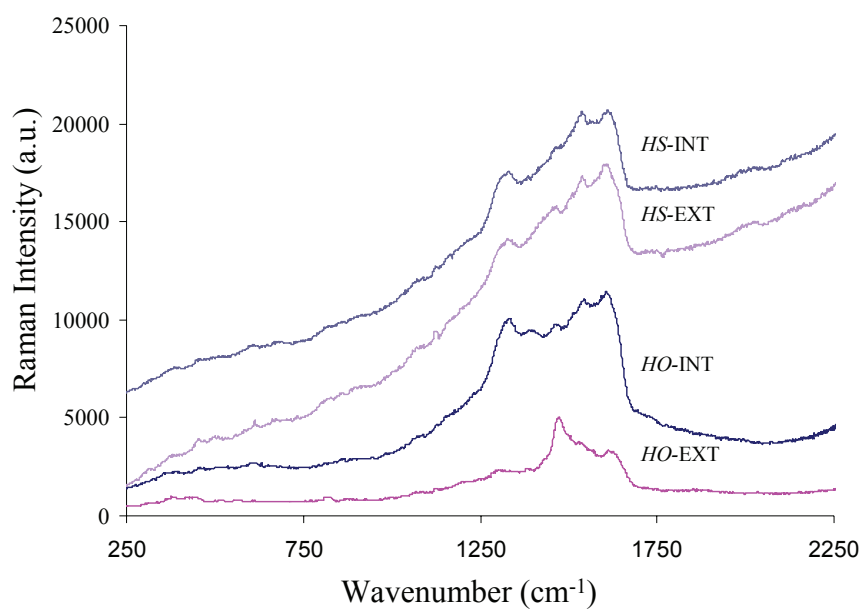


Fig. 9. Comparison of the in vitro Raman spectra recorded on purified blue pigments of *H. ostrearia* (HO) and *H. silbo* sp. nov. (HS). INT means intracellular form, EXT means extracellular form.

<i>H. ostrearia</i> INT	<i>H. ostrearia</i> EXT	<i>H. silbo</i> INT	<i>H. silbo</i> EXT
1616	1625	1619	1616
1539	1569 1470	1542	1541
1457 1400		1459	1458
1332		1312	1322

Table 8: Summary of identifiable Raman bands (wavenumber cm^{-1}) observed in the Raman spectra of purified blue pigments of *H. ostrearia* and *H. silbo*. INT means intracellular form, EXT means extracellular form. The most intense band is shown in bold.

Molecular markers

The sequences of the ribosomal box ITS1-5.8S-ITS2 present in the two blue diatoms were comparable in length, ranging from 1102 to 1105 bp, and from 1086 to 1097 bp, for *H. ostrearia* and *H. silbo* sp. nov., respectively. *H. silbo* sp. nov. was characterized by a lot of insertion higher than 10 pb, the zones localized at the position 800-810, 940-961, 978-988 and 994-1011, which were completely absent in *H. ostrearia*. In contrast, the sequences were shorter, by ca. 586 pb, in *H. crucigera*, which was used as an out-group. *H. karadagensis* exhibited longer sequences, up to 1394 pb. The percentages of identity calculated for the ITS sequences are showed in Table 6.

	<i>H. ostrearia</i> NCC 345	<i>H. ostrearia</i> NCC 148.75	<i>H. silbo</i> sp. nov. SCCAP 1283	<i>H. karadagensis</i> NCC 313	<i>H. crucigera</i> NCC 185
<i>H. ostrearia</i> NCC 345	-	97%	58.5%	55%	41.5%
<i>H. ostrearia</i> NCC 148.75	-	-	59%	56.5%	42%
<i>H. silbo</i> sp. nov. SCCAP 1283	-	-	-	47%	36%
<i>H. karadagensis</i> NCC 313	-	-	-	-	31%
<i>H. crucigera</i> NCC 185	-	-	-	-	-

Table 6. Percentages of homology between the ITS box sequences of the clones NCC... Average values made on two distinct sequencing. Values obtained by Genedoc after alignment using ClustalW.

DISCUSSION

The new species of blue diatom described in this work was sampled on the shores of La Gomera Island, located west of the archipelago of the Canary Islands, which comprises 7 principal islands. These are volcanic islands, located *ca.* 100 km west of the African coast but geologically distinct from the African continental shelf, and they belong to Spain. A compendium of the diatom flora of Spain and Spanish Islands has been published by Aboal et al. (2003). Although principally dedicated to freshwater diatoms, it contains also some marine or brackish species, but only one species from the genus *Haslea* is described, *Haslea spicula*, observed in many places in Spanish and Portuguese coastal waters. Another diatom *Haslea balearica*, can be found in the Spanish Islands of the Mediterranean Sea. However, none of this two species could be confounded with *Haslea silbo* sp. nov., as their frustule presents a pseudo-stauros, a thickening of its central ribs visible even under optical microscope, absent in the Canarian diatom, and it does not produce any blue pigment. Until the description of *H. karadagensis*, *H. ostrearia* was considered the only microalga to produce a blue pigment like marennine. In the present work, different and complementary approaches were used to describe the third species of blue diatoms ever identified to produce such a blue pigment.

The morphological approach

For decades, a lanceolate shape and blue apices were two characters seemingly relevant enough to identify a pennate diatom as being *H. ostrearia*. The possibility to grow several strains of blue diatoms originating from different geographic areas in the

laboratory allowed to make direct comparisons of their cellular and blue pigment characteristics, and to question such an *a priori* statement. Indeed, just by using light microscopy and biometry, slight differences could be noticed between *H. ostrearia* and the diatom collected in the Canary Islands. Cells of *H. silbo* sp. nov. appear more ‘stocky’ than *H. ostrearia*, with apices less acute, an observation confirmed by the differences noticed in the angles formed between equidistant point from the apex in the frustule. As to SEM pictures, they demonstrate that both density of striations and shape of the areolae clearly differ between the two blue diatoms, two sets of characters often used to distinguish separated species of Bacillariophyta (Mann, 1999, Chepurnov et al. 2004). Moreover, among these series of characters, two of them (lower density of striations and square shape of the areolae) present values closer to those observed in two *Haslea* species which do not produce blue pigment, *H. crucigera* and *H. salstonica* (Massé et al. 2001), than in *H. ostrearia*.

On the other hand, in spite of significant differences in the morphology of the frustule, interbreeding experiments can sometimes confirm two diatom cells belong to a same species (e.g., Vanormelingen et al. 2008, Kaczmarska et al. 2009, Mann 2010), relativizing the importance that can be attributed to morphology in diatom taxonomy. However, due to the lack of any sexually compatible clones of *H. silbo* sp. nov. presently in culture, or to unknown favourable conditions, some key information are missing, regarding the life cycle and cardinal points of the species. Indeed, our attempts to induce auxosporulation in *H. silbo* sp. nov. were not successful, and no information is available on the sexually inducible size region, nor on the initial cell size region.

Could blue pigment be used as taxonomic marker?

Marennine has long been considered as the trade mark of *H. ostrearia*, and as such, could be considered as a trivial taxonomic marker, given the supposed singularity of this diatom. The recent discovery of a blue diatom which produces a pigment different from marennine has shown that marennine-like pigment could be used as markers for chemical taxonomy in a more general context, since these two pigments can be distinguished from each other, using micro-Raman spectroscopy *in-vivo* (Gastineau et al. submitted). Pouvreau et al. (2006) demonstrated that marennine exists in two different forms, intracellular and extracellular, differing in their spectroscopic characteristics. In contrast with *H. karadagensis*, *H. silbo sp. nov.* produces a pigment which is similar to intracellular marennine, according to their Raman and UV-visible spectra. For example, no significant differences were observed between the absorbance peaks in the visible part of the spectra, for the intracellular forms of the two pigments. Some discrepancies in the wavelengths of isobestic points or absorbance peaks at the lowest pH can be observed when compared to data published in Pouvreau et al (2006) (Table 4). However, the differences are less than a few nm and they could result from changes in the material (spectrophotometers, cells, buffers), or in biomass or pigment batches used for the experiments. Last, regarding the putative process of externalization of the pigments outside the cells, if the extracellular form of marennine shows absorption peaks at wavelengths distinct from those of the intracellular form, especially in the UV-C band, as already noticed by Pouvreau et al. (2006), no differences were observed between intracellular and extracellular forms of the pigment produced by the Canarian diatom. Thus it can be concluded that *H. silbo sp. nov.* produces a pigment

similar to the intracellular form of marennine, and that this pigment is not cleaved during its excretion by exocytosis (Nassiri et al. 1998), in contrast with *H. ostrearia*.

In vitro Raman spectroscopy confirmed that intracellular marennine was similar to the pigment of *H. silbo sp. nov.*, and that the pigment produced by this species did not exist under two different forms, intracellular and extracellular, in contrast to marennine. This technique thus confirms its potential for revealing pigment differences and identifying species, in the line of previous works (e.g., Wu et al. 1998, Wood et al. 2005).

What does the molecular approach say?

The discovery of a new species of blue diatom gave rise to several questions. In comparison with *H. ostrearia*, *H. silbo sp. nov.* originates from the same part of the Atlantic Ocean, produces a similar pigment, but strongly differs, as morphologically as genetically. Molecular phylogeny based on the sequencing of the ribosomal box ITS1-5.8S-ITS2 is a method which has become routine to study the genetic characteristics of diatoms (Medlin & Kooistra 2010). The ribosomal box ITS1-5.8S-ITS2 sequenced in all strains of *H. ostrearia* and *H. silbo sp. nov.* were similar in sizes, compared with results obtained on species of the *Sellaphora* complex (Behnke et al. 2004). The sequences are highly diverging in their composition, revealing 40% of differences, mostly due to insertion-deletion. This degree of difference is correlated with the reproductive isolation. For example, it has been shown that in diatoms, sexual reproduction was possible when genetic divergence, based on ITS1 and ITS2 sequencing, was lower than 10% to 12% (Vanormelingen et al. 2008). Pairing of cells, the first stage of sexual

reproduction, can sometimes occurs between strains having up to 29% of divergence in ITS regions (Behnke et al. 2004). Despite sharing the capacity to synthesize possibly the same blue pigment, according to molecular data, *H. ostrearia* and *H. silbo* sp. nov are different species, which confirms the differences observed in the morphology between these two organisms.

In conclusion, the present work demonstrates the existence of a third blue diatom from the genus *Haslea*, which has the capacity to produce a marennine-like pigment. We report for the first time the *in vivo* and *in vitro* Raman spectra of the same diatom's pigments and show that *H. ostrearia* is able to produce an external form of the pigment different from the internal one, contrarily to *H. silbo* sp. nov.. As vibrational bands are sensitive to the chemical and physical environment of the chemical functional groups associated with the molecular vibration, Raman spectrometry is therefore a powerful tool for distinguishing between pigments that are probably similarities in chemical structure. To the notable exception of *H. wawriake*, diatoms from the genus *Haslea* are benthic organisms. This may restrain the possibility of natural transport or movement of *Haslea* species far from coasts through deep sea. Once installed on the shores of Canary Islands, blue diatoms could be separated from coastal continuity and marine flux, so that speciation could occur. Hence *H. silbo* sp. nov could be a species resulting from the geographical isolation of an ancestral population of blue diatoms, the major (*i.e.*, the best known) representing species today being *H. ostrearia*. Whatever the origin of the Canarian blue diatom, *H. silbo* sp. nov, and its phylogenetic relations with other blue diatoms, this finding opens new avenues of research, *e.g.*, regarding the elucidation of the chemical structure of these marennine-like pigments, the description of their metabolic pathways and externalization processes, the distribution of the capacity to

synthesize such peculiar pigments in algae, especially in species that are morphologically, reproductively and genetically diverging.

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III.6.3 Conclusion et perspectives

H. silbo est donc la première nouvelle espèce de diatomée bleue décrite capable de synthétiser un pigment proche, si ce n'est identique, à la marennine. Peu de temps avant de terminer la rédaction de ce manuscrit, une autre souche de diatomée bleue originaire de l'île de Gran Canaria, enregistrée sous la référence SCCAP 1584, nous a été fournie par le Dr. Hansen. L'approche moléculaire préliminaire a déjà montré que cette diatomée présente de nettes différences génétiques avec *H. silbo*, et cette diatomée a reçu le nom provisoire d'*Haslea acoran*. Elle n'a pas jusqu'à présent montré d'interfécondité avec *H. silbo*. L'identification spécifique de cette diatomée est toujours en cours. L'archipel des Canaries pourrait être une zone géographique qui, par sa fragmentation, aurait un fort pouvoir de spéciation allopatrique sur les diatomées benthiques.

III.7 Boulouris et *Haslea provincialis*

III.7.1 Introduction

Dans la revue de bibliographie présentée en début de manuscrit sur la dispersion géographique d'*H. ostrearia*, plusieurs références font état de la présence d'*H. ostrearia* en Mer Méditerranée. Sauvageau (1906) en aurait ainsi observée vivante comme épiphytes sur l'algue *Liebmannia leveillei*. D'autres références en font état en différents points des côtes italiennes, sur le continent comme dans les eaux insulaires (Solazzi et Tolomio, 1976 ; Tolomio, 1978 ; Barone, 1979). Enfin, d'autres ont été observées dans la proche Mer Adriatique et dans la baie de Trieste (Molisch, 1903 ; Höfler *et al.*, 1956).

Un premier clone isolé par le Dr. Gert Hansen et répertorié SCCAP 1343 nous a été fourni. Ce clone provenait d'échantillonnages réalisés à Saint Raphaël, sur la côte méditerranéenne. Par la suite, cet endroit s'est révélé propice par la suite à la recherche de ces diatomées, quatre échantillonnages ayant pu être opérés sur une durée d'un an, avec des prélèvements contenant systématiquement des diatomées à apex bleus. L'identification de cette population localement pérenne de diatomées a été entreprise, et elle est toujours en cours au moment du dépôt de ce manuscrit.



Figure 21. A : La commune de Saint Raphaël, avec Boulouris entourée en noir. B : les plages de Boulouris, lieux des prélèvements. C : *H. provincialis*, la diatomée bleue de Méditerranée.

III.7.2 Caractéristiques morphologiques d'*H. provincialis*

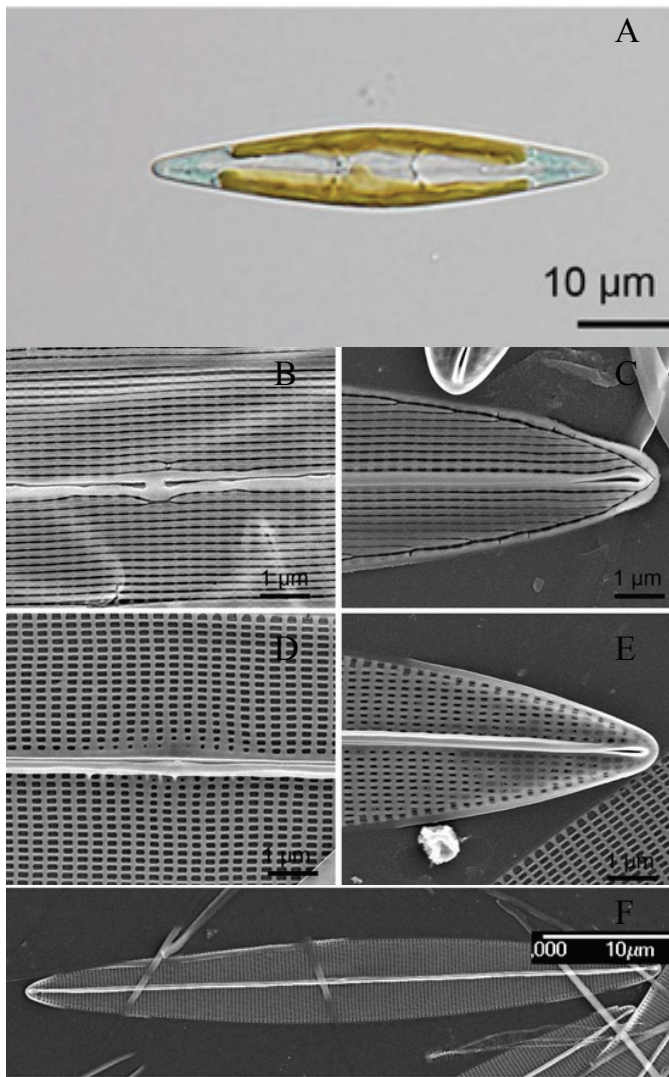


Figure 22. Les aspects morphologiques de *H. provincialis*. A : vue au microscope optique (grossissement x 1000) d'une cellule d'*H. provincialis*. B : vue au MEB d'une extrémité centrale du raphé sur la face externe de la valve. C : vue au MEB d'une extrémité terminale du raphé sur la face externe de la valve. D : vue au MEB d'une extrémité centrale du raphé sur la face interne de la valve. E : vue au MEB d'une extrémité terminale du raphé sur la face interne de la valve. F : vue d'une cellule entière au MEB.

En microscopie optique, les cellules d'*H. provincialis* montrent une forme pennée avec des apex fins, remplis d'un pigment bleu (Fig.A). Elles possèdent deux chloroplastes pariétaux en vue valvaire (Fig.A). En microscopie électronique à balayage, le frustule apparaît être constitué de bandes longitudinales de silice sur sa face externe (B et C), et présente l'aspect alvéolé caractéristique du genre *Haslea* en vue interne (D et E). Les aréoles sont rectangulaires. Les extrémités centrales du raphé sont droites (B et D). Les extrémités terminales du raphé sont droites et peu prononcées (C et E). La densité d'aréoles en vue interne est transapicalement de $34,9 \pm 0,6$ pour $10 \mu\text{m}$ (moyenne \pm erreur standard, n=10) et transversalement de $63,5 \pm 1,1$ (moyenne \pm erreur standard, n=10). Lors de sa réception, la longueur moyenne du clone SCCAP 1343 était de $66,9 \mu\text{m} \pm 0,4$ (moyenne \pm erreur standard, n=5) et la largeur de $7,4 \pm 0,2$ (moyenne \pm erreur standard, n=5).

III.7.2 Aspects reproductifs

Des expériences préliminaires, suivant le protocole défini au premier chapitre et basé sur Mouget et al. (2009), ont été réalisées entre les clones d'*H. provincialis* ainsi qu'avec diverses combinaisons de couples sexuellement compatibles d'*H. ostrearia* (NCC 148.78 et NCC 158.4) et d'*H. karadagensis* (NCC 313 et NCC 316). Jusqu'à présent, aucune forme de reproduction n'a été observée dans aucun des croisements réalisés.

III.7.3 Aspects moléculaires

Le protocole appliqué pour caractériser moléculairement *H. provincialis* est décrit en détail dans la partie traitant de phylogénie, et ne sera donc pas repris ici.

A la place sont fournis un tableau listant les pourcentages d'homologies entre *H. ostrearia*, *H. karadagensis*, *H. silbo*, *H. crucigera* et *H. provincialis* pour deux des trois marqueurs génétiques utilisés (les séquences de la cassette ribosomale ITS1-5,8S-ITS2 pour *H. provincialis* étant en cours d'acquisition au moment de la rédaction).

Ces tableaux ont été construits à partir des logiciels ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) et Genedoc (<http://www.nrbcs.org/gfx/genedoc/>).

	<i>H. karadagensis</i> NCC 313	<i>H. provincialis</i> NCC 332	<i>H. ostrearia</i> NCC 235.1	<i>H. silbo</i> SCCAP 1283
<i>H. karadagensis</i> NCC 313		98%	98%	97%
<i>H. provincialis</i> NCC 332			98%	97%
<i>H. ostrearia</i> NCC 235.1				97%
<i>H. silbo</i> SCCAP 1283				

Tableau 1. Les pourcentages d'homologies pour les séquences *rbcL* des quatre espèces de diatomées bleues étudiées.

	<i>H. provincialis</i> NCC 332	<i>H. silbo</i> SCCAP 1283	<i>H. ostrearia</i> NCC 235.1	<i>H. karadagensis</i> NCC 313
<i>H. provincialis</i> NCC 332		98%	91%	91%
<i>H. silbo</i> SCCAP 1283			90%	90%
<i>H. ostrearia</i> NCC 235.1				90%
<i>H. karadagensis</i> NCC 313				

Tableau 2. Les pourcentages d'homologies pour les séquences *cox1* des quatre espèces de diatomées bleues étudiées.

Des résultats préliminaires obtenus par la biologie moléculaire, il ressort que des différences génétiques corrélerent l'isolement reproductif et les différences morphométriques évoquées précédemment. Les différences au niveau des deux marqueurs entre *H. provincialis* et *H. ostrearia* sont d'un ordre de grandeur similaire à celui pour les mêmes marqueurs considérés entre *H. ostrearia* et *H. karadagensis* ou *H. silbo*.

III.7.4 Aspects pigmentaires

Des méthodes similaires à celles employées pour la description de *H. silbo* ont été appliquées au pigment d'*H. provincialis* afin d'avoir des résultats comparatifs préliminaires.

III.7.4.1 Matériel et méthodes

La spectrophotométrie UV-visible

Le pigment d'*H. provincialis* a été purifié suivant la méthode de Pouvreau (2006), et deux fractions internes et externes ont été obtenues sous forme lyophilisée. Elles ont été solubilisées à concentration de 10 mg mL⁻¹ dans de l'eau distillée, puis 50 µL de cette solution furent mélangés dans des cuves spectrophotométriques en quartz Hellma dans du tampon phosphate Na₂HPO₄ / NaH₂PO₄ 0,25 M ayant une gamme de pH allant de 2 à 12 par pas de 2, préalablement filtré à la seringue sur filtre 0,22 µm. Les spectres d'absorption furent acquis par un spectrophotomètre Thermo electron Helios gamma pour une gamme de longueurs d'ondes allant de 220 à 800 nm, le témoin ayant été réalisé à partir du tampon phosphate seul.

Spectrométrie Raman

Toutes les acquisitions de spectres furent faites avec un spectromètre confocal T64000 (Horiba, Jobin Yvon) couplé avec un détecteur CCD refroidi à l'azote liquide. Les spectres furent enregistrés dans la région comprise entre 250 et 2250 cm⁻¹, mais ne seront présentés ici que dans la zone 1000 - 2000 cm⁻¹. Le laser incident était de longueur d'onde monochromatique de 514,5 nm. La puissance employée fut au maximum de 0.1 mW *in vivo* et au maximum de 0,3 mW *in vitro*. Tous les échantillons *in vivo* comme *in vitro* furent repérés sur un écran grâce à une caméra couplée à un

microscope utilisé avec un objectif $\times 100$. Le diamètre du laser fut de $0,8 \mu\text{m}$. Les temps d'acquisition furent de moins de 10 secondes *in vivo* jusqu'à 10 minutes *in vitro*.

Pour la spectrométrie Raman *in vivo*, les suspensions d'algues, prélevées dans des cultures en phase exponentielle de croissance et soumises à un fort éclairage ($150 \text{ mol photons m}^{-2} \text{ s}^{-1}$), furent déposées dans des boîtes de Petri au moins deux heures avant expérimentation. Ceci avait pour but de leur laisser le temps de sédimenter et donc de réduire leur mobilité, les cellules se déplaçant lorsque visées par le rayon laser. Le faisceau laser fut dirigé sur les apex colorés des diatomées, et les moyennes furent réalisées sur 10 acquisitions.

Pour la spectrométrie Raman *in vitro*, de faibles quantités de pigment purifié, de l'ordre de $0,5 \text{ mg}$, furent placées sur des lames en verre. Un volume de $10 \mu\text{L}$ de tampon phosphate $\text{Na}_2\text{HPO}_4 / \text{NaH}_2\text{PO}_4$ $0,25 \text{ M}$ de pH 8 fut déposé dessus. Les lames furent laissées à sécher à température ambiante. Lorsque les agrégats de pigment furent secs, l'acquisition des spectres Raman fut réalisée comme décrit précédemment.

III.7.4.2 Résultats

Spectrophotométrie UV-visible

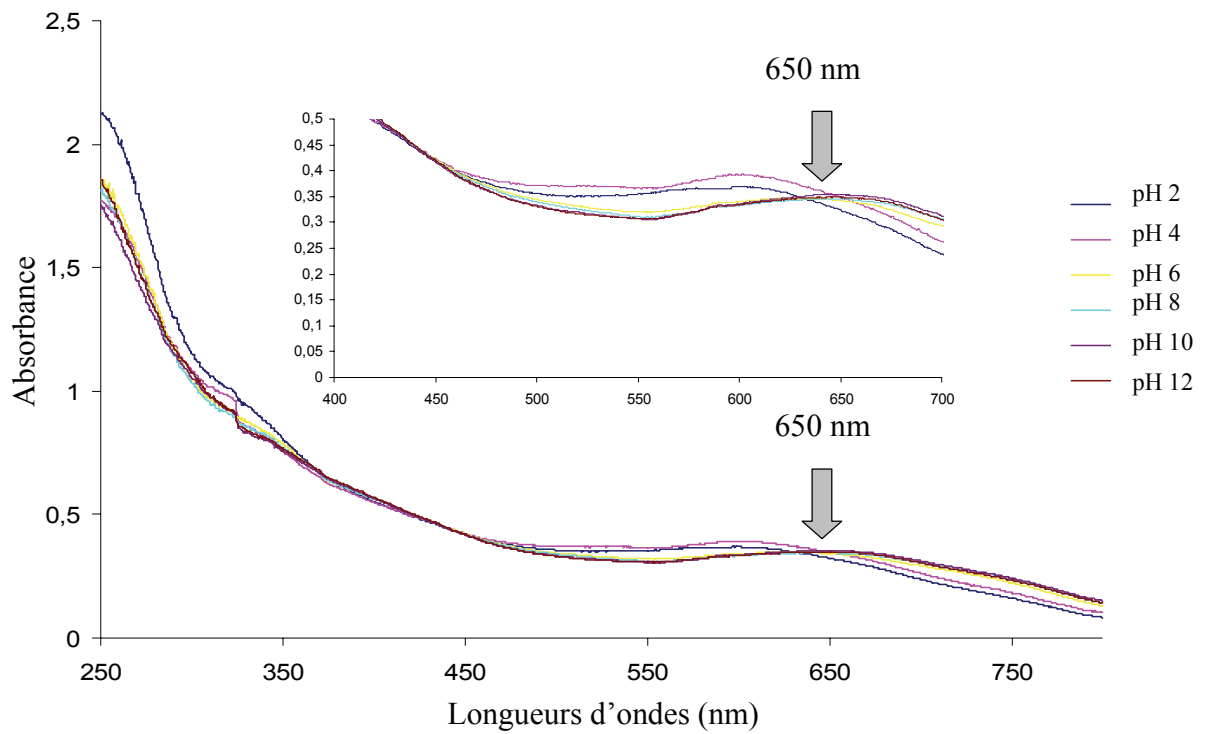


Figure 23. Spectre d'absorption de la forme interne du pigment de *H. provincialis* en fonction du pH de 250 à 800 nm, avec en insert la région des 400 à 700 nm.

La figure 23 montre que le spectre d'absorption de forme interne du pigment de *H. provincialis* en fonction du pH présente un point isobestique aux alentours de 650 nm. Le pic d'absorption dans le domaine du visible augmente avec le pH.

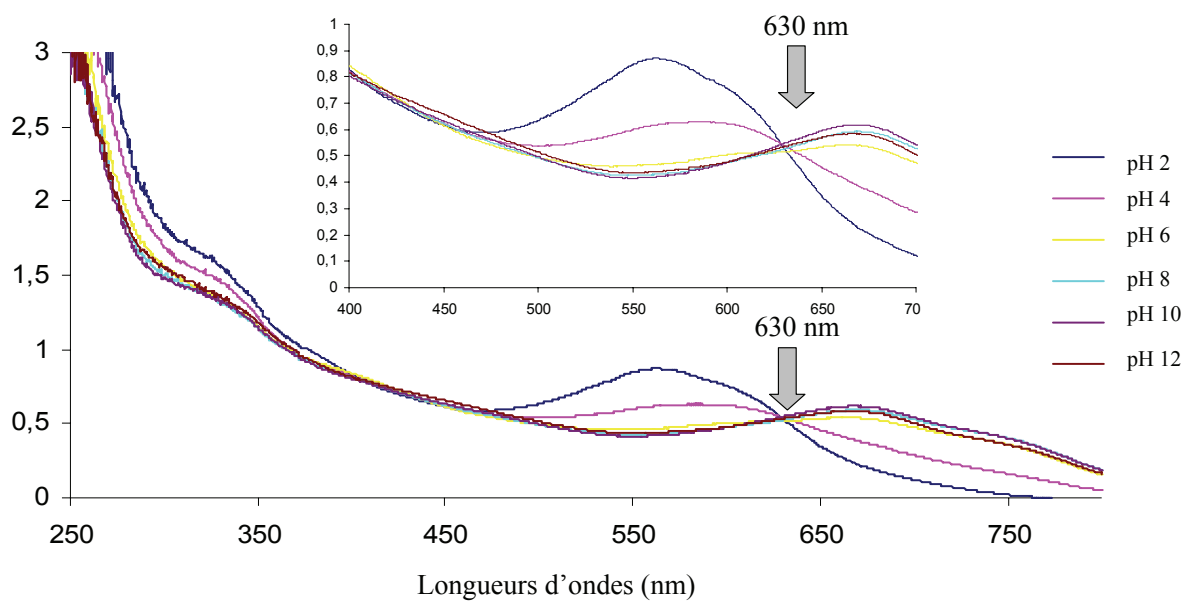


Figure 24. Spectre d'absorption de la forme externe du pigment de *H. provincialis* en fonction du pH de 250 à 800 nm, avec en insert la région des 400 à 700 nm.

La figure 24 montre que le spectre d'absorption de la forme externe du pigment de *H. provincialis* en fonction du pH présente un point isobestique aux alentours de 630 nm. Le pic d'absorption dans le domaine du visible augmente avec le pH. La présence d'un autre point isobestique aux alentours des 490 nm n'est pas à écarter.

Spectrométrie Raman

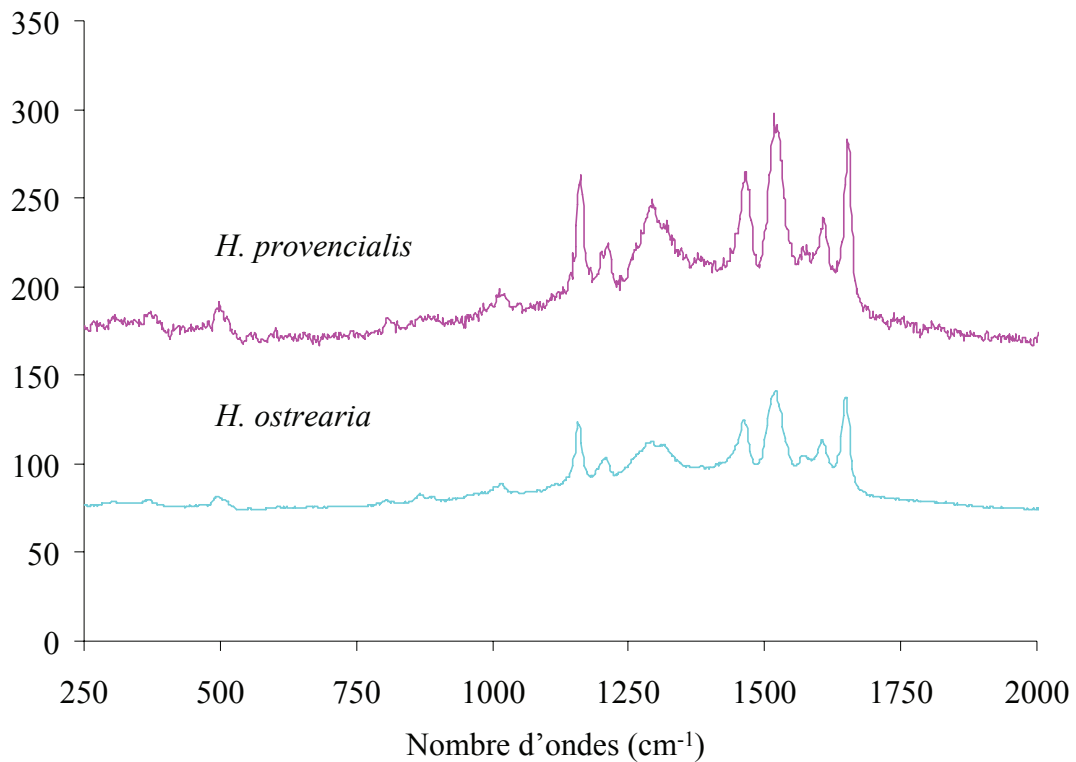


Figure 25. Spectre Raman obtenu *in vivo* à 514,5 nm sur les apex pigmentés d'*H. ostrearia* et *H. provencialis*.

Le spectre Raman obtenu *in vivo* (Fig.) montre des pics d'intensité aux mêmes positions pour les signaux obtenus en dirigeant le laser sur les apex pigmentés d'*H. ostrearia* et d'*H. provencialis*.

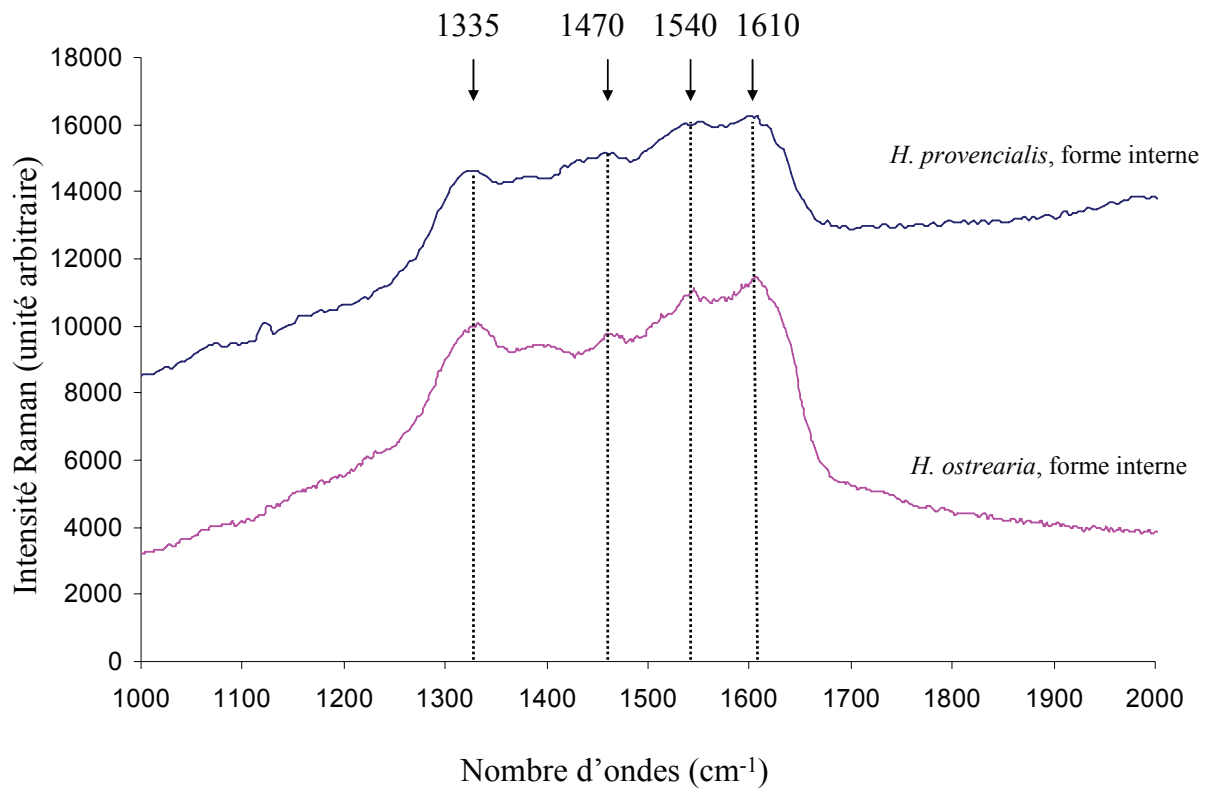


Figure 26. Spectre Raman obtenu sur la forme interne de la marennine et du pigment d'*H. provencialis* à 514,5 nm.

Le spectre Raman obtenu sur les formes internes purifiées de la marennine et du pigment d'*H. provencialis* chargées à pH 8 (figure 26) montre une allure globale des spectres similaires et des pics d'intensité à des positions identiques pour les deux pigments.

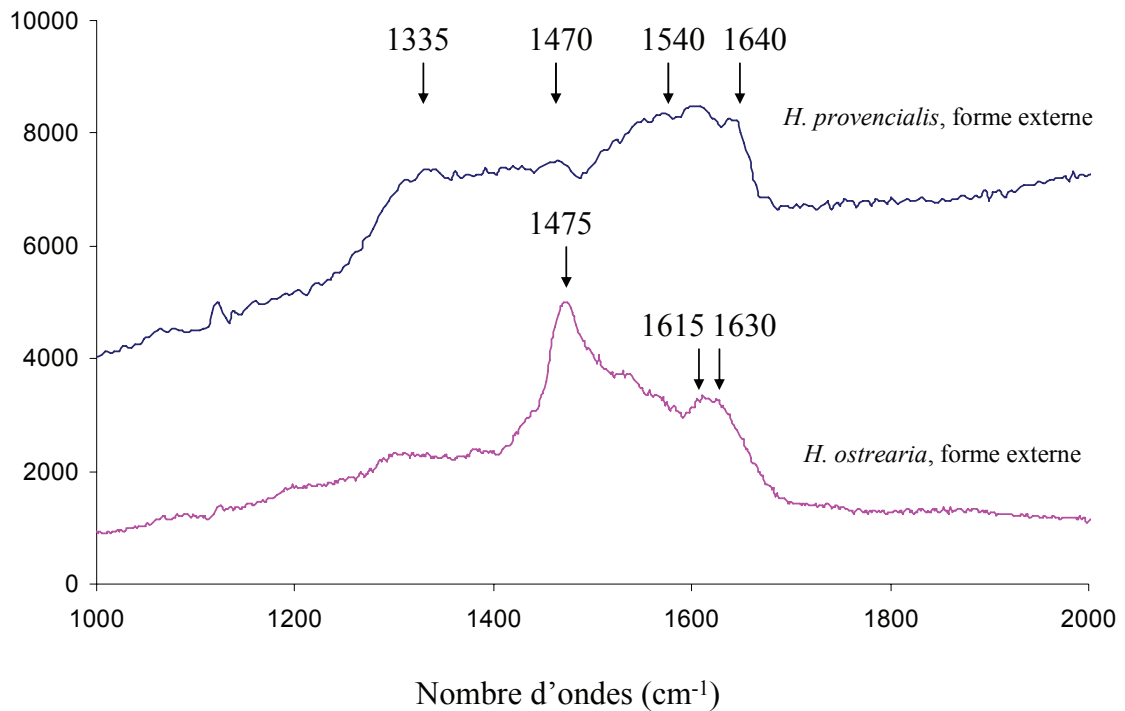


Figure 27. Spectre Raman obtenu sur la forme externe de la marennine et du pigment d'*H. provencialis* à 514,5 nm.

Le spectre Raman obtenu sur les formes externes purifiées de la marennine et du pigment d'*H. provencialis* chargées à pH 8 (figure 27) montre une allure globale différente pour les deux pigments. Deux des pics d'intensité (1470-1475 cm⁻¹ et 1630-1640 cm⁻¹) caractéristiques de la marennine externe sont potentiellement à des positions identiques pour les deux pigments. Cependant, le pic présent à 1470 cm⁻¹ pour la marennine externe a une intensité relative par rapport aux autres signaux très supérieure à son homologue supposé du pigment d'*H. provencialis*.

III.7.5 Conclusion et perspectives

Il reste encore des travaux à réaliser pour terminer la description d'*H. provincialis*. La possibilité de procéder régulièrement à des échantillonnages dans la zone de Boulouris fait entrevoir l'éventualité d'obtenir un nombre conséquent de clones de cette diatomée afin d'étudier ses mécanismes reproductifs.

Les premières données sur le pigment laissent à penser que sa forme interne est très similaire à la marennine, comme suggéré par la spectrophotométrie UV-visible et par la spectrométrie Raman, *in vivo* comme *in vitro*. Pour sa forme externe, le doute subsiste. Si la spectrophotométrie UV-visible laisse supposer la présence potentielle d'un point isobestique dans la zone des 490 nm comme pour la marennine externe (Pouvreau, 2006), le spectre Raman montre plutôt de fortes similitudes avec la forme interne. La forme caractéristique du spectre de la marennine externe, découlant probablement d'un changement de structure par rapport à la forme interne, dû à un clivage opéré lors de l'excrétion, ne se trouve pas non plus chez *H. silbo*. Les propriétés du spectre Raman de la marennine externe ont été observées sur plusieurs lots de pigments et ne sauraient être dues à un artefact. Dans le doute, et comme pour *H. silbo*, nous ne saurions pour l'instant conclure quant à l'existence d'une forme externe du pigment différente de l'interne chez *H. provincialis*. La spectrométrie de masse serait indiquée pour obtenir des résultats complémentaires quant à ces formes pigmentaires.

De même, l'investigation moléculaire devrait rapidement se poursuivre par le séquençage du marqueur moléculaire manquant, auquel pourront être adjoints d'autres gènes au besoin. L'étude du marqueur UPA, codant pour une région variable de l'ADNr

23S, a ainsi été évoquée, et des expériences ont été planifiées (Sherwood et Presting, 2007 ; Hamsher *et al.*, 2011).

La Mer Méditerranée est séparée de l'Océan Atlantique, d'où proviennent la majorité des espèces de diatomées bleues découvertes, par le détroit de Gibraltar, long de 55 km, large de 14 km et profond de 280 m (Garibaldi et Caddy, 1998). Au cours des temps géologiques, à la fin du Miocène, la Méditerranée a connu une période d'assèchement due à la fermeture du détroit (la crise de salinité messinienne), suivie d'une remise en eau par l'Océan Atlantique au début du Pliocène, événements qui furent à l'origine de modifications de la faune et de la flore aquatique, que ce soit par le changement de salinité consécutif ou bien du fait de la colonisation par de nouvelles espèces (Hsü *et al.*, 1977). Une étude phylogénétique basée sur l'horloge moléculaire et la vitesse d'évolution des diatomées permettrait peut être de comparer le moment supposé de divergence entre *H. provincialis* et les espèces présentes dans l'Océan Atlantique, par rapport à l'époque d'ouverture de la Mer Méditerranée. Cette étude pourrait être basée sur le gène 18S de la petite sous unité du ribosome (Kooistra et Medlin, 1996 ; Sorhannus, 2007). La présence de diatomées bleues dans la Mer Méditerranée constituerait peut être un exemple de spéciation parapatrique par rapport aux espèces présentes sur l'arc atlantique, où la colonisation se serait faite à partir d'espèces océaniques. De même, rien n'est pour l'instant su de l'éventuelle présence de cette espèce ou d'une autre diatomée bleue dans les parties les plus occidentales de la Mer Méditerranée, dans la Mer d'Alborán, le Golfe de Cadiz et sur les façades atlantiques de l'Espagne, du Portugal ou du Maroc.

La Mer Méditerranée tient lieu d'interface entre l'Océan Atlantique et la Mer Noire, d'où est originaire *H. karadagensis*. D'autres échantillonnages dans des parties

plus orientales de la Méditerranée, ou dans les autres mer adjacentes (Mer de Marmara, Mer Egée, Mer Adriatique), permettraient peut être de découvrir d'autres espèces, plus particulièrement des espèces présentant des pigments bleu-gris comme *H. karadagensis*.

IV

Chapitre 3

Méthodes moléculaires

appliquées à l'étude des diatomées bleues

IV.1 La phylogénie du genre *Haslea* et des diatomées bleues

IV.1.1 Introduction

Longtemps, *H. ostrearia* a été considérée comme la seule diatomée « bleue ». La découverte de plusieurs espèces nouvelles appartenant au genre *Haslea* et produisant des pigments bleus a fait s'interroger sur l'organisation de ce genre, la position au niveau phylogénétique des différentes espèces « bleues » entre elles ainsi qu'avec les espèces du genre *Haslea* dépourvues de ce type de pigments surnuméraires bleus. Le but de l'étude suivante fut de répondre à ces interrogations multiples. A cette fin, l'étude de trois marqueurs génétiques différents fut entreprise.

Tout d'abord, la cassette ribosomale ITS1-5,8S-ITS2. Les amorces utilisées se fixent dans les régions très conservées des gènes 18S et 26S. Elles permettent d'amplifier par PCR un fragment contenant le gène 5,8S, très conservé, bordé par deux fragments dits ITS, pour Internal Transcribed Spacer, qui sont eux très variables. Les séquences obtenues peuvent présenter un fort polymorphisme du fait de la haute variabilité des parties ITS. Les ITS ont couramment été employés pour établir une phylogénie des diatomées (Medlin et Kooistra, 2010).

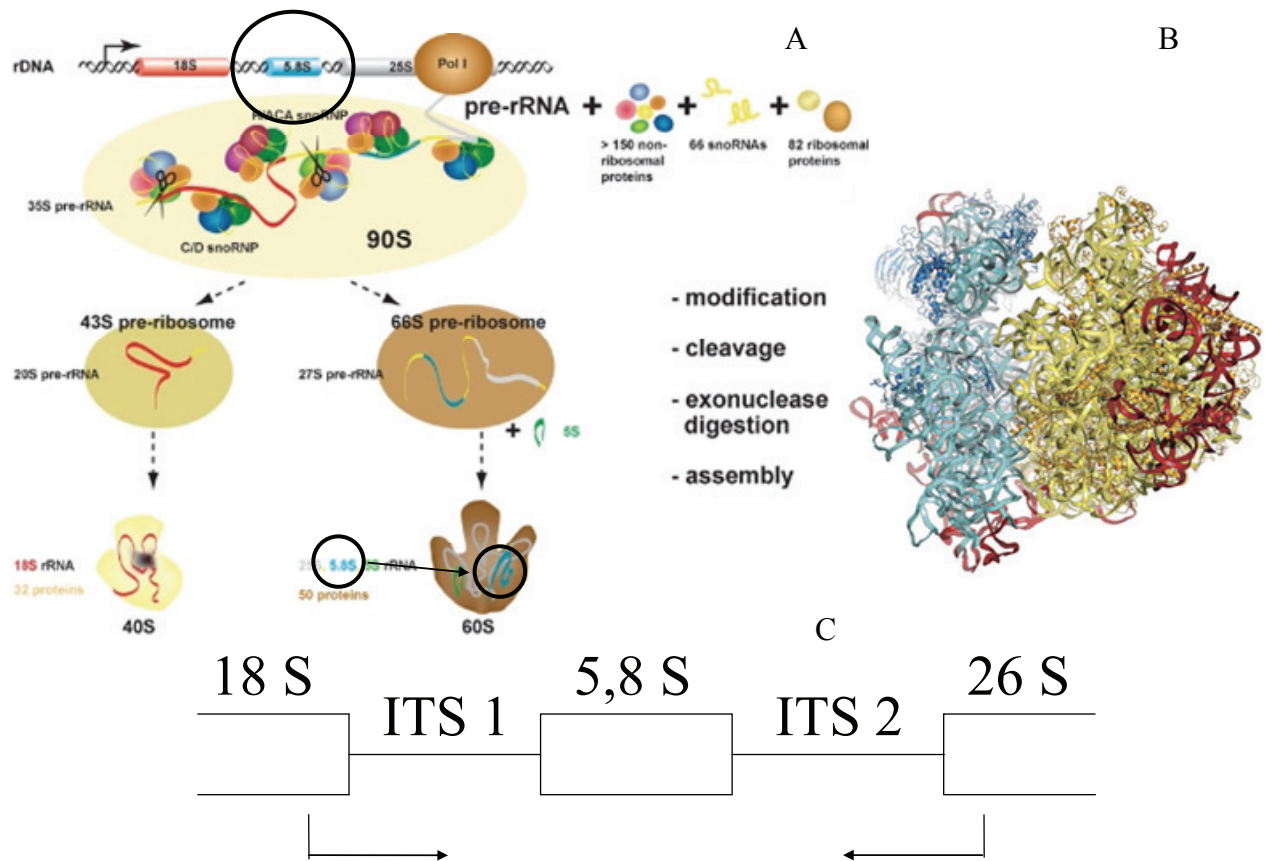


Figure 28. Le marqueur moléculaire de la cassette ribosomale ITS1-5,8S-ITS2. A : la chaîne de synthèse d'un ribosome chez les eucaryotes (entourée en noir, le gène 5,8 S et l'ARNr correspondant). B : représentation en 3 dimensions d'un ribosome d'eucaryote (levure) d'après Ben-Shem *et al.* (2010). C : schéma de fixation des amorces utilisées dans cette étude permettant l'obtention d'un fragment complet de longueur variable.

Le gène *rbcL* code pour la grande sous-unité de l'enzyme Ribulose 1,5-Biphosphatase Carboxylase/Oxygénase, ou RuBisCO, qui intervient au début du cycle de Benson Calvin et Bassham pour la fixation du gaz carbonique. Les amorces qui ont été utilisées furent définies pour se fixer en amont et en aval du gène, permettant d'obtenir un fragment de 1545 pb contenant une séquence complète de 1473 pb codant pour une protéine fonctionnelle de 491 acides aminés.

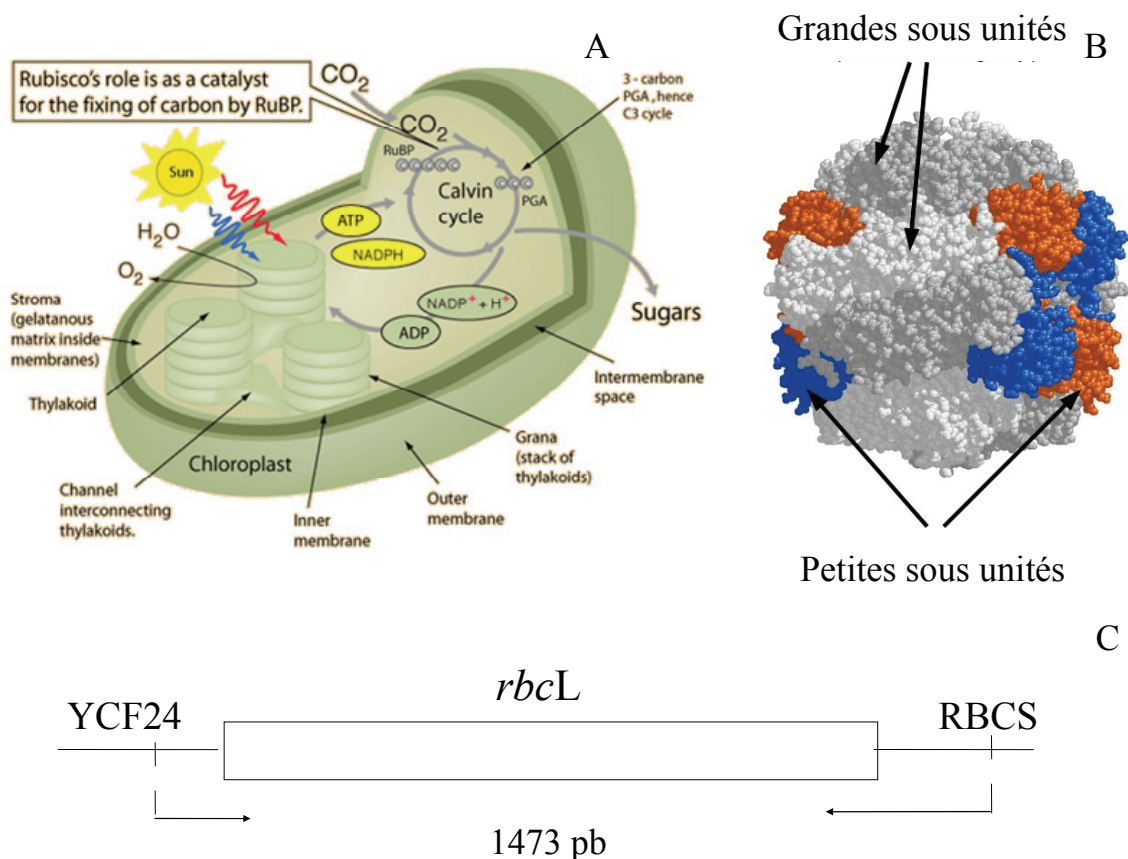


Figure 29. Le marqueur moléculaire *rbcL*. A : schéma d'un chloroplaste de végétal supérieur avec représentation du cycle de Calvin et du rôle de la Rubisco. B : représentation en 3 dimensions de la protéine enzymatique de la Rubisco, en gris les grandes sous-unités codées par le gène *rbcL*. C : schéma de fixation des amorces utilisées dans cette étude permettant l'obtention d'un fragment complet de 1473 pb.

Le gène *cox1* code pour la première sous-unité de l'enzyme Cytochrome Oxydase, qui est une protéine transmembranaire intervenant en bout de chaîne respiratoire pour catalyser la conversion du dioxygène en molécules d'eau. Les amorces utilisées dans cette étude furent définies à partir de séquences du gène *cox1* de trois diatomées, *Nitzschia frustulum* (AB020225), *Cylindrotheca closterium* (AB020222) et

Fragilaria striatula (AB020224) (Ehara *et al.*, 2000). Ces amorces furent profilées dans le but d'obtenir des séquences partielles d'à peu près 775 pb.

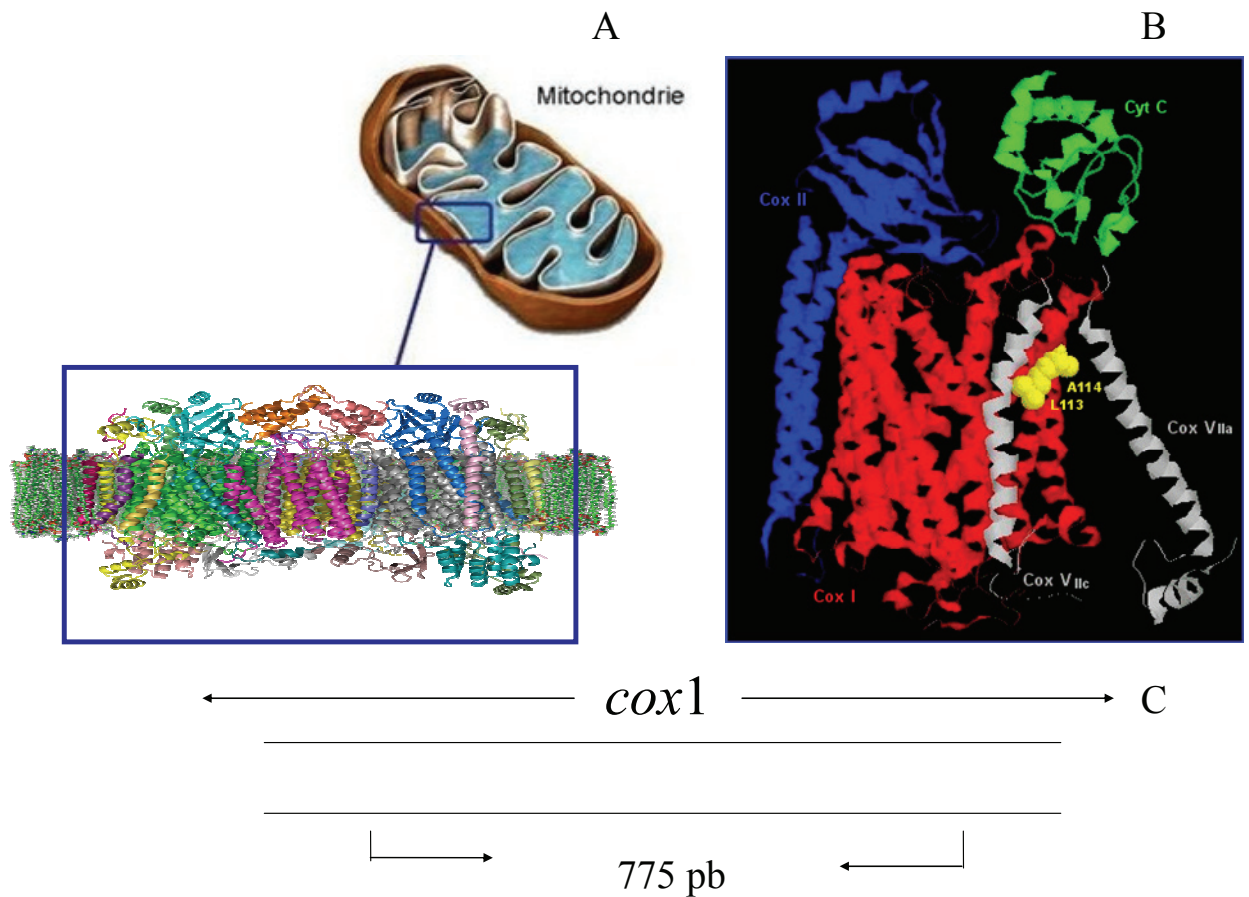


Figure 30. Le marqueur moléculaire *cox1*. A : la mitochondrie et ses protéines transmembranaires impliquées dans la chaîne respiratoire. B : le complexe de la cytochrome oxydase, en rouge la partie codée par le gène *cox1*. C : schéma de fixation des amorces utilisées dans cette étude permettant l'obtention d'un fragment de 775 pb.

Les résultats obtenus ont été rédigés sous la forme d'une version préliminaire d'un article, dont la mise aux normes suit les consignes de la revue Protist.

IV.1.2 Molecular phylogeny of the *Haslea* spp. (pennate diatoms, Naviculaceae): “To be or not to be blue diatom” Is it the same?

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Abstract

The *Haslea* genus (pennate diatoms, Naviculaceae) includes more than twenty species. This genus is known by the existence of *Haslea ostrearia* which produce a blue pigment used in the aquaculture to the “greening oyster”. “Blue diatoms” were observed in different geographic localizations but any analysis has been developed to confirm if these blue microalga represent the same species “*H. ostrearia*”. Molecular investigation was performed using the mitochondrial Cytochrome Oxidase subunit I (*cox1*), the ribosomal box ITS1-5.8S-ITS2 and the chloroplastic Ribulose biphosphate Carboxylase Oxygenase (RubisCO) large sub-unit gene (*rbcL*) gene to determine the phylogenetic relationships existing between several isolates from “blue *Haslea*” and “not blue *Haslea*” collected in Atlantic (Canary Islands, France, Sweden) Ocean, Black Sea (Ukraine) and Mediterranean Sea (France). The ITS box revealed a high length polymorphism allowing easily distinction between the distinct species (for example *H. crucigera*, *H. karadagensis* and *H. ostrearia*) but it was not appropriate to establish the phylogeny. Our phylogenetic analyses (ML, MP), based on *rbcL* and *cox1*, showed two large clades defining the “blue *Haslea*” and “not blue *Haslea*”. Inside the “blue *Haslea*” cluster, three branches (Canary Islands, Ukraine, France/Sweden) lead to suggest a progressive colonization from the Atlantic Ocean to the internal Sea (Mediterranean Sea, Black Sea).

Keywords: *Haslea*, diatom, phylogeny, *cox1*, *rbcL*, ITS, blue pigments

Introduction

Diatoms are commonly classified in three groups: centric, raphids and araphids which are sub-divided into many genera, mainly on the basis of the morphology of their siliceous cell wall, the frustule (Round et al., 1990). Many other criteria were proposed to support a classification of diatoms, such as the tolerance to pH fluctuation (Hustedt, 1939), the shape and number of chloroplasts (Mereschkowsky, 1903; Cox, 1981) or the pyrenoids structure (Schmid, 2001). Diatoms are essentially considered as brown microalgae, a colour resulting from their main accessory photosynthetic pigments, fucoxanthin, diatoxanthin and diadinoxanthin, which mask the chlorophylls (a and c). One species derogates to this statement, the pennate diatom *Haslea ostrearia* (Bory/Simonsen, 1824/1974), which produces during growth a water-soluble non-photosynthetic blue pigment, transiently stored at cell apices (Robert et al., 2002). This specific pigment has been called marennine, in reference to the French region of Marennes-Oléron (Lankester, 1886), a place of intense oyster farming. In oyster ponds during blooms of *H. ostrearia*, which occur unpredictably, marennine released in the water can be fixed on oyster gills, which turn green. Green oysters represent an added value for producers, increasing their price and, according to connoisseurs, their palatability, a phenomenon which has been known for centuries (e.g., Sprat, 1669; Gaillon, 1820). Recently marennine got increased attention, and progresses were made regarding its characterization and his proprieties (Pouvreau et al., 2006; 2007; 2008).

Concerning his taxonomic statue, the blue diatom *H. ostraria* was first named *Vibrio ostrearius* by (Gaillon, 1820), then it was referred to as *Navicula ostrearia* (Bory, 1824), and finally transferred to the *Haslea* genus (Simonsen, 1974). The diagnosis and characters retained for this transfer were based on morphological features

needing Scanning Electron Microscopy to be observed. Longitudinal siliceous slits parallel to the raphe on the external side of the valve, quadrangular areolae on internal side, are two criteria mainly retained to classify a diatom as belonging to the *Haslea* species. In its description of the genus *Haslea*, *H. ostrearia* is used as the type model species (Simonsen, 1974). *H. ostrearia* has long been considered a cosmopolitan species because many occurrences of this diatom were noticed in almost all Seas and Oceans (Table 1).

However, very few precise studies were based on SEM technology and recent molecular methods. In the last three years, four new species of pigmented diatoms resembling *H. ostrearia* were described using morphological and molecular markers: *Haslea karadagensis* from the Black Sea (close to Karadag on the Crimean Coast) which produces a blue-grey pigment; *Haslea silbo*, a blue diatom isolated in the Canary Islands (La Gomera Island), producing a pigment very similar to marennine; a new species temporarily named *Haslea provincialis* found in the western Mediterranean Sea and a fourth species of “blue diatom” recently observed in the Canary Islands (Gran Canaria Island) and temporarily named *H. acoran*. Apart from these pigmented diatoms, the genus *Haslea* comprises species with strong morphological, physiological and ecological differences. For example, *Haslea nipkowitzii* has a sigmoid shape (Poulin et al., 2004), *H. gigantean*, which is a highly morphologically similar to *H. specula*, presents a mesoplanktonic size (Simonsen, 1974; Lange-Bertalot, 1997); *H. salstonica* (Massé et al., 2001) have a thickening of the central ribs called a pseudo-stauros, and their related species *H. crucigera* forms tubes of mucilage (Simonsen, 1974). A preliminary phylogeny of the genus *Haslea* has already been made using the 16S gene but it based

on limited number of *Haslea* species (Poulin et al., 2004; Sinnighe-Damste et al., 2004).

Geographical area of observation	Bibliographical reference	Geographical area of observation	Bibliographical reference
Colchester oyster ponds, United Kingdom	Sprat, 1669	Belgian coast, North Sea, Belgium	M'harzi et al., 1998
Plymouth, Channel Sea, United Kingdom	Hustedt and Aleem, 1951	Norwegian coast, North Sea, Norway	Hendey, 1964
Banyuls, Mediterranean Sea, France	Sauvageau, 1906	Sweden, North Sea	Wulff et al., 2000 Cleve-Euler, 1955 Aleem, 1973 Kuylenstierna, 1990
Sardinia, Mediterranean Sea, Italy	Solazzi and Tolomio, 1976 Tolomio, 1978	Denmark, North Sea	Petersen, 1916
Palermo, Sicilia, Mediterranean Sea, Italy	Barone, 1979	Baltic Sea	Snoeijs et al. Kasperovičienė, 1996
Trieste, Adriatic Sea, Italy	Molisch, 1903	Rhode Island, Atlantic Ocean	Kennett and Hargraves, 1991
Suez Canal estuary, Egypt	Madkour et al., 2009	Virginia, Atlantic Ocean	Mitchell and Barney, 1918
North Caucasus, Black Sea, Russia	Proshkina-Lavrenko, 1964	New Jersey, Atlantic Ocean	Sullivan, 1977
Indian Ocean	Simonsen, 1974	Australia, Pacific Ocean	Ricard, 1987 Volkman et al., 1994
Japan, North Pacific Ocean	Ranson, 1937	San Juan Islands	Hardy, 1973

Table 1. Occurrences of *H. ostrearia* around the world with references.

As complex species of “blue *Haslea*” was recently described with putative four new species (*H. acoran*, *H. karadagensis*, *H. provincialis*, *H. silbo*), we carried out a molecular investigation to establish precisely the phylogenetic relationship existing inside the *Haslea* genus. Three independent molecular markers were analysed: The ribosomal Internal Transcribed Spacer (ITS1), chloroplastic Ribulose biphosphate Carboxylase Oxygenase (RubisCO) large sub-unit gene (*rbcL*) and mitochondrial Cytochrome Oxidase first subunit gene (*cox1*). Ten species of the *Haslea* genus were included in this study (table 2 ; figure 1).

Species	Origin	Provider	References
<i>H. acoran</i>	Gran Canaria, Canary Island Spain	SCCAP	SCCAP 1584
<i>H. pseudostrearia</i>	Robben Island, South Africa	SCCAP	SCCAP 1594
<i>H. crucigera</i>	Etier du Pouliguen, France	NCC	NCC 185.0
<i>Haslea</i> sp. 1	Piriac sur Mer, France	NCC	NCC 189
<i>Haslea</i> sp. 2	Noirmoutier, France	NCC	NCC 266
<i>Haslea</i> sp. 3	Boulouris, France	NCC	NCC 330

Species	Origin	Provider	References
<i>H. ostrearia</i> population 1	Bay of Bourgneuf, France	NCC	NCC 148.76 NCC 235.1 NCC 156.1
<i>H. ostrearia</i> population 2	L'houmeau, France	NCC	NCC 345
<i>H. ostrearia</i> population 3	Kattegat Detroit, Sweden	NCC	NCC 318 NCC 320 NCC 321
<i>H. karadagensis</i>	Karadag Coast, Crimea, Ukraine	NCC	NCC 313 NCC 316
<i>H. silbo</i>	La Gomera, Canary Island Spain	SCCAP	SCCAP 1283 SCCAP 1285 SCCAP 1289
<i>H. provencialis</i>	Boulouris, France	SCCAP NCC	SCCAP 1343 NCC 323

Table 2. Species' names, geographical origins, providers and references of the strains.

NCC means Nantes Culture Collection and SCCAP Scandinavian Culture Collection of Algae and Protists.

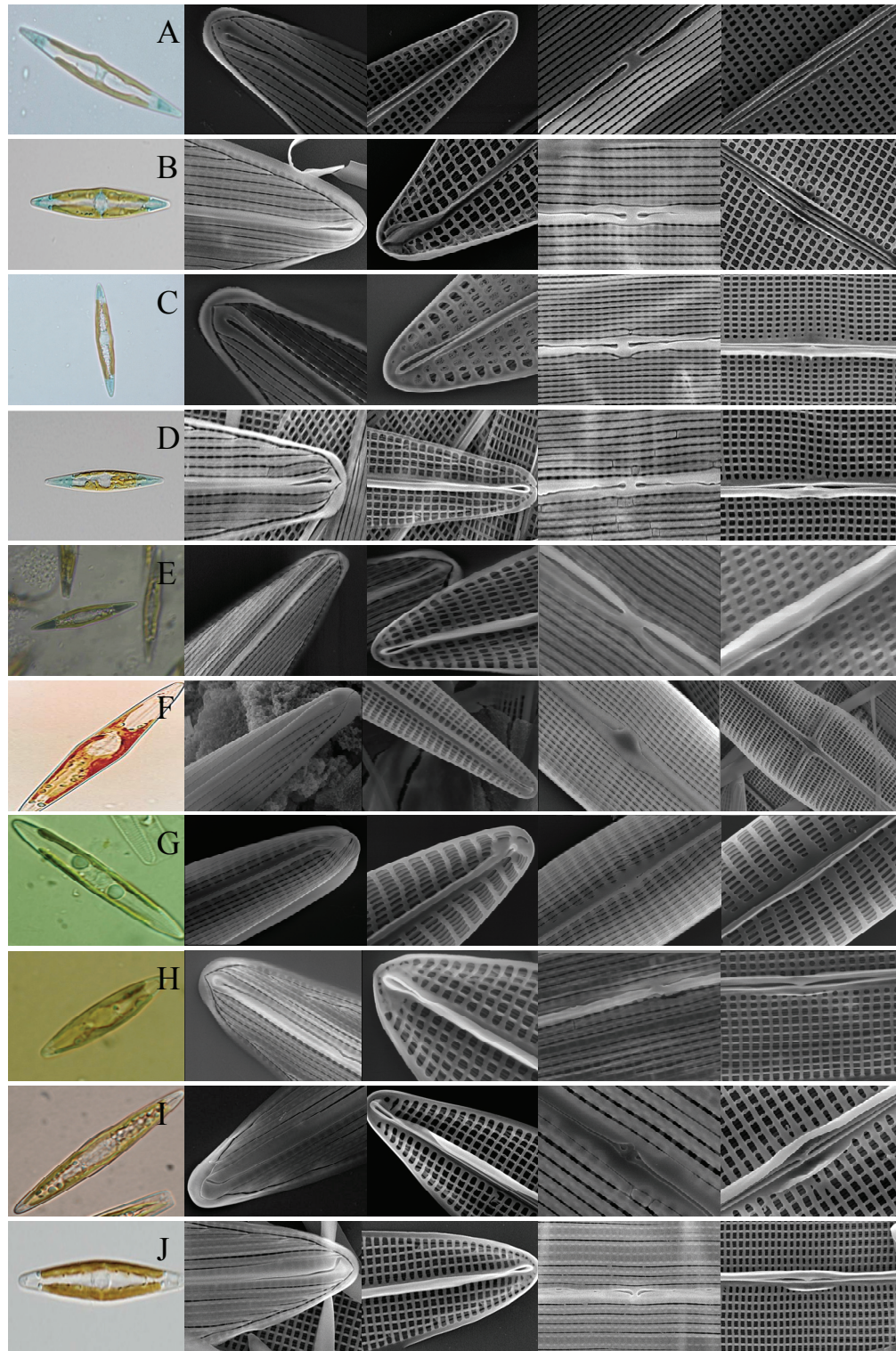


Figure 1. The species of diatoms used for phylogeny. A: *H. ostrearia*. B: *H. silbo*. C: *H. provencialis*. D: *H. acoran*. E: *H. karadagensis*. F: *H. crucigera*. G: *Haslea* sp.1 NCC 189. H: *Haslea* sp. 2 NCC 266. I: *Haslea* sp. 3 NCC 320. J: *H. pseudosteraria*. From left to right : view of living cells with optical microscope, apical raphe ending-external

view, apical raphe ending-internal view, central raphe ending-external view, central raphe ending-internal view.

Results

The respective primers, COI1/COI6, DPrbcL1/DPrbcL7 and ITS1/ITS4 allowed getting respectively fragment from *cox1* (775 bp), *rbcL* (1473 bp, complete ORF) and the complete ribosomal box ITS1-5.8S-ITS2, for all species tested. The length of the ribosomal box was highly variable, ranged from 586 bp to 1370 bp, according to the species studied. This high polymorphism not allowed using the ITS1 for the phylogenetical analyses. The best evolutionary model estimated by jModeltest was the GTR (general time-reversible model) for the three molecular markers (Lanave et al., 1984; Rodriguez et al., 1990).

For the *cox1* gene: Phylogenetical topology obtained for the MP (PAUP) and ML (MEGA5) trees was similar. In the MP analysis, on 775 characters, 457 were constant, 120 were parsimony uninformative and 198 were parsimony informative. For the consensus MP tree, the consistency index was 0.693 and the retention index 0.755. Length was 600. Tree was built under the 50% majority rule consensus of 6 trees. With Maximum Likelihood, the negative neperian Likelihood logarithm was 2986.72.

For the *rbcL*: the typology of the ML and MP trees was equally the same. In the MP approach, on the 1473 characters, 1141 were constant, 176 were parsimony uninformative and 156 were parsimony informative. For the Mp tree, the consistency index was 0.707 and the retention index 0.624. Length was 532. Tree was built under the 50% majority rule consensus of 6 trees. With Maximum Likelihood, the negative

neperian Likelihood logarithm was 3861.75. ‘Blue’ diatoms always belong to a main cluster.

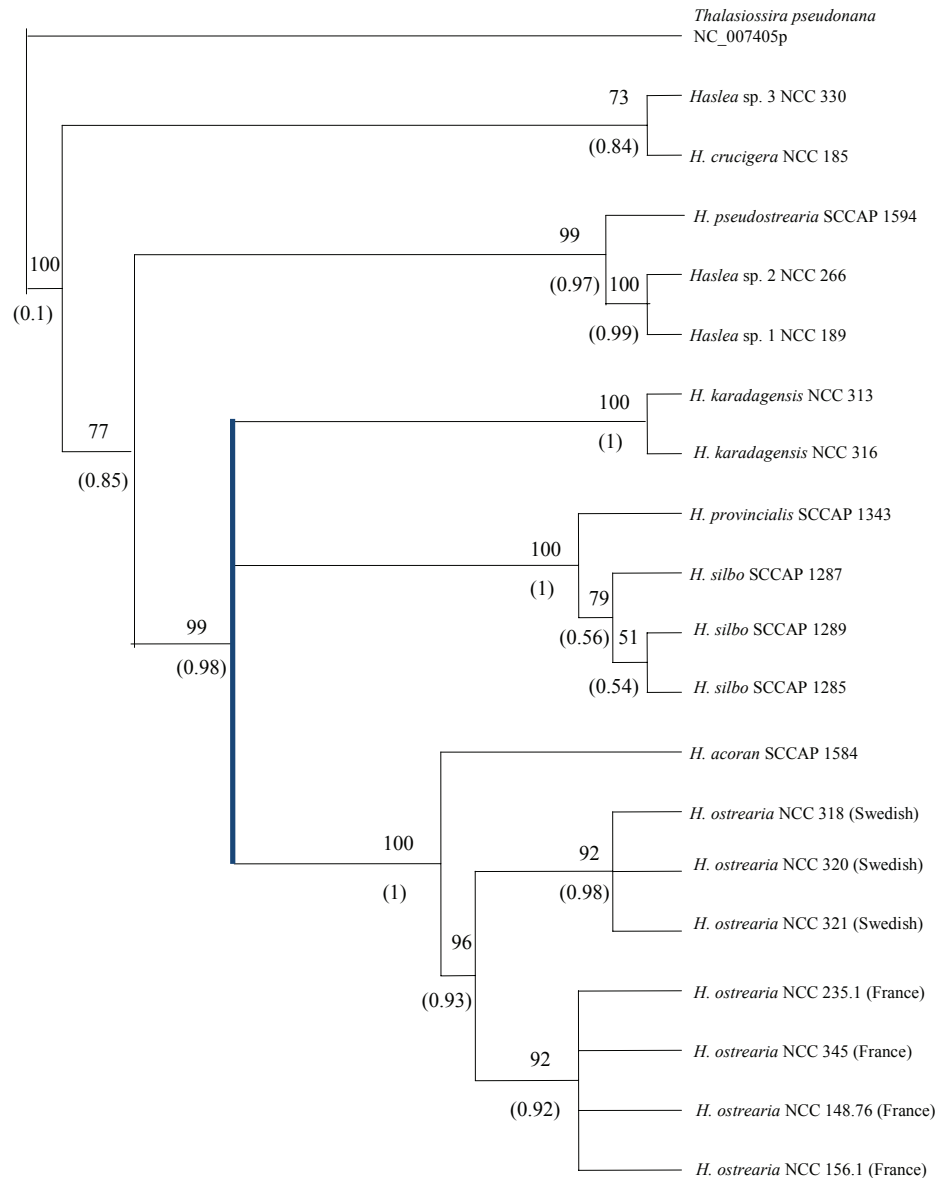


Figure 2. Phylogenetic tree obtained with the *cox1* gene, constructed after PAUP (Length=600, CI=0.693, RC=0.755) and Mega5 (-lnL=2988.72). Bootstrap values after 1000 replications for Maximum Parsimony are given as entire number up the clades. Bootstrap values after 1000 for Maximum Likelihood are given in brackets below the clades. Blue clade bar indicates the ‘blue diatoms’.

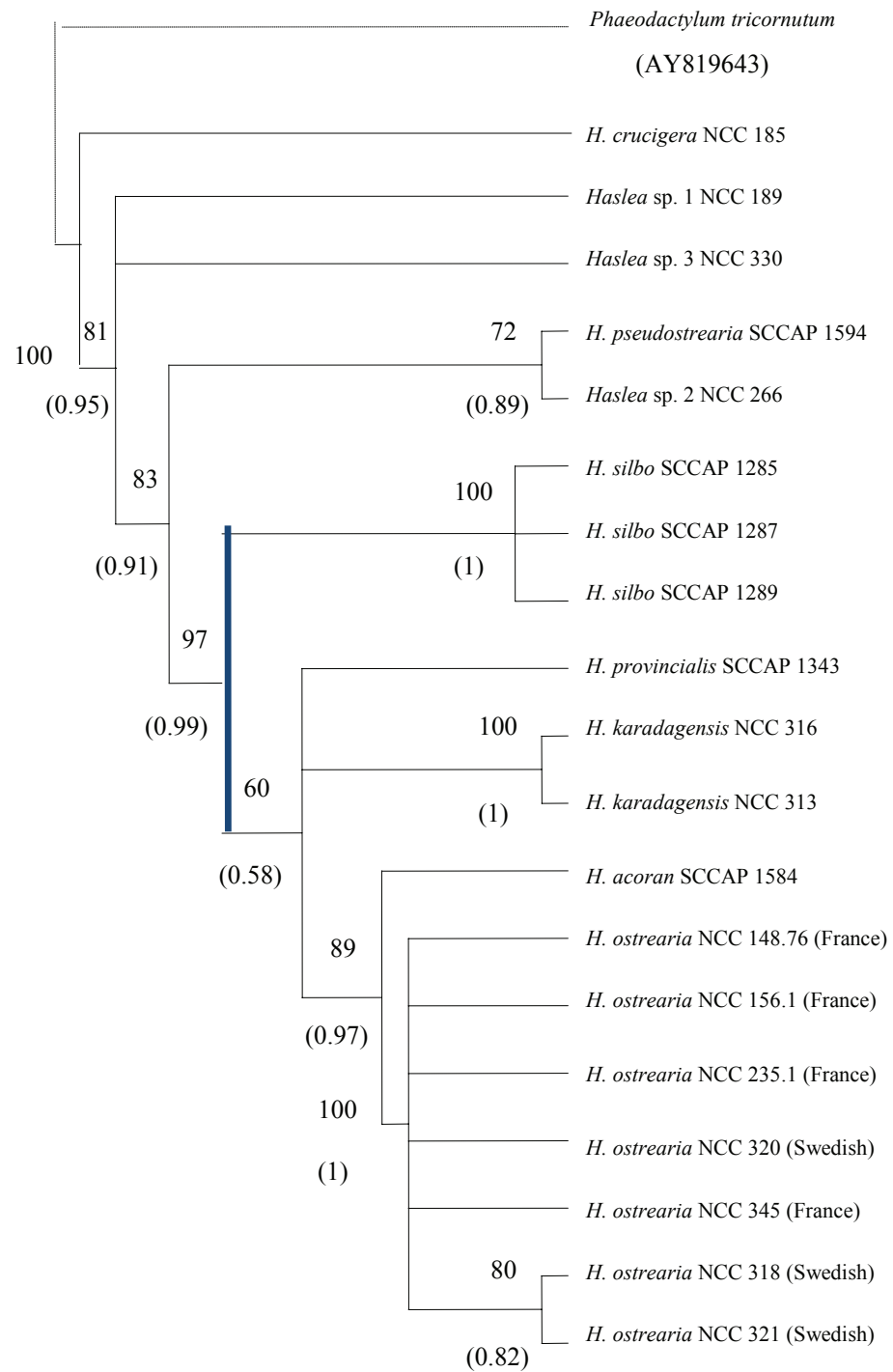


Figure 3. Phylogenetic tree obtained with the *rbcL* gene, constructed after PAUP (Length=532, CI=0.707, RC=0.624) and Mega5 (-lnL=3861.75). Bootstrap values after 1000 replications for Maximum Parsimony are given as entire number up the clades. Bootstrap values after 1000 for Maximum Likelihood are given in brackets below the clades. Blue clade bar indicates the 'blue diatoms'.

Name of the species	<i>H. crucigera</i>	<i>Haslea</i> sp. 1 NCC 189	<i>Haslea</i> sp. 2 NCC 266	<i>Haslea</i> sp. 3 NCC 330	<i>H. pseudostrearia</i>	<i>H. silbo</i>	<i>H. acoran</i>	<i>H. ostrearia</i>	<i>H. karadagensis</i>
Average lengths of ITS1 box (with primers) in bp	586	679	819	833	1181	1097	1122	1104	1370

Table 3. Average length of ITS box given in bp value for the different *Haslea* species

Discussion

We now know that the species *H. ostrearia* is not the only ‘blue diatom’, marennine is not the only blue pigment synthesized by diatoms. Our Phylogenetical analyses, based on the *coxI* and *rbcL*, clearly showed that the pigmented *Haslea* (blue and blue-grey *Haslea*) constitute a robust clade, independent that the “not blue *Haslea*” (figure 2 and 3). These results are congruent with the analyses of other authors (Evans et al., 2007). The ITS1 sequences revealed highly polymorphs (Table 3). For the “blue *Haslea*”, ITS box length was always beyond 1000 bp, whereas it was below for all the ‘not blue *Haslea*’ species, excepted for *H. pseudostrearia*.

Haslea ostrearia, historically the first species of blue diatom

For reasons related to history, as well as economy or gastronomy, *H. ostrearia* and its marennine were intrinsically associated to oysters, and first observations mainly

came from either England or France (Briée, 2010). The presence of *H. ostrearia* elsewhere in Swedish waters has been deduced by Wulff et al. (2000) on the basis of blue apices in a pennate diatom. Our molecular study (*cox1*, ITS1, *rbcL*) equally showed that French and Swedish samples may include in same species *H. ostrearia*. The strains genetically close may be inferred from their ability to interbreed. The strains from the French Atlantic coast, and the strains from the Kattegat Detroit live in similar environmental conditions, regarding to changes in salinity and their tolerance to, due to mixing between salted and brackish waters. Salinity in the Marennes-Oléron basin, which varies between 24 psu to 35 psu (Héral et al., 1983), can be compared with the salinity in the Kattegat Detroit, which can vary between *ca.* 23.5 to 34 psu (Håkanson, 2009). Average temperatures in these areas are however different, varying in Marennes Oléron from 8 °C to 26 °C over a year (Héral et al., 1983), but reaching 34 °C at a sediment surface in summer (Vouvé et al., 2000), and from *ca.* 3 °C to 17 °C in the Kattegat Detroit (Håkanson, 2009). *H. ostrearia* is clearly known to be euryhaline (Neuville et Daste, 1978; Wraige et al., 1998), and widespread in temperate to tropical waters (Round *et al.*, 1990), although there is no ground evidence to support this assertion.

Then there was Haslea karadagensis

H. karadagensis, the diatom from the Black Sea, is the first new blue diatom, different from *H. ostrearia*, ever isolated and described (Gastineau et al., submitted). Inside the ‘blue lineage’, position of the species *H. karadagensis* needs to be precise. Indeed, this diatom produces a blue-grey pigment, which is clearly different from those synthesized by the other blue diatoms. Our Phylogenetical analyses (MP, ML), and our

interbreeding test, confirmed that *H. karadagensis* and *H. ostrearia* may be considered as two distinct species. The Black Sea is separated from the Atlantic Ocean by the Bosphorus Strait, the Sea of Marmara, the Strait of the Dardanelles, the Aegean Sea, the Mediterranean Sea and finally the Gibraltar Strait. All these separations may have been factors maximizing the allopatric speciation process probability among blue diatoms.

***Haslea silbo* and *H. acoran*, the Canarian connexion**

Blue diatoms isolated from the Canary Islands correspond to two different periods of sampling, and two different islands in the archipelago. Both present morphological, as well as molecular characters that allow differentiating them from *H. ostrearia*. Despite the fact that the Canarian blue diatoms do not form a cluster, they present some morphological characteristics which are different from the other blue diatoms, *H. ostrearia*, *H. karadagensis* and *H. provincialis*. The areolae of *H. acoran* and *H. silbo* have a square shape, whereas the frustule of the other three species is perforated by rectangular areolae. Square areolae seem most common in non-pigmented species like *H. crucigera*, *H. pseudostrearia*, *Haslea* sp. NCC 226 and *Haslea* sp. NCC 330.

A majority of species from the *Haslea* genus are benthic (with the notable exception of *Haslea wawrikan*). *H. ostrearia* is known to be tytopelagic, meaning it can sometimes become planktonic (Robert, 1983). But without a coastal continuity (like between France and Sweden), an archipelago like the Canary Islands may have been a favourable place for isolation of the genetic flux and allopatric speciation process. Moreover, the presence of two closely related but different species in the coastal waters

of a same archipelago illustrates the peculiar interest of the Canary Islands for microevolution studies (Juan et al., 2000). Specific diversity across Canary Islands has been noticed in such various organisms as insects (Contreras-Díaz et al., 2007; Emerson et al., 1999; Juan et al., 1997; Moya et al., 2006), angiosperms (Meimberg et al., 2006), mammals (Vogel et al., 2002), birds (Suárez et al., 2009; Kvist et al., 2005). Although microevolution of marine organisms may follow different rules, our observations could support the hypothesis that despite they are marine micro-organisms with possibly a strong dispersal, diatoms can encompass endemic species (Vanormelingen et al., 2008). Gran Canaria Island is closer to the African Coast than La Gomera Island, which may be congruent with the in-between phylogenetic position of *H. acoran* with regards to *H. ostrearia* and *H. silbo*, and appeals for further investigations (figure 2 and 3).

***Haslea provincialis*, a possible missing link**

H. provincialis, which has been sampled in Mediterranean Sea in an area distant of ca. 2000 km from the Gibraltar Detroit, can be another example of parapatric speciation. According to preliminary results, the blue pigment produced by this diatom sampled in the French coast of the Mediterranean Sea seems more related to marennine or to the pigments produced by *H. silbo* and *H. acoran*, than to the pigment produced by the Ukrainian blue diatom, *H. karadagensis*. Regarding the molecular markers, the tree obtained using the *rbcL* gene underlined a close proximity between *H. provincialis* and *H. karadagensis*. The *cox1* tree, in contrast, associates *H. provincialis* to *H. silbo*. Geological history of the Mediterranean Sea includes periods of closing/opening on the Atlantic Ocean, consequently followed by changes in the flora and fauna, by settlement of foreign species among other factors (Hsü et al., 1977). Hence, the history of the

colonization of this place by blue diatoms possibly originating from the Atlantic Ocean has still to be unravelled, but is an intriguing topic. Sampling facilities and collection uncertainties made us unable to get a continuum of strains from the Gibraltar Detroit to the Black Sea. If such a species collection is ever available, it could make possible to study the continuity of the blue diatom dispersal and diversity, and to pinpoint where and when blue-grey diatoms arose. The paleogeographic, paleogeologic and climatic changes, the variations of salinities in these areas because of fluvial streams, are all factors mainly reported to be possible explanation of speciation process in aquatic organisms, or for the occurrences of biological species (Stefanni et al., 2002; Mejri et al., 2011; Audzijonyte et al., 2008; Mäkinen and Merilä, 2007; Faria et al., 2006; Jesse et al., 2011).

Information retrieved from the non-blue Haslea

Among other diatoms from the genus *Haslea* which do not have the peculiarity to synthesize a marennine-like pigment (“not blue *Haslea*”), the case of the unidentified *Haslea* sp. 1 NCC 189 raises different questions regarding their phylogenetic relations. The frustule of this species exhibits externally the longitudinal silica slits, relevant to the *Haslea* genus, as evidenced by SEM. However, the shape of the areolae strongly differs, looking more similar to the main features of *Navicula* species. Moreover, under optical microscopy, two spherical shapes are present, but their nature is unclear. Among all the *Haslea* species tested in this work, *Haslea* sp. 1 NCC 189 is the only one having this feature, which can occasionally be observed in other pennate diatom genera, being assimilated to volutine grains. According to the phylogenetic trees presented here, this diatom does not form an outgroup, and always belongs to a same cluster together with

Haslea sp. 2 NCC 266 and *H. pseudostrearia*. Given the data afforded by molecular biology, the differences observed in this species morphology as compared to other species, are not sufficient to position it outside the genus *Haslea*. In a phylogenetic tree based on the 16S gene, Poulin et al. (2004) showed that *H. crucigera* and *H. salstonica* form a cluster that is relevant as both species have a pseudo-stauros. Unfortunately, none of the species tested in this work did have a pseudo-stauros, to the exception of *H. crucigera*. And there is not such morphological evidence to link *H. crucigera* with *Haslea* sp. 3 NCC 330. Nevertheless, the *cox1* marker tree, with both PAUP and MEGA5 calculation, distributes the two species inside a single cluster, with relevant bootstrap values.

In conclusion, for decades, *H. ostrearia* was meant an exception among the entire Bacillariophyceae class. Now, it appears that there is a whole collection of species, inside a unique genus, which shares the ability of producing blue pigments. Since these pigments are promising in putative valorisations, they earn extensive awareness from specialists, and new assays are today performed in the aim of elucidating their structures and natures. From the moment their chemical structure will be known, the question of their anabolic pathway and their dispersal among the genus will be raised. Since we now possess several strains of ‘blue *Haslea*’ and ‘not blue *Haslea*’ diatoms for comparisons, there is an exciting field of research in physico-chemistry, biochemistry, physiology and molecular biology opening for us.

Material and methods

Biological material and experimental culture

Some *Haslea* samples are available either in the Nantes Culture Collection (NCC, Nantes, France) or in the Scandinavian Culture Collection of Algae and Protist (SCCAP, Copenhagen, Denmark). Diatoms whose specific identification has not been done yet, are only referenced after their number of classification in the culture collection. Cells were grown in controlled conditions in a culture cabinet. Sterile vessels were used with artificial sea water (Mouget et al., 2009). They were cultivated under an illumination of 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ distributed 14h/10h L/D cycle, measured with a Li-Cor LI-189 quantum meter coupled with a 2II Li-Cor Q21284 quantum sensor. Light was provided by fluorescent tube Philips TLD 36W/965. Growth temperature was 15 ± 1 °C.

DNA extraction and PCR conditions

Biomass of cultures in exponential phase of growth was collected by gentle centrifugation using a Sigma 3K15 model from Bioblock scientific, and then crushed with liquid nitrogen. DNA extraction was made using a Qiagen DNeasy Plant mini Kit. Primers for the ribosomal box were the universal ITS1 and ITS4 primers (White et al., 1990). Their sequences are 5'-TCCGTAGGTGAACCTGCGG-3' (Forward) for ITS1 primer and 5'-TCCTCCGCTTATTGATATGC-3' (Reverse) for ITS4. Primers for the *rbcL* gene were DPrbcL1 and DPrbcL7 (Daugbjerg and Andersen, 1997; Jones et al., 2005). Their sequences are 5'-AAGGAGAAATHAATGTCT-3' (Forward) for DPrbcL1 and 5'-AARCAACCTTGTGTAAGTCTC-3' (Reverse) for DPrbcL7. Primers for the *cox1* gene were manually designed using a multiple alignment based on the sequences available in GenBankTM database and isolated from *Nitzschia frustulum*

(AB020225), *Cylindrotheca closterium* (AB020222) and *Fragilaria striatula* (AB020224) (Ehara et al., 2000). Their sequences are 5'-GTWATGCCWAYTTTRATWGG-3' (Forward) for COI1 and 5'-CCCATAAWGTHGCTAACC-3' (Reverse) for COI6. The PCR mixtures were done as following: 5 µL of 5 X green GoTaq[®] flexi buffer, 2.5 µL of MgCl₂ 25 mM, 0.5 µL of primers, 0.5 µL PCR nucleotide mix (10 mM each), 15.38 µL of ultra-pure water provided by a Synergy Millipore system, 0.12 µL of Taq polymerase and 1 µL of DNA, final volume 25 µL. The Eppendorf Mastercycler Gradient thermocycler conducted 40 cycles of polymerisations. PCR products were separated by agarose gel electrophoresis (1% agarose, 110 volts, 30 min). Revelation was made under UV light using a CN-1000 Darkroom by Vilber Lourmat.

Cloning and sequencing

Elution of PCR products was made with a Wizard[®] SV Gel and PCR Clean-up System (Promega). DNA was integrated into pGEM[®] plasmid, with a T Easy Vector System I (Promega). The recombined vector was integrated into competent *Escherichia coli* cells NEB-5-alpha (New-England Biolabs). White /blue coloration serve to identify positive colonies. Sequencing reactions were provided by Beckmann Coulter Genomics (United-Kingdom, <http://www.cogenicsonline.com>) using SP6 and T7-promoters.

Phylogenetic analyses

Reliability of sequences and potential contaminations were assessed with NCBI Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Preliminary Alignment were made using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and visualised using Genedoc software (<http://www.nrbsc.org/gfx/genedoc/>). The best evolutionary model was determined using jModeltest (<http://darwin.uvigo.es/software/jmodeltest.html>). Maximum Parsimony analysis was built using PAUP* 4.0 software (<http://paup.csit.fsu.edu/>) and Maximum Likelihood approach was established using MEGA 5 software (<http://www.megasoftware.net/>). Outgroup sequences (indicated on figures) were extracted from GenBankTM database (<http://www.ncbi.nlm.nih.gov/genbank/>).

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IV.2 Héritabilité de l'ADN mitochondrial chez *H. ostrearia*

IV.2.1 Introduction

Peu de choses sont connues quant à l'héritabilité de l'ADN mitochondrial chez les diatomées (Evans *et al.*, 2007). Chez une macro-algue brune comme *Scytosiphon lomentaria* (Kato *et al.*, 2006) et chez une chlorophycée unicellulaire comme *Chlamydomonas reinhardtii* (Aoyama *et al.*, 2006), des études ont montré une transmission uniparentale des mitochondries. Chez les diatomées en revanche, il y a un grand manque d'informations. Comme dit précédemment, *H. ostrearia* est une diatomée dont la reproduction est isogame. Il est donc au premier abord impossible de parler de clones mâles ou femelles, aucune donnée phénotypique n'allant dans le sens d'un dimorphisme sexuel, même au niveau des gamètes. De plus, comme décrit dans Davidovich *et al.* (2009), les gamètes formés par deux clones compatibles d'*H. ostrearia* entrés en auxoporation ne montrent pas de différences de comportement.

L'inter-fécondité entre les souches d'*H. ostrearia* provenant de la Baie de Bourgneuf et celles du détroit de Cattéat a déjà été évoquée précédemment. En réalisant la phylogénie du genre *Haslea*, il est apparu qu'il existait dans les séquences des fragments du gène *cox1* des bases, au nombre de 7, qui se révélèrent systématiquement discriminantes entre les deux populations. Ceci nous a permis de rechercher le mode de transmission de l'ADN mitochondrial chez *H. ostrearia*, par l'obtention d'hybrides entre les populations pré-citées. Un premier clone hybride, aujourd'hui perdu, avait été isolé suite à un épisode d'auxoporation intervenu entre les clones NCC 234 (baie de Bourgneuf) et NCC 321 (détroit de Cattéat). Des expériences préliminaires ont par la

suite permis de mettre en place deux couples de clones compatibles, les couples NCC 148.78 (France) x NCC 320 (Suède) et NCC 158.4 (France) x NCC 321 (Suède).

Le propos de ces expériences a été d'obtenir plusieurs hybrides afin d'analyser leurs séquences partielles de *cox1*, et de les comparer à celles des clones parentaux. Au moment de la rédaction de ces lignes, 8 séquences ont été obtenues pour 10 hybrides provenant de l'auxosporulation du couple NCC 148.78 x NCC 320, ainsi que 3 séquences de 4 hybrides provenant de l'auxosporulation du couple NCC 158.4 x NCC 321.

Les résultats obtenus ont été rédigés sous la forme d'une version préliminaire d'un article, dont la mise aux normes suit les consignes de la revue Protist.

IV.2.2 Inheritance of the mitochondrial DNA in the pennate diatom *Haslea ostrearia* (Naviculaceae) during auxosporulation suggests a uniparental transmission

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Abstract

Inheritance of the mitochondrial DNA in diatom was not investigated yet. We present the first study developed on the mtDNA transmission in diatoms after auxosporulation by the focus on the pennate diatom *Haslea ostrearia* (Naviculaceae). A fragment of the cytochrome oxydase subunit I (*cox1*) was obtained on 16 strains from French Atlantic Coast and Swedish Detroit of Kattegat. Seven nucleic substitutions inside the *cox1* sequences were discriminating between the French and Swedish *H. ostrearia*. We obtained eleven hybrid cells by auxosporulation after inbreeding between French and Swedish strains. All the hybrids presented only one *coxI* parental haplotype. This result clearly suggests that the mitochondrial DNA transmission in *H. ostrearia* may be uniparental. Based on these results, several implications and new topics of investigation are discussed.

Keywords: *Haslea ostrearia*, diatoms, uniparental, mitochondrial DNA, inheritance

Introduction

Diatoms are unicellular organisms, characterized by a cell wall composed of silica, the frustule, and a unique mode of vegetative division. Frustule is made of two valves, the epitheca (outer) and the hypotheca (inner) that are held in place by connecting (girdle) bands. Mitotic division consists in the separation of the two valves of the mother cell. Each daughter cell inherits one of the two valves of the frustule from the mother cell, and synthesises a new inner valve. Hence, one of the daughter cells is identical in size to the parental cell, the other is smaller. Because of successive divisions, the average cell size of a population of diatoms decreases, a phenomenon usually called the MacDonald-Pfitzer rule (MacDonald, 1869; Pfitzer, 1869). At the end of the life history the cells become critically small, they may acquire deformities and unable to divide indefinitely (for a review, see Kociolek and Stoermer, 2010). Occurrence of the sexual reproduction phase in the life history makes the problem solvable, when cell sizes are restored due to the process of auxoporation (*e.g.*, Geitler, 1932; Chepurnov et al., 2004). Cells become sexually inducible when their length (in pennate) or diameter (in centric) decrease to *ca.* 45% to 55% of the maximum species-specific cell size, sexual reproduction can occur (Davidovich, 2001). Mating system can differ depending on species, in term of triggering factors, gamete and zygote formation, copulation mode or sexual compatibility (Drebes, 1977; Chepurnov et al. 2004). When the process is completed, the size of initial cells resulting from auxosporulation is close to the species-specific maximum one, and a shape of the first descendants is characteristic to the species (in opposition with the parental cell deformities) (Cox, 2010).

Among diatoms, *Haslea ostrearia* (Bory/Simonsen, 1974) has long been known due to its specific blue pigment, called marennine (a name given in reference to the Bay of Marennes-Oléron, France, an area dedicated to oyster farming) (Lankester, 1886). Indeed, *H. ostrearia* and marennine are responsible for the greening of oysters, a phenomenon which has an important local economical impact in French seafood industry and gastronomy, although not completely predictable by oyster producers (Gaillon, 1820; Dyer, 1877; Ranson, 1927). Since the last decade, *H. ostrearia* and marennine received increasing attention in our laboratory. Characterisation of the pigment has been supported with new elements on its chemical nature and molecular weight (Pouvreau *et al*, 2006). For instance, a possible ecological role of marennine, by an allelopathic or a screening effect has been observed (Pouvreau *et al* 2007), as well as its antioxidant and biological activities (Pouvreau *et al.* 2008).

Recently, into the European project BIOVADIA, two major topics related to *H. ostrearia* were addressed: reproduction and biodiversity. First, investigations on its sexual behaviour resulted in the description of a heterothallic reproductive mechanism as an Ib2a type (Geitler, 1973; Davidovich *et al.* 2009), which corroborated and completed a previous study describing homothallic reproduction (Neuville and Daste, 1975; Neuville and Daste, 1979). Thus it was shown that *H. ostrearia* is a dioecious species, with two different sexual types, able to reproduce heterothallically as well as homothallically, in accordance with recent theory on dioecy in diatoms (Roschchin and Chepurnov, 1999; Chepurnov *et al.* 2004). Furthermore, light was demonstrated a main triggering factor which induce the heterothallic auxosporulation in compatible clones of *H. ostrearia* (Mouget *et al.* 2009). The biogeographic molecular studies have led to the description of new *Haslea* species producing marennine-like blue pigments, namely *H. karadagensis* and *H. silbo* (Gastineau *et al.* personal communication).

Presently, it is supposed that *Haslea ostrearia* seems to possess a cosmopolitan repartition. We isolated some strains collected in the Marennes-Oléron Bay and the Bourgneuf Bay in France (two oyster production area in France), and the Kattegat Detroit (Sweden). Cells from these populations revealed morphologically similar, as evidenced by SEM, and able to interbreed. These two features are sufficient to argue for a single species localized in France and Sweden. We analyzed three molecular markers (ribosomal Internal Transcribed spacer I; mitochondrial Cytochrome Oxidase subunit I and the plastidial *rbcL*) which showed that our French and Swedish populations are clearly members of *H. ostrearia* species (personal data not shown). Nevertheless, the *cox1* sequences obtained in our strains exhibited seven nucleic substitutions discriminating the French and Swedish populations. Exploiting these discriminating substitutions inside the *cox1*, we studied the inheritance of mitochondrial DNA after auxosporulation between French and Swedish inbreeding in pennate diatom *Haslea ostrearia*. In other algae, an uniparental transmission of mtDNA has been observed in the phaeophyceae *Scytosiphon lomentaria* (Kato et al. 2006), *Laminaria angustata* (Motomura 1990), *Fucus vesiculosus* (Brawley et al. 1976), *Fucus distichus* (Motomura, 1994), and in the chlorophyceae *Ulva compressa* (Kagami et al. 2008), and *Chlamydomonas reinhardtii* (Aoyama et al. 2006). Nevertheless, to our knowledge, no molecular data demonstrating this phenomenon exist in diatoms yet, as underlined by Evans et al. (2007). For the present work, hybridisations through mating experiments between monoclonal strains originating from two different areas, the Bourgneuf Bay (France) and the Kattegat Detroit (Sweden) were performed. Resulting initial cells were isolated, grown in monoclonal cultures, and their *cox1* fragments amplified, sequenced and compared.

Results

Discriminating substitutions into *cox1* between French and Swedish *H. ostrearia*

A *cox1* fragment (712 bp), obtained using COI1/COI2 primers, matched with the *cox1* gene from the diatoms *Synedra acus* (Ravin et al. 2010; NC_013710.1), *Thalassiosira pseudonana* (Armbrust et al. 2004; NC_007405.1) and *Phaeodactylum tricornutum* (Bowler et al. 2008; Oudot Le Secq and Green, 2011; HQ840789.1), and with the gene portions obtained in the *Sellaphora* complex (Evans et al. 2007, e.g. FJ042920.1). In particular, it matches with the second exon of the *cox1* gene described for *T. pseudonana* (Armbrust et al. 2004). This fragment was analyzed from 16 clones of *H. ostrearia* originating from different geographic areas (Table1).

References	Geographical origins	Date of isolation
NCC 148.76	Bourgneuf Bay, France (Homothallic reproduction progeny)	November 2007
NCC 148.78	Bourgneuf Bay, France (Homothallic reproduction progeny)	November 2007
NCC 148.14a	Bourgneuf Bay, France (Homothallic reproduction progeny)	July 2008
NCC 148.14b	Bourgneuf Bay, France (Homothallic reproduction progeny)	July 2008
NCC 148.14c	Bourgneuf Bay, France (Homothallic reproduction progeny)	July 2008
NCC 156.1	Bourgneuf Bay, France (Heterothallic reproduction progeny)	June 2005
NCC 158.4	Bourgneuf Bay, France (Heterothallic reproduction progeny)	June 2005
NCC 235.1	Bourgneuf Bay, France (natural populations)	November 2007
NCC344	Marennes-Oléron, France (natural populations)	October 2010
NCC 345	Marennes-Oléron, France (natural populations)	October 2010
NCC 346	Marennes-Oléron, France (natural populations)	October 2010
NCC 317	Kattegat Detroit, Sweden (natural populations)	October 2010
NCC 318	Kattegat Detroit, Sweden (natural populations)	October 2010
NCC 319	Kattegat Detroit, Sweden (natural populations)	October 2010
NCC 320	Kattegat Detroit, Sweden (natural populations)	October 2010
NCC 321	Kattegat Detroit, Sweden (natural populations)	October 2010

Tab. 1 Origins of the strains of *H. ostrearia*

It was identical between the two strains from the French Atlantic Coast (Marennes-Oléron Bay, Bourgneuf Bay) but differed by 7 substitutions (noted as follow: French type-postion-Swedish type): T-138-A, C-189-T, C-231-T, C-277-A, A-333-G, C-357-T, G-369-T; as compared with the Swedish clones (Fig. 1). The translation of these *cox1* fragments showed that these discriminative mutations are silent mutations (Fig. 2). BlastP allowed comparing this amino-acid sequence, which was found to be related to functional proteins in other diatoms as *Thalassiosira nitzschoides*

BAA86613 (Ehara *et al.*, 2000), max and total scores of 415, query coverage of 100%, e-value of 2e (first match).

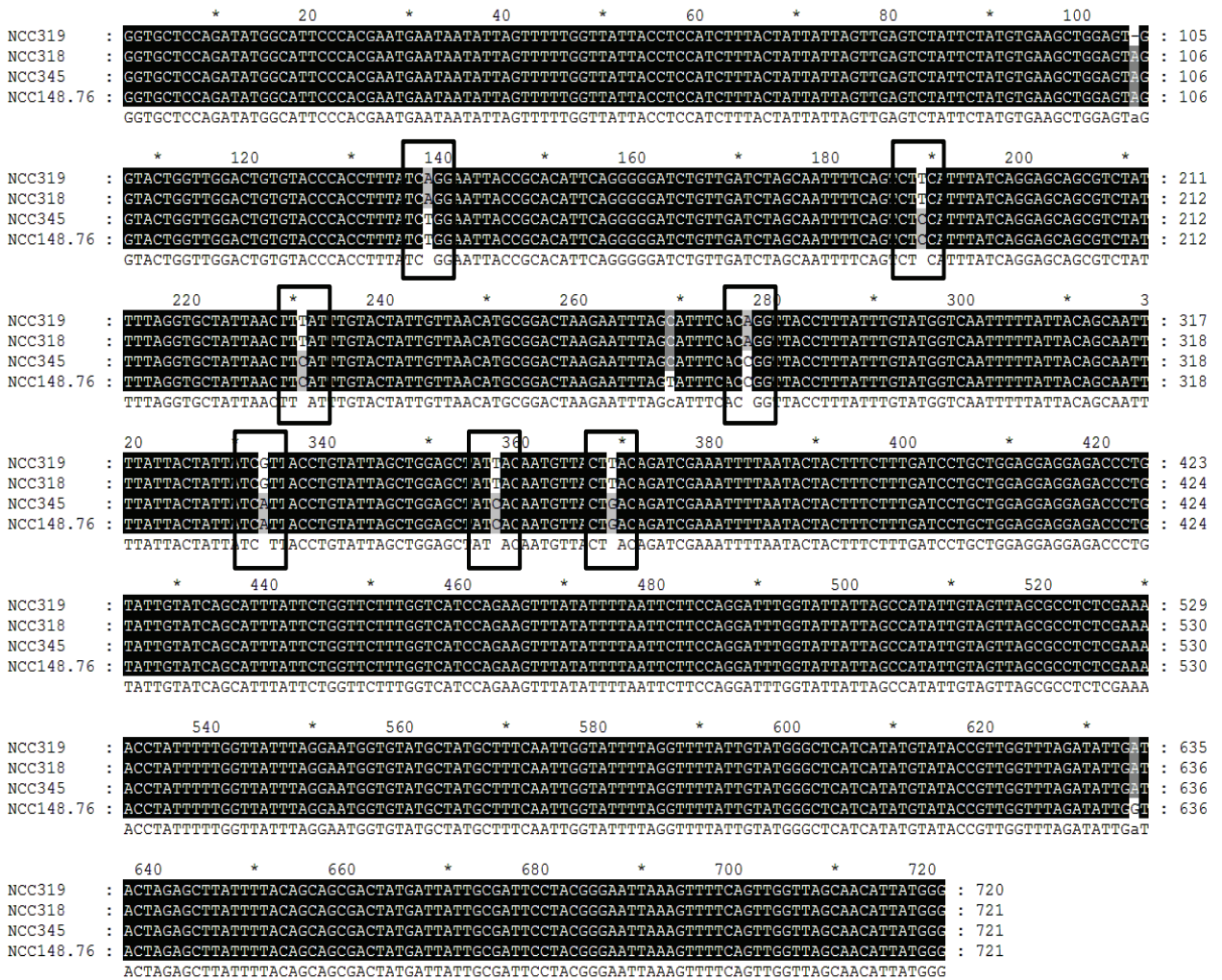


Fig. 2. Multiple alignment of *cox1* sequences from two Swedish clones (NCC 318 and NCC 319) and two French clones (NCC 148.76 and NCC 345) of *H. ostrearia*. The seven discriminating bases are surrounded in black.

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      *      20      *      40      *      60      *      80      *      100      *
NCC 158.4 PRMNNISFWLLPPSLLLLVESILCEAGVGTGWTVYPPPLSGITAHSGGSVDLAI FSLHLSGAASILGAINFICTIVNMRTKNLAFHRLPLEFVWSIFITAILLLSLPVLAGA : 111
NCC 320 PRMNNISFWLLPPSLLLLVESILCEAGVGTGWTVYPPPLSGITAHSGGSVDLAI FSLHLSGAASILGAINFICTIVNMRTKNLAFHRLPLEFVWSIFITAILLLSLPVLAGA : 111
      *      120      *      140      *      160      *      180      *      200      *      220
NCC 158.4 ITMLLTDNRNFNTFFDPPAGGGDPVLYQHLEWFFGHPEVYIILPGFGIISHIVVSASRKPIFGYLGVMVYAMLSIGILGFIVWAHHMYTVGLDIDTRAYFTAATMIIAIP TG : 222
NCC 320 ITMLLTDNRNFNTFFDPPAGGGDPVLYQHLEWFFGHPEVYIILPGFGIISHIVVSASRKPIFGYLGVMVYAMLSIGILGFIVWAHHMYTVGLDIDTRAYFTAATMIIAIP TG : 222
      *
NCC 158.4 IKVFSWLATLW : 233
NCC 320 IKVFSWLATLW : 233
      IKVFSWLATLW

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Fig. 3. Alignment between the COXI fragments obtained in French (NCC 158.4) and Swedish (NCC 320) strains of *H. ostrearia*. The complete identity indicated that the nucleic signature substitutions between the French and Swedish strains noted inside the *cox1* are silent.

Mating experiments, auxosporulation and transmission of *cox1* fragments

In accordance with to the studies from Kraan and Guiry (2000), Coyer et al. (2002) and Leviaaldi-Ghiron et al. (2008), we produced in experimental conditions some F1 hybrids from *Haslea ostrearia* between French and Swedish strains. Up to 50 mating experiments were run with the two pairs of compatible clones, followed by *ca.* one hundred isolation attempts. Auxosporulation was heterothallic, that was proved by the difference in the sizes of gametangia, each coming from one of the partners (Fig.3). In the end, eleven initial cells, all resulting from different sexual events happened in separated Petri dishes, were isolated. Eight were the offspring of the couple NCC 148.78xNCC 320 and three of NCC 158.4xNCC 321 (Tab.2). Isolation was sometimes unsuccessful, probably because of the mechanical stress caused to diatoms. However,

after isolation, no massive death of hybrid clones was observed. Effectively, only one of the hybrids so far isolated being lost.

Clone name	NCC 148.78	NCC 158.4	NCC 320	NCC 321
NCC 148.78 (French origin)	-			
NCC 158.4 (French origin)	+	-		
NCC 320 (Swedish origin)	+	-	-	
NCC 321 (Swedish origin)	-	+	+	-

Table 2. Crossing chess showing the sexual behaviour of the parental couple of clones.

The sign + signifies the ability to mate.

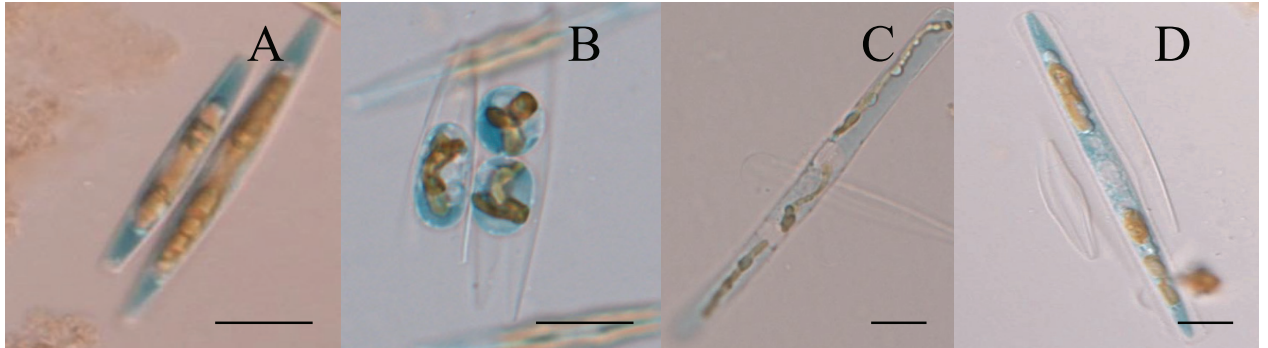


Figure 3. The auxosporulation of *H. ostrearia* in the sexually compatible pair of clones NCC 148.78 (smaller cell) and NCC 320 (larger cell). A: Pairing of the parental cells. B: Two gametes (right) and one zygote (left) close to dehisced parental frustule. C: An expanding auxospore. D: Initial cell inside its perizonium. Scale bar are 10 μm .

Heterothallic reproduction assessed from the different sizes of gametangia or their remaining frustules in each pair.

To evaluate the mitochondrial DNA transmission inside the *Haslea ostrearia*, we developed an approach focused on the observation of discriminative substitutions noted into the *cox1* from French and Swedish strains. We amplified and directly sequenced our *cox1* PCR products to estimate if the F1 hybrid possesses one or two parental haplotypes. In the case of the hybrid inherit the mitochondrial DNA from their two parents, we would observe at the positions 138, 189, 231, 277, 333, 357, 369 inside the *cox1*, the two putative bases. The fragment obtained from the F1 hybrids revealed that the mitochondrial DNA present in the hybrid seems always inherits from only one parent (Fig.4; 5). For example, the couple constituted with NCC 148.78 (France) x NCC

320 (Sweden), the hybrid inherited the NCC 148.78 type, whereas for the couple NCC 158.4 (France) x NCC 321 (Sweden), it was the NCC 321 clone which transmitted his *cox1*. As referred to the mating chess of the Table 2, NCC 148.78 and NCC 321 clones are of the same sexual type.

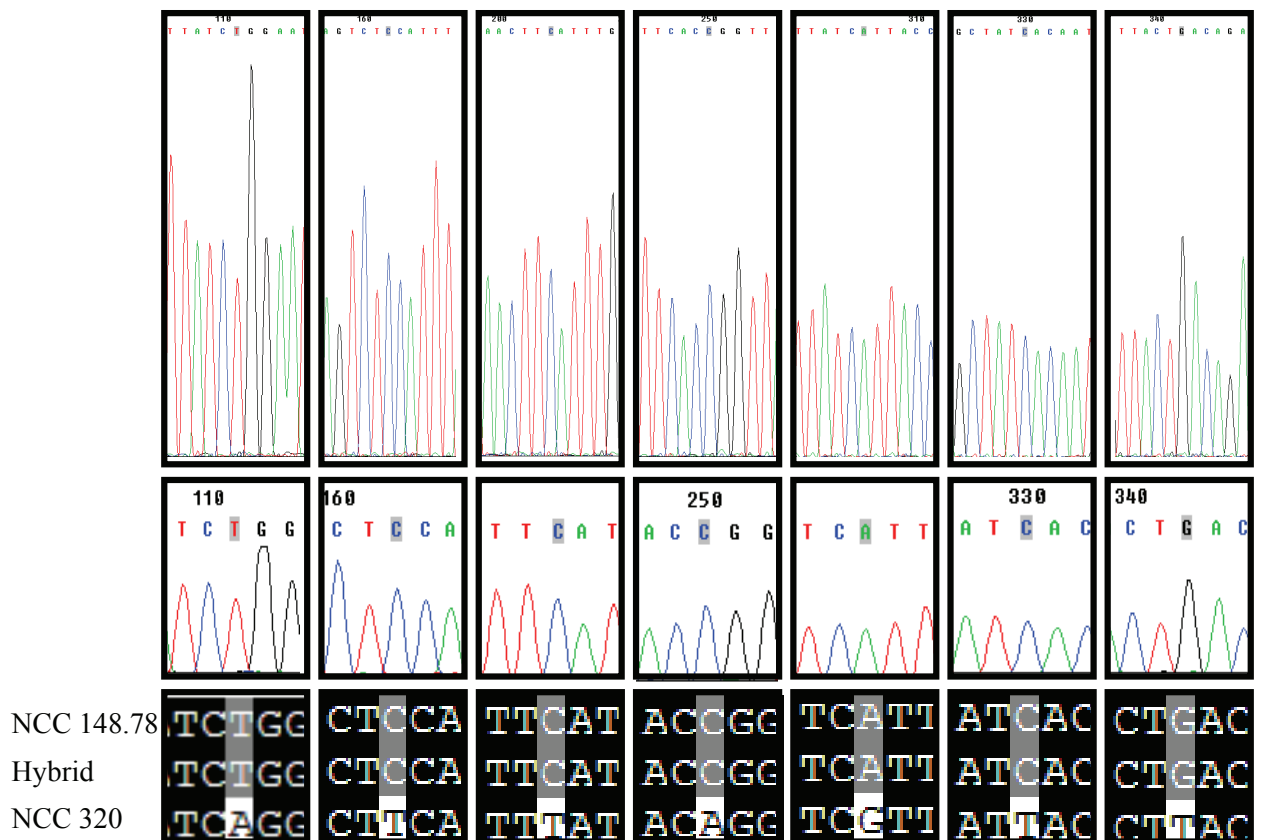


Fig. 4. Example of chromatogram obtained after sequencing an F1 hybrid offspring of NCC 148.78 x NCC 320 *cox1* gene fragment. The chromatogram shows no sign of homoplasmy in the position of the discriminating bases. For the hybrid illustrated in this figure, the *cox1* gene was inherited from the clone NCC 148.78.

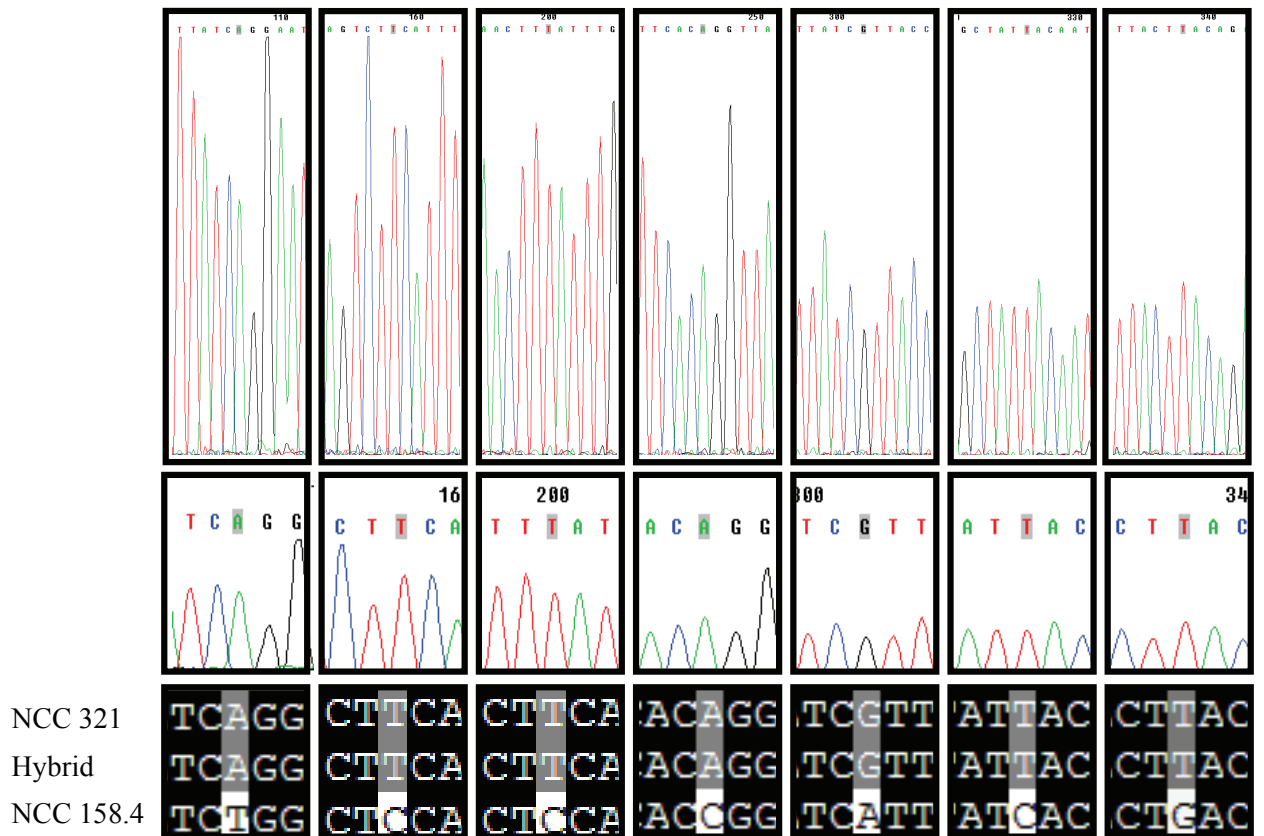


Fig. 5. Example of chromatogram obtained after sequencing an F1 hybrid offspring of NCC 158.4 x NCC 321 *cox1* gene fragment. The chromatogram shows no sign of homoplasmy in the position of the discriminating bases. For the hybrid illustrated in this figure, the *cox1* gene was inherited from the clone NCC 321.

Discussion

Application of the auxosporulation protocol of *H. ostrearia* (Davidovich et al. 2009; Mouget et al. 2009) led to the obtention of several zygotes, auxospores and initial cells. Most of the monoclonal strains derived from these initial cells were viable on the long term, as for strains isolated from natural populations. Hence, no pre- or post-zygotic isolation seem to exist between French and Swedish strains. Thus it can be inferred that all the *H. ostrearia* strains tested corresponded to different populations of a same species. Based on the particularity of the seven signature substitutions discriminating the *coxI* sequences from the French and Swedish populations, we evaluate the transmission of mitochondrial. Presently, the inheritance or modification of characters following auxosporulation has been studied only in a few diatom species. Belt et al. (2002) showed that the ability of *Rhizosolenia setigera* to synthesize highly branched isoprenoids was influenced by the happening of sexual events. Molecular methods have been applied to study the inheritance of chloroplasts in the progeny of *Pseudo-nitzschia delicatissima* (Levialdi-Ghiron et al. 2008), by sequencing of the Ribulose biphosphate Carboxylase Oxygenase large subunit gene *rbcL*. During auxosporulation in *P. delicatissima*, Levialdi-Ghiron et al. (2008) demonstrated that, chloroplast inheritance is bi-parental, both vegetative cells and gametes containing two plastids, and a zygote, four. The entire process further involves either a mechanism by which two of the four plastids are eliminated inside the zygote, or a random segregation during the first mitotic division of the initial cell, combined to an absence of plastid division. This was indirectly confirmed by Casteleyn et al. (2009), who observed the occurrence of hybridisation process in natural populations of *Pseudo-nitzschia pungens*. As to mitochondria, their inheritance in diatoms during sex is still an open field of

investigation, due to the absence of data in the literature (Evans et al. 2007). Our results established on the *cox1* sequences noted in F1 hybrid (interbreeding France x Sweden) showed clearly that the hybrids possess only one *cox1* sequence. Effectively, the chromatograms did not allow detecting any sign of heteroplasmy of the mitochondrial gene due to different *cox1* haplotypes inside a monoclonal diatom population (hybrid). Transmission of the mitochondrial DNA seems thus uniparental and sex-dependent in the diatom *H. ostrearia*, whatever the geographical origin of the parental cells. However, one possible criticism to the present study could pertain to the number of F1 hybrids so far investigated, which could be considered insufficient, when compared to experiments made on the chloroplastic DNA transmission in *P. delicatissima*, for which 96 hybrids were studied (Levialdi-Ghiron et al. 2008). Nevertheless, despite a lower number of F1 hybrids analyzed, the results obtained in *H. ostrearia* suggest univocally a uniparental mode of mitochondrial DNA transmission during auxosporulation.

According to the literature, uniparental inheritance of mitochondria has already been demonstrated in some algae; *H. ostrearia* being the first diatom so far studied. Indeed, the phaeophyceae *Scytosiphon lomentaria* (Kato et al. 2006), *Laminaria angustata* (Motomura 1990), *Fucus vesiculosus* (Brawley et al. 1976), *F. distichus* (Motomura, 1994) and the chlorophyceae *Chlamydomonas reinhardtii* (Aoyama et al. 2006) exhibited uniparental transmission of their mtDNA. Like *H. ostrearia*, *S. lomentaria* and *C. reinhardtii* present an isogamous mode of reproduction, even if *C. reinhardtii* is also an unusual model of paternal mtDNA inheritance (e.g., Boynton et al. 1987, Gillham et al. 1987, Beckers et al. 1991, Nakamura et al. 2003, Aoyama et al. 2006). Note that in some of these previous studies, mitochondrial inheritance statement relied on microscopy observations of the disappearance of mitochondria in gametes, not on the use of molecular markers.

This is commonly admitted that in a majority of organisms, mitochondria are ‘maternally’ inherited (Barr et al. 2005). Two major mechanisms are usually invoked to explain this phenomenon. The first mechanism is the loss of mitochondria during sperm formation (Myamura et al. 1987; Sodmergen et al. 2002), the second one is the degradation of the paternal mitochondrial DNA (Mogensen, 1996). As *H. ostrearia* undergoes an isogamous reproduction without sperm formation (Davidovich et al. 2009), it can be hypothesized that this second mechanism should be considered.

Uniparental sex-dependent mode of organelle transmission led to question the rationale behind the differentiation of parental cells into two sexual types in an isogamous pennate diatom like *H. ostrearia*, in which, possibly both gametangia and gametes are morphologically and physiologically similar. For instance Davidovich et al. (2009), for *H. ostrearia*, never noticed any behavioural differences between gametes, but differentiate two different sexual types according to progeny ability to mate with compatible partners. Furthermore, for diatom sexuality, the mechanisms involved in the triggering of auxosporulation, especially intrinsic factors like sex recognition and compatibility between partners remain to be investigated at the molecular level. Indeed, according to the literature, it exists a limited number of works aimed to study these questions, as the sequencing of different sexually induced genes (especially *sig1*, accessorially *sig2* and *sig3*), thought to play a role in sperm-egg recognition during auxosporulation in the centric diatom *Thalassiosira weissflogii* (Armbrust, 1999) and present in other species of the same genus (Armbrust and Galindo, 2001; Sorhannus and Kosakovsky-Pond, 2006; Sorhannus et al. 2010).

The present study raised another question regarding auxosporulation modalities. The clone NCC 148.78 resulted from homothallism. Homothallic reproduction was the first sexual process described in *H. ostrearia* (Neuville and Daste, 1975, 1979).

Apparently, these authors were unable to induce auxosporulation, as homothallism occurred unpredictably in some of their cultures, and the same is true for our strains, in which populations presenting a bimodal size distribution are observed from time to time. After homothallic reproduction, "clonal" cultures are constituted of small cells and bigger cells with a length close to the species-specific maximum. Until now, homothallic reproduction occurred only in monoclonal cultures that share the same sexual type than NCC 148.7. Moreover, to the possible exception of one strain for which we lack data, all the clones resulting from homothallic reproduction inherited the same parental sexual type, and the same ability to reproduce homothallically. We are currently collecting data to confirm these observations, and working on other molecular markers, e.g., from chloroplastic DNA, to study cDNA and plastid inheritance (markers different from the *rbcL* gene, which is completely conserved between French and Swedish populations, personal data not shown). The species *H. ostrearia* has definitely revealed an interesting model to study sex and genetics in diatoms.

Methods

Cultures and mating experiments: Monoclonal strains of *Haslea ostrearia* used for this study come from the Nantes Culture Collection (NCC, University of Nantes), and are listed in Table 1, along with data on their origin and date of isolation. For experiments, cultures were grown in sterile flasks with artificial sea water (ASW) (Perkins et al. 2006) in a culture chamber with controlled temperature (15 ± 1 °C), at an irradiance of $60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (14h/10h light/dark cycle). Illumination was provided by fluorescent tube Philips TLD 36W/965 and measured with a Li-Cor LI-189 quantum meter and a 2Π Li-Cor Q21284 quantum sensor. Regular refreshment of the medium kept the cultures in exponential phase of growth.

Mating, and hybridization: Hybrids were obtained by crossing two couples of compatible clones, NCC 148.78 (France) x NCC 320 (Sweden) and NCC 158.4 (France) x NCC 321 (Sweden), whose mating types are shown in Table 2. Each couple is made of one strain from Bourgneuf Bay (France) and one of Kattegat Detroit (Sweden). Mating experiments were conducted following the protocole described in Davidovich et al. (2009) and Mouget *et al.* (2009). Mixtures of compatible clones were made in sterile polystyrene Petri dishes, by mixing cells in exponential phase of growth at a final concentration of 2000 cells mL⁻¹, completed up to 5 mL with fresh ASW medium. Petri dishes were surrounded by Parafilm to avoid desiccation, and then placed in a culture box at 15±1 °C and an illumination of 25 µmol photons m⁻² s⁻¹ with a photoperiod of 6h/18h, light/dark cycle. After one week incubation, mixtures were observed with an inverted microscope Nikon TS100. Initial cells were isolated under inverted microscope using an Eppendorf 100 µL pipette. Cells were re-isolated at least three times before the cultures being considered as monoclonal. Only one initial cell per Petri dishes of mating experiments was retained to initiate F1 hybrid cultures.

DNA analyses: DNA of all clones was extracted using a Qiagen DNeasy Plant mini Kit. Polymerising Chain Reactions (PCR) were carried out using a GoTaq[®] Flexi DNA Polymerase kit from Promega. COI1 and COI2 primers were manually designed using an multiple alignment based on the sequences of *Nitzschia frustulum* (AB020225), *Cylindrotheca closterium* (AB020222) and *Fragilaria striatula* (AB020224). Their sequences are 5'-GTWATGCCWAYTTTRATWGG-3' (Forward) for COI1 and 5'-CCCATAAWGTHGCTAACC-3' (Reverse) for COI2. The Eppendorf Mastercycler Gradient thermocycler conducted 39 cycles of polymerisations, consisting in the initial denaturing (94 °C, 1 min), annealing (48 °C, 1 min) and extension (72 °C, 1 min 30 s) steps. A final elongation defined as 72°C during 10 minutes finalized the PCR program. PCR products were separated by agarose gel electrophoresis (1%; migration : 110 volts during 20 min). Revelation was made under UV light using a CN-1000 Darkroom by Vilber Lourmat. Elution was made with a Wizard[®] SV Gel and PCR Clean-up System (Promega). Elution product was sent for sequencing

to Beckmann Coulter Genomics (United-Kingdom, <http://www.cogeniconline.com>) using the COXINT1 primer, whose sequence is 5'-GGTGCTCCAGATATGGCATT-3'.

Chromatograms were carefully checked to detect the presence of multiple peaks in the nucleotide positions, using Chromas software (<http://www.technelysium.com.au/chromas.html>). The fragments were identified with NCBI Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Alignment were made using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and visualized using the Genedoc software (<http://www.nrbsc.org/gfx/genedoc/>). Translations were done using ExPASy Translate Tool (<http://expasy.org/tools/dna.html>),

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IV.3 Conclusion et perspectives

La phylogénie du genre *Haslea* ici présentée est encore parcellaire. Elle pourrait être complétée par l'introduction de nouvelles espèces. Au moment de la rédaction de cette thèse a émergé la possibilité d'acquérir une souche de l'espèce *Haslea spicula*. Dans le même temps, des prélèvements réalisés par un chercheur associé sur la côte Est des Etats-Unis ont montré contenir des diatomées bleues. Plusieurs autres prélèvements sont en cours en Méditerranée, entre autres sur la côte d'Afrique du Nord.

L'étude sur les mitochondries présentée sous forme d'un article a besoin d'être complétée par l'ajout d'autres séquences d'hybrides, afin d'obtenir un échantillon d'au moins vingt séquences du fragment de gène *cox1* correspondant à autant d'hybrides, provenant à part égale de chacun des deux couples féconds utilisés. Néanmoins, les résultats obtenus permettent d'ores et déjà d'émettre l'hypothèse que la transmission du gène mitochondrial *cox1*, et par la même celle de l'ADN mitochondrial et des mitochondries, est uniparentale chez *H. ostrearia*. Ces résultats pourraient aussi être corroborés par l'étude de marqueurs génétiques mitochondriaux supplémentaires, avec le risque que les séquences des clones français et suédois ne présentent pas les différences régulières qui furent observées avec les fragments du gène *cox1*. La question de l'héritabilité de l'ADN chloroplastique s'est également posée. Une étude sur l'espèce *Pseudo-nitzschia delicatissima* existe déjà (Levialdi-Ghiron *et al.*, 2008) et une étude sur des hybrides naturels a été menée par Casteleyn *et al.* (2009) sur l'espèce *Pseudo-nitzschia pungens*. Contrairement à ces travaux, des expériences similaires n'ont pu être testées chez *H. ostrearia* du fait de la très forte homologie des séquences du gène *rbcL* entre les souches françaises et suédoises. Un essai de définition de nouvelles amorces

permettant d'amplifier par PCR des zones plus variables de l'ADNc a été mené, sans succès. Il serait intéressant toutefois de continuer cette étude. En effet, les résultats potentiels pourraient différer de ceux obtenus avec les espèces du genre *Pseudo-nitzschia*. Les zygotes formés à la suite de l'auxosporulation de *Pseudo-nitzschia delicatissima* par exemple contiennent en effet quatre chloroplastes, avant que leur nombre ne soit ensuite réduit à deux. Ceci ne semble pas être le cas chez *H. ostrearia* (Davidovich *et al.*, 2009).

V

Chapitre 4

Les pigments bleus

des diatomées du genre *Haslea*

et leur valorisation

V.1 Introduction

La découverte de plusieurs nouvelles espèces de diatomées bleues, possédant pour au moins l'une d'entre elles (*H. karadagensis*) un pigment clairement différent de la marennine, a provoqué un regain d'intérêt pour ces substances, leurs propriétés et les utilisations potentielles qui en découleraient. Différentes activités biologiques avaient déjà été mises en évidence par le passé à partir d'extraits aqueux contenant de la marennine (Bergé *et al.*, 1999; Carbonelle *et al.*, 1999), avant la mise au point d'un protocole d'extraction du pigment. Plus récemment, sur des échantillons purifiés de marennine, d'autres propriétés biologiques du pigment ont été détectées (Pouvreau, 2006 ; Pouvreau *et al.*, 2008). Le présent chapitre traite tout d'abord de l'étude des activités antivirales de la marennine et du pigment d'*H. karadagensis*. Puis sont abordés les activités antibactériennes et antifongiques, sur des organismes terrestres et marins. Enfin, un dispositif permettant de comparer la fixation de ces pigments sur les branchies de deux espèces d'huîtres est décrit. Les résultats obtenus seront présentés sous la forme de deux publications.

V.2 Activités antivirales

V.2.1 Introduction

D'anciennes études, réalisées avec des extraits aqueux obtenus à partir d'*H. ostrearia*, avaient suggéré l'existence d'un effet antiviral de composés contenus dans cette diatomée (Bergé *et al.*, 1999). Ces expériences furent faites avant le développement par Pouvreau (Pouvreau *et al.*, 2006) de son protocole de purification. Il

a été décidé de réitérer à partir d'échantillons purifiés de marennine les expériences précédemment citées. Ces nouvelles expériences prirent en compte deux spécificités récemment décrites, à savoir l'existence de deux formes, internes et externes, de marennine, ainsi que la disponibilité d'un pigment apparenté, celui présent chez *H. karadagensis*.

V.2.2 Matériel et méthodes

Les expériences ont été réalisées à Vannes, dans le laboratoire de biotechnologie et chimie marines (EA 3884) de l'Université de Bretagne-Sud, sous la direction du professeur Nathalie Bourgougnon et la supervision de Christel Marty. Ces expériences ont aussi bénéficié de l'aide technique de Catherine Tardy-Laporte, stagiaire de l'Université de Rimouski, Québec. Les tests furent réalisés sur une souche virale d'herpèsvirus HSV1, à l'aide de cultures cellulaires issues de singes verts (*Chlorocebus* sp.) et en prenant comme drogue de référence le Zovirax.

Les pigments des deux espèces ont montré une activité antivirale, variable quant à l'espèce et la forme du pigment (interne ou externe) considérées. Ces résultats sont repris dans les articles présentés plus loin.

V.2.3 Discussion et perspectives

Plusieurs axes peuvent d'ores et déjà être dégagés quant à une suite à donner à ces expériences. Les activités antivirales observées encouragent à la poursuite d'expérimentations. En effet, l'herpèsvirus HSV1 n'est pas seulement un virus de

référence utilisé en routine. Il est aussi responsable de 95% des herpès faciaux et de 20% à 40% des herpès génitaux. La prévalence de ce virus dans la population française serait de 67% (Malkin *et al.*, 2002). De plus, les herpèsvirus sont fréquemment associés au HIV, dont ils semblent augmenter le rythme de transcription génétique (Aymard, 2002). Les résultats obtenus peuvent s'inscrire dans une stratégie de recherche de composés bioactifs d'origine marine. Les herpèsvirus ont récemment été érigés en ordre, celui des *Herpesvirales* (Davison, 2010). Cet ordre contient en son sein *Ostreid herpesvirus 1*. Celui-ci est apparu ces dernières années comme un des pathogènes émergent des huîtres, responsable de mortalités importantes (Hine *et al.*, 1992 ; Nicolas *et al.*, 1992 ; Renault *et al.*, 1994 ; Arzul *et al.*, 2001 ; Arzul et Renault, 2002 ; Schikorski *et al.*, 2011). Il serait intéressant d'étudier une éventuelle activité antivirale des pigments ici testés sur ce virus. Des contacts ont été pris en ce sens. Enfin, ces expériences ont mis en évidence un effet cytotoxique des formes internes des deux pigments. Cet effet reste à préciser. Nous ne pouvons pas extrapoler d'un effet similaire sur d'autres tissus. Mais, s'il est un organisme dont les organes peuvent être en contact prolongé avec de la marennine, ce sont les huîtres de claire. Bien que les concentrations de marennine relevées dans les claires soient nettement inférieures aux C_{50} mesurées ici ($5 \mu\text{g. mL}^{-1}$ d'après Turpin, 1999), il n'en reste pas moins que ces pigments pourraient fragiliser les branchies et les palpes labiaux des huîtres verdies. Aucun rapport ne semble avoir été fait sur une possible surmortalité d'huîtres au cours du processus de verdissement. Concomittamment aux expériences de coloration des branchies d'huîtres décrites plus haut, une approche moléculaire se basant sur la PCR quantitative a été initiée au laboratoire, afin de détecter la surexpression de gènes de stress liés à la réponse immunitaire ou à l'apoptose chez des huîtres colorées par l'action d'*H. ostrearia* ou d'*H. karadagensis*. La mise au point d'une bibliothèque de séquences et

d'amorces spécifiques à l'huître *C. gigas* et *O. edulis* fit l'objet d'un stage de Master 1 réalisé par Mme Nafissa Tekaya-Abdelmalek, qui travailla à cette occasion sur le protocole de coloration déjà évoqué.

V.3 Activités antibactériennes

V.3.1 Introduction

La nature polyphénolique de la marennine a été suggérée par Pouvreau (2006). Or, de nombreux polyphénols possèdent des activités bactéricides. Dans sa thèse, Pouvreau avait testé d'éventuelles activités sur un échantillon de trois espèces de bactéries et un mycète.

	<i>Staphylococcus aureus</i>	<i>Escherichia Coli</i>	<i>Vibrio anguillarum</i>	<i>Candida tropicalis</i>
EMn	0	0	0	0
IMn	12	0	11	0

Tableau IV-5: activités antimicrobiennes des deux formes de marennine exprimées en diamètre d'inhibition (mm) pour un dépôt de 100 µg par disque de 6 mm de diamètre.

Figure 31. Les activités antibactériennes et antifongiques des deux formes de la marennine d'après Pouvreau (2006).

Les activités observées par Pouvreau ne semblaient s'appliquer qu'à certains pathogènes, et seul la forme interne de la marennine, celle qui n'est pas excrétée, semblait active. La découverte d'*H. karadagensis* et de son pigment, la possibilité d'en obtenir des formes purifiées de concert avec de la marennine, et les facilités d'accès à

deux laboratoires qualifiés, ont conduit à mener une étude sur un spectre plus large de micro-organismes. Cette étude s'est déroulée en deux temps. Tout d'abord, les activités sur des micro-organismes marins ont été testées à l'Université de Portsmouth, sous la direction du Dr. Claire Hellio. Les recherches sur des pathogènes terrestres se sont quant à elles déroulées au Laboratoire Départemental de la Sarthe, service de bactériologie, sous la direction des docteurs Sylvie Poliak et Jean-Michel Mainguet.

V.3.2 Matériel et méthode

Plusieurs souches d'organismes marins ont été fournies par l'Université de Portsmouth. Trois bactéries, *Polaribacter irgensii*, *Pseudoalteromonas elyakowii* et *Vibrio aesturianus* y ont été cultivés et testés. Les cultures de bactéries étaient maintenues dans un bouillon nutritif placé dans une étuve à 37°. Elles étaient maintenues en croissance exponentielle par des dilutions régulières. Le bouillon nutritif était fait à partir d'eau de mer filtrée et stérilisée mélangée à de la peptone à 5 g. L⁻¹. La densité cellulaire des cultures était évaluée par mesure de densité optique. Les cultures étaient ensuite diluées de façon à obtenir la concentration d'usage de 10⁶ CFU. mL⁻¹. Les boîtes de pétri faites d'eau de mer stérilisée et gélosée étaientensemencées avec 1 mL des cultures pré-diluées. Ces boîtes étaient placées à sécher en milieu axénique durant 30 minutes, puis les disques de papier chargés étaient disposés dessus. Des disques de papier non enduits de solution pigmentaire étaient utilisés comme témoins. Les boîtes étaient mises à incuber deux jours en milieu stérile. Au bout de des deux jours, une évaluation du rayon d'un halo d'inhibition était faite, comme représenté figure 32.

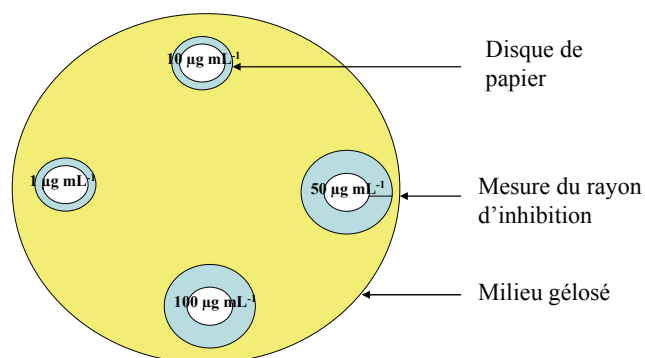


Figure 32. La méthode de diffusion par disques de papiers sur gel d'agar utilisée au cours de l'étude sur les activités antibactériennes

Pour les pathogènes terrestres, dix souches de bactéries et une souche de mycètes ont été fournies par le Laboratoire Départemental de la Sarthe. Leur liste et les conditions de cultures sont détaillées dans le tableau 3. Du fait d'un épuisement de nos stocks de pigments, ces expériences n'ont pu être menées qu'avec la marennine, formes internes et externes.

Après insémination, les disques de papier furent déposés sur les boîtes de pétri (marque Biomérieux). Ces boîtes furent placées dans les enceintes dévolues à leur culture, à savoir deux Incucell cabinet (Fisher Bioblock) pour les cultures à 37° et 41°, et un Galaxy 170S (New Brunswick Eppendorf) pour les bactéries cultivées en atmosphère enrichie en CO₂. Les boîtes furent sorties au bout de 48 heures, et la présence d'un halo d'inhibition est recherchée.

	Type Gram	Milieu de culture	Température de culture	Atmosphère de culture	Turbidité utilisée pour l'ensemencement (en Mac Farland)	Origine
<i>Bacillus cereus</i>	+	Mueller Hinton à 5% de sang de mouton	37°	normal	0,5 10 ⁻²	ATCC 10876
<i>Campylobacter jejuni</i>	-	Mueller Hinton à 5% de sang de mouton	41,5°	micro aérophilie	0,5 10 ⁻¹	ATCC 33291
<i>Clostridium perfringens</i>	+	Mueller Hinton à 5% de sang de mouton	37°	anaérobie	0,5 10 ⁻¹	ATCC 13124
<i>Enterococcus faecalis</i>	+	Mueller Hinton à 5% de sang de mouton	37°	CO ₂	0,5 10 ⁻³	ATCC 29212
<i>Escherichia coli</i>	-	Mueller Hinton à 5% de sang de mouton	37°	normal	0,5 10 ⁻³	ATCC 25922
<i>Legionella pneumophila</i> sérotype 1	-	BCYE			Non-applicable	Institu Pasteur
<i>Listeria monocytogenes</i>	+	Mueller Hinton à 5% de sang de mouton	37°	normal	0,5 10 ⁻¹	ATCC 19115
<i>Proteus mirabilis</i>	-	Mueller Hinton à 5% de sang de mouton	37°	normal	0,5 10 ⁻³	Souche interne
<i>Pseudomonas aeruginosa</i>	-	Mueller Hinton à 5% de sang de mouton	37°	normal	0,5 10 ⁻³	ATCC 104009
<i>Salmonella typhimurium</i>	-	Mueller Hinton à 5% de sang de mouton	37°	normal	0,5 10 ⁻³	ATCC 14028
<i>Staphylococcus aureus</i>	+	Mueller Hinton à 5% de sang de mouton	37°	CO ₂	0,5 10 ⁻²	ATCC 25923

Tableau 3. Les souches de pathogènes terrestres utilisées au cours de l'étude, avec les conditions de culture et les références fournisseur.

V.3.3 Résultats

Pour les micro-organismes marins, les expériences ont montré que tous les pigments utilisés ont, au moins pour les concentrations les plus élevées, un effet inhibiteur sur la croissance des bactéries et mycètes. Certaines formes pigmentaires ont même montré une activité à des très faibles concentrations, comme par exemple les deux formes de marennine sur *V. aesturianus*. Pour les micro-organismes terrestres en revanche, il n'a pas été possible d'observer le moindre effet inhibiteur des pigments dans toutes les expériences menées au Laboratoire Départemental de la Sarthe. Les expériences sur *L. pneumophila* ont dû être abandonnées, car il fut impossible d'obtenir sur les boîtes de milieu charbonné BCYE le tapis bactérien homogène formé de colonies confluentes nécessaire pour des expériences de type antibiogramme.

V.3.4 Conclusion et perspectives

Dans son manuscrit de thèse, Pouvreau avait indiqué que la marennine interne était inhibitrice de la croissance de *Vibrio anguillarum*. Il était important pour nous de refaire des expériences sur *V. aesturianus*. En effet, *V. aesturianus* est un important pathogène des huîtres, responsable d'épizooties massives, sur les côtes françaises en particulier (Garnier *et al.*, 2008 ; Lago *et al.*, 2009). Le genre *Vibrio* contient nombre d'autres pathogènes d'espèces animales fréquentes en aquaculture, et il est tristement célèbre pour abriter *Vibrio cholerae*, le bacille du choléra. L'idée que la marennine, libérée dans le milieu, pourrait être une sorte d'antibiotique à disposition des huîtres, avait déjà été suggérée par Pouvreau. Nos études montrent, que des pathogènes des huîtres peuvent être concernés par ce phénomène. Nos études montrent aussi que la

marennine, sous toutes ses formes, de même que le pigment d'*H. karadagensis*, agissent à des concentrations nettement inférieures à celles utilisées par Pouvreau. Certaines de ces concentrations actives sont d'ailleurs plus faibles que le seuil déjà cité des 5 µg. mL⁻¹ de marennine présente dans les claires, comme dosée par Turpin (1999). Les quantités de marennine libérées lors d'un épisode de verdissement des claires seraient donc suffisantes pour contrer le développement de bactéries pathogènes. Il faut à cela ajouter que plusieurs espèces du genre *Pseudoalteromonas* sont elles aussi pathogènes d'animaux marins. Des résultats obtenus par ailleurs avec les mycètes marins, et présentés dans la publication plus loin, encouragent aussi à la poursuite d'études en ce sens, sur un plus grand nombre d'organismes. Les espèces ici testées peuvent être incriminées dans les phénomènes de biofouling marin, phénomène économiquement coûteux, et qui consiste en l'encroûtement des structures immergées par la colonisation de différents organismes, au nombre desquels les mycètes marins. Mais il existe, parmi les mycètes marins, d'autres espèces connues pour être pathogènes des huîtres.

Des modèles expérimentaux ont été proposés afin de voir l'effet protecteur des pigments bleus de diatomées sur des huîtres subissant *in vitro* une infection par des pathogènes comme ceux ici utilisés. Au moment de la rédaction de ces lignes, l'obtention d'une souche de *V. aesturianus* est en bonne voie.

A contrario, aucune activité n'a été détectée chez les pathogènes terrestres testés, ni sur le champignon *Candida albicans*. Pouvreau n'avait observé que de faibles activités de la marennine sur *Staphylococcus aureus*, et aucune sur le mycète *Candida tropicalis*. Les raisons qui ont prévalu au choix de ces organismes et à cette étude sont au nombre de deux. Tout d'abord, en tant qu'organismes filtreurs, les huîtres peuvent

concentrer de grandes quantités de micro-organismes, au nombre desquels des entérobactéries présentes dans les milieux en contact avec des eaux usées, comme *E. coli* ou *P. mirabilis*. Un effet antibactérien de la marennine aurait pu être un élément garantissant une meilleure hygiène de la consommation d’huîtres verdies. L’autre raison est que la marennine a pu être pressentie pour devenir un colorant alimentaire bleu. A l’exception de certains anthocyanes dont la couleur peut varier du rouge au violet en passant par le bleu, les colorants alimentaires bleus sont majoritairement des produits de synthèse. Il y a une forte demande d’alternatives naturelles (Zhang et al., 2006) (Cai et al., 2011), rôle que la marennine pourrait avoir. Dans cette optique, un pouvoir inhibiteur sur des bactéries présentes dans l’alimentation à l’instar de *B. cereus*, *S. typhimurium* ou *L. monocytogenes*, aurait ajouté à l’intérêt de développer la marennine comme ingrédient alimentaire. Le mycète *C. albicans* avait été choisi pour son rôle dans les candidoses, maladies de la peau qu’il déclenche chez les individus immunodéprimés. Enfin, il nous avait semblé intéressant de tester l’action de la marennine sur *L. pneumophila* sérotype 1, bactérie responsable de la légionellose. Ces expériences n’auront pu être menées à bien pour les raisons techniques déjà invoquées.

V.4 Fixation des pigments sur les organes des huîtres

V.4.1 Introduction

Expérimentalement, Dyer (1877), Puysegur (1880), Lankester (1886) ainsi que Bornet et Chatin (1895), avaient prouvé l’aptitude de la marennine à se fixer aux branchies et palpes labiaux d’huîtres à partir de cultures d’*H. ostrearia* viables. Ranson (1927) prouvera que le même phénomène peut se dérouler dans un milieu riche en

marennine solubilisée dans l'eau et dénué de cellules algales. Gaillon (1820) avait déjà signalé que la marennine avait aussi la propriété de colorer les organes d'autres animaux marins, tels les crabes, anémones de mer, littorines et moules. La découverte d'*H. karadagensis* ainsi que de son pigment a conduit à s'interroger sur la capacité de celui-ci à se fixer sur les organes des huîtres.

V.4.2 Matériel et méthodes

Des expériences seront menées sur l'huître creuse *Crassostrea gigas*, ainsi que sur l'huître plate *Ostrea edulis*. Ces huîtres ont été maintenues en élevage au laboratoire dans des aquariums en verre de dimensions 34x19x25cm, remplis de 5 litres de milieu d'eau de mer artificielle Red Sea Salt (Red Sea), à pH 8,4 et de salinité 30. Les aquariums furent placés dans une chambre de culture thermostatée à 16°, sous un dispositif lumineux produisant un éclairage de 25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ de façon continue. Les huîtres furent acclimatées à ce traitement durant 15 jours, l'eau des aquariums étant changée une fois par semaine. Durant cette période, elles furent nourries 3 fois par semaine avec des inoculums contenant les diatomées *Skeletonema costatum* (NCC 53) et *Entomoneis paludosa* (NCC 18), toutes deux très fréquentes dans les claires ostréicoles (Rincé, 1978). Au moment de changer leur alimentation, l'eau des huîtres est renouvelée une dernière fois. Dans un aquarium, les huîtres témoins sont nourries avec un nouvel inoculum de *S. costatum* aux concentrations définies précédemment. Dans les deux autres aquariums ont été distribués des inoculums de *H. ostrearia* et *H. karadagensis* correspondant à des concentrations finales de 120 000 cellules mL^{-1} . Des huîtres sont prélevées régulièrement afin d'établir leur état de coloration.

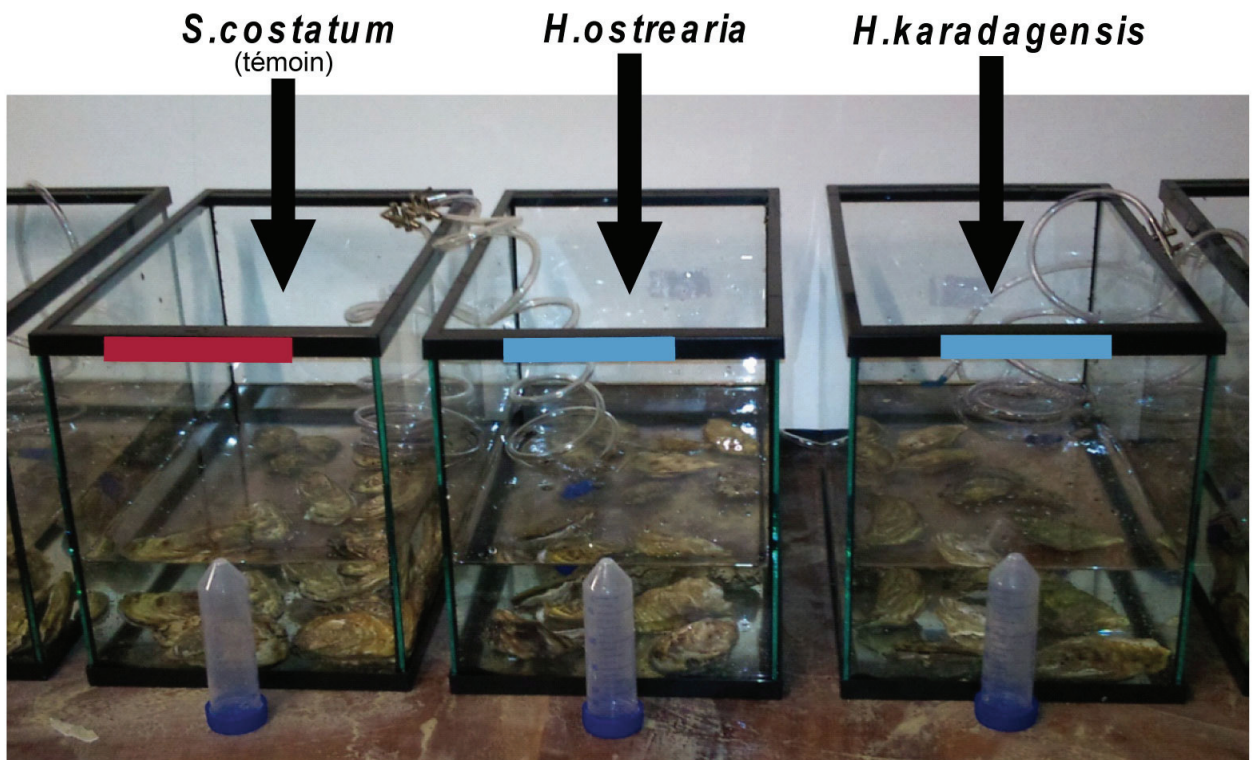


Figure 33. Le dispositif d'aquariums utilisé pour les expériences de colorations des branchies d'huîtres.

V.4.3 Résultats, conclusion et perspectives

Ce protocole a permis d'obtenir au bout d'une semaine une nette coloration des branchies des huîtres, pour *H. ostrearia* comme pour *H. karadagensis*. Les huîtres nourries avec *H. ostrearia* présentaient la teinte verte caractéristique des huîtres de claires verdies. Les huîtres nourries avec *H. karadagensis* présentaient quant à elles un teinte grise. Les résultats de ces expériences et les photos des huîtres colorées sont contenus dans l'article présenté plus loin.

V.5 Biological activities of purified marennine, the blue pigment responsible for the greening of oysters

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ABSTRACT

Marennine, the blue pigment produced by the diatom *Haslea ostrearia*, has been purified and the two different forms of the pigment, internal and external, were assessed for their biological activities (antibacterial, antiviral and antiproliferative). Both forms of marennine inhibit development of several marine bacteria, including the infamous *Vibrio aesturianus* at concentrations as low as 1 $\mu\text{g mL}^{-1}$, but do not actually showed any effect on terrestrial and alimentary bacteria. They exhibited antiviral activities against the HSV1 herpes virus too, with EC_{50} of 24.0 $\mu\text{g mL}^{-1}$ for internal marennine and 27.0 $\mu\text{g mL}^{-1}$ for external marennine. Moreover, they were effective enough to slow down the proliferation of cancerous cells lineage. This study confirms Marennine as a potentially active compound, which may have protective effect for the bivalves that filter it. Since marennine is absorbed by human due to green oysters' consumption and can have nutraceutical effects, applications in food engineering and chemistry as natural blue pigment are discussed.

Keywords: marennine, green oysters, *Haslea ostrearia*, *Vibrio*, HSV, antiproliferative

INTRODUCTION

Haslea ostrearia is a pennate diatom, considered worldwide in distribution, which produces a water-soluble blue pigment called “marennine” (1). This name refers to the Marennes-Oléron Bay, an area of intensive oyster farming since the end of the XVIIIth century, which is now the most important oyster rearing area in Europe. Before commercialisation, part of the oyster stocks from the Marennes-Oléron Bay undergoes a fattening process called ‘affinage’, which mostly takes place in ancient salt marshes where the ponds are located. These ponds called ‘claires’ are used for oyster fattening (2). The duration of the fattening period and the oyster density in the ponds are regulated according to specific norms (3). In some ponds, *H. ostrearia* can outcompete other microalgae and become seasonally dominant, a phenomenon responsible for the ‘greening’ of the ponds, and as a consequence, of the oysters. Indeed marennine released in the seawater during *H. ostrearia* blooms is filtered by oysters and remains on the gills and labial palps, giving them a specific but unusual green colour. This phenomenon has long been known, and was first mentioned in England (4). The greening phenomenon is not restricted to oysters, as it seems to occur with other invertebrates like sea-anemone (5), polychaete, crabs, littorina or mussels (6).

A second consequence is that marennine has been indirectly consumed by some humans for centuries. The ‘green oysters’ (‘vertes de claires’ in French), although more expensive by *ca.* 20% compared to ordinary fattened oysters (‘fines de claires’), are traditionally eaten during festive periods, which has participated to the reputation of local oyster farmers. Greening of oyster gills is a reversible phenomenon, and it is supposedly concomitant with changes in oyster organoleptic properties (7), although the direct role of marennine has not been assessed (8) except for color (9). For decades, the

chemical nature of marennine remained unknown, and many hypothesis were suggested, such as anthocyanid (10), chromoprotein (11) or a chlorophyll degradation product (12). Our knowledge of marennine chemical nature recently increased with the publication of a method for its extraction and purification (13). Preliminary characterization has suggest that marennine is a polyphenolic compound of medium molecular weight (*ca.* 10 kDa) (14), but its chemical structure has not yet been established. Two forms of the pigment were described, one intracellular (IMn), accumulating mainly at the apical regions of the cells, and one extracellular (EMn), released in the culture medium. It has been demonstrated that intracellular and extracellular forms of marennine differ in their molecular weight and spectroscopic characteristics (13).

Both forms of purified marennine have been shown to display allelopathic (15) and antioxidant properties (16). Moreover, antiproliferative and antitumoral activity (17), as well as antiviral and anticoagulant activities (18) have been observed, when aqueous crude extracts of *H. ostrearia* were tested. All these types of biological activities are usually recorded with various polyphenolic marine substances (19), which is in accordance with the likely polyphenolic nature of marennine (20). Hence the two goals of this work were 1) to confirm the antiviral, antiproliferative and antitumoral activities observed using crude extracts of *H. ostrearia*, but using purified marennine, and 2) to extend our knowledge of the biological activities of this peculiar blue pigment, by testing its activity against bacteria, major components in the microfouling phenomenon. Some of the bacteria studied belong to the genus *Vibrio*, and are known for being pathogens of bivalves (21). In complement, antibacterial activities were also studied on some pathogenic strains of bacteria possibly present on food products.

MATERIALS AND METHODS

Cultivation of algae and biomass collection. The different strains of *H. ostrearia* used for biomass production and pigment extraction were provided by the Nantes Culture Collection (NCC, University of Nantes). They were derived from samples collected in the Bourgneuf Bay, France (46° 59' 19" N / 2° 14' 14" W). Algae used to test antiproliferative and antitumoral activities, were grown in the MMS Nantes laboratory with modified Provasoli medium (ES Provasoli1/3) at 16°C and subjected to an irradiance of 100 $\mu\text{mol photon. m}^{-2} \cdot \text{s}^{-1}$, with illumination provided by cool-white fluorescent tubes in a 14/10 light/dark cycle (22). First culture was done in a 250 mL Erlenmeyer flask then was used to inoculate a 2 L flask which was then used to inoculate a 22 L flask in the same conditions. For antibacterial and antiviral analyses, algae were cultured in the MMS Le Mans laboratory in Erlenmeyer flasks containing modified artificial sea water (23), under controlled conditions (15±1 °C, irradiance of 200 $\mu\text{mol of photons m}^{-2} \text{ s}^{-1}$, 14h/10h L/D cycle). Illumination of the culture cabinet was provided by fluorescent tube Philips TLD 36W/965. Irradiance was measured using a Li-Cor LI-189 quantum meter coupled with a 2II Li-Cor Q21284 quantum sensor. Cultures were harvested regularly during their exponential phase of growth. For all cultures, cells and supernatant were separated by centrifugation with a Sigma 3K15 centrifuges (Bioblock scientific). Freeze dried cells were stored in a freezer until extraction. Supernatant was stored in the dark in a temperature-controlled room at 6±1 °C until analysis.

Pigment extraction and purification. After centrifugation, pigments were extracted and purified following (13). Cells were crushed in liquid nitrogen, with a mortar and a

pestle, in 250 mMol NH_4HCO_3 buffer solution (pH 8). The crude extract containing the internal form was then centrifuged, and the supernatant collected. For the external form of the pigment, 20 litres of coloured culture supernatant were use directly after filtration on a 0.45 μm filter. Then, for both extracts, a two-step ultrafiltration process performed. Cartridges fitted with regenerated cellulose spiral membranes (Prep/Scale-TFF cartridge 0.23 m², Millipore) were used with first a 30 kDa cut-off membrane and then a 3kDa cut-off membrane. At the end of the process, the retentate was collected. Pigment was then separated by anion-exchange chromatography, using an UV-visible detector to identify the fraction, thanks to its spectroscopic properties. The fraction collected was dialysed and last freeze dried. Purified pigments were stored in the dark at -20°C.

Antibacterial activities Three marine bacterial strains, *Polaribacter irgensii* (ATCC 700398), *Pseudoalteromonas elyakovii* (ATCC 700519) and *Vibrio aesturianus* (ATCC 35048), were cultured in the Biological Sciences University of Portsmouth. They were maintained in an incubator at 25°, with regular refreshment of their medium. This medium was made of steam-sterilized sea water mixed with peptone, final concentration of 5g L⁻¹. Antibacterial testing of marennine was performed by the disc diffusion technique in agar plated Petri dishes, using a modified version of the method described in Hellio *et al.* (24). Whatman filter paper discs of 6 mm diameter were sterilised at 15 atm pressure for 15 min. They were loaded with 10 μL of internal or external marennine solutions at four concentrations of 1, 10, 50 and 100 $\mu\text{g}/\text{mL}$. They were dried in an axenic place at room temperature for 6 hours. A dilution of the bacterial culture in nutrient broth was made one day before experiments. A volume of 0.1 mL samples of the culture (10⁶ CFU/mL) was used for agar plate insemination. After incubation for 2

days at 25°C, the activity was evaluated by measuring the ray of the inhibition zone around the discs. All inhibition assays were made in triplicate.

Pathogenic bacteria. Several strains of terrestrial bacteria were obtained and cultured in the Laboratoire Départemental de la Sarthe (*Proteus mirabilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium perfringens*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Enterococcus faecalis*, and *Legionella pneumophila*). All strains were referenced at ATCC (accession number on demand), except to the exception of *Proteus mirabilis*, a strain isolated in the Laboratory. Cultures in exponential phase of growth were diluted to the McFarland concentration traditionally used to realise antibiograms, using a Densimat densitometer (Biomérieux) and sterile flasks of NaCl 5% water (Biomérieux). Petri plates of Mueller Hinton 2 agar 5% sheep blood were inoculated with 1 mL of diluted suspension of bacteria. Sterile Whatman filter paper discs of 6 mm diameter, prepared as above with 10 µL of IMn or EMn solutions at four different concentrations (1, 10, 50 and 100 µg/mL), were put on the Petri plates. Plates were placed in culture cabinet for two days, Incucell cabinet of Fisher Bioblock for bacteria cultured in normal atmosphere at 37° and 41° (for *C. jejuni*), and Galaxy 170S by New Brunswick Eppendorf for *E. faecalis* and *S. aureus* grown with CO₂. *C. jejuni* was cultured in micro aerophily and *C. perfringens* in anaeroby (nom du kit). After an overnight incubation, the diameters of the inhibition zone around the discs were measured. All inhibition assays were made in triplicate.

Antiviral activities. African green monkey kidney cells (Vero cell line n°ATCC CCL81) were grown in Eagle's minimum essential medium (MEM, Laboratory

Eurobio) supplemented with 8% fetal calf serum (FCS), to which was added 1% of PCS (penicillin 10 000U, colimycine 25 000U, streptomycine 10 mg). Cells were routinely passaged every 3 days. A virus stock of Herpes simplex virus type 1 (HSV-1), wild 17 strain ACVS and PFAS was obtained from Pr. Billaudel, Laboratoire de Virologie de Nantes (France). Subsamples of virus stock were prepared by incubating Vero monolayers (75 cm² culture flasks seeded with 350.000 cells/mL) at low multiplicity and incubating at 37°C, in a 95% air, 5% CO₂ (v,v) atmosphere. Two or three days after infection, the cultures were frozen and thawed twice times, before clearing the preparation by centrifugation at low speed to remove cell debris. The resulting supernatant aliquot was stored at -70°C until use. Virus titrations were performed by the Reed and Muench dilution method (25), using 10 wells on 96-wells microtiter plates per dilution. The virus titer was estimated from cytopathogenicity and expressed as 50% infectious doses per milliliter (ID₅₀/mL). As an antiviral reference drug, the sodium salt Zovirax IV, 25 mg/mL was purchased from the Wellcome Foundation Ltd.

Dilutions of Zovirax and pigments (50 µL aliquots) were prepared in Eagle's MEM supplemented with 8% FCS and distributed into the wells of a 96-well microtest III tissue culture plate (Nunclon, Intermed). A series included eight assays ranging from extreme concentrations of pigments of 200, 50, 10, 5 and 1 µg/mL (4 wells per concentration). 100µL of cellular suspension (3.5 x 10⁵ Vero cells/mL) in Eagle's MEM containing 8% FCS were distributed into the wells using a multichannel Titertek® pipette and infected with HSV-1. Fifty microliters of mock- and virus-infected cell suspensions at multiplicity of infection (MOI) of 0.001 ID₅₀/cells (2 x 10^{6.5} ID₅₀/mL) were then transferred into each well containing the dilution compound and were incubated for 3 days without change of the medium, at 37°C in 5% CO₂. Cells and virus controls were run simultaneously.

After microscopic examination to check the growth of virus, 50 μ L of neutral red dye (0.15% in saline, pH 5.5) was added to each well and the cultures were incubated for 45 min at 37°C (26). Excess dye was removed by rinsing with phosphate buffered saline (PBS, pH 7.2; Biomérieux) and the neutral red incorporated by the viable cells was eluted into 100 μ L/well of citrate ethanol buffer. After shaking the tray for 20 min, whereby cell monolayers were completely disorganized, the absorbance (OD) of the wells was read in a multichannel spectrophotometer (Packard Spectra CountTM) at 540 nm. The OD was directly related to the percentages of viable cells that were inversely proportional to CPE ratio. The straight line of the regression was determined for each assay and for each plate on the basis of cell controls (0% CPE) and virus controls (100% CPE) (27). The 50% cytotoxic concentration (CC50) of the test pigment was defined as the concentration of marennine that reduced the absorbance of mock-infected cells to 50% of that of controls. The 50% antiviral effective concentration (EC50) was expressed as the concentration that achieved 50% protection of virus-infected cells from the HSV-induced destruction. The percent protection was calculated by the following formula:

$$[(OD_t)_{HSV} - (OD_c)_{HSV}] / [(OD_c)_{MOCK} - (OD_c)_{HSV}] \times 100 (\%)$$

where (OD_t)_{HSV} is the absorbance of the test sample (marennine), (OD_c)_{HSV} is the absorbance of the virus-infected control (no marennine), and (OD_c)_{MOCK} is the absorbances of the mock-infected control. The ratio (OD_c)_{HSV} / (OD_c)_{MOCK} is expressed as percent of control.

Antiproliferative activities. Internal and external marennine were solubilized in RPMI 1640 medium at 1 mg mL^{-1} . Solutions were sterilized by filtration on $0.2 \text{ }\mu\text{m}$ filtre (RC-Millipore). Dilutions were conducted in PBS medium under axenic conditions, to obtain concentrations of 1, 10, 50 and $100 \text{ }\mu\text{g mL}^{-1}$. The cell lineages used were derived from human tumorous lineage. M113 is melanoma cells, SKOV3 and SHIN3 are from ovarian cancer, SW116 is colon cancer, R3111 from kidney cancer, 1355 from lungs and MCF7 from breast. They were maintained at the INSERM U463 (University of Nantes). Another lineage of human bronchopulmonar cells NSCLC-N6 was kept in the marine pharmacological laboratory of the University of Nantes.

Cultures were made on RPMI 1640 medium with 5 % fetal bovine serum, 100 UI mL^{-1} penicillin, $100 \text{ }\mu\text{g mL}^{-1}$ streptomycin and 2 mMol/L glutamin. Cultures were placed in an incubator at $37 \text{ }^\circ\text{C}$ in a CO_2 enriched atmosphere at 5 %. Regular refreshments of the medium kept cultures in exponential growth phase. An optical method was used to estimate cellular growth by a viability test based on the conversion of the MTT in formazan by the mitochondria of living cells (28). Sterile 96-wells cell culture plates were incubated with $50 \text{ }\mu\text{L}$ of cell culture in exponential growth phase. Then $50 \text{ }\mu\text{L}$ -aliquots of marennine solutions or medium were added. Some wells were filled with pure medium or only marennine solutions for blank settlement. Absorbance of the wells was checked the first day using a Titertek Multiscan MKII microplate reader at 570 nm ($A_{j0[0]}$). After 72 h, $50 \text{ }\mu\text{L}$ of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) dissolved in phosphate buffer saline at 2.5 mg mL^{-1} were added in each well. After 4 h of incubation at 37° , $100 \text{ }\mu\text{L}$ of a lysate buffer made with SDS 12.5%, N,N-dimethylformamide 30%, HCl $2,4 \text{ mmol L}^{-1}$ and acetic acid 1%, were added in each well. After 12 h of incubation at room temperature, a new lecture of the absorbance at 570 nm was done ($A_{j3[\text{Mn}]}$) or growth control without

marennine ($A_{j3[0]}$). Absorbance of assays with marennine was corrected by marennine control at three days without cells ($A_{j3[Mn]c}$), and the percentage growth (G%) of control was determined as follows:

$$G\% = \left\{ \frac{A_{j3[Mn]} - A_{j3[Mn]c} - A_{j0[0]}}{A_{j3[0]} - A_{j0[0]}} \right\} \times 100.$$

This was used to determine IC50, the minimum inhibitory concentration of marennine necessary to cause 50% growth inhibition compared to the control. IC50 values, obtained by plotting inhibition rate ($I\% = 1 - G\%$) versus marennine concentration, were calculated by sigmoidal dose-response model with a four-variables slope from the dose-response curve. Statistical analyses were applied at a maximum significance level of 5% by one-way analyses of variance (ANOVA) followed by Student-Newman-Keuls's post-hoc multicomparison test (SNK). Data were computed using the software SigmaPlot version 9.0 for Windows.

RESULTS

Antibacterial activities. Both forms of marennine, intracellular (IMn) and extracellular (EMn), significantly inhibited the growth of marine bacteria around the paper discs. Size of the inhibition zone diameter generally increased with the concentrations used to load discs. Concentrations as low as $1 \mu\text{g mL}^{-1}$ were effective to induce the apparition of inhibition rings (Table 1 and 2). No inhibition zone was observed in any culture of terrestrial pathogenic bacteria, whatever marennine form or concentration considered (not shown).

	Gramm type	Solid medium	Temperature of growth	Atmosphere	1 $\mu\text{g. mL}^{-1}$	10 $\mu\text{g. mL}^{-1}$	50 $\mu\text{g. mL}^{-1}$	100 $\mu\text{g. mL}^{-1}$
<i>Polaribacter irgensii</i>	-	Sea water and agar	room temperature (20°)	normal	2 \pm 0	2.3 \pm 0,5	2.7 \pm 0,9	3.3 \pm 1,2
<i>Pseudoalteromonas elyakowii</i>	-	Sea water and agar	room temperature (20°)	normal	2.7 \pm 0,5	3 \pm 0,8	3.3 \pm 1,2	25 \pm 0,5
<i>Vibrio aesturianus</i>	-	Sea water and agar	room temperature (20°)	normal	2.7 \pm 0,9	2.7 \pm 0,9	3 \pm 1,4	3.3 \pm 1,2

Tab 1. Antibacterial activities of purified internal marennine on marine bacteria expressed in mm of inhibition ray (\pm S.E) around the loaded paper discs

	Gramm type	Solid medium	Temperature of growth	Atmosphere	1 $\mu\text{g. mL}^{-1}$	10 $\mu\text{g. mL}^{-1}$	50 $\mu\text{g. mL}^{-1}$	100 $\mu\text{g. mL}^{-1}$
<i>Polaribacter irgensii</i>	-	Sea water and agar	room temperature (20°)	normal	2.7 \pm 0.6	2.7 \pm 0.6	3.3 \pm 0.4	3.3 \pm 0.4
<i>Pseudoalteromonas elyakowii</i>	-	Sea water and agar	room temperature (20°)	normal	2.5 \pm 0.4	2.5 \pm 0.4	2.5 \pm 0.4	2.5 \pm 0.4
<i>Vibrio aesturianus</i>	-	Sea water and agar	room temperature (20°)	normal	2.3 \pm 0.4	2.3 \pm 0.4	2.3 \pm 0.4	2.3 \pm 0.4

Tab 2. Antibacterial activities of purified external marennine on marine bacteria expressed in mm of inhibition ray (\pm S.E) around the loaded paper discs

Antiviral activity and cytotoxicity. After 3 days of treatment, microscopically visible alteration of normal cell morphology was observed and viability assays showed destruction of cell layers. No cytotoxic effect of the compounds on the Vero cells was observed in the range of the concentrations assayed for the extracellular pigment extract (Table 3). Both forms of marennine, EMn and IMn, presented an effective antiherpetic activity with EC₅₀ ranging between 23 and 62 µg/mL. For a MOI of 0.001 ID₅₀/cells, 100 % cellular protection was obtained for 200 µg/mL of EMn and IMn pigment extracts at 72 h after infection.

Code	CC ₅₀ µg/ml	EC ₅₀ µg/ml
Zovirax	> 200	0.2
Internal marennine	107.2	24.0
External marennine	> 200	27.0

Tab 3. Antiviral activities of purified internal and external marennine.

CC₅₀: The 50% Cytotoxic Concentration. The concentration that reduced the absorbance of mock-infected cells to 50% of that of controls. EC₅₀: The 50% antiviral Effective Concentration: concentration that achieved 50% protection of virus-infected cells from the HSV-induced destruction

Antiproliferative activity. Cells incubated with the intracellular (IMn) or the extracellular (EMn) form of marennine showed a different morphology compared to the controls. Cells were no longer adhesive to the plates and get a globular shape. They grew as piles, floating in the medium. Only the SHIN 3 lineage, which was not initially adhesive, did not present shape modification. During experiments, no degenerative or toxic cultures were observed (negative growth).

The inhibition of growth is dependent on cell lines (Tab.). SHIN3 line, non-adherent, is insensitive at the doses tested. The proliferation of cell lines SKOV-3, SW116 and M113 is inhibited at higher doses (100 $\mu\text{g ml}^{-1}$, 31 \pm 9%, 36 \pm 4% and 49 \pm 3% respectively) by EMn and only SW116 by IMn (100 $\mu\text{g ml}^{-1}$, 38 \pm 7%). The antiproliferative activities are between 0.79 and 82.7 $\mu\text{g mL}^{-1}$ for marennine (Tab). These activities are not significantly different between the EMn and the IMn (ANOVA, SNK, $P < 0.05$).

cell line		IC ₅₀ ($\mu\text{g. mL}^{-1} \pm \text{SE}$)	
		EMn	IMn
lung cancer	1355	1.1 \pm 0.56 ^d	0.79 \pm 0.35 ^d
lung cancer	NSCLC-N6	14.4 \pm 9.8 ^{cd}	nd
breast cancer	MCF-7	6.5 \pm 3.5 ^{cd}	22.2 \pm 8.7 ^{bc}
kidney cancer	R3III	25.9 \pm 9.8 ^{bc}	36.7 \pm 1.1 ^b
melanoma	M113	>100	82.7 \pm 33 ^a
ovarian cancer	SKOV-3	>100	no
ovarian cancer	SHIN3	no	no
colon cancer	SW116	>100	>100

Tab 4. Antiproliferative activities of internal and external marennine on human cancer cell line. IC₅₀, the minimum inhibitory concentration of marennine necessary to cause

50% growth inhibition compared to the control. Statistical analysis ANOVA, SNK, $P < 0.05$. no, no inhibition observed; nd, not determined

DISCUSSION

With respect to algae and human affairs, marennine, the blue pigment responsible for the greening of oysters, is a real paradox. On the one hand, it has been named officially two centuries ago (1) but possibly consumed for a much longer time, on the other hand, its chemical structure has not been elucidated yet, in spite of a preliminary characterization recently achieved and a polyphenolic nature hypothesized (14). Furthermore, aqueous blue extracts of *H. ostrearia*, thus containing marennine, have been shown to demonstrate some biological activities (17-18), but the role of marennine could not be demonstrated before a method of extraction and purification was available. Following the development of such a methodology (13), the present work was aimed to confirm different valuable biological activities of marennine, in addition of allelopathic (15) and antioxidant properties (16).

Antibacterial activities. The two forms of marennine, intracellular and extracellular, inhibited the growth of the three strains of marine bacteria tested, a result which is relevant for aquaculture (e.g., for stocks production or the interactions between microorganisms and fauna in oyster ponds) and food industry. As for example, some species from the genus *Pseudoalteromonas* are highly virulent (29), *P. nigrifaciens* on holothurians (30) or *P. atlantica* on crabs (31), causing skin ulcers of holothurians to quick death of crabs. Other species of *Pseudoalteromonas* may degrade mussel polypeptidic threads (32), or polysaccharides from macroalgae (33), and can affect

different components of marine ecosystems. As to the genus *Vibrio* it contains many species which are pathogenic for different animals, and can affect stocks of shrimps (34-35), clams (36-39), and oysters (e.g., 40). The *Vibrio* genus also contains one of the deadliest bacteria for mankind, *V. cholerae*, for which few antibiotic treatments exist (41). In a preliminary work it was first observed that IMn, the intracellular form of marennine, could inhibit the growth of *V. anguillarum* and that EMn had no effect. This experiment consisted in a unique deposit of 100 µg of pigment per discs. The present work confirms the marennine activity with another *Vibrio* strain, and extends it to lower concentrations, demonstrating that both IMn and EMn can inhibit growth of *V. aesturianus*, which is especially encouraging for aquaculture. Indeed, in the last years, *V. aesturianus* has been involved in high seasonal mortality of the oyster *Crassostrea gigas* in France (40), and also in other countries, as Japan, Korea, United States and Australia. For reason not explained yet, our work shows that marennine extracellular form EMn is as active as IMn. EMn accumulates in the seawater during algal growth and culture cycle (42), and it should be the principal agent of the greening of oysters (Pouvreau, unpublished). Marennine produced by *H. ostrearia*, and released in oyster ponds during the greening phenomenon at concentrations up to 5 µg mL⁻¹ (43), could thus act as a natural antibiotic or bacteriostatic compound for oysters, counteracting the effect of pathogenic bacteria.

Oysters are filter feeders and their gills filter out huge amounts of seawater to gather, and then select food particles. As a consequence, they may accumulate many viruses or bacteria, at concentration possibly a thousand times higher than those of the surrounding water (44). Among these pathogens, some are responsible for diseases and alimentary intoxication after shellfish consumption, such as enteric and faecal bacteria *Enterococcus faecalis* or *Escherichia coli*. In a preliminary work (Pouvreau,

unpublished), it has been observed that only IMn was active, and inhibited the growth of *S. aureus*, but not of *E. coli*. In our study, no activity was detected, whatever the form of marennine or the strains tested. To circumvent possible interactions of the bacterial culture medium with marennine, two strains *E. faecalis* and *P. mirabilis* were grown on normal Mueller Hinton solid medium and blood agar, before reiterating experiments, but once again no activity was noticed. Thus it seems that marine and ‘terrestrial’ pathogenic bacteria differ from each others, regarding a wide range of morphological, chemical and physiological features, including a higher sensitivity to the antibacterial activity of marennine in marine bacteria.

Antiviral activities. Many plants (45) and a wide diversity of marine organisms, including macro- and microalgae produce different compounds displaying antiviral activities (46). In higher plants, the active compounds are commonly polyphenolic molecules (see for example 47-50). In algae, most of the time, active compounds are polysaccharides, eventually highly sulfated (51). These are the kind of compounds whose effects were once observed by Bergé *et al.* (18) using *H. ostrearia* aqueous extracts. In our study, both forms of marennine, intracellular and extracellular, exhibited antiviral activities. IMn reveals to be cytotoxic at a concentration which was half the CC_{50} cytotoxic concentration of EMn and Zovirax. Differences in the CC_{50} for both forms of marennine could be explained by the different structures they have, as evidenced by Pouvreau *et al.* (14). Antiviral activities, as expressed by their EC_{50} , were consequently lower than those of Zovirax.

Antiproliferative activities. Bioactive compounds from algae have got more and more attention, in particular for their antiproliferative potential. For example, pigments from

brown algae showed different activities (52), and some algal polyphenols were found to have antiproliferative effects against cancerous cells from organs of the digestive system (53). Various macroalgae being edible and eaten on a daily basis in some Asian countries, they could be considered as functional food. Some microalgal extracts are also known to have potential antiproliferative properties (54). In *H. ostrearia*, the two forms of marennine showed activities against diverse lineages of human cancerous cells. In particular, the main activity observed on the bronchopulmonar lineage NSCLC-N6, confirms a previous observation made using *H. ostrearia* aqueous crude extracts (17). The CE₅₀ observed with purified pigment was sensibly (two time) lower than values given by Carbonelle *et al.* (17), and these antiproliferative activities are comparable with those of other polyphenols present in food products (55-57). Whatever IMn or EMn form, when converted in specific activity, CE₅₀ observed are in the range of µmol/L, comparable or inferior to the CE₅₀ measured with different polyphenolic compounds against melanom or colon cancer cells (58-59).

Marennine is a water soluble polyphenolic compound, with a high molecular weight (Pouvreau *et al.* 2006a). Its mechanisms of action may thus differ from those of other polyphenols commonly described in pharmacology, most of them being amphiphilic small molecules. These molecules can pass through the cellular membranes and reach their target, for example to induce apoptosis (60). Polyphenols are also responsible for a tanning action and astringency (61), leading to the precipitation and denaturation of proteins, and as a consequence, to a lower digestibility of some plant products (62). This mechanism is possibly as a way of defence against grazing organisms (63). Marennine activity does not seem specific with regard to the cellular lineages. The morphological changes observed in cell cultures in presence of both marennine forms, in combination with the antiproliferative activities, could be the

consequence of a tanning effect of proteins in cell membranes or as proanthocyanidins interact with insulin-like growth factor binding protein-3 in NSCLC cells (64). This tanning effect results in the alteration of the proteins at the cell surface, and changes of the cellular matrix and cell ability to adhere to the substrate or to each other. This hypothesis is in accordance with the low inhibition and occasional stimulation of the growth of cells from the non-adherent lineage, and complete the explanation formerly proposed of an activation of apoptotic pathways (17) via interaction with the extracellular matrix.

Since they are consumed by human, marennine-filled oysters were not known to provoke any specific disease. In contrary, observations made in this publication, coupled with what is known of marennine's antioxidant capacities, tend to prove that marennine can be a healthy component of food. Big polyphenols are commonly metabolized and fractioned before absorption, resulting in small molecules with other properties, tough most of the time bactericide to (65). We cannot assume what may be the degradation products of marennine after digestion, as its structure is unknown. However, marennine seems very resistant against biotic aggression and enzymatic digestion (Pouvreau, unpublished). Many micro algae product substances that are bioactive substances, commonly used in food industry since recent times (66). Maybe one day, marennine will be used as a natural blue pigment for food industry. In that way, results here exposed will probably be helpful.

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V.6 The blue-grey pigments of *H. karadagensis* (Naviculaceae): the Putative utilization to colour the oysters and the Biological properties

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Abstract

The diatom *H. karadagensis* (species newly described) produces blue-grey pigment. We demonstrated that these pigment i) like the marennine produced by *H. ostrearia*, may remain on the gills of the two economical oysters *Crassostrea gigas* and *Ostrea edulis*; and ii) have distinct biological activities as the inhibition of marine mushrooms, *Herpes simplex virus type 1* (HSV-1) and *Vibrio aesturianus* (bacteria). As high prevalence of *Herpes* and *Vibrio* seems correlated with the high mortality period of oysters, the valorisation and putative applications of the pigments of *H. karadagensis* in aquaculture are discussed.

Keywords : *H. karadagensis*, oyster farming, *Vibrio*, *Herpesvirus*, fouling, biological activities, probiotic

Introduction

Greening of oysters is a natural phenomenon which has long been known (Sprat, 1669) and dependant from the presence of diatoms producing a pigment, which may remain in gills of bivalves (Gaillon, 1920). Greening today mainly occurs in the Atlantic Coast of France, (Marennes-Oléron Bay and Bourgneuf Bay). Special ponds, usually old salt marshes, are actively colonized by microalgae, and used for oyster maturation and fattening. In some of these oyster ponds named 'claires', the diatom *Haslea ostrearia* (Gaillon) Simonsen (1974) can eventually outcompete other microalgae, and be consumed in quantity high enough to sustain growth of oysters, as experimentally demonstrated (Barillé et al. 1993, Piveteau 1999, Cognie 2001). The diatom *H. ostrearia* is known for the water-soluble blue pigment it produces and accumulates at its apices, the so-called marennine (or marennin, Lankester, 1886), in reference to Marennes-Oléron. When *H. ostrearia* makes a bloom and releases marennine in the water, the oyster pond possibly becomes dark bluish-green. The bivalves turn green as well, by feeding on *H. ostrearia* cells, and/or fixing the pigment released in water on their gills, as reproduced experimentally (Lankester in Dyer, 1877; Puységur 1880; Sauvageau 1907). In the literature, apart from France, greening of oysters naturally occurring (i.e., not provoked by bivalve producers), was first described in Great-Britain (Sprat, 1669), and also observed in Denmark (Petersen, 1916), and the United-States (Ryder, 1884; Mitchell and Barney, 1918).

Marennine structure is still undefined, but it seems it can exist in two forms, intracellular which accumulates in the cell, and extracellular which is excreted in the medium (Pouvreau et al., 2006a). These two forms have different molecular weight and spectral characteristics. Note that the presence of algal cells is not necessary, as

greening has been demonstrated in the laboratory using supernatant (crude extract) of *H. ostrearia* cultures (Ranson 1925; Neuville & Daste 1971), or a solution of purified marennine (Pouvreau 2006).-Due to the unpredictability of the greening phenomenon and the consumer demand, their price of the “greening oyster” is ca. 20% higher than those of other oysters. Furthermore, marennine present antioxidant properties (Pouvreau et al., 2008), and could exert an ecological allelopathic effect on other diatoms in oyster ponds (Pouvreau et al., 2007).

For decades, *H. ostrearia* was believed the solely diatom able to produce marennine, itself considered an original and unique pigment. Hence, this diatom was ascribed worldwide in distribution, given to either direct evidence of optical microscopy observations of blue diatoms, or indirectly by observation of a greening effect on bivalves. The recent discovery and the study of a pennate diatom with blue-grey apices in the Crimean shores of the Black Sea, different from *H. ostrearia*, resulted in the description of a new species belonging to the genus *Haslea*, named *Haslea karadagensis*-(Gastineau et al., submitted). The pigment synthesized by *H. karadagensis* , differs from marennine, especially regarding *in vivo* UV-visible spectrophotometry and Raman spectroscopy. Moreover, as for marennine, this new pigment also exists in two forms, intracellular, and extracellular (unpublished data).

This discovery brings into question the worldwide distribution and the supposedly ubiquity of *H. ostrearia*, as well as the unicity of marennine as an original microalgal blue pigment. Hence the possibly specific pigment produced by the newly discovered blue diatom initiated the study of their possible effects on bivalves, since for centuries, no microalga other than *H. ostrearia* , and no other pigment than marennine were ascribed to colour oysters. The first objective of the present work was thus to

compare the colouring potential of the pigments produced by the two species of blue diatoms, on two different economically important species of oysters, *Crassostrea gigas* and *Ostrea edulis*, which were given a diet composed either of *H. ostrearia* or *H. karadagensis*. The second objective of this work was to screen for biological activities (antifungal, antibacterial and antiviral properties) of the internal and external forms of *H. karadagensis*' pigment with an emphasis on marine micro-organisms.

Material and methods

Two different economical species of oysters were used for these experiments. The Pacific oysters *Crassostrea gigas* (Thunberg, 1793) originated from Saint Germain sur Ay, Normandy, France, and the plate oysters *Ostrea edulis* (Linnaeus, 1758) from Saint-Malo, Brittany, France. Oysters were acclimated in 35x25x20 cm aquaria, containing 5 L of artificial sea water, oxygenized by air bubbling (Rena[®] Air 100 air pump). An illumination of 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ was provided from above by fluorescent tube Philips TLD 36W/965, as measured with a Li-Cor LI-189 quantum meter coupled with a 2II Li-Cor Q21284 quantum sensor. Temperature of the water in aquaria was 12 ± 1 °C. During a one week acclimation phase, oysters were fed with regular inoculums of *Skeletonema costatum* and *Entomoneis paludosa* cultures. Both microalgae are common diatoms encountered in Atlantic oyster ponds (Rincé, 1978). Furthermore, it has been shown that *Skeletonema* and *Entomoneis* species have already been used experimentally for oyster feeding (Piveteau, 1999; Lefebvre et al., 2000; Soletchnik et al., 2001; Knuckey et al., 2002; Barillé et al., 2003). For the two species inoculums were designed to make final concentration of $1.5 \cdot 10^6$ cells L^{-1} . At the start of the experiments, oysters were immersed in aquaria containing 120 000 cells mL^{-1} of *H.*

ostrearia and *H. karadagensis* for one week before observation. Some oysters remained fed with *S. costatum* and *E. paludosa* as controls and stock oysters. Algal strains were provided by the Nantes Culture Collection (University of Nantes, V. Méléder): NCC 18 (*E. paludosa*), NCC 53 (*S. costatum*), NCC 148.14c (*H. ostrearia*) and NCC 313 (*H. karadagensis*). Prior to experimentation, algae were grown in sterile Erlenmeyer flasks with modified artificial sea water (Mouget et al., 2009), in a controlled-culture room (60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 14h/10h L/D cycle, provided by fluorescent tube Philips TLD 36W/965).

Pictures of the diatoms were taken using a Zeiss Axiostar plus microscope with a Zeiss Acroplan 100x/1.25 Oil objective and a Zeiss Axiocam ICc1 camera. Pictures of oysters were taken using a Canon EOS 550 D camera with a Canon Zoom Lens EF 24-105 mm 1: 4 objective.

The pigment of *H. karadagensis* was extracted and purified according to Pouvreau et al. (2006b). The intracellular and extracellular forms were obtained from algal pasta and supernatant, respectively, after gentle centrifugation of algal suspensions (10 min at 900g, with an acceleration/deceleration of 2/2 at 4°, using a Sigma 3K15 model from Bioblock scientific). Extraction was made by crushing algal biomass in liquid N₂ with a 250 mM L⁻¹ NH₄HCO₃ buffer at pH 8 (Pouvreau et al. 2006b). Purification consisted in a two step ultrafiltration process made on 30 kDa and 3 kDa cut-off membrane, with a Prep/Scale-TFF cartridge 0.23 m² from Millipore, and a peristaltic pump 313S from Watson Marlow. It was followed by anion-exchange chromatography, performed with a dual piston pump WatersTM 626 coupled with a

tunable absorbance detector Waters™ 486, and with the help of DEAE Sepharose Fast Flow resin from Amersham Biosciences. Dialysis was made with Spectra/por Regenerated Cellulose (Spectrum) 3.5 kDa cut-off membrane. Finally, the pigment was freeze-dried on a Heto FD3 apparatus.

Antibacterial and antifungal activities of the pigment of *H. karadagensis* were studied using different strains of marine bacteria (*Vibrio aestuarianus*, *Pseudoalteromonas elyakowii*), two bacteria commonly used in biological assays (Plouguerné et al., 2010), and fungi (*Corollospora maritima*, *Lulworthia* sp., *Dendryphiella salina*), following modified protocol after Hellio et al (2000). Stock cultures of bacteria were maintained in an incubator at 37°, regularly diluted with fresh medium, which was made of steam-sterilized sea water mixed with peptone for a final concentration of 5 g L⁻¹. The disc diffusion technique in agar plated Petri dishes was slightly modified according to Hellio et al. (2001), using sterilised Whatman filter paper discs wade-cut to a 6 mm diameter. The purified pigment (both forms) of *H. karadagensis* was diluted in sterile distilled water at concentrations ranging from 0.01 to 100 µg mL⁻¹, filtered on a 0.22 µm filter. Then, 10 µL aliquots of the solutions were used to load the paper discs, which were dried in axenic conditions at room temperature for 6 h before use. A dilution of the bacterial culture in nutrient broth was made one day before experiments. Aliquots (0.1 mL) of the culture (10⁶ CFU mL⁻¹) were used for agar inoculation. After incubation for 2 days at 20°C, the activity was estimated by measuring the diameter of the inhibition zone around the discs. All inhibition assays were made in triplicate.

Stock cultures of fungi were maintained on maize meal agar (Oxoid) slopes. Pigments were diluted in distilled water, filtered on 0.22 μm filters, and mixed with 6 mL of maize meal agar 12% (Sigma) at pH 6. The Petri plates were inoculated with that mix in axenic condition, in order to get an agar plug of 8 mm. Inhibiting activity was measured after 3 week of cultures at 20 °C by measuring the diameter of the fungal colonies, in comparison to pigment-free experiments. Assays were all made in triplicate.

Evaluation of cytotoxicity and antiviral activity of drugs by cell viability (neutral red dye method) were described by McLaren et al. (1983) and Langlois et al. (1986). Experiments were conducted using Vero cell lines n°ATCC CCL81 of African green monkey kidney. Virus stock of *Herpes simplex* virus type 1, wild 17 strain ACV^S and PFA^S was obtained from Pr. Billaudel, Laboratoire de Virologie de Nantes (France). The reference drug was sodium salt Zovirax IV, 25 mg mL⁻¹, purchased from the Wellcome Foundation Ltd. A series included eight assays ranging from extreme concentrations of drugs of 200, 50, 10, 5 and 1 $\mu\text{g mL}^{-1}$ were done, after dilution of the pigments in Eagle's MEM supplemented with 8% FCS. The plates of 96 wells containing the dilution compound and virus-infected cell suspensions were incubated for 3 days without change of the medium, at 37°C in 5% CO₂. Cells and virus controls were run simultaneously. At the end of the experiments, the 50% cytotoxic concentration (CC₅₀) of the test compound was defined as the concentration that reduced the absorbance of virus-infected cells to 50% of that of controls. The 50% antiviral effective concentration (EC₅₀) was expressed as the concentration that achieved 50% protection of virus-infected cells from the HSV-induced destruction.

Results

In the present work, the protocol used for the colouration of oysters was inspired from Pouvreau (2006), who used *H. ostrearia* cultures of 120 000 cells mL⁻¹, and marennine concentration of *ca.*, 2 µg mL⁻¹. These concentrations of *Haslea* cells corresponded to the density observed in cultures in late exponential growth of phase, and it revealed enough to provoke a modification of the gill colour in less than one week. In comparison with the controls (*S. costatum* + *E. paludosa* diet), after one week of the experimental diet composed of blue diatoms, gills and mantle of oysters fed with a suspension either of *H. ostrearia* or of *H. karadagensis* cells, were coloured (Fig. 1). In the mean time, the gills and mantle of oysters fed with *S. costatum* and *E. paludosa* kept their yellowish initial colour. Oysters fed with *H. ostrearia* had organs strongly coloured in green, whereas oysters fed with *H. karadagensis* exhibited blue-grey organs, and by the naked eye, at a lighter colouration. Whatever the diet and diatom specific composition, no mortality of oysters occurred.

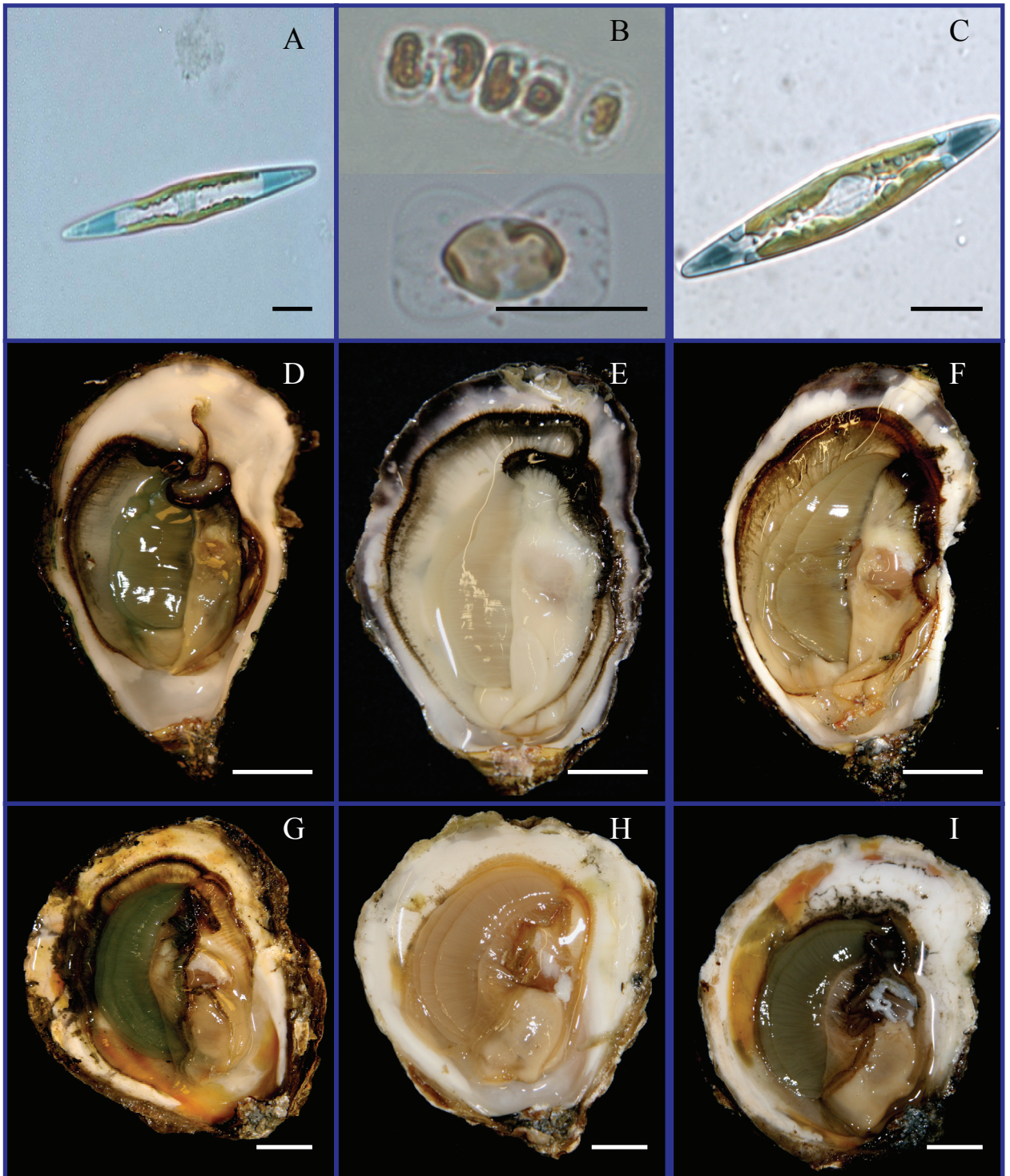


Figure 1. Colouring oyster's gills. A: *H. ostrearia*. B: *S. costatum* (up) and *E. paludosa* (down). C: *H. karadagensis*. D: *C. gigas* fed with *H. ostrearia*. E: *C. gigas* fed with *S. costatum* and *E. paludosa*. F: *C. gigas* fed with *H. karadagensis*. G: *O. edulis* fed with

H. ostrearia. H: *O.edulis* fed with *S. costatum* and *E. paludosa*. I: *O. edulis* fed with *H. karadagensis*. Scale bar are 10 μm (A to C) and 1 cm (D to I).

The effects of the pigment produced by *H. karadagensis* on two marine bacteria, *Vibrio aesturianus* and *Pseudomonas elyakowii*, are illustrated in Table 1. Both forms of the pigment inhibited the growth of the two bacteria, up to the concentration of 0.1 $\mu\text{g mL}^{-1}$. The minimal inhibiting concentrations of the pigment for the growth of the three species of marine fungi tested are shown in Table 2, and are ranging 0.1 - 1 $\mu\text{g mL}^{-1}$ (Table 2).

	I. F 0.01 $\mu\text{g. mL}^{-1}$	E. F 0.01 $\mu\text{g. mL}^{-1}$	I. F 0.1 $\mu\text{g. mL}^{-1}$	E. F 0.1 $\mu\text{g. mL}^{-1}$	I. F 1 $\mu\text{g. mL}^{-1}$	E. F 1 $\mu\text{g. mL}^{-1}$	I. F 10 $\mu\text{g. mL}^{-1}$	E. F 10 $\mu\text{g. mL}^{-1}$	I. F 50 $\mu\text{g. mL}^{-1}$	E. F 50 $\mu\text{g. mL}^{-1}$	I. F 100 $\mu\text{g. mL}^{-1}$	E. F 100 $\mu\text{g. mL}^{-1}$
<i>Vibrio aesturianus</i>	0	+	+	++	+	+	++	+	+++	++	+++	++
<i>Pseudoalteromonas elyakowii</i>	+	+	++	+	+++	++	+++	++	+++	+++	++++	+++

Table 1: Evaluation of the antibacterial activities of *H. karadagensis* internal and external pigments. I. F means internal form and E. F means external form. Evaluation is made by measuring the ray of the inhibition's halo. 0 means no inhibition, + an average halo's size comprised between 0 and 1 mm, ++ between 1 and 2 mm, +++ between 2 and 3 mm and ++++ upper 3 mm. Average values obtained from triplicates.

	<i>Corollospora maritima</i>	<i>Lulworthia sp</i>	<i>Dendryphiella salina</i>
Internal form of the pigment	0.1	1.0	0.1
External form of the pigment	1.0	1.0	1.0

Table 2. Evaluation of the minimum inhibitory concentration of *H. karadagensis* pigments for marine fungi. Results are minimum inhibitory concentration expressed in $\mu\text{g mL}^{-1}$.

Cytotoxicities and antiviral anti-HSV-1 activities of the pigment are shown in Table 3. No cytotoxic effect of the extracellular form of the pigment on the Vero cells was observed in the range of the concentrations assayed. After 3 days of treatment, no microscopically visible alteration of normal cell morphology was observed even at $200.0 \mu\text{g mL}^{-1}$. In addition, viability assay showed no destruction of cell layer. For a MOI of 0.001 ID₅₀/cells, the extracellular form of the pigment exhibited antiviral activity with EC₅₀ of $23 \mu\text{g mL}^{-1}$. In contrast, the intracellular form presents a lower antiviral activity with an EC₅₀ of $62 \mu\text{g mL}^{-1}$. This form appears more cytotoxic with a CC₅₀ of $87 \mu\text{g mL}^{-1}$.

Compound tested	CC ₅₀ µg/ml	EC ₅₀ µg/ml
Zovirax	> 200	0.2
Internal form of the pigment	87.0	62.0
External form of the pigment	> 200	23.0

Table 3: Evaluation of anti-HSV activity on Vero cell line of *H. karadagensis*' pigments using neutralised red dye method. The 50% Cytotoxic Concentration (CC₅₀) is the concentration that reduced the absorbance of mock-infected cells to 50% of that of controls. The 50% antiviral Effective Concentration (EC₅₀) is the concentration that achieved 50% protection of virus-infected cells from the HSV-induced destruction.

Discussion

Colouring potential of the pigment produced by H. karadagensis

Usually, the appearance of a green colouration in oyster gills can reflect two diametrically opposite phenomena, when considering consequences for Human affairs. On the one hand, changes in oyster colour, especially when it turns to green, is usually the alerting sign of a disease caused by metal (e.g., copper, iron) pollution (Lin and Hsieh, 1999), sometimes lethal for the bivalve (Lee et al., 1996). On the other hand, mostly in France today, greening of oysters is the consequence of the fixation on gills of marennine, the pigment produced by *H. ostrearia*, the diatom upon which the bivalves feed in the shallow oyster ponds named 'claires'. This documented phenomenon is usually expected by oyster producers to get the Red Label 'fine de claire verte' given by the French Ministry of Agriculture (AFNOR 1985), although it has revealed

unpredictable, for reasons as yet unknown. For decades, this unpredictability appealed for more research on the causal links between the bloom of blue diatoms and the greening of bivalves, and on the factors controlling such blooms. On the other hand, a change in colour is not restricted to oysters, as it has been observed that a greening-like action also occurs in other animals possibly present in the oyster ponds (Chaux-Thevenin, 1939), in polychaete, crabs, littorina, mussels (Ranson 1927) or sea-anemone (Gaillon 1820). And for all these organisms, as for oysters, specific interactions between microalgal pigments and gills, labial palps or any coloured organs of the bivalves still remain an opened field for biochemical and histological investigations.

In the line of preliminary experiments concerning the greening of *C. gigas* fed with *H. ostrearia* cultures in the laboratory (Pouvreau 2006), and whatever the mechanisms involved in the greening phenomenon, the present work demonstrates for the first time that feeding oysters with the newly-described blue diatom *H. karadagensis* gave their gills a blue-grey colour, different from the more or less intense green obtained with *H. ostrearia* and its marennine. Furthermore, although French oyster production in ponds is now mainly dedicated to *C. gigas*, both species of oysters used in these experiments demonstrated their capacity to fix marennine-like pigments. Indeed, both the Pacific oysters *C. gigas* and the plate oysters *O. edulis* were coloured by the pigments produced by the two blue diatom species, revealing possible similarities in their mode of action or interactions with the physiology of oysters.

Biological activities of H. karadagensis pigment

Both forms of the pigment produced by *H. karadagensis*, intracellular and extracellular, exhibited antibacterial and antifungal activities, at concentrations $\leq 0.1 \mu\text{g mL}^{-1}$. These concentrations are in the range of those tested with marennine produced by *H. ostrearia* (Gastineau et al., submitted). To our knowledge, no data about marennine concentration in oyster ponds during a natural greening event has ever been measured, but in a series of experiments on outdoor mass production of *H. ostrearia*, the maximum concentration of marennine measured in 25 m³ ponds was 2.4 $\mu\text{g mL}^{-1}$ (Turpin et al. 1999), 3.4 and 7.1 $\mu\text{g mL}^{-1}$ for 500 L tanks and 10 m³ ponds, respectively (Turpin et al. 2001).

Among marine bacteria, the growth of which was inhibited by *H. karadagensis* pigment, *Vibrio aesturianus* is especially relevant for aquaculture. *Vibrio* species and isolates are common bacteria encountered in aquaculture systems, able to infect a wide variety of hosts (Vandenberghe et al., 2003). They are responsible for diseases in different organisms as pleuronectiformes (Planas et al., 2005) and anguilliformes fishes (Esteve-Gassant et al., 2004), shrimps (e.g., Sudheesh and Xu, 2001), holothurians (Zhao et al., in press) or high valued molluscs like abalone (Cheng et al., 2004). Among all *Vibrio* species, *V. aesturianus* and *V. splendidus* are of particular concern for oyster industry as they are possibly involved in most of the severe summer mortality events observed in the last decade (Garnier et al. 2008, Lago et al. 2009). The results dealing with growth inhibition in *V. aesturianus* observed using *H. karadagensis* pigment confirm previous observations with marennine (Gastineau et al., unpublished/submitted), and are encouraging for present aquaculture challenges

regarding mortality events due to pathogenic bacteria. On the other hand, the marine bacteria *Pseudoalteromonas elyakovii* has been involved in damages caused to seaweed production in aquaculture (Narita et al., 2001), and a related species, *Pseudoalteromonas peptidolytica*, has depredate action on mussel threads (Venkateswaran and Dohmoto, 2000).

Since forty years (Farley et al., 1972; Farley, 1974), one of the major threats associated with oyster farming has been identified as a virus called OsHV-1, which belongs to the recently defined order of *Herpesvirales* (Davison et al. 2005; Davison, 2010) and is responsible of numerous oysters' decimation worldwide (Hine et al., 1992; Nicolas et al., 1992). Concerning France, OsHV-1 is also involved in most of the severe summer mortality events recorded in many French oyster production sites (Renault et al., 1994; Arzul et al., 2001; Arzul and Renault, 2002; Schikorski et al., 2011).

An antiviral effect of a water soluble fraction obtained with *H. ostrearia* was first hypothesized by Bergé et al. (1999). Later, antiviral properties of marennine were demonstrated (Gastineau et al., submitted). The present work demonstrates that purified pigment of *H. karadagensis* has also an antiviral activity and thus confirms the potential of marennine-like molecules as prophylactic natural compounds.

Regarding antifungal properties, both forms of *H. karadagensis* pigment displayed a growth inhibitory activity. Marine fungi such as those used in this study are commonly involved in the phenomenon of biofouling. They can adhere to immersed structures like shells or oyster trays and cages, leading to a loss of time and a waste of money for cleaning (Sala et al., 2008). Moreover are they corroding agents of immersed

surfaces, along with marine bacteria (Little et al., 1999). Another fungus, *Ostracoblabe implexa*, is an important causal agent of oyster disease (Bower et al., 1994). In the continuity of the antifungal effects observed in the present work, investigations could be made on *O. implexa*, because of the involvement of this organism in oyster mortality in the Black Sea (Pirkova and Demenko, 2008).

In this study, the antiviral evaluation consisted of testing oyster pigments on a mammalian fibroblastic cell line (Vero cells) infected by HSV-1. This model is currently used for the screening of antiviral molecules from marine organisms (Bergé et al., 1999; Yasin et al., 2000; Maier et al., 2001). The choice of a heterologous model was imposed by the lack of bivalve cell lines (Prescott et al. 1966; Li et al., 1972; Azumi et al., 1990; Tamamura et al., 1993; Lee and Maruyama, 1998).

In cell from that lineage, the pigment presents also a cytotoxicity, especially the intracellular form, as for marennine. Nevertheless, the highest marennine concentration measured in conditions similar to those prevailing in oyster ponds was $< 10 \mu\text{g mL}^{-1}$ (Turpin et al., 1999, 2001), a value lower than the CC_{50} measured with the intracellular form of *H. karadagensis* pigment, which revealed the most cytotoxic. During a greening episode, it can be hypothesized that the pigment fixed on the gills is the extracellular form, which is the less cytotoxic, but it also must be considered that, if the intracellular form of the pigment is supposed to remain inside the algal cell, it can be released at its death (e.g., when blue diatoms are used as a diet for oysters). Moreover, green oysters have been consumed for centuries, and to our knowledge, have never been of special concern for gourmets, nor have marennine-like pigments produced by *H. ostrearia*-like diatoms ever been involved in whatever oyster pathology.

In a local context more pertaining to *H. karadagensis* and the Black Sea, regarding oyster cultivation, the Karadag area is not comparable to Marennes-Oléron or Bourgneuf Bay. Experiments on acclimation and farming of *C. gigas* in Karadag have been successfully conducted in the past (Silkin et al., 2001), but without any follow-up or commercial exploitation. Some biological factors have been shown to potentially limit oyster farming in the Black Sea, one being *Rapana venosa*, an invasive carnivorous gastropod which feeds on bivalves (Miossec et al., 2009). *R. venosa* is recognized for the destruction of most of the living stock of *O. edulis* in the Black Sea, and it has been recorded to feed on *C. gigas* specimens too. A parasitic protist *Bonamia ostreae* has also been suspected to participate in the decline of oyster populations (Todorova et al., 2009). The marine fungus cited above, *O. implexa*, is also a cause of shell diseases in Black Sea oysters (Pirkova and Demenko, 2008).

However, the results of the present work allow hypothesizing that the development of oyster farming in the Black Sea could be helped using *H. karadagensis* as a diet for the bivalves, acting also as a prophylactic treatment. Illustrating this interest, prophylactic treatments based on microalgal diet received some attention in the last two decades (Naviner et al., 1999), also due to the fact that diatoms or other algae used for feeding can sometimes be themselves a source of various bacterial introduction (Nicolas et al., 2004; Nakase and Eguchi, 2007). Such possible valorisation of the biological activities of pigments produced by blue diatoms is definitely a new field of research, which does not preclude investigating before the innocuity and organoleptic qualities of any new coloured oysters, as well as their acceptability by consumers, two of the major conditions retained for the viability of probiotics or probiotic-sources in aquaculture (Verschuere et al., 2000).

In conclusion, the biological activities of the pigment produced by the newly-discovered *H. karadagensis* reinforce the interest on the family of diatom blue pigments as a source of natural bio-active compounds. Potential applications could concern aquaculture, by exploiting *H. ostrearia*-like specie as prophylactic diet for bivalves. In particular, a next challenge will be to demonstrate that marennine-like pigments produced by *H. ostrearia*-like diatoms can be used as natural prophylactic compounds for bivalves, in particular for juvenile or adult oysters, the requirement of new probiotics in aquaculture being especially topical (Kesarcodei-Watson et al., 2008).

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VI

Conclusion générale

Longtemps considérée comme l'unique diatomée capable de synthétiser un pigment bleu, *H. ostrearia* s'est vue adjoindre plusieurs espèces nouvellement décrites partageant cette aptitude. Ainsi, ce sont deux espèces, *H. karadagensis* et *H. silbo*, qui ont déjà été décrites. Deux autres espèces, nommées provisoirement *H. provincialis* et *H. acoran*, restent encore à caractériser complètement. La maîtrise du déclenchement de la reproduction sexuée d'*H. ostrearia*, grâce au protocole perfectionné décrit dans le deuxième chapitre de cette thèse, prenant notamment en compte des effets de la concentration cellulaire, de la qualité lumineuse et du pré-conditionnement lumineux des cultures, se révéla primordiale pour l'étude de ces nouvelles espèces et la caractérisation de leur cycle de vie.

En plus des méthodes morphométriques et reproductives, la physico-chimie fut d'une grande aide dans la caractérisation des pigments bleus particuliers à ce consortium d'espèces. La spectrophotométrie UV-visible, et plus encore la spectrométrie Raman, ont permis de mettre en évidence l'existence chez *H. karadagensis* d'un pigment bleu-gris distinct de la marennine. Des expériences complémentaires, basées notamment sur la résonance magnétique nucléaire, sont en cours ou devront être menées, afin d'élucider la structure de ces molécules.

Les liens phylogénétiques entre ces espèces, ainsi que d'autres appartenant au même genre, ont été définis sur la base des résultats obtenus à partir de trois marqueurs moléculaires. Ces résultats laissent supposer une grande parenté génétique chez les diatomées bleues du genre *Haslea*, et l'existence de voies métaboliques communes conduisant à la synthèse de ces pigments bleus.

Les mêmes méthodes moléculaires, couplées au protocole d'induction de l'auxosporulation préalablement mis au point, auront permis d'apporter les premières preuves expérimentales de l'héritabilité de l'ADN mitochondrial chez l'espèce modèle *H. ostrearia*, dont la transmission semble uniparentale.

Enfin, plusieurs activités biologiques de la marennine et du pigment d'*H. karadagensis* ont été mises en évidence, au nombre desquelles des activités bactéricides, fongicides et viricides. Les éventuelles voies de valorisation, en aquaculture comme dans le domaine agro-alimentaire, ont été abordées.

VII

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Annexes

Publications en tant que premier auteur

Gastineau R., Davidovich N. A., Bardeau J. F., Davidovich O I, Rincé Y., Gaudin P., Cox E. J., Mouget J. L. *Haslea karadagensis* sp. nov. (Bacillariophyta), a new diatom from the Black Sea producing a novel blue pigment (soumis à la revue European Journal of Phycology)

Gastineau R., Jacquette B., Bardeau J. F., Hansen G., Caruso A., Leignel V., Morançais M., Fleurence J., Gaudin P., Cox E., Davidovich N. A., Mouget J. L. *Haslea silbo* sp. nov. (Bacillariophyta), a new diatom from the Canary Islands producing a marennine-like pigment evidenced by spectrometric methods. (Soumis à la revue Journal of Phycology)

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 produced by the diatom *Haslea ostrearia* and responsible for the greening of oysters. (Soumis à la revue Journal of Agriculture and Food Chemistry).

Gastineau R., Hardivillier H., Leignel V., Morançais M., Fleurence J., Hellio C., Bourgougnon N., Davidovich N. A., Tekaya N., Gaudin P., Mouget J. L. Darkening oysters' gills with the new diatom *Haslea karadagensis* and its biologically active pigment, with probable prophylactic properties. (Terminé, sous réserve d'être soumis à la revue Aquaculture).

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Prix scientifiques obtenus

1^{er} prix du poster étudiant, 28^{ème} colloque de l'Association des Diatomistes de Langue Française.

1^{er} prix du projet innovant, 1ères doctoriales du PRES UNAM.

Communications

Gastineau R., Davidovich N. A., Gaudin P. & Mouget J.-L.

Déterminisme de la reproduction sexuée chez la diatomée *Haslea ostrearia*

Septembre 2008

27^{ème} colloque de l'Association des Diatomistes de Langue Française

Dijon

Gastineau R., Davidovich N. A., Bardeau J.-F., Gaudin P., Davidovich O., Gryniou V.
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Éléments de comparaison entre la diatomée verdissante *Haslea ostrearia* et une diatomée pennée aux apex pigmentés, *Haslea ostrearia conformis*, isolée en Mer Noire.

Décembre 2008

Colloque Algimac 2008, Société Phycologique de France

Concarneau

Gastineau R., Davidovich N. A., Gaudin P. & Mouget J.-L.

Sexual reproduction in *Haslea ostrearia*

Janvier 2009

British Phycological Society 57th winter meeting

Londres

Gastineau R., Bardeau J.-F., Rincé Y, Fleurence J., Morançais M., Gaudin P.,
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Nouveaux éléments dans l'étude des diatomées possédant un pigment bleu : avancées dans l'identification d'une nouvelle espèce pigmentée du genre *Haslea*

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Gastineau R., Davidovich N., Gaudin P., Morançais M., Fleurence J., Rincé Y., Hellio C., Bardeau J.F., Mouget J.L.

Advances in the knowledge of diatoms with blue apices

Janvier 2010

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Oban

Gastineau R., Bardeau J.F., Gaudin P., Morançais M., Fleurence J., Davidovich N., Mouget J.L.

Can Raman spectrometry discriminate between 'blue' diatoms?

Janvier 2010

British Phycological Society 58th winter meeting

Oban

Gastineau R., Jacquette B., Caruso A., Leignel V., Hardivillier Y., Bardeau J. F., Gaudin P., Rincé Y., Bounous L., Hansen G., Wulff A., Davidovich N. A., Mouget J. L.

Biodiversité des « diatomées bleues » : approches morphologiques, biochimiques et moléculaires

Septembre 2010

30ème Colloque de l'Association des Diatomistes de Langue Française (ADLaF)

Québec

Gastineau R., Jacquette B., Leignel V., Caruso A., Hardivillier Y., Gaudin P., Rincé Y., Tardy-Laporte C., Bounous L., Hansen G., Wulff A., Davidovich N., Cox E., Mouget J. L.

A first molecular approach to the phylogeny of blue diatoms from the genus *Haslea*

Janvier 2011

British Phycological Society 59th winter meeting

Cardiff

Gastineau R., Jacquette B., Bardeau J. F., Hansen G., Caruso A., Leignel V., Morançais M., Fleurence J., Gaudin P., Cox E., Davidovich N. A., Mouget J. L.

Haslea silbo sp. nov., une nouvelle diatomée produisant un pigment bleu de type marennine, provenant des îles Canaries

30ème Colloque de l'Association des Diatomistes de Langue Française (ADLaF)

6 - 8 septembre 2011

Boulogne-sur-Mer

Gastineau R., Pouvreau J. B., Hardivillier Y., Leignel V., Morançais M., Fleurence J., Hellio C., Bourgougnon N., Davidovich N. A., Tekaya N., Gaudin P., Mouget J. L.

Activités biologiques des pigments bleus produits par les diatomées *Haslea ostrearia* et *H. karadagensis*

30ème Colloque de l'Association des Diatomistes de Langue Française (ADLaF)

6 - 8 septembre 2011

Boulogne-sur-Mer

Gastineau R., Jacquette B., Bendahmane D., Leignel V., Wulff A., Gaudin P., Mouget J. L.

Etude de l'héritabilité du gène mitochondrial *cox1* lors de l'auxosporulation chez la diatomée *Haslea ostrearia*

30ème Colloque de l'Association des Diatomistes de Langue Française (ADLaF)

6 - 8 septembre 2011

Boulogne-sur-Mer

France

Gastineau R., Jacquette B., Caruso A., Leignel V., Hardivillier Y., Bardeau J. F., Gaudin P., Hansen G., Wulff A., Davidovich N. A., Mouget J. L.

Phylogénie moléculaire du genre *Haslea* et de ses diatomées présentant un pigment bleu complétée par l'étude des marqueurs génomiques, chloroplastiques et mitochondriaux.

30ème Colloque de l'Association des Diatomistes de Langue Française (ADLaF)

6 - 8 septembre 2011

Boulogne-sur-Mer

Stages scientifiques effectués

Ukraine – Station biologique de la réserve de Karadag, Crimée

Mai 2008

Isolement de souches inconnues de diatomées

Royaume-Uni – Université de Portsmouth, laboratoire de biologie

Août 2009

Evaluations et comparaisons des activités bactéricides et anti-fouling des pigments marennine et marennine like

France – Laboratoire départemental de la Sarthe, Le Mans

Juin 2010

Evaluations des activités bactéricides de la marennine sur des pathogènes terrestres