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Justine Castrec

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Par

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Impacts des efflorescences du dinoflagellé toxique *Alexandrium minutum* sur la reproduction et le développement de l'huître *Crassostrea gigas*

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Chapitre 1

Introduction

Rappels bibliographiques

1. Les efflorescences de dinoflagellés toxiques

Le terme micro-algue désigne des organismes unicellulaires, microscopiques, photosynthétiques et vivant en suspension dans la colonne d'eau. Les efflorescences de micro-algues sont des phénomènes de développement rapide et soudain d'une ou de plusieurs espèces phytoplanctoniques qui peuvent alors atteindre des valeurs de concentration extrêmement fortes. Le phytoplancton est à la base de la chaîne trophique au sein des océans. Néanmoins, certaines efflorescences phytoplanctoniques sont nuisibles pour les organismes marins. Ces phénomènes sont appelées « Harmful algal blooms » ou HAB en anglais. Les efflorescences algales massives causent des phénomènes d'eaux colorées aussi nommées « marées rouges » (Figure 1) (Anderson et al., 2012b). Certaines efflorescences représentent une forte biomasse qui entraîne l'hypoxie, voir l'anoxie du milieu, et privent le fond et la colonne d'eau de lumière (Sellner et al., 2003). Certaines espèces de micro-algues produisent des toxines appelées phycotoxines (Hallegraeff et al., 2003).



Figure 1. Efflorescence majeure d'*Alexandrium monilatum* dans la baie de Chesapeake (Etats-Unis) en août 2015.

Ces efflorescences nuisibles de micro-algues sont de plus en plus fréquentes et intenses et leur étendue géographique ne cesse d'augmenter (Anderson et al., 2012b). Les facteurs avancés pour expliquer ce phénomène sont principalement l'eutrophisation des eaux continentales,

l'augmentation des échanges commerciaux qui favorisent la dispersion des espèces marines et les changements climatiques globaux (Hallegraeff et al., 2003; Lassus et al., 2016).

Les efflorescences de micro-algues productrices de toxines sont à l'origine d'importants problèmes pour les écosystèmes aquatiques, l'aquaculture, la pêche, le tourisme et la santé humaine (Gobler et al., 2017). Les poissons et les bivalves, par leur activité de filtration, accumulent les toxines produites par ces micro-algues et deviennent alors impropres à la consommation humaine. Ces efflorescences affectent notamment des bivalves d'intérêt commercial et causent alors la fermeture de la pêche ou des interdictions de vente entraînant des pertes économiques (Sellner et al., 2003). Les composés toxiques produits par ces micro-algues affectent aussi la physiologie des organismes marins, et peuvent provoquer des mortalités de poissons, de coquillages, de mammifères et d'oiseaux marins (Landsberg, 2002). Ces toxines sont bioaccumulées et leurs effets néfastes peuvent se répercuter sur l'ensemble de la chaîne trophique. Les conséquences néfastes de ces phénomènes vont probablement s'intensifier par l'accroissement de l'utilisation des eaux côtières pour l'aquaculture et la conchyliculture dans le monde (Lassus et al., 2016).

1.1. Le genre *Alexandrium*

Les micro-algues appartenant à la classe des *Dynophyceae*, aussi appelées dinoflagellés, représentent 75 à 80 % des espèces toxiques (Hallegraeff et al., 2003; Smayda, 1997). Parmi les dinoflagellés responsables des efflorescences toxiques, le genre *Alexandrium* (Halim 1960) est l'un des plus importants en termes de distribution, d'intensité et de diversité à l'échelle mondiale (Anderson et al., 2012a).

Les dinoflagellés du genre *Alexandrium* appartiennent à l'ordre des *Gonyaulacales*, à la famille des *Ostreopsidaceae*, et comprennent une trentaine d'espèces décrites (Lassus et al., 2016). Ce sont des eucaryotes unicellulaires biflagellés, majoritairement autotrophes. Des cas de mixotrophie ont cependant été décrits (Gribble et al., 2005; Jacobson and Anderson, 1986; Jeong et al., 2010). Ces micro-algues sont protégées par une thèque composée de plaques cellulose rigides. Le cycle de vie des dinoflagellés comporte un stade végétatif haploïde, où la cellule peut se diviser et être mobile dans la colonne d'eau grâce à des flagelles et un stade diploïde appelé planozygote associé à la reproduction sexuée (Figure 2). Dans certains cas, le planozygote peut directement entrer en méiose et redonner des cellules végétatives, sans passer par un enkystement (Figueroa et al., 2015).

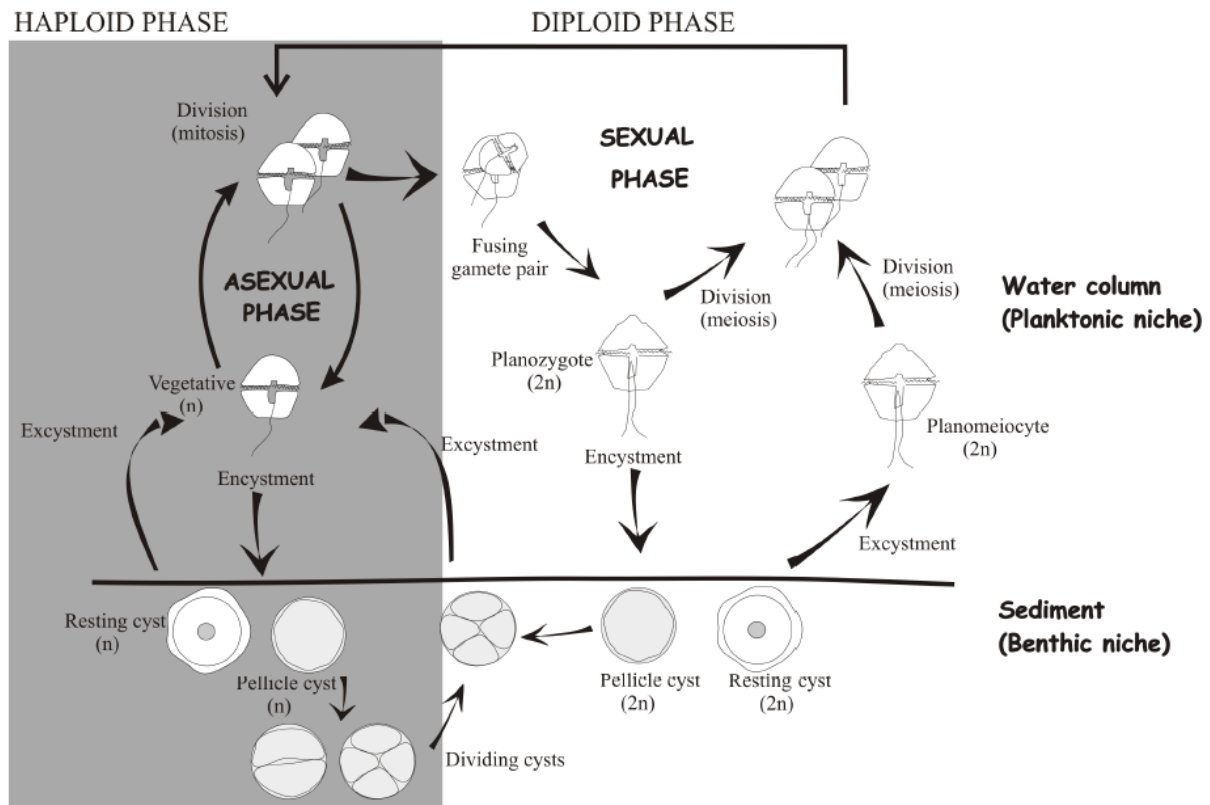


Figure 2. Schéma représentant les processus pouvant se produire lors du cycle de vie des dinoflagellés dont *A. minutum*, d'après Bravo et al. (2014).

Les dinoflagellés du genre *Alexandrium* ont la capacité de former deux types de kystes : les kystes de résistance et les kystes temporaires. Ce sont des cellules qui ont une morphologie différente des cellules végétatives avec des parois particulières, elles ne sont pas mobiles et sédimentent. Les kystes de résistance sont issus de la reproduction sexuée après fusion des gamètes, en réponse à des facteurs endogènes et exogènes. La formation de ces kystes, notamment observée lors de la terminaison de l'efflorescence, permet aux cellules de résister aux conditions hivernales (phase de dormance) (Brosnahan et al., 2017). La durée de maturation des kystes varie entre 1 mois et 12 mois en fonction des espèces et de l'écosystème (Anderson et al., 2012a). Lorsque la maturation des kystes est terminée, ceux-ci peuvent germer si les conditions environnementales sont favorables (Ní Rathaille and Raine, 2011). Les kystes présents dans le sédiment forment des « banques » de kystes, qui favorisent la réapparition des efflorescences l'année suivante (Cosgrove et al., 2014). Les kystes temporaires sont formés très rapidement en réponse à un stress environnemental comme la turbulence, la présence de parasites, les variations de température, ou le passage par le tractus digestif des organismes brouteurs (Anderson et al., 2012a). Les kystes qui se forment après ingestion par les bivalves peuvent résister à la digestion et sont retrouvés viables dans les fèces (Hégaret et al., 2008;

Laabir et al., 2007). Contrairement aux kystes de résistance, ces kystes temporaires n'ont pas de phase de maturation obligatoire et peuvent se retransformer en cellules végétatives dès lors que les conditions sont favorables.

Les espèces du genre *Alexandrium* sont présentes dans les régions tropicales, tempérées et subantarctiques (Tillmann et al., 2016). Le réchauffement continu des océans a facilité l'expansion de certaines espèces toxiques, comme *Alexandrium fundyense*, et pourrait favoriser l'intensification et l'expansion des efflorescences de cette espèce et probablement d'autres espèces toxiques dans le monde (Gobler et al., 2017). Les côtes françaises sont régulièrement touchées par des efflorescences toxiques de dinoflagellés *Alexandrium* spp (Lassus et al., 2004). Les efflorescences d'*Alexandrium* sont susceptibles d'avoir des impacts sanitaires, économiques et écologiques importants. En effet, elles peuvent provoquer des intoxications alimentaires humaines via la consommation de coquillages contaminés, des fermetures de la pêche et des interdictions de vente de poissons et de coquillages mais aussi induire la mort des organismes et des modifications de la structure trophique des écosystèmes marins (Anderson et al., 2012a; Bricelj et al., 2005; Bricelj and Shumway, 1998).

Les espèces d'*Alexandrium* sont capables de produire des toxines paralysantes regroupées sous le terme de « Paralytic Shellfish Toxins » (PST) (Tableau 1), qui ont une toxicité particulièrement aigue (Kodama, 2010). Les PST sont aussi produites par les dinoflagellés *Gymnodinium catenatum* et *Pyrodinium bahamense*, ainsi que plusieurs genres de cyanobactéries (Wiese et al., 2010). Généralement, un organisme producteur de PST synthétise un panel de toxines composé de plusieurs analogues PST (Wiese et al., 2010) et la production d'un certain profil toxinique est vraisemblablement fixée de manière génétique pour chaque souche d'*Alexandrium*. Les profils de PST produites peuvent être très variables selon les souches d'une même espèce (Anderson et al., 2012a). De plus, certaines souches sont incapables de produire des PST (John et al., 2014).

Les PST sont des alcaloïdes hydrosolubles composés de saxitoxine (STX) et de dérivés de la saxitoxine : les carbamates (Néo-STX, GTX1-GTX4), les N-sulfocarbamoyles (B1, B2, C1-C4) et les dérivés décarbomoyl (dcSTX, dcGTX1-dcGTX4). Les PST se fixent aux canaux sodium voltage-dépendant et inhibent l'augmentation voltage-dépendante de la conductance des ions sodium qui génère le potentiel d'action des fibres nerveuses et musculaires (Bricelj et al., 2005). Cette fixation entraîne un blocage de la conduction nerveuse provoquant une paralysie neuromusculaire. Depuis les années 1970, la répartition géographique des zones touchées par les PST s'est globalement étendue. Actuellement, les efflorescences de micro-

algues productrices de PST ont été rapportées en Europe, en Amérique du Nord, au Japon mais aussi en Amérique du Sud, en Australie, en Asie du Sud-Est et en Afrique du Sud (Van Dolah, 2000) (Figure 3).

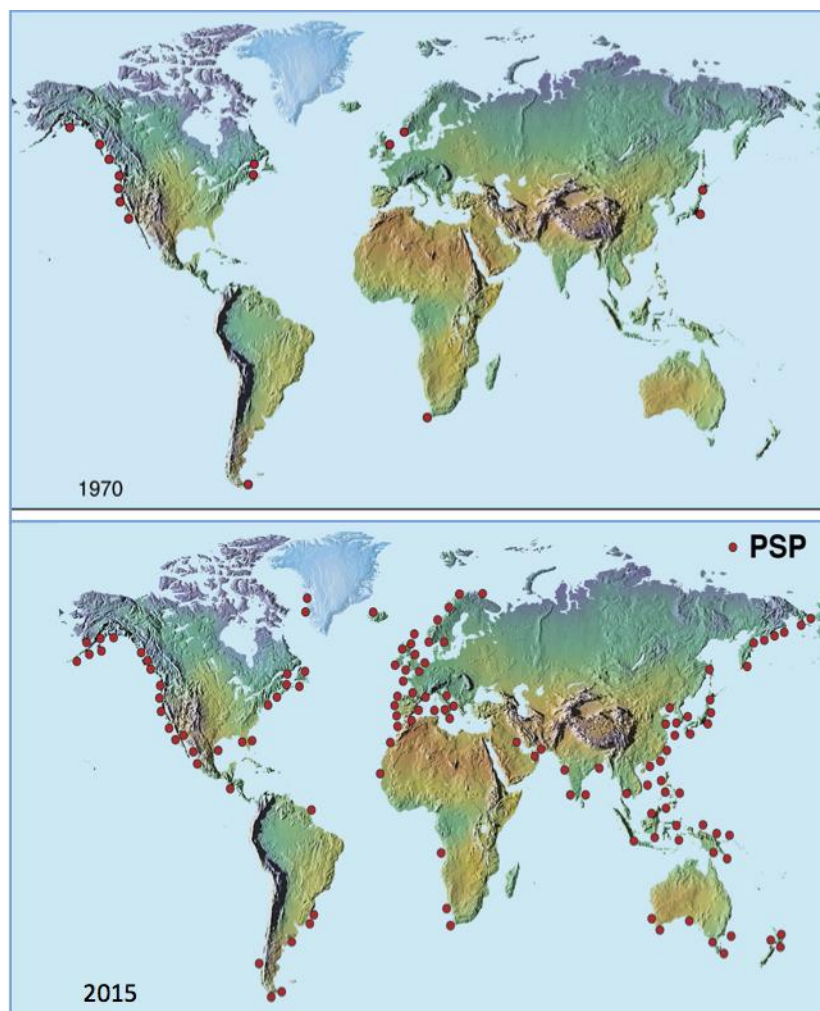


Figure 3. Distribution géographique des zones où la présence de PST avait été relevée en 1970 et 2015. Source : U. S. National Office for Harmful Algal Blooms, Woods Hole Oceanographic Institution.

Les organismes filtreurs, comme les mollusques bivalves, peuvent accumuler une grande quantité de PST dans leurs tissus, principalement au niveau de la glande digestive. C'est via la consommation de coquillages et de poissons contaminés que les efflorescences toxiques à PST nuisent à la santé humaine, entraînant des pathologies regroupées sous le terme « Paralytic Shellfish Poisoning » ou PSP. Les symptômes associés sont variables : perte de coordination motrice, paresthésie du visage, des bras puis des jambes dans la demi-heure suivant l'ingestion, et paralysies pouvant conduire à la mort (Manfrin et al., 2012). Dans l'Union Européenne, la charge en PST est soumise à une réglementation sanitaire interdisant la vente des coquillages présentant plus de 80 µg équivalent STX/100 g de chair (Lassus et al., 2004).

Les PST ne sont pas les seules molécules toxiques qui contribuent à la toxicité des efflorescences d'*Alexandrium*. Quelques espèces, comme *Alexandrium ostenfeldii*, peuvent produire également des molécules lipophiles du groupe des amines cycliques appelées spirolides (SPX) (Cembella et al., 2000). Ces spirolides sont composés de plusieurs analogues dont les spirolides 13, 13-desmethyl-C, 13-desmethyl-D et 19-didesmethyl-C qui sont accumulés par les bivalves. Aucun cas d'intoxication humaine liée à la consommation de coquillages contaminés par les spirolides n'a été reporté et les études toxicologiques menées sur ces toxines n'ont à ce jour pas justifié une réglementation de leur concentration dans la chair des bivalves (Anderson et al., 2012a). Néanmoins, les spirolides sont surveillés car elles entraînent la mort rapide de souris injectées par voie intrapéritonéale (Otero Paz et al., 2009). Le mode d'action des spirolides est encore inconnu, mais des expériences indiquent que les récepteurs muscariniques et nicotiniques pourraient être les cibles de ces toxines neurotoxiques (Gill et al., 2003; Munday et al., 2012). Les souches d'*A. ostenfeldii* produisent soit des PST, soit des spirolides et rarement les deux en même temps (Anderson et al., 2012a). Deux autres espèces, *Alexandrium monilatum* et *Alexandrium hiranoi*, produisent des goniodomines dont la goniodmine A qui cause la paralysie et la mort de poissons, mais qui ne semblent pas être toxiques pour l'Homme (Hsia et al., 2006).

En plus de ces toxines produites intracellulairement et contenues très majoritairement dans les cellules algales mais également en partie excrétées (Lefebvre et al., 2008; Persson et al., 2012), différentes espèces d'*Alexandrium* peuvent aussi produire des composés extracellulaires bioactifs (Bioactive Extracellular Compounds ou BEC) de nature inconnue qui présentent des propriétés ichtyotoxiques (Mardones et al., 2015; Ogata and Kodama, 1986), cytotoxiques (Borcier et al., 2017; Lush and Hallegraeff, 1996), hémolytiques (Eschbach et al., 2001; Yang et al., 2010) ou allélopathiques (Lelong et al., 2011; Long et al., 2018a; Tillmann et al., 2008; Zheng et al., 2016). A l'heure actuelle, la nature chimique de ces composés est inconnue et leurs modes d'action restent très mal caractérisés (Lelong et al., 2011; Long et al., 2018a), car ils sont notamment difficiles à isoler et peuvent être labiles. Ces composés affectent un grand nombre de protistes marins, autotrophes et hétérotrophes (Cho and Matsuoka, 2000; Hansen et al., 1992; Kim et al., 2016; Matsuoka et al., 2000; Tillmann and John, 2002), des crustacés, artémies et copépodes (Bagoien et al., 1996; Huntley et al., 1986; Lush and Hallegraeff, 1996) mais aussi des poissons (Aldrich et al., 1967; Mardones et al., 2015). Ils peuvent aussi être hémolytiques pour des globules rouges de mammifères (Chen et al., 2015; Emura et al., 2004; Simonsen et al., 1995). Une exotoxine cytotoxique et thermolabile (Lush et al., 2001), une

exotoxine protéolytique présentant une activité hémolytique (Emura et al., 2004) et un polysaccharide cytotoxique (Yamasaki et al., 2008) ont été récemment isolés à partir de cultures d'*A. minutum*, d'*Alexandrium taylori* et d'*Alexandrium tamarense*. Les principales espèces d'*Alexandrium* citées dans cette thèse sont présentées dans le Tableau 1, qui récapitule leur distribution géographique ainsi que leur toxicité en termes de production de toxines connues (PST et SPX) et de molécules bioactives exytracellulaires inconnues.

1.2. *Alexandrium minutum*

Le dinoflagellé *A. minutum* se trouve dans les ports abrités, les lagunes, les estuaires ou les baies, où la stratification est fréquente (Lewis et al., 2018). Cette micro-algue, mesurant entre 15 et 30 μm (Figure 4), est assez tolérante aux variations de température et de salinité (Anderson et al., 2012a; Touzet et al., 2007).

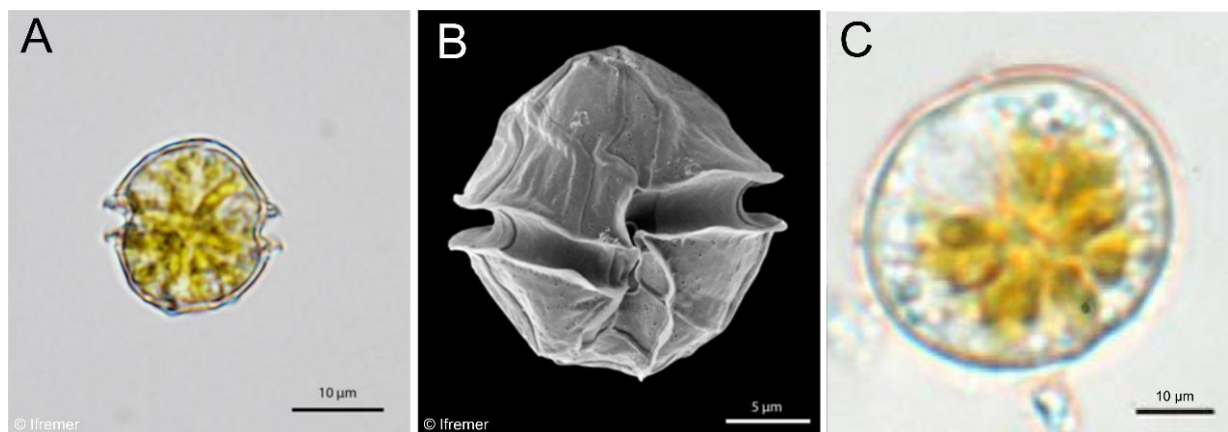


Figure 4. Cellules végétatives d'*Alexandrium minutum* observées par microscopie optique (A) et par microscopie électronique à balayage (B) et kyste temporaire observé par microscopie optique (C).

Comme d'autres espèces d'*Alexandrium*, cette espèce est capable de produire des PST (Haberkmorn et al., 2010a; Pousse et al., 2018a) et des BEC qui sont nocifs pour d'autres espèces de phytoplancton (Arzul et al., 1999; Fistarol et al., 2004; Lelong et al., 2011; Long et al., 2018a, 2018b), des crustacés (Bagoien et al., 1996; Lush and Hallegraeff, 1996) et des mollusques comme la coquille Saint Jacques *Pecten maximus* (Borcier et al., 2017). La première détection et description d'*A. minutum* (Halim, 1960) a eu lieu dans le port d'Alexandrie en Egypte, d'où l'origine du nom de genre. Depuis, la présence de cette espèce a été reportée dans de nombreuses zones dans le monde (Figure 5): Australie, Irlande, France, Espagne, Taiwan, Nouvelle-Zélande, Suède, Inde et Malaisie (Lewis et al., 2018).

Tableau 1. Principales espèces de dinoflagellés du genre *Alexandrium* citées dans cette thèse : distribution, production de toxines connues, et activités toxiques mises en évidence et reliées à la production de composés extracellulaires bioactifs (BEC) non caractérisés.

Espèce	Distribution géographique	Toxines connues	Activité toxique liée à la production de BEC	Références bibliographiques
<i>A. minutum</i>	Europe, Amérique du Nord et du Sud, Afrique, Asie, Asie du Sud-est, Océanie	PST	Allélopathique, hémolytique	Arzul et al. (1999), Borcier et al. (2017), Fistarol et al. (2004), Lelong et al. (2011), Tillmann et al. (2008), Tillmann and John (2002), Yang et al. (2010)
<i>A. catenella</i>	Europe, Amérique du Nord et du Sud, Afrique, Asie, Océanie	PST	Allélopathique, hémolytique, ichtyotoxique	Arzul et al. (1999), Dorantes-Aranda et al. (2015), Mardones et al. (2015), Ogata and Kodama (1986), Tillmann et al. (2008)
<i>A. fundyense</i>	Amérique du Nord, Afrique, Océanie	PST	Allélopathique, hémolytique	Eschbach et al. (2001), Hattenrath-Lehmann and Gobler (2011), Lyczkowski and Karp-Boss (2014)
<i>A. tamarense</i>	Europe, Amérique du Nord et du Sud, Afrique, Asie, Asie du Sud-Est, Océanie, îles Antarctiques et Subantarctiques	PST	Allélopathique, hémolytique, ichtyotoxique	Arzul et al. (1999), Chen et al. (2015), Eschbach et al. (2001), Ogata and Kodama (1986), Simonsen et al. (1995), Tillmann et al. (2008), Yamasaki et al. (2008), Yang et al. (2010), Zheng et al. (2016)
<i>A. affine</i>	Europe, Amérique du Nord, Asie, Asie du Sud-Est, Océanie	PST	Allélopathique	Nguyen-Ngoc (2004), Tillmann and John (2002)
<i>A. ostenfeldii</i>	Europe, Amérique du Nord et du Sud, Afrique, Asie, Océanie	PST, Spirolides (SPX)	Allélopathique, hémolytique	(Cembella et al., 2000; Eschbach et al., 2001; Hakanen et al., 2012, 2014; Hansen et al., 1992; Tillmann et al., 2007, 2008; Tillmann and John, 2002)
<i>A. taylori</i>	Europe (France, Grèce), Espagne, Asie (Japon)	PST	Allélopathique, hémolytique (exotoxines non-protéiques)	Emura et al. (2004), Tillmann et al. (2008)
<i>A. monilatum</i>	Europe (Mer Noire), Amérique du Nord (Mexique, Floride), Amérique Centrale (Costa-Rica)	Goniodomine A	Hémolytique, ichtyotoxique	Aldrich et al. (1967), Anderson et al. (2012a), Hsia et al. (2006)

BEC : composés extracellulaires bioactifs (Bioactive Extracellular Compounds) ; PST : toxines paramysantes (Paralytic Shellfish Toxins).

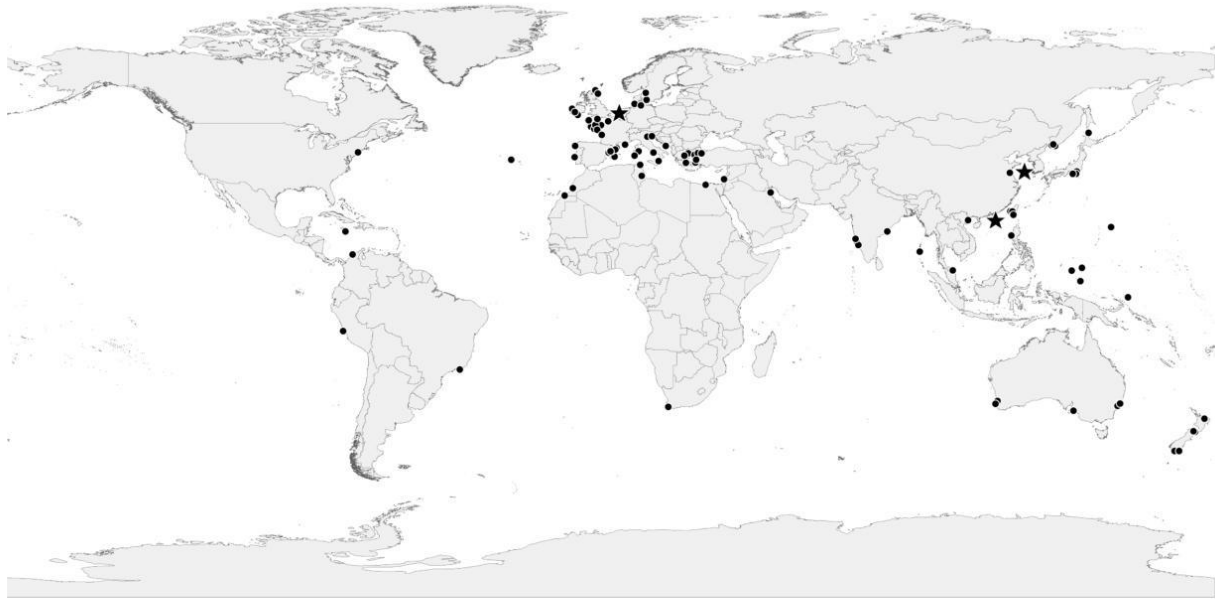


Figure 5. Distribution des zones géographiques où la présence d'*A. minutum* a été confirmée (points noirs) ou rapportée dans la littérature mais de manière incertaine (étoile noire), d'après Lewis et al. (2018).

Des traces génétiques (ADN ancien) d'*A. minutum* ont récemment été retrouvées dans des sédiments datant de la fin du 19^{ème} siècle dans la rade de Brest (Klouch et al., 2016). Ces résultats suggèrent que cette espèce serait présente en rade de Brest depuis plus de 150 ans, et aurait récemment proliféré soudainement du fait de l'action de l'homme et/ou du changement de l'écosystème (Klouch et al., 2016).

En France, les efflorescences d'*Alexandrium minutum* ont été rapportées pour la première fois dans les années 1980 en Bretagne nord, dans l'Aber-Wrac'h et l'Aber-Benoît (Bec et al., 2016). Dans la rade de Brest (Bretagne, France), *A. minutum* a été détecté depuis 1990 par le réseau de monitoring REPHY (Réseau de surveillance et d'observation du Phytoplancton et des Phycotoxines, http://envlit.ifremer.fr/surveillance/phytoplancton_phycotoxines/presentation). Dans les années 1990, son abondance était plutôt faible ($< 2 \times 10^3$ cellules L⁻¹) mais elle a très fortement augmenté lors des efflorescences observées ces dernières années. Une efflorescence remarquable d'*A. minutum* s'est produite dans la rade de Brest en juillet 2012, avec une concentration de plus de 40 millions de cellules L⁻¹ (Chapelle et al., 2015). Cette efflorescence remarquable a conduit à l'accumulation de PST par les coquillages, causant une fermeture de vente et des pertes économiques. Depuis cette date, les efflorescences d'*A. minutum* sont quasi-récurrentes tous les printemps/été en rade de Brest.

La phénologie et les caractéristiques des efflorescences d'*A. minutum* observées sur les côtes françaises, dans la manche et l'Atlantique Nord-Est, ont récemment été étudiées (Guallar et al.,

2017). Les efflorescences sont toujours annuelles (1 fois par an) et peuvent atteindre des concentrations supérieures à un million de cellules L⁻¹. La période des efflorescences s'étend d'avril à octobre et la durée des efflorescences varie entre 2 semaines et 6 mois. Les principaux facteurs environnementaux qui influent sur la magnitude, le taux de croissance et la durée des efflorescences sont la température de l'eau de mer au niveau régional et le débit des rivières à un niveau plus local. En Irlande et en France, les efflorescences se développent lorsque la température de l'eau de mer dépasse 15°C. Le développement de l'efflorescence est influencé par les courants hydrodynamiques locaux créés par le vent, la marée et le débit des rivières (Anderson et al., 2012a; Laanaia et al., 2013; Sourisseau et al., 2017). Ces facteurs peuvent moduler les concentrations algales par dilution. La terminaison des efflorescences est le résultat de différents processus incluant la limitation en nutriments (Sourisseau et al., 2017), la prédation (Calbet et al., 2003) et le parasitisme (Chambouvet et al., 2008).

2. L'huître creuse *Crassostrea gigas*

2.1. Biologie de l'espèce

L'huître creuse *Crassostrea gigas* (Thunberg, 1793), aussi appelé huître du Pacifique, occupe la position systématique suivante : Phylum : *Mollusca*, Classe : *Bivalvia*, Ordre : *Ostreoida*, Famille : *Ostreidae*, Genre : *Crassostrea*, Espèce : *gigas*. Le genre *Crassostrea* regroupe une vingtaine d'espèces, dont l'huître creuse portugaise *Crassostrea angulata* (Lamarck, 1819) et l'huître creuse américaine *Crassostrea virginica* (Gmelin, 1791). Récemment, Salvi and Mariottini (2017) ont proposé de classer *C. gigas* dans le genre *Magallana*. A l'heure actuelle, cette nouvelle classification ne fait pas consensus au sein de la communauté scientifique (Bayne et al., 2017). Dans ce manuscrit, j'ai donc fait le choix de conserver le nom *C. gigas*.

L'huître est un organisme sessile et ectotherme, présent dans les écosystèmes marins et estuariens, donc soumis à d'importantes variations des conditions du milieu. Ce bivalve est suspensivore et filtre l'eau de mer pour récupérer les particules nutritives mais aussi le dioxygène nécessaire à sa respiration. L'huître se nourrit essentiellement de phytoplancton, mais le zooplancton, le microphytobenthos, les bactéries, le nanoplancton et les particules détritiques font aussi partie de son alimentation (Bayne, 2017a).

Native du Nord-Est de l'Océan Pacifique et de la mer du Japon, l'huître *C. gigas* est un des invertébrés marins les plus cosmopolites. L'huître creuse a été introduite avec succès dans diverses régions du monde à des fins d'aquaculture pour remplacer les stocks d'huîtres

indigènes sévèrement atteints par les épizooties, la surexploitation, ou pour créer une nouvelle industrie. Au début des années 1970, des quantités importantes de juvéniles et d'adultes ont été importés du Japon et du Canada vers la France pour relancer l'aquaculture, après des mortalités massives de *Crassostrea angulata* dues à une épizootie (Buestel et al., 2009).

Les huîtres du Pacifique dominent la production de bivalves en aquaculture dans de nombreuses régions du monde. Sa production au niveau mondial est d'environ 626 000 tonnes en 2014 (FAO, 2018a). En France, 64 200 tonnes ont été produites en 2016, représentant une valeur de 360 400 USD (FAO, 2018b). Des populations d'huîtres sauvages s'établissent de manière croissante, ce qui pourrait causer le déplacement d'espèces natives et modifier les écosystèmes existants (Herbert et al., 2016).

2.2. Anatomie générale

Les huîtres creuses sont des organismes à corps mou protégé par une coquille formée par deux valves asymétriques articulées autour d'une charnière. Un ligament élastique permet de réunir les deux valves et un puissant muscle adducteur permet la fermeture des valves. Le muscle adducteur est composé d'un muscle strié blanc et opaque assurant la fermeture prolongée, et d'un muscle lisse qui intervient dans les fermetures rapides et les micro-fermetures (Figure 6).

Le manteau enveloppant les organes de l'huître est formé de deux lobes soudés dorsalement près de la bouche, délimitant ainsi la cavité palléale. Le manteau intervient dans la formation de la coquille et du ligament, joue un rôle dans la perception sensorielle et tactile grâce à de nombreuses terminaisons nerveuses, et permet aussi le stockage de réserves principalement sous forme de glycogène (Berthelin et al., 2000).

Les branchies ont une double fonction respiratoire et alimentaire en assurant une circulation d'eau de mer par le mouvement de cils branchiaux. Les branchies sont composées de deux lames ou lamelles, elles-mêmes formées de deux feuillettes. Un courant inhalant d'eau de mer est généré par les cils branchiaux latéraux. Les particules en suspension dans l'eau de mer sont retenues à la surface des branchies, collées à un mucus et conduites vers la bouche par l'action des cils latéro-frontaux puis des cils frontaux.

L'appareil digestif de l'huître comprend une bouche entourée de deux paires de palpes labiaux qui trient et conduisent les particules nutritives vers la bouche. Les particules non-ingérées sont rejetées dans la chambre inhalante sous forme d'agrégats enrobés de mucus appelés pseudo-fèces. La production de pseudo-fèces est donc un mécanisme de tri pré-ingestif, il est aussi considéré comme un mécanisme de régulation secondaire de l'ingestion chez les bivalves

marins (Navarro and Widdows, 1997; Shumway and Cucci, 1987; Wildish et al., 1998). Les particules ingérées passent par un court œsophage, menant à l'estomac entouré des diverticules digestifs. Dans l'estomac, la digestion mécanique et enzymatique des aliments fait intervenir le stylet cristallin, structure longiligne d'un à deux centimètres. Il se dissout pour libérer les enzymes digestives au moment de l'arrivée du bol alimentaire et se reconstitue entre les épisodes digestifs (Galtsoff, 1964). L'estomac s'ouvre sur l'intestin et se termine par un anus débouchant juste au-dessus du muscle adducteur. Les fèces produites par la digestion sont expulsés dans le courant exhalant.

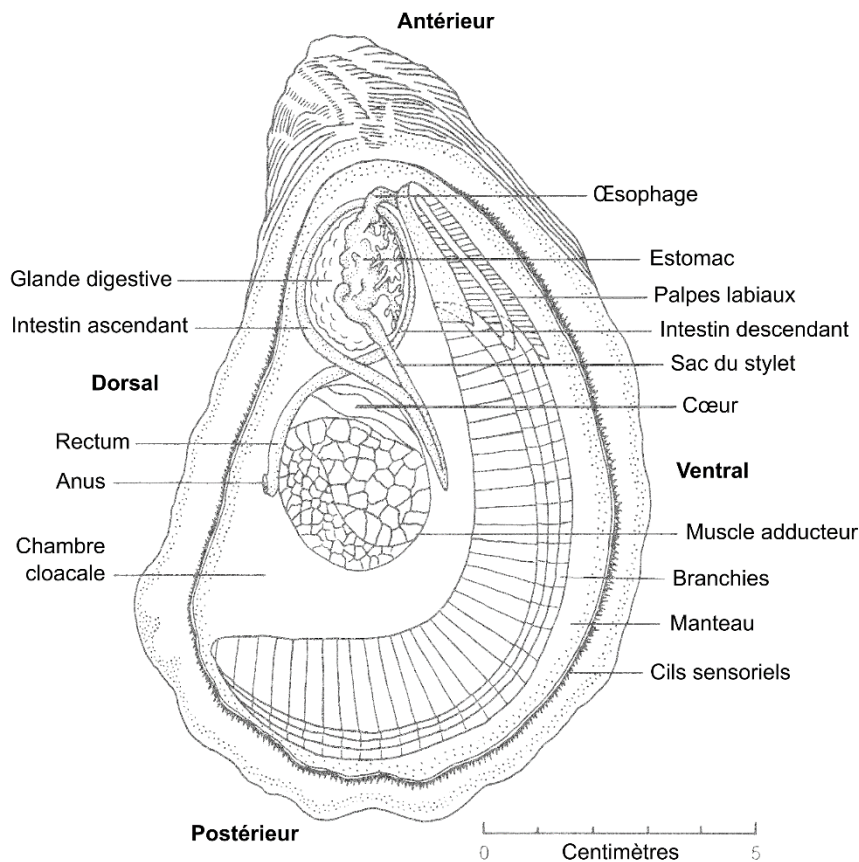


Figure 6. Anatomie générale de l'huître creuse présentée dans sa valve gauche, d'après Kennedy et al. (1996). Les axes d'orientation sont indiqués en caractères gras.

L'huître possède un système circulatoire semi-ouvert. L'excrétion est assurée par un rein constitué d'une glande tubulaire. Le fluide circulant ou hémolymphe, constitué de cellules circulantes appelées hémocytes et de plasma, baigne les organes. L'hémolymphe circule via des vaisseaux vers le cœur qui est composé d'un ventricule et de deux oreillettes.

Les hémocytes sont des cellules qui assurent d'importantes fonctions immunitaires chez les huîtres, jouant notamment un rôle essentiel dans la réaction inflammatoire, la réparation des

tissus endommagés, ainsi que dans la phagocytose et l'encapsulation de corps étrangers (Cheng, 1981; Cima et al., 2000; Dyachuk, 2016; Hine, 1999; Wang et al., 2017). Ces cellules immunitaires sont capables de franchir les barrières épithéliales par diapédèse et d'infiltrer les tissus, pour se rendre sur les sites lésés ou infectés. Les hémocytes constituent la première ligne de défense de par leur fonction de phagocytose, reconnaissant, absorbant et détruisant les agents pathogènes et les particules étrangères (Burgos-Aceves and Faggio, 2017). Ils produisent également des facteurs humoraux et des espèces réactives de l'oxygène (Donaghy et al., 2012). Les hémocytes sont aussi impliqués dans la nutrition, la formation et la réparation de la coquille (Donaghy et al., 2009; Mount et al., 2004).

Le système nerveux de l'huître est relativement simple et décentralisé. Il est composé de deux paires de ganglions nerveux, les ganglions cérébraux près de la bouche et les ganglions viscéraux situés sous le muscle adducteur (Marteil, 1976). Un réseau de nerfs permet l'intégration des informations entre les ganglions, notamment grâce à la commissure cérébrale et aux connectifs cérébroviscéraux.

3. Reproduction de l'huître

L'huître du Pacifique est une espèce hermaphrodite protandre de type alternatif irrégulier (Amemiya, 1929; Galtsoff, 1964). Cela signifie qu'elle est généralement mâle lors de la première saison de reproduction, et que des changements de sexe peuvent se produire irrégulièrement lors des cycles suivants chez un même individu (Guo et al., 1998). Néanmoins, il existe un faible pourcentage d'huîtres hermaphrodites simultanées (Amemiya, 1929), et tous les individus ne subissent pas forcément de changement de sexe. Le déterminisme du sexe chez l'huître creuse est déterminé par des facteurs génétiques et environnementaux qui sont encore mal connus (Hedrick and Hedgecock, 2010).

3.1. Gamétogenèse

L'activité de gamétogenèse chez l'huître suit un rythme saisonnier dans les zones tempérées : la gonade n'est pas un organe permanent, elle se développe annuellement puis régresse après l'émission des gamètes (Fabioux et al., 2005). Un cycle comprend : (1) l'initiation de la gamétogenèse à la fin de l'hiver, (2) la phase active de gamétogenèse caractérisée par une intense multiplication et différenciation des cellules sexuelles pendant le printemps, (3) la maturation et l'émission des gamètes qui a lieu en été, (4) une éventuelle résorption des gamètes non émis pour permettre le rétablissement d'un nouveau cycle (Marteil, 1976; Pouvreau and Le

Pennec, 2006). La période de « repos sexuel » se déroule durant l'automne et le début de l'hiver (Lango-Reynoso et al., 2006).

Les gamètes sont produits dans le tissu gonadique par un épithélium germinale à l'intérieur de follicules enveloppés dans un tissu conjonctif. La gonade se développe autour de la glande digestive et occupe une grande partie de la cavité palléale lorsqu'elle est mature. A la fin du cycle de gamétogenèse, l'émission des gamètes est déclenchée en réponse à un ensemble de stimuli extérieurs, liés à la température, la marée, les conditions météorologiques, les efflorescences phytoplanctoniques (Bernard et al., 2016).

Les ovocytes matures de *C. gigas* sont majoritairement constitués de protéines (Boulaïs et al., 2015). Les protéines et lipides sont accumulés pendant l'ovogenèse chez l'huître *C. gigas* (Li et al., 2000). Ces nutriments sont essentiels pour l'embryogenèse et le développement précoce car ils constituent la source d'énergie disponible pour l'embryon et les jeunes larves, qui sont autotrophes jusqu'au stade larve D.

3.2. Cycle de vie

Le cycle de vie de l'huître creuse est divisé en deux périodes : une phase embryo-larvaire pélagique et une phase benthique sessile de l'huître juvénile et adulte, après métamorphose et fixation des larves (Figure 7). La fécondation est externe : les gamètes matures, ovocytes et spermatozoïdes, sont transportés via les gonoductes vers le canal génital, puis sont émis dans l'eau de mer. Après émission des globules polaires, l'œuf hétérolécite subit plusieurs clivages pour donner une blastula, 5 heures post-fécondation (hpf) pour *C. gigas* à 25°C, puis donne naissance à la gastrula par épibolie 7 hpf (Fabioux et al., 2004).

Le premier stade larvaire est la larve trochophore (16-18 hpf), en forme caractéristique de toupie, capable de nager grâce à des cils présents à la périphérie et au niveau apical. Puis la larve devient véligère, la première coquille se forme (prodissoconque I) et le vélum se développe, c'est la larve D, qui présente un tube digestif fonctionnel (24-48 hpf). La larve se développe ensuite en larve véligère à umbo (7 jours post-fécondation, jpf), caractérisée par l'acquisition d'un crochet ou umbo au niveau de la charnière et par une coquille plus calcifiée (prodissoconque II). Le velum, constitué de deux plis semi-circulaires portant de grands cils sur leur périphérie, est un organe natatoire, respiratoire et nourricier. Pendant la nage, le velum est projeté entre les valves de la coquille et à la moindre perturbation, cet organe contractile se rétracte entre les valves. De plus petits cils présents à la base du vélum conduisent les particules

alimentaires vers la bouche. Le système digestif, relativement complexe, est composé d'un œsophage, d'un estomac relié au sac du stylet cristallin et aux diverticules digestifs, d'un intestin, et d'un rectum qui se termine par un anus s'ouvrant dans la cavité palléale.

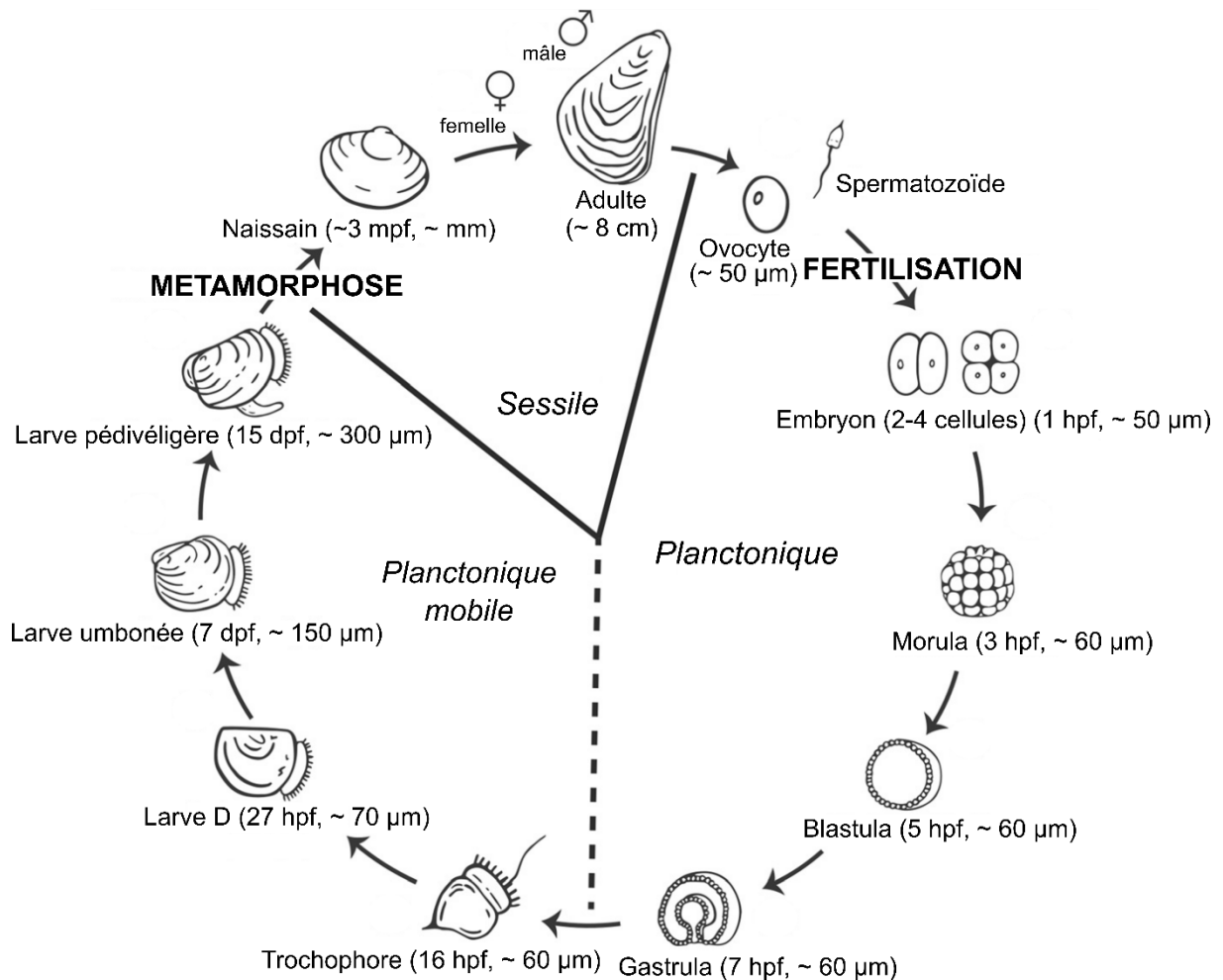


Figure 7. Cycle de vie de l'huître *Crassostrea gigas*, d'après Vogeler et al. (2016).

Lorsque la larve approche de la métamorphose, une tache sombre apparaît au niveau de la première ébauche branchiale : la larve est dite « œillée ». Cette larve est décrite comme compétente ou apte à la métamorphose, et mesure environ 300 µm. Vers le 15^{ème} jour post-fécondation, lorsque la larve dépasse la taille de 300 µm, elle devient capable de ramper (activité de « crawling ») pour explorer le sédiment grâce à un pied, c'est la larve pédivéligère (Figure 8). Le pied est un organe cilié sensoriel et glandulaire, permettant la sélection du substrat de fixation et la sécrétion du ciment lors de la fixation. Le vélum est encore fonctionnel pour la nutrition et la nage.

Le passage de la vie pélagique à la vie benthique comporte deux événements essentiels : la fixation de la larve pédivéligère sur le support (Figure 8C) et la métamorphose. Le phénomène

complexe de la métamorphose a lieu après la fixation : les différents tissus et organes se réorganisent pour donner une huître juvénile appelée naissain ou post-larve (Troost, 2010). Le vélum et le pied régressent, les palpes labiaux se forment, les branchies et les palpes labiaux se développent et la coquille définitive est sécrétée, la dissoconque.

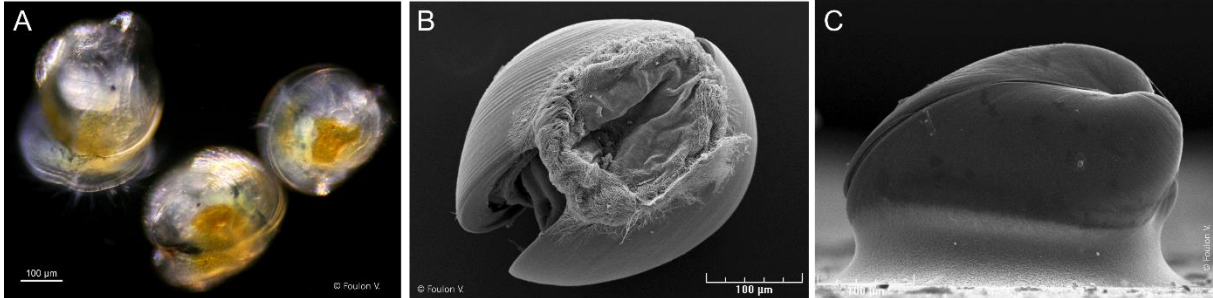


Figure 8. Larves pédivéligères de *C. gigas*, en phase de nage (A, B) et venant juste de se fixer sur le substrat (C) ; observées par microscopie optique (A) et microscopie électronique à balayage (B).

Au stade de larve D, il y a passage de la phase endotrophe (utilisation des réserves vitellines provenant de l'ovocyte), à la phase exotrophe (capture de la nourriture).

En se basant sur l'activité de nutrition des larves, la phase larvaire est caractérisée par 3 phases distinctes (Rico-Villa et al., 2009) :

- une période initiale de mixotrophie caractérisée par une ingestion faible pendant les premiers jours de développement larvaire (du stade larve D au stade larve umbonée de longueur $\approx 110 \mu\text{m}$) où la larve commence à acquérir de l'énergie via la filtration mais dépend encore des réserves provenant de l'ovocyte,
- une phase exotrophique caractérisée par une augmentation marquée de l'ingestion (de la larve umbonée à la larve œillée de longueur $\approx 300 \mu\text{m}$) où l'apport d'énergie se fait exclusivement via l'alimentation,
- et enfin une 3^{ème} phase durant laquelle le taux d'ingestion des larves $\geq 300 \mu\text{m}$ décroît pendant la métamorphose.

3.3. Paramètres influant sur la reproduction

L'huître *C. gigas*, comme de nombreux mollusques marins, présente une très forte fécondité, qui est caractéristique de la stratégie démographique de type « r ». L'huître produit un grand nombre de gamètes qui sont émis dans un environnement hostile, ce qui est souvent perçu comme une stratégie évolutive en réponse à une mortalité importante et imprévisible des stades précoces de développement (Huvet et al., 2015). La gamétogenèse est une fonction majeure en

termes d'investissement énergétique chez *C. gigas*. L'huître creuse peut émettre entre 20 et 100 millions d'ovocytes (Marteil, 1976) et environ 30×10^9 spermatozoïdes par gramme de gonade chez le mâle (Dong et al., 2005). La proportion de l'énergie acquise par les huîtres allouée soit à la croissance somatique soit au développement de la gonade est la résultante d'une balance entre les coûts et les bénéfices pour garantir le succès de la reproduction tout au long de leur vie (Bayne, 2017b).

Le succès de la reproduction de l'huître creuse dépend du déroulement de deux phases distinctes : la phase de gamétogenèse saisonnière qui conduit à la formation des gamètes matures et la phase de vie larvaire. L'abondance et durée des phases de la reproduction de l'huître creuse dépend de nombreux facteurs environnementaux comme le phytoplancton, la température de l'eau de mer, la marée, le vent, la salinité ou la présence de contaminants toxiques (Figure 9).

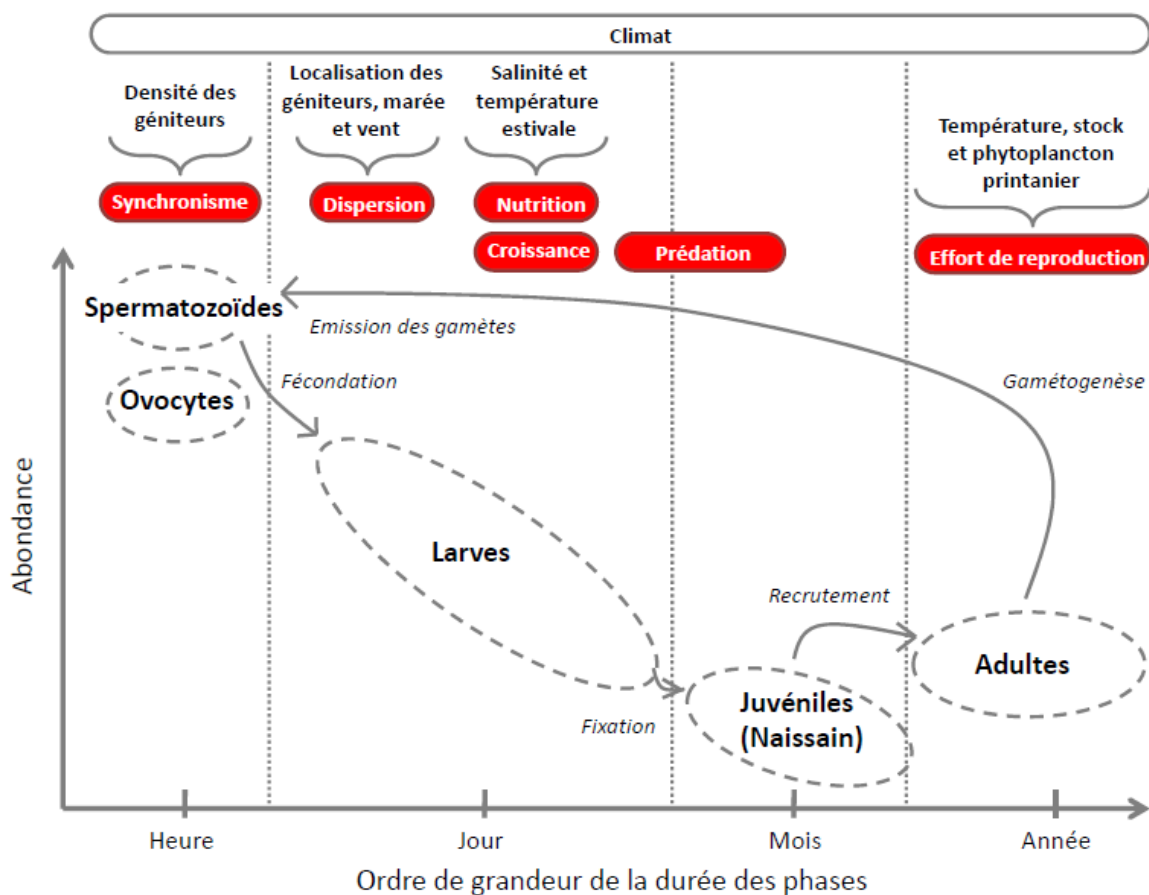


Figure 9. Facteurs influençant l'abondance et durée des phases de la reproduction de l'huître creuse, d'après Bernard (2011). Les facteurs influençant directement l'abondance des phases sont représentés en rouge et les facteurs indirects sont indiqués dans les accolades.

La durée de la phase larvaire varie entre 2 et 4 semaines sur la côte atlantique, en fonction de la température et de la nourriture disponible (Rico-Villa et al., 2006). La nutrition est un facteur important influençant la croissance des larves, expliquant une plus grande proportion de la variabilité que d'autres facteurs comme la température ou la salinité (His et al., 1989). L'abondance et la composition du phytoplancton influencent la croissance et la survie des larves de bivalves marins (Bricelj and MacQuarrie, 2007; Chícharo and Chícharo, 2001). La larve doit accumuler suffisamment de réserves durant la phase exotrophique, pour faire face aux forts besoins énergétiques requis pendant le processus de métamorphose et d'assurer leur survie (Haws et al., 1993).

4. Réponses des bivalves marins aux efflorescences d'*Alexandrium*

Les dinoflagellés du genre *Alexandrium* affectent le comportement et une large gamme de fonctions physiologiques des bivalves marins.

Le manteau, les branchies et les palpes labiaux des bivalves sont en contact avec les cellules d'*Alexandrium* spp. et leurs toxines extracellulaires lors de la filtration. Les organes internes vont être affectés lors du passage des micro-algues toxiques dans le tube digestif. Lors de la digestion, une partie des cellules de micro-algues sont lysées et leurs toxines paralysantes intracellulaires sont alors libérées dans la glande digestive. La glande digestive est l'organe où les PST sont initialement accumulées chez les bivalves (Bricelj and Shumway, 1998), représentant 65 à 95 % du contenu toxinique total (Guéguen et al., 2008).

De récentes études ont démontré la toxicité d'*A. minutum* sur les rythmes biologiques de *C. gigas*. Le rythme de l'activité valvaire, l'activité circadienne globale ainsi que le transcriptome temporel des branchies sont ainsi perturbés par une exposition à la micro-algue (Mat et al., 2013; Payton et al., 2017; Tran et al., 2015).

Le comportement des bivalves est perturbé par *Alexandrium* spp. Ainsi, l'exposition à *Alexandrium* spp. induit soit la fermeture des valves comme chez la moule *Mytilus edulis* exposée à *A. tamarense* (Shumway and Cucci, 1987), soit une augmentation de la durée d'ouverture valvaire, du nombre de micro-fermetures (fermetures partielles et rapides des valves) et une diminution de l'amplitude d'ouverture valvaire comme chez *C. gigas* exposée à *A. minutum* (Haberhorn et al., 2011; Tran et al., 2010).

L'exposition à *Alexandrium* spp. provoque une diminution partielle de l'activité de filtration, une production moindre de bio-dépôts (Bardouil et al., 1993; Bougrier et al., 2003; Laabir et

al., 2007; Lassus et al., 2004, 1999) et une production accrue de mucus par les branchies des bivalves (Haberkorn et al., 2010b; Shumway and Cucci, 1987) et perturbe donc la fonction de nutrition. Dans les branchies d'huîtres *C. gigas* exposées à *A. minutum*, une répression de l'expression de gènes impliqués dans l'horloge interne, l'immunité et les processus de détoxification ont été observées (Haberkorn et al., 2010b; Mat et al., 2013; Payton et al., 2017). La fonction et la structure de la glande digestive de *C. gigas* est aussi modifiée en réponse à *A. minutum* : une modification de la composition lipidique de ce tissu et une forte réponse inflammatoire, caractérisée par des infiltrations (forte concentration d'hémocytes dans un tissu) et des diapédèses d'hémocytes (passage des hémocytes entre les cellules de l'épithélium depuis le tissu conjonctif vers la lumière du tractus digestif ou inversement) ont été décrits (Haberkorn et al., 2010b).

L'exposition à *A. minutum* affecte aussi le système immunitaire des huîtres. Des études *in vitro* d'exposition d'hémocytes de *C. gigas* à *A. minutum* ont démontré une diminution significative de la production d'espèces réactives de l'oxygène (ERO ou Reactive Oxygen Species, ROS) par les hémocytes ainsi qu'une diminution de la phagocytose de ces cellules (Hégaret et al., 2011; Mello et al., 2013). La production de ROS par les hémocytes, liée à leur activité de phagocytose, est un mécanisme important pour la défense contre les pathogènes. Par ailleurs, l'exposition à des micro-algues du genre *Alexandrium* peut également modifier la réponse à des bivalves à des infections parasitaires (Lassudrie et al., 2014, 2015b) ou à des pathogènes (Abi-Khalil et al., 2016; Lassudrie et al., 2015a, 2016). L'exposition *in vitro* d'hémocytes d'huîtres à différents analogues de la saxitoxine (STX et C1 / C2) provoque l'apoptose des hémocytes, processus faisant intervenir l'activation de la caspase (Abi-Khalil et al., 2017).

Une grande partie des effets d'*Alexandrium* spp. sur les bivalves et notamment sur *C. gigas* ont été reliés aux toxines paralysantes produites par ces micro-algues. Néanmoins, des effets néfastes sont également observés chez les bivalves exposés à des souches d'*Alexandrium* non productrices de PST. Il a par exemple été démontré que le filtrat d'une souche d'*A. tamarense* non productrice de PST réduit l'adhérence et la phagocytose des hémocytes de la palourde *Ruditapes philippinarum* et de la mye *Mya arenaria* (Ford et al., 2008). Une souche d'*A. minutum* (souche CCMI1002), non-productrice de PST mais productrice de composés extracellulaires bioactifs, diminue la croissance, la filtration et altère les tissus de juvéniles de coquille Saint Jacques *Pecten maximus* (Borcier et al., 2017). Cette même souche d'*A. minutum* modifie aussi fortement l'activité de nutrition de *C. gigas* et de *R. philippinarum*, probablement du fait de ces BEC (Contreras, 2011; Lambert et al., 2003). Ces résultats suggèrent que les

composés extracellulaires bioactifs jouent également un rôle non négligeable dans la toxicité d'*Alexandrium* sur les bivalves.

Dans le milieu naturel, les bivalves peuvent être exposés aux efflorescences de micro-algues toxiques durant différentes périodes de leur cycle de reproduction. Les effets des dinoflagellés du genre *Alexandrium* sur la physiologie des bivalves sont relativement bien détaillés, néanmoins, les conséquences de ces micro-algues sur la reproduction des bivalves sont encore mal connues.

5. Effets des efflorescences d'*Alexandrium* sur la reproduction des bivalves marins

Les expériences en laboratoire menées sur le sujet ont consisté le plus souvent à exposer certains stades du cycle de vie des bivalves à *Alexandrium* spp. afin de voir les effets directs sur ces mêmes stades, ou les conséquences sur le développement pour les larves ou sur la suite de la reproduction pour les adultes. Les stades exposés lors de ces études sont : les géniteurs, les gamètes, les embryons et les larves.

5.1. Exposition des géniteurs

Une exposition d'huîtres *C. gigas* adultes matures pendant 4 jours à 5×10^3 cellules mL⁻¹ d'*A. minutum* (qui produit des PST et des BEC), induit une diminution de la mobilité et de la teneur en ATP des spermatozoïdes, comparés aux individus témoins nourris avec des micro-algues non toxiques (Haberkorn et al., 2010b). L'ATP jouant un rôle dans la mobilité des spermatozoïdes, Haberkorn et al. (2010b) ont émis l'hypothèse que la diminution de la mobilité des spermatozoïdes serait liée à la diminution de la teneur en ATP. De manière surprenante, la mortalité des spermatozoïdes est significativement plus faible chez les huîtres exposées que chez les huîtres témoins (Haberkorn et al., 2010b).

Les spermatozoïdes issus d'huîtres *C. gigas* matures exposées à $2,5 \times 10^2$ cellules mL⁻¹ d'*Alexandrium catenella* pendant 9 jours présentent un potentiel membranaire mitochondrial (PMM) significativement plus élevé et que les spermatozoïdes issus d'huîtres témoins nourries avec un dinoflagellé non-toxique *Heterocapsa triquetra* (Le Goïc et al., 2013).

Des huîtres *C. gigas* exposées à des souches d'*Alexandrium* productrices de PST (*A. minutum* et *A. catenella*) produisent des spermatozoïdes qui ont un marquage moyen de l'ADN (mesuré par la fluorescence du fluorochrome SYBR-14 ou Sybr Green par cytométrie en flux) plus

variable que les huîtres nourries avec des micro-algues non-toxiques (Haberhorn et al., 2010b; Le Goïc et al., 2013). Ces changements de marquage de l'ADN des huîtres exposées pourraient être le signe d'anomalies des chromosomes ou de modifications de condensation de l'ADN (Haberhorn et al., 2010b; Le Goïc et al., 2013).

Lors d'une expérience d'exposition de moules *Mytilus edulis* adultes à *Alexandrium fundyense*, certaines moules ont pondu après 6 jours d'exposition (Galimany et al., 2008). Après 7 jours d'exposition, les moules exposées présentent une prévalence de gonades anormales plus élevée que les moules non-exposées. Les gonades anormales présentent des ovocytes en dégénérescence et des débris cellulaires dans la lumière des follicules gonadiques (Galimany et al., 2008). Après la ponte, le statut physiologique des moules peut être affecté, avec notamment une diminution de la masse corporelle et une immunodépression, en lien avec un statut énergétique plus faible (Delaporte et al., 2003; Hendriks et al., 2003). De même, après la ponte, les huîtres semblent être moins capables de résister aux effets des toxines produites par *A. fundyense*, peut-être à cause d'un statut énergétique plus faible (Bricelj et al., 1993).

5.2. Exposition des gamètes

L'effet d'*A. minutum* (souche AM89BM, produisant des PST et des BEC) sur la reproduction de *C. gigas* a également été étudié en exposant directement les ovocytes aux algues toxiques ou au filtrat de culture (Le Goïc et al., 2014). Les ovocytes exposés à des cultures d'*A. minutum* âgées de 7 ou 11 jours présentent une production d'espèces réactives de ROS plus importante que les ovocytes exposés à l'eau de mer. Le Goïc et al. (2014) suggèrent que cet effet serait lié aux BEC produits par *A. minutum*, qui sont présents en plus grande quantité dans les cultures vieillissantes (Lelong et al., 2011). L'exposition au surnageant de culture n'induit qu'une augmentation non-significative de la production de ROS par les ovocytes (Le Goïc et al., 2014). Ce résultat pourrait s'expliquer par la dégradation rapide des composés toxiques extracellulaires dans le surnageant (Flores et al., 2012), alors que les cultures contiennent des cellules vivantes qui produisent et excrètent continuellement ces composés extracellulaires. Le contact entre les cellules algales et les ovocytes est potentiellement nécessaire pour provoquer la production de ROS par les ovocytes en réponse à *Alexandrium*.

L'exposition des gamètes de l'huître perlière japonaise, *Pinctada fucata martensii* à *A. catenella* et *Alexandrium affine* réduit la vitesse de nage des spermatozoïdes et la viabilité des ovocytes (Banno et al., 2018). La souche d'*A. affine* non productrice de PST induit des effets négatifs plus drastiques sur les gamètes que la souche d'*A. catenella* productrice de PST,

confirmant une toxicité indépendante de la production de PST. Banno et al. (2018) suggèrent que les BEC produits par les deux souches utilisées dans cette étude jouent un rôle dans la toxicité sur les gamètes de l'huître perlière japonaise, ces deux souches présentant une activité cytotoxique sur des cellules Vero de mammifères (lignée cellulaire isolée à partir de cellules épithéliales de rein extraites d'un singe vert africain).

5.3. Exposition des embryons

Une exposition d'embryons de la moule *Mytilus edulis* à *A. minutum* et *A. ostensfeldii* diminue le développement et la survie des embryons, après 5 jours d'exposition (2.5×10^3 cellules mL⁻¹) (De Rijcke et al., 2016). Le taux de développement des embryons du pétoncle *Chlamys farreri* en larves D est fortement inhibé après 36 h d'exposition à *A. tamarense*, et ce, de manière dépendante de la concentration algale (Yan et al., 2001).

L'exposition des embryons de *C. gigas* à *A. catenella* (souche ACDH, isolée en Chine) a un effet délétère sur le clivage (ou segmentation) et entraîne un retard de développement des embryons par rapport à la condition témoin (eau de mer filtrée) (Mu and Li, 2013) : une partie des embryons se sont développés en larves D dans la condition témoin 8 heures post-fécondation (hpf) alors que des gastrulas sont encore observées lorsque les embryons sont exposés à *A. catenella*. Cette souche entraîne aussi une augmentation de la mortalité 8 hpf et diminue leur développement en larves D 32 hpf (Mu and Li, 2013). Pour de fortes concentrations en *A. catenella* ($> 10^3$ cellules mL⁻¹), certaines larves trochophores ne se sont pas développées en larves D 32 hpf. L'exposition des embryons de *C. gigas* à *A. catenella* provoque aussi des anomalies des embryons : gonflements des membranes, écoulements de cytoplasme, pouvant entraîner la lyse des cellules (Mu and Li, 2013). Cependant, aucune anomalie n'a été détectée chez les embryons de l'huître *P. fucata martensii* exposés à *A. catenella* (Basti et al., 2015). Basti et al. (2015) suggèrent que cette différence pourrait être due à une sensibilité différente des deux espèces d'huîtres ou une toxicité différente des deux souches d'*A. catenella*.

Le développement embryonnaire de l'huître perlière japonaise *P. fucata martensii* est aussi affecté par l'exposition à *A. catenella* et *A. affine*. Les deux souches inhibent le 2^{ème} clivage des embryons 2 hpf, pour une concentration de $2,5 \times 10^2$ cellules mL⁻¹ d'*A. affine* ou 5×10^2 cellules mL⁻¹ d'*A. catenella* (Basti et al., 2015). Le développement en larves D est affecté de manière plus importante par *A. affine* que par *A. catenella* (inhibition totale avec 5×10^2 cellules mL⁻¹ pour *A. affine*, 2×10^3 cellules mL⁻¹ pour *A. catenella*) (Basti et al., 2015). Ces résultats

indiquent que l'espèce non-productrice de PST *A. affine* induit plus d'effets délétères sur les embryons de l'huître perlière qu'*A. catenella* (Basti et al., 2015), comme cela a été observé pour les gamètes de cette espèce (Banno et al., 2018). Une exposition à une faible concentration en *A. affine* ($2,5 \times 10^2$ cellules mL^{-1}) provoque des anomalies chez 50-80% des embryons de l'huître perlière japonaise en causant des gonflements anormaux de la membrane, des écoulements de cytoplasme, qui peuvent entraîner la lyse des œufs fécondés et des blastomères. Les toxines et autres métabolites produits indépendamment des PST par *A. affine*, ont une activité lytique très puissante qui cible l'intégrité de la membrane (Basti et al., 2015).

Comme il n'y a pas d'activité de nutrition chez les embryons et que les PST sont très majoritairement intracellulaires, l'effet néfaste d'*Alexandrium* spp. n'est probablement pas lié aux PST mais plutôt à des molécules situées à la surface des cellules de l'algue (Mu and Li, 2013; Yan et al., 2001), ou des composés excrétés dans l'eau de mer (Basti et al., 2015). De plus, l'exposition des embryons de *C. farreri* au contenu cellulaire d'*A. tamarensis* ou à la saxitoxine purifiée n'affecte pas le taux de développement des embryons de *C. farreri*, par rapport au contrôle (Yan et al., 2001).

Les stades premiers stades embryonnaires semblent très sensibles aux composés bioactifs produits par *Alexandrium* spp. Plus l'exposition des œufs de *C. farreri* à *A. tamarensis* est initiée tôt après la fécondation (plus l'embryon est jeune), plus la probabilité d'un effet inhibiteur sur le développement est forte ; si l'exposition débute avant le stade blastula (16 hpf), aucun œuf ne se développe en larve D (Yan et al., 2001).

Un autre paramètre qui fait varier la toxicité des cellules d'*Alexandrium* spp sur le développement embryonnaire est l'âge de la culture. Par exemple, l'exposition d'œufs fécondés de *C. farreri* à une culture d'*A. tamarensis* en début de phase exponentielle inhibe de manière plus importante le développement des embryons par rapport à des cultures plus âgées ou sénescents (Yan et al., 2001).

5.4. Exposition des larves

Une exposition de larves trochophores de *C. gigas* (17 hpf) à 10^7 cellules mL^{-1} d'*A. tamarensis* (souche AT91, isolée au Japon, productrice de PST) ou à 2×10^6 cellules mL^{-1} d'*A. taylori* (souche ATY-IN, isolée en Indonésie, non productrice de PST) induit une mortalité de 92 et 100% respectivement après 8h d'exposition (Matsuyama et al., 2001). Matsuyama (2001) suggère que les effets néfastes observés seraient causés par des composés produits par certaines souches d'*Alexandrium* indépendamment des PST et localisés à la surface des cellules de

dinoflagellés. Néanmoins, il est peu probable d'observer une concentration d'*A. tamarensis* de 10^7 cellules mL^{-1} dans la nature (Bricelj et al., 2010).

L'exposition de larves D de *M. mercenaria* âgées de 24h à *A. fundyense* ($4,9 \times 10^2$ cellules mL^{-1}) induit une mortalité (5 %) des larves après 72h d'exposition (Tang and Gobler, 2012). Mu et Li (2013) ont exposé des larves D (âgées de 3 jours) et de jeunes larves umbonées de *C. gigas* à *A. catenella* en plus d'un apport de *T. lutea* pendant 4 jours. La croissance des larves D et des larves véligères est significativement réduite pour des concentrations en *A. catenella* supérieures à 3×10^2 cellules mL^{-1} . Les larves D paraissent plus sensibles que les larves umbonées : la survie des larves D et des larves véligères est réduite pour une concentration 6×10^2 cellules mL^{-1} et 2×10^3 cellules mL^{-1} d'*A. catenella*, respectivement (Mu and Li, 2013).

Une exposition de jeunes larves D du pétoncle *Chlamys farreri* à de fortes concentrations ($> 3 \times 10^3$ cellules mL^{-1}) en *A. tamarensis* (souche ATHK isolée en Chine, 11 pg STXeq cellule⁻¹) augmente la mortalité des larves après 6 jours d'exposition (Yan et al., 2001). Cette même souche d'*A. tamarensis* inhibe l'activité des larves D de deux espèces de pétoncles, *C. farreri* et *Argopecten irradians concentricus*, après 48h d'exposition à 3×10^3 cellules mL^{-1} et 10^4 cellules mL^{-1} d'*A. tamarensis*, respectivement (Yan et al., 2003, 2001). Les jeunes larves D de ces deux espèces de pétoncles étant incapables d'ingérer les cellules d'*A. tamarensis* à cause de sa taille relativement grande (20-28 μm) (Yan et al., 2003, 2001), Yan et al. (2003) suggère que l'effet délétère observé serait plutôt lié aux composés bioactifs excrétés par *A. tamarensis*, et non aux PST.

La micro-algue *Alexandrium monilatum* produit des endotoxines qui ont des propriétés neurotoxiques et hémolytiques (Aldrich et al., 1967). La goniodomine A est une endotoxine produite par *A. monilatum* (Hsia et al., 2006), qui interagit directement avec l'actine et affecte la réponse contractile en modifiant l'activité de l'ATPase actomyosine (Matsunaga et al., 1999). La toxicité d'*A. monilatum* (souche AM03) sur les larves véligères de *Crassostrea virginica* et de *Mercenaria mercenaria* (âgées de 10-14 jours, longueur moyenne de $240 \pm 11 \mu\text{m}$) a été testée en laboratoire (May et al., 2010). L'exposition des larves aux cellules lysées d'*A. monilatum* (après sonication) affecte la survie des larves : seulement 38% des larves de *M. mercenaria* et 90% des larves de *C. gigas* survivent après 2h d'exposition (May et al., 2010) (May et al., 2010). En revanche, l'exposition des larves à la culture d'*A. monilatum* ou à des cellules contenues dans un boudin de dialyse, n'a pas d'effet sur la mortalité (May et al., 2010). Les auteurs suggèrent que l'effet létal d'*A. monilatum* provient des endotoxines qui peuvent être libérées de manière modérée dans l'eau environnante et de manière plus importante durant

la sénescence ou la fin de l'efflorescence, ou bien dans des situations de stress (Landsberg, 2002).

L'exposition de larves véligères de *Mya arenaria* âgées d'une semaine à une souche très toxique d'*A. tamarense* (souche PR18b, 35.7 pg STX_{eq} cellule⁻¹) à 10² cellules mL⁻¹ additionnée à une algue fourrage *Pavlova pinguis* pendant 7 jours ne modifie pas la croissance et la mortalité des larves, comparé à une condition témoin où les larves sont uniquement nourries avec *P. pinguis* (Bricelj et al., 2010). L'hypothèse avancée par Bricelj et al. (2010) est que les cellules d'*A. tamarense* (35 µm) sont trop grandes pour être ingérées par les larves véligères de *M. arenaria* (180-240 µm) et que l'excrétion potentielle des PST dans l'eau de mer par *A. tamarense* est insuffisante pour induire des effets délétères.

Mu et Li (2013) ont étudié en laboratoire l'effet d'une exposition à *A. catenella* pendant 4 jours sur la fixation de larves œillées (âgées de 16 jours) de *C. gigas*, en plus d'un apport de *T. lutea*. La survie et la fixation des larves œillées sont significativement plus faibles par rapport à la condition témoin (eau de mer filtrée), après exposition à 2 × 10³ cellules mL⁻¹ et 3 × 10² cellules mL⁻¹ d'*A. catenella*, respectivement (Mu and Li, 2013).

L'exposition de larves œillées d'*A. irradians concentricus* (196 × 181 µm) à *A. tamarense* (souche ATHK) n'affecte pas les taux de métamorphose et de mortalité des larves mais réduit la taille des juvéniles après 14 jours d'exposition à des concentrations supérieures à 5 × 10² cellules mL⁻¹ (Yan et al., 2003). Selon les auteurs, les PST pourraient contribuer aux effets délétères d'*A. tamarense* sur les larves œillées et les juvéniles, car ces stades de développement sont potentiellement capables d'ingérer les cellules algales et donc d'accumuler les PST (Yan et al., 2003). Les composés bioactifs inconnus produits par *A. tamarense* semblent jouer un rôle dans la toxicité sur les larves D et embryons de *C. farreri* (Yan et al., 2001), et pourraient donc affecter la croissance des juvéniles d'*A. irradians concentricus*.

Les larves pédivéligères (âgées de 21 jours) d'huître perlière *P. fucata martensii* sont plus sensibles à une exposition à *A. catenella* (producteur de PST) ou *A. affine* (non-producteur de PST) que les larves D de cette même espèce (Basti et al., 2015). L'activité des larves pédivéligères est réduite de manière significative après 24 h d'exposition à 2,5 × 10² cellules mL⁻¹ d'*A. affine* ou à 10 cellules mL⁻¹ d'*A. catenella* (Basti et al., 2015). Les larves D de l'huître perlière sont incapables d'ingérer des cellules d'*A. catenella*, contrairement aux larves pédivéligères. Comme les PST sont majoritairement intracellulaires, la diminution de la mobilité des larves pédivéligères en réponse à *A. catenella* pourrait être liée à la libération des

PST dans les tissus des larves suite à la rupture des cellules d'*A. catenella* lors de la digestion, qui entraînerait une diminution de la mobilité par paralysie et/ou par une altération de l'homéostasie cellulaire (Basti et al., 2015). Basti et al. (2015) suggèrent que les larves pédivéligères sont : (1) soit plus sensibles aux PST produits par *A. catenella*, (2) soit plus sensibles aux composés bioactifs produits par *A. catenella* que ceux produits par *A. affine*, (3) soit plus sensibles à la synergie des PST et des composés bioactifs inconnus d'*A. catenella*.

L'effet d'*Alexandrium* spp. sur les larves de bivalves marins semble dépendre du stade de développement. Par exemple, la toxicité d'*A. catenella* dépend du stade de développement de l'huître *P. fucata martensii* (Basti et al., 2015). La micro-algue *A. catenella* a des effets délétères mais non-lytiques sur les embryons en division retardant la métamorphose en larve D, un effet lytique sur les larves trochophores à de fortes concentrations, et une activité non lytique sur les larves pédivéligères qui diminue leur mobilité (Basti et al., 2015). Les auteurs de cette étude suggèrent qu'*A. catenella* produit des substances lytiques secrétées dans le milieu extérieur ou situées à la surface des cellules et que les stades de développement de l'huître perlière japonaise sont plus ou moins sensibles aux PST et/ou autres composés bioactifs qui ont sûrement des modes d'action et des cibles différents (Basti et al., 2015).

Les huîtres *Crassostrea gigas* produites le long du littoral français sont exposées à des efflorescences d'*Alexandrium minutum* et d'*Alexandrium catenella*, respectivement sur les côtes Atlantique et Méditerranée (Guallar et al., 2017; Laanaia et al., 2013), ce qui peut entraîner des interdictions de vente de coquillages.

Contexte scientifique et objectifs

Les efflorescences de micro-algues toxiques sont de plus en plus fréquentes et intenses et leur étendue géographique ne cesse d'augmenter, notamment en lien avec les changements climatiques globaux (Anderson et al., 2012b). Les recherches sur les micro-algues toxiques se sont principalement focalisées sur la production de phycotoxines dangereuses pour la santé humaine, l'identification, la quantification de ces toxines ainsi que les mécanismes d'accumulation et d'élimination de ces toxines par les bivalves.

Néanmoins, ces efflorescences ont des conséquences à plus large échelle. Elles affectent négativement l'écologie des écosystèmes marins côtiers, les populations marines, la physiologie des organismes marins et les traits d'histoire de vie d'espèces exploitées (Harvell et al., 1999; Landsberg, 2002). Les efflorescences toxiques causées par les dinoflagellés *Alexandrium* spp., producteurs de toxines paralysantes, jouent un rôle écologique important en influençant la distribution et l'abondance des organismes marins et le comportement alimentaire d'espèces « clé de voûte » (Kvitek and Bretz, 2004; Zimmer and Ferrer, 2007).

Ces efflorescences toxiques apparaissent de plus généralement pendant la période de reproduction de la plupart des bivalves. Même s'il est impossible actuellement de prouver que les efflorescences de micro-algues toxiques sont directement responsables de défauts de recrutement de bivalves, cette hypothèse a été formulée à partir d'observations de terrain (Bricelj and MacQuarrie, 2007; Granmo et al., 1988; Leverone et al., 2006; Summerson and Peterson, 1990; Tang and Gobler, 2009). Des défauts de recrutement pourraient modifier la structure de populations sauvages mais aussi affecter l'exploitation d'espèces d'intérêt commercial et écologique, comme les bivalves. L'étude des effets potentiels des HAB sur la reproduction des bivalves apparaît donc cruciale.

L'huître du Pacifique, *Crassostrea gigas*, domine la production de bivalves en aquaculture dans de nombreuses régions du monde. En France, elle est régulièrement touchée par des efflorescences toxiques causées par des dinoflagellés du genre *Alexandrium* producteurs de toxines paralysantes (PST), dont l'espèce cosmopolite *Alexandrium minutum*. Toutes les étapes du cycle de vie des huîtres peuvent être exposées aux efflorescences d'*A. minutum*.

Les études portant sur l'interaction entre *A. minutum* et *C. gigas* se sont principalement intéressées aux cinétiques d'accumulation, de détoxification et de dépuration des PST (Guéguen et al., 2011, 2008; Lassus et al., 2004), ou les effets d'*A. minutum* sur le comportement, la physiologie, et les rythmes biologiques de *C. gigas* (Haberhorn et al., 2010a, 2011; Mat et al.,

2013; Payton et al., 2017; Tran et al., 2010, 2015). Récemment, des études ont mis en évidence qu'*A. minutum*, comme certaines autres micro-algues toxiques, produit également d'autres composés bioactifs dont la nature chimique est inconnue, qui semblent parfois plus toxiques que les PST pour les espèces marines (Borcier et al., 2017; Lelong et al., 2011; Long et al., 2018a). De plus, l'effet d'*Alexandrium minutum* sur la reproduction de *C. gigas* reste encore peu étudié et les expériences réalisées sur le sujet sont surtout des expositions aiguës. Quelques études ont démontré des effets délétères d'*A. minutum* sur les gamètes directement exposés à la micro-algue toxique (Le Goïc et al., 2014) ou après exposition d'huîtres adultes matures (Haberhorn et al., 2010b). A notre connaissance, aucune étude ne s'est intéressée aux effets d'*A. minutum* sur le développement embryo-larvaire de *C. gigas*.

Dans ce contexte, le but principal de cette thèse est de caractériser les effets d'*Alexandrium minutum* sur la reproduction et le développement embryo-larvaire de l'huître creuse *Crassostrea gigas* et d'appréhender les potentielles conséquences sur le recrutement.

Le premier objectif de la thèse était d'étudier les effets d'une exposition à long terme à *A. minutum* sur la gamétogenèse des huîtres et leurs conséquences sur le développement précoce de la descendance.

Le deuxième objectif de ce travail était de discriminer les effets respectifs des PST et des BEC produits par *A. minutum* sur la physiologie des huîtres adultes, des gamètes et des larves.

Le troisième objectif était d'étudier les effets d'une exposition des gamètes à *A. minutum*.

Le quatrième objectif était de déterminer les conséquences d'une efflorescence d'*A. minutum* sur le développement embryo-larvaire de l'huître.

Après ce **premier chapitre** d'introduction générale présentant l'état des connaissances actuelles concernant les impacts des efflorescences de micro-algues toxiques, et plus particulièrement les effets des dinoflagellés du genre *Alexandrium* sur les bivalves marins, la suite de ce travail de thèse est structurée en 4 chapitres visant à répondre aux objectifs fixés et aux questions suivantes :

- **Chapitre 2 :**

Quels sont les effets d'une efflorescence d'*A. minutum* sur la gamétogenèse des huîtres adultes et les potentielles conséquences sur le développement de la descendance ? Quelles

sont les conséquences d'une exposition des huîtres pendant le développement embryonnaire ? (objectifs 1 et 4)

Ce chapitre fait l'objet d'un article soumis à Environmental Pollution :

Castrec, J., Hégaret, H., Picard, M., Soudant, P., Alunno-Bruscia, M., Petton, B., Boulais, M., Suquet, M., Quéau, I., Ratiskol, D., Le Grand, J., Huber, M., Le Goïc, N., Mingant, C., Fabioux, C. (submitted) The dinoflagellate *Alexandrium minutum* affects development of the oyster *Crassostrea gigas*, through parental or direct exposure.

- **Chapitre 3 :**

Quels sont les effets respectifs des PST et des BEC produits par *A. minutum* sur la physiologie et le comportement des huîtres adultes ? (objectif 2)

Ce chapitre fait l'objet d'un article publié dans Aquatic Toxicology :

Castrec J., Soudant P., Payton L., Tran D., Long M., Miner P., Lambert C., Le Goïc N., Huvet A., Quillien V., Bouillot F., Zouher A., Fabioux C, Hégaret H. (2018) Bioactive extracellular compounds produced by the dinoflagellate *Alexandrium minutum* are highly detrimental for oysters. *Aquatic Toxicology*, 199, 188-198.

- **Chapitre 4 :**

Quelle est la sensibilité des gamètes libres de *C. gigas* à *A. minutum* et à ses toxines ? (objectifs 2 et 3)

Ce chapitre fait l'objet d'un article en cours de préparation :

Castrec J., et al. (in prep-a) The toxic dinoflagellate *Alexandrium minutum* affects gametes and fertilization of the oyster, *Crassostrea gigas*.

- **Chapitre 5 :**

Quels sont les stades de développement de l'huître les plus sensibles à *A. minutum* ? Quels sont les effets respectifs des PST et des BEC produits par *A. minutum* sur le développement embryonnaire de l'huître ? (objectifs 2 et 4)

Ce chapitre fait l'objet d'un article en cours de préparation :

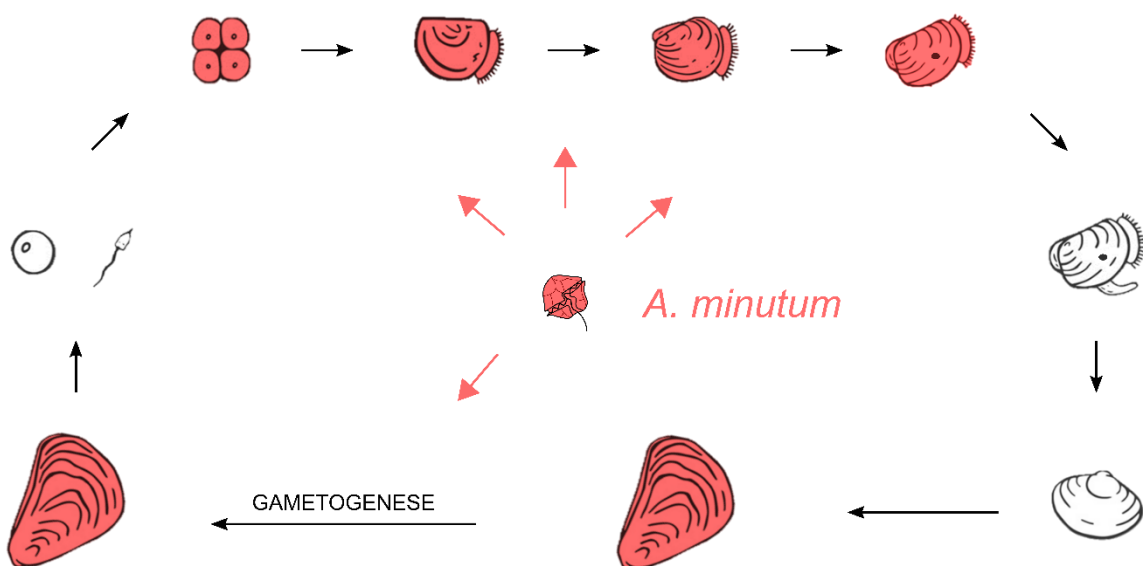
Castrec J., et al. (in prep-b) Stage-specific detrimental effects of the dinoflagellate *Alexandrium minutum* and its toxins on development of the oyster, *Crassostrea gigas*.

Enfin, un sixième et dernier chapitre de synthèse permettra de discuter des résultats présentés et de les mettre en perspective.

Chapitre 2

Quels sont les effets d'une efflorescence d'*A. minutum* sur la gamétogenèse des huîtres adultes et les potentielles conséquences sur le développement de la descendance ? Quelles sont les conséquences d'une exposition des huîtres pendant le développement embryon-larvaire ?

La reproduction des bivalves peut être perturbée suite à l'exposition des adultes ou des jeunes stades de vie à certaines micro-algues toxiques. Dans le cadre de la présente étude, nous avons choisi dans un premier temps de déterminer l'impact d'une exposition de 2 mois d'huîtres adultes à *A. minutum* sur la gamétogenèse et l'effort reproducteur. L'effet d'*A. minutum* sur le développement embryon-larvaire a ensuite été étudié en exposant la descendance pendant 1 mois supplémentaire. Pour cette expérience en laboratoire de 3 mois, nous avons choisi d'exposer les huîtres à une souche d'*A. minutum* produisant des PST et des composés extracellulaires bioactifs (BEC), ces deux types de toxines pouvant être produites lors des efflorescences. Les paramètres étudiés étaient : le poids, la croissance, l'activité de nutrition, le stade de maturité, l'occupation gonadique des huîtres adultes, la qualité et les caractéristiques cellulaires, moléculaires et biochimiques des gamètes, ainsi que la croissance, la survie et la fixation des larves.



Les hypothèses avancées étaient :

1. La quantité et/ou la qualité des gamètes est affectée suite à une exposition de 2 mois des huîtres à *A. minutum*,
2. L'exposition des parents peut affecter le développement embryo-larvaire de la descendance,
3. L'exposition directe des larves à *A. minutum* altère leur développement.

Ce chapitre fait l'objet d'un article publié dans Environmental Pollution :

Castrec, J., Hégaret, H., Alunno-Bruscia, M., Picard, M., Soudant, P., Petton, B., Boulais, M., Suquet, M., Quéau, I., Ratiskol, D., Foulon, V., Le Goïc, N., Fabioux, C., 2019. The dinoflagellate *Alexandrium minutum* affects development of the oyster *Crassostrea gigas*, through parental or direct exposure. Environ. Pollut. 246, 827–836.

The dinoflagellate *Alexandrium minutum* affects development of the oyster *Crassostrea gigas*, through parental or direct exposure

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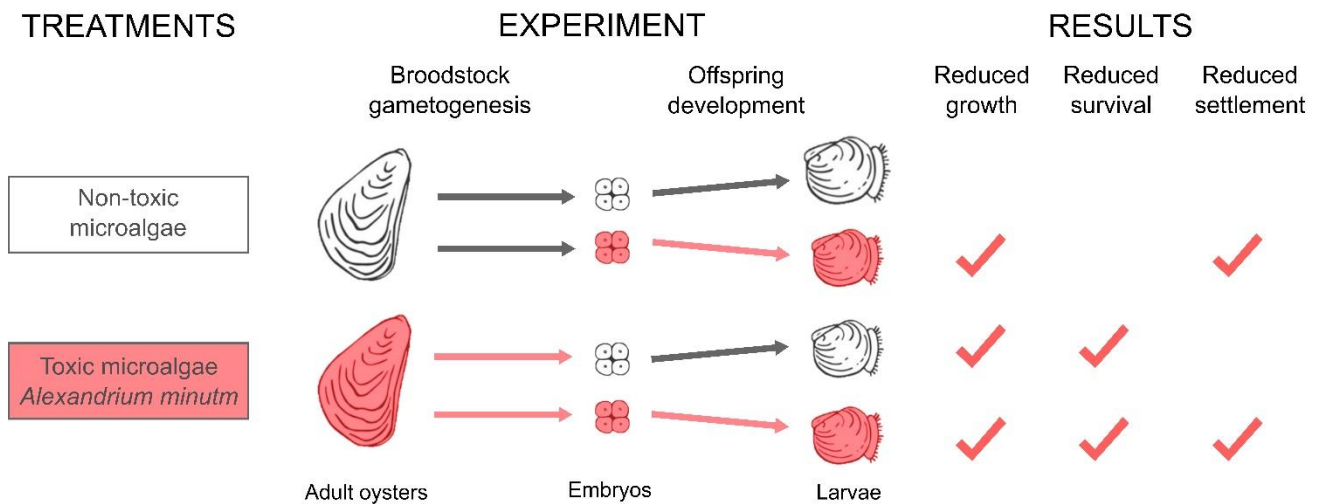
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Highlights

- Two-month exposure of adult oysters to *Alexandrium minutum* decreased sperm motility.
- Oocytes and spermatozoa of exposed oysters contained paralytic shellfish toxins.
- Larvae derived from these gametes showed reduced growth and survival.
- Exposure of oyster larvae to *A. minutum* altered larval growth and settlement.
- Adult exposure influenced offspring response to *A. minutum* exposure.

Graphical abstract



Capsule:

The toxic dinoflagellate *Alexandrium minutum* affected larval development of the oyster *Crassostrea gigas*, directly through exposure of larvae and indirectly through parental exposure during gametogenesis.



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The dinoflagellate *Alexandrium minutum* affects development of the oyster *Crassostrea gigas*, through parental or direct exposure[☆]

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ABSTRACT

Harmful algal blooms are a threat to aquatic organisms and coastal ecosystems. Among harmful species, the widespread distributed genus *Alexandrium* is of global importance. This genus is well-known for the synthesis of paralytic shellfish toxins which are toxic for humans through the consumption of contaminated shellfish. While the effects of *Alexandrium* species upon the physiology of bivalves are now well documented, consequences on reproduction remain poorly studied. In France, *Alexandrium minutum* blooms have been recurrent for the last decades, generally appearing during the reproduction season of most bivalves including the oyster *Crassostrea gigas*. These blooms could not only affect gametogenesis but also spawning, larval development or juvenile recruitment. This study assesses the effect of toxic *A. minutum* blooms on *C. gigas* reproduction. Adult oysters were experimentally exposed to *A. minutum*, at environmentally realistic concentrations (10^2 to 10^3 cells mL⁻¹) for two months during their gametogenesis and a control group, not exposed to *A. minutum* was fed with a non-toxic dinoflagellate. To determine both consequences to next generation and direct effects of *A. minutum* exposure on larvae, the embryo-larval development of subsequent offspring was conducted with and without *A. minutum* exposure at 10^2 cells mL⁻¹. Effects at each stage of the reproduction were investigated on ecophysiological parameters, cellular responses, and offspring development. Broodstock exposed to *A. minutum* produced spermatozoa with decreased motility and larvae of smaller size which showed higher mortalities during settlement. Embryo-larval exposure to *A. minutum* significantly reduced growth and settlement of larvae compared to non-exposed offspring. This detrimental consequence on larval growth was stronger in larvae derived from control parents compared to offspring from exposed parents. This study provides evidence that *A. minutum* blooms, whether they occur during gametogenesis, spawning or larval development, can either affect gamete quality and/or larval development of *C. gigas*, thus potentially impacting oyster recruitment.

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1. Introduction

Harmful algal blooms (HABs) have increasingly disrupted coastal ecosystems for the last few decades (Kudela et al., 2015). Among toxic species, blooms of microalgae producing paralytic shellfish toxins (PST) represent an important threat for marine

ecosystems, human health and the economy in coastal areas. Blooms of *Alexandrium* affect marine ecosystems by disrupting community and food web structures (Hallegraeff, 2010), and causing death of seabirds and mammals (Hattenrath-Lehmann et al., 2017). The resulting PST can affect humans via shellfish poisoning events (Anderson et al., 2012a), as filter-feeding bivalves accumulate PST in their flesh by feeding on PST-producing microalgae (Bricelj and Shumway, 1998). Prohibition of shellfish harvesting, impairment of tourism and recreational activities are direct socio-economic consequences of PST-producing microalgae blooms in coastal regions (Anderson et al., 2012b). Detrimental effects of

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those phenomena are expected to intensify as the geographical distribution of HABs and the use of coastal waters for aquaculture are increasing worldwide (Lassus et al., 2016).

In marine ecosystems, outbreaks of Paralytic Shellfish Poisonings (PSPs) are mainly caused by the globally distributed dinoflagellate genus *Alexandrium* (Rossini, 2014). Blooms of *Alexandrium* spp. affect the physiology of bivalves including exploited species such as *Mytilus edulis*, *Pecten maximus*, *Venerupis philippinarum*, and *Crassostrea gigas* (Borcier et al., 2017; Bricelj et al., 1993; Haberkorn et al., 2010a; Lassudrie et al., 2014). *Alexandrium minutum* modifies valve behavior, disrupts biological rhythms, affects defense responses and digestion of *C. gigas* (Haberkorn et al., 2010b, 2011; Mat et al., 2013; Mello et al., 2013; Payton et al., 2017; Tran et al., 2010).

In France, *A. minutum* blooms usually occur from April to November and can reach high concentrations ($>10^7$ cells L^{-1}) (Guallar et al., 2017; Chapelle et al., 2015). In French coastal waters, *A. minutum* blooms may last several months (Guallar et al., 2017) and are often concomitant with breeding period of bivalves, including the oyster *C. gigas* (Pouvreau et al., 2016; Suquet et al., 2016; Ubertini et al., 2017). Marine bivalves are ecosystem engineers and a key resource in coastal areas (Ekstrom et al., 2015). In France, 64,200 tons of Pacific oysters were produced in 2016, representing a value of 360,400 USD (FAO, 2018). In France, the natural recruitment of oyster spat constitutes a large part of cultivated stocks and repeated recruitment failures could have negative impacts on local oyster aquaculture. Given the economic importance of oysters, an understanding of the effects of *Alexandrium* blooms on the reproduction of *C. gigas* is critically important. Adult Pacific oysters which reproduce during the summer are frequently exposed to the harmful algae during gametogenesis. Gametes, embryos and larvae, the free-living stages of oysters, also directly experience the toxicity of *Alexandrium* with potential consequences on recruitment.

Short-term exposure of mature Pacific oysters to *Alexandrium* spp. affected spermatozoa quality, and *in vitro* exposure of oocytes to *A. minutum* increased reactive oxygen species production in oocytes (Haberkorn et al., 2010b; Le Goïc et al., 2013). Knowledge regarding potential effects of adult oyster exposure to ecological concentrations of HAB species on the next generation remains scarce (Rolton et al., 2018, 2016; Vasconcelos et al., 2010). Adult eastern oysters *Crassostrea virginica* exposed to a natural bloom of the brevetoxin producer, *Karenia brevis*, produced smaller larvae with higher mortality than non-exposed oysters (Rolton et al., 2016). Northern quahogs *Mercenaria mercenaria* exposed to *K. brevis* showed reduced gonadal allocation and fertilization success, and the early development of the subsequent offspring was also affected (Rolton et al., 2018). Concerning direct exposure of larvae, acute short-term exposures of *Alexandrium* spp. negatively affected survival, growth, activity, and settlement of bivalve larvae, including *C. gigas*, *Pinctada fucata martensii*, *Mercenaria mercenaria*, *Chlamys farreri*, and *Argopecten irradians concentricus* (Basti et al., 2015; Matsuyama et al., 2001; Mu and Li, 2013; Tang and Gobler, 2012; Yan et al., 2003, 2001). However, the effects of *A. minutum* blooms on oyster larvae remain unknown.

The present study investigated the effects of a 3-month exposure to toxic *A. minutum* at concentrations similar to natural bloom events on the gametogenesis of adult *C. gigas* and on their offspring. We assessed the weight (total, shell, and flesh), feeding, sex and gametogenesis stage of adult oysters, the quality and cellular, molecular and biochemical characteristics of gametes, and the growth, survival and settlement of larvae. The hypotheses tested were: (1) Gamete quality will be negatively affected by a long-term exposure to *A. minutum*, (2) Parental exposure may adversely affect subsequently produced offspring, and (3) Direct exposure of larvae to *A. minutum* will alter larval development.

2. Materials and methods

2.1. Biological materials

2.1.1. Oysters

Adult oysters *Crassostrea gigas* (*Magallana* gen. nov.; Salvi and Mariottini, 2017) were produced and cultured in controlled conditions according to a standardized protocol (Petton et al., 2015), in Ifremer experimental facilities (Argenton and Bouin, France). Oysters were never exposed to any harmful algal bloom. Histological inspection at the start of the experiment in April 2016 showed that oysters (12 months old, mean total weight 18.0 ± 0.4 g) were in early gametogenesis (stage 1), according to Steele and Mulcahy (1999).

2.1.2. Algal cultures

Tisochrysis lutea (formerly *Isochrysis* sp., T. iso strain, CCAP 927/14) and *Chaetoceros* sp. (formerly *Chaetoceros neogracile*, strain CCAP 1010-3) were used as non-toxic food for oysters. They were cultured with continuous light ($200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in separated 300-L cylinders enriched with Conway medium (Walne, 1970), and with silicium for *Chaetoceros* sp. The dinoflagellates *Alexandrium minutum* (Halim, AM89BM strain) and *Heterocapsa triquetra* (Ehrenberg, HT99PZ strain) were grown in filtered seawater ($0.2 \mu\text{m}$) supplemented with L1 medium (Guillard and Hargraves, 1993) and kept in exponential growth phase. Cultures were maintained at 17 ± 1 °C. This *A. minutum* strain produced a quantity of Paralytic Shellfish Toxins (PST) equivalent to 1.3 ± 0.1 pg eq. STX per cell in the exponential growth phase (Haberkorn et al., 2010a,b). This strain also produces bioactive extracellular compounds (BECs), which have allelopathic and cytotoxic activities (Borcier et al., 2017; Castrec et al., 2018; Lelong et al., 2011). The non-toxic *H. triquetra* was chosen as a control because of its similarity to *A. minutum* in terms of size and shape (Tran et al., 2015).

2.2. Experimental exposure of adult oysters to *A. minutum*

The flow chart of experimental procedure is presented in Fig. 1. After acclimation, the oysters were placed in six experimental 50-L tanks (46 oysters per tank) supplied with filtered running seawater ($1\text{-}\mu\text{m}$ filtered and UV-treated (FSW); 17.0 ± 0.1 °C, pH 8.3 ± 0.1 , and 35.1 ± 0.1 PSU; 12.5 L h^{-1}), and fed continuously on an algal mixture (1:1 equivalent volume) of *T. lutea* and *Chaetoceros* sp. (Tiso/Chaeto) at a daily ratio equal to 6% dry mass algae/dry mass oyster (Petton et al., 2015). The toxic *A. minutum* was continuously added to Tiso/Chaeto in 3 replicate tanks (exposed treatment) at a concentration of 10^2 cells mL^{-1} , except during the seventh week of the experiment where the concentration was gradually increased to 10^3 cells mL^{-1} for 4 days, mimicking an *A. minutum* bloom event (Chapelle et al., 2015). The 3 other tanks (control treatment) were exposed to the same concentrations of the non-toxic dinoflagellate *H. triquetra*. Algal concentrations and exposure time correspond to environmentally realistic conditions (Chapelle et al., 2015). To prevent algal sinking, the water inflow was pressurized to create recirculating flow in the tank and air bubbling was used. The oysters were conditioned for 8 weeks to reach ripeness.

2.3. Algal consumption of adult oysters

For each algal treatment, a fourth tank was deployed without oysters to evaluate algal sinking. Once a day, inflow and outflow seawater was sampled from each tank (20 mL). Phytoplankton counts were made using an electronic particle counter (Multisizer 3 equipped with a $100\text{-}\mu\text{m}$ aperture tube) to provide 56 days of continuous data. Algal sinking (S) was evaluated in percentage of

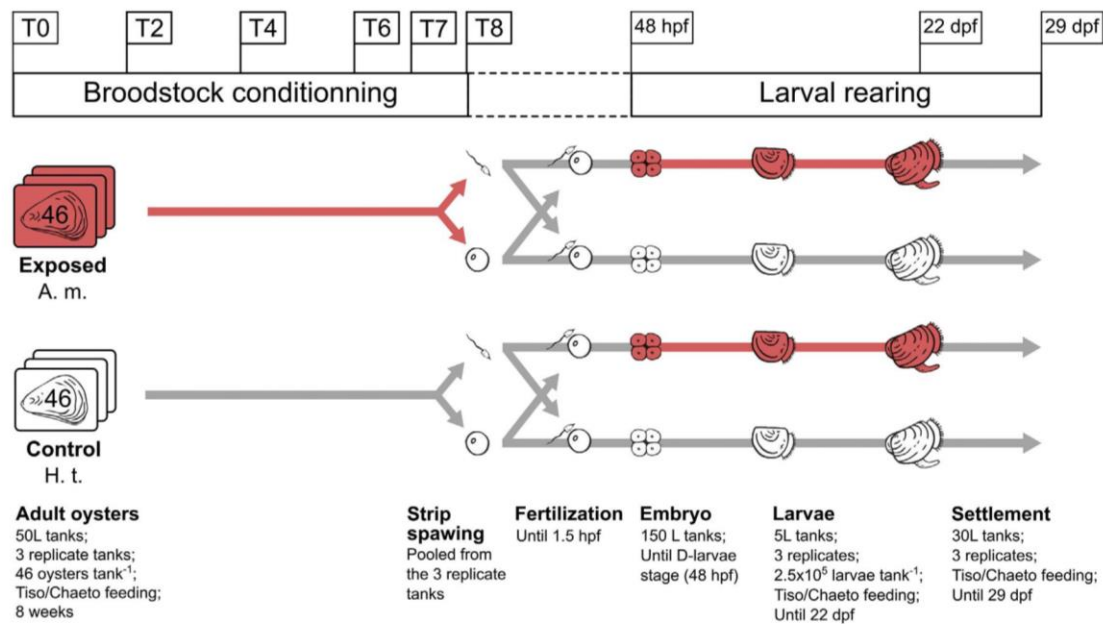


Fig. 1. Flow chart of the experiment. For broodstock conditioning, oysters in red are exposed to the toxic *Alexandrium minutum* (A. m.), whereas oysters in white are fed with non-toxic *Heterocapsa triquetra* (H. t. = control treatment). During adult oyster exposure, oysters were sampled every two weeks (T0, T2, T4, T6 and T8) for PST accumulation and histological analyses to study gametogenesis. Gamete cellular analyses were conducted on oysters sampled after 7 weeks of exposure (T7). For larval rearing, embryos and larvae in red are exposed to the toxic *A. minutum*, whereas stages in white are non-exposed. Tiso/Chaeto feeding: *Tisochrysis lutea* and *Chaetoceros* sp. feeding *ad libitum*; hpf: hours post-fertilization; dpf: days post-fertilization. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

microalgae retained in the tank without oysters: $S = [(V_0 - V_i) / V_i] \times 100$; V_i being the number of algae at the inlet of the control tank, and V_0 the number of algae at the outlet. Algal sinking was low (<4%) and similar in the two treatments. Total algal consumption, i.e. dinoflagellate and Tiso/Chaeto, was expressed in algal cell volume per oyster per hour ($\mu\text{m}^3 \text{ oyster}^{-1} \text{ h}^{-1}$), as in Sussarellu et al. (2016).

2.4. Sampling

At 2, 4, 6, and 8 weeks after the beginning of exposure (corresponding to T2, T4, T6 and T8, respectively), five animals per tank were sampled for flesh mass, and a transversal section of the gonadic area for histological examination (Fig. 1). The remainder of the digestive gland was flash frozen in liquid nitrogen and stored at -20°C for subsequent PST quantification. At the beginning (T0) and at the end (T8) of the experiment, 15 oysters per algal treatment were sampled to measure biometric parameters (total wet mass (TWM), wet shell mass (WSM), and dry flesh mass (DFM)). Condition index was calculated as: $\text{DFM} \times 100 / (\text{TWM} - \text{WSM})$.

Six control and 6 exposed oysters of each sex were collected after 7 weeks of broodstock conditioning (T7) for gamete quality measurements (see section 1.6).

Gametes were collected at T8 in the remaining control and exposed animals (45 oysters per treatment, 15 oysters per tank) by stripping entirely the gonad, and pooling gametes of oysters from the same tank, for each sex. Oocytes were suspended in FSW, filtered in a 100- μm sieve, and oocyte concentrations were determined by flow cytometry (FCM) according to Le Goïc et al. (2014). Oocytes were transferred in 2 mL tubes for PST quantification and biochemical composition measurements (2×10^6 oocytes for each analysis, stored at -20°C), and for RNA analyses (4×10^6 oocytes, stored in liquid nitrogen). Sperm samples for PST content and biochemical composition were obtained by direct pipetting the incised male gonad. For larval rearing, gametes were pooled for each treatment

and each sex (Fig. 1).

2.5. Histology

A 3-mm cross section of the visceral mass was excised in front of the pericardial region and immediately fixed in modified Davidson's solution (Latendresse et al., 2002) for 24 h at 4°C . Tissues were processed, stained with Harris' hematoxylin–eosin, and observed as described by Hermabessiere et al. (2016). Tissue sections were examined under a light microscope (Leica DMIRB) equipped with a digital camera (Imaging RETIGA 2000R) to determine the sex and gametogenesis stage of each oyster according to the reproductive scale reported by Steele and Mulcahy (1999). In addition, measurement of reproductive effort was determined by image software (Adobe® Photoshop®): gonadal occupation index is the percentage of whole gonadal area in relation to the total transverse section area (Fabioux et al., 2005).

2.6. Gamete cellular analyses

2.6.1. Gamete collection

Oocytes and spermatozoa were collected at T7 for each oyster by stripping entirely the gonad, according to Boulais et al. (2017) for spermatozoa and Le Goïc et al. (2014) for oocytes. Spermatozoa were collected in 10 mL of FSW at 19°C and sieved through 60 μm mesh. Oocytes were suspended in FSW, sieved through 100 μm to remove pieces of gonad tissue and concentrated on a 20- μm sieve. Oocyte and spermatozoa concentrations were determined by FCM according to Le Goïc et al. (2013, 2014) and adjusted to 5×10^4 oocytes mL^{-1} and 1×10^7 spermatozoa mL^{-1} , for FCM and cellular analyses.

2.6.2. Cellular parameters by flow cytometry

FCM measurements were performed using an EasyCyte Plus cytometer (Guava Millipore) equipped with standard optics and a

488 nm argon laser. Analyses of gamete relative size and complexity, viability, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) production were performed according to Le Goïc et al. (2013, 2014) for spermatozoa and oocytes.

2.6.3. Oocyte morphological measurements

Oocyte circularity (ranging from 0 to 1, where a value of 1 indicates a perfect circle) and Feret's diameter (Ferreira and Rasband, 2012) were measured under microscope, using ImageJ software ($n = 30$ oocytes for each female) according to Boulais et al. (2015a).

2.6.4. Characterization of spermatozoa movement

Spermatozoa movement was triggered using a two-step dilution in an activating solution (FSW, 5 g L^{-1} bovine serum albumin, Tris 20 mM, pH 8.1, dilution rate 1:30) and analyzed under microscope using a CASA plug-in for ImageJ software. The percentage of motile spermatozoa and their velocity (VAP: Velocity of the Average Path) were assessed on a minimum of 30 spermatozoa for each male, according to Boulais et al. (2015b).

2.6.5. Quantification of ATP content in spermatozoa

Intracellular ATP content of spermatozoa was estimated in triplicates using 5×10^6 spermatozoa for each male as described in Boulais et al. (2015b), by bioluminescence (kit ATPlite, Perkin Elmer) using a plate reader (EnSpire™ 2300 Multilabel Reader, PerkinElmer).

2.7. Quantification of total RNA in oocytes

Total RNA was isolated using Tri-reagent (Sigma), treated with rDNase (Macherey-Nagel), purified using affinity chromatography (Nucleospin RNA kit, Macherey-Nagel) according to the manufacturer's instructions, and assayed for concentration using a ND-1000 spectrophotometer (Nanodrop Technologies).

2.8. Protein, lipid, and carbohydrate compositions of gametes

Total lipids and carbohydrates were analyzed as described by Bligh and Dyer (1959), and Dubois et al. (1956), respectively. Total protein content was assayed as described by Da Costa et al. (2015). Dry weights were determined with a 400 μL aliquot of the first fraction distributed in pre-weighed capsules and dried at 80°C for 48 h. Content of each constituent was expressed as: biochemical content of each constituent in $\text{mg} \times 100/\text{dry mass of gonad in mg}$.

2.9. Toxin quantification

PST extraction was performed individually following manufacturer instructions: digestive gland tissue was homogenized in HCl 0.1 M (1:1, w:v) using a Precellys®24 beads-grinder, then boiled for 5 min. For the sampling times T2, T4, and T8, individual homogenates were pooled for each tank ($n = 3$ pools of 5 oysters for each sampling time). The PST digestive gland content was analyzed individually just before (T6, $n = 15$) and after (T7, $n = 12$) the high dinoflagellate bloom simulated in this experiment. Toxin quantification was performed by spectrophotometry using the Abraxis ELISA PSP kit (Novakits, France; see methods in Lassudrie et al. (2015)). Toxin load was expressed in $\mu\text{g STX } 100 \text{ g}^{-1}$ of wet digestive gland weight (DG) or gonad weight.

2.10. Larval rearing

To test the influence of parental exposure on offspring, fertilization was performed for each treatment; a pool of 15×10^6

oocytes (from 33 control females or 29 exposed females) were fertilized separately in a beaker using a pool of sperm (from 12 control males or 16 exposed males) using a non-limiting sperm to oocyte ratio (100:1) (Fig. 1). Embryos were transferred 1.5 h post-fertilization (hpf) in 150-L tanks in UV-treated FSW at a concentration of 50 embryos mL^{-1} and maintained 48 h at 21°C . D-larvae were then transferred to 5-L cylindrical triplicate tanks at the density of 50 larvae mL^{-1} , and maintained in a flow-through rearing system (100% seawater renewal h^{-1} , 21°C , 35 PSU) (Fig. 1). The larvae were fed continuously with Tiso/Chaeto as described by Asmani et al. (2016).

To test for influence of offspring exposure, fertilization and larval rearing were performed for each algal treatment as described above, but the subsequent offspring were continuously exposed to *A. minutum* (10^2 cells mL^{-1}), from 4-cell embryos (2.5 hpf) to veliger larvae (22 days post-fertilization, dpf) (Fig. 1).

Larvae were sampled every 2–3 days and fixed in a 0.1% formaldehyde-seawater solution until image analysis for size monitoring. Larval size was assessed by measuring shell length using image analysis on at least 30 larvae per tank per day of sampling (WinMager 2.0 and ImageJ software for image capture and analysis, respectively).

A small complementary experiment was carried out on oyster larvae raised on the same conditions as the control larvae to test the capacity of larvae to ingest *A. minutum* cells. Umbonate larvae (mean shell length $150 \pm 22 \mu\text{m}$) and eyed larvae (mean shell length $304 \pm 15 \mu\text{m}$) were exposed to *A. minutum* (AM89BM strain) and the presence of *A. minutum* cells in larvae digestive tract and feces was checked under light microscope.

2.11. Larval survival and settlement

All the tanks were drained and the total number of larvae was determined at 22 dpf (Fig. 1), when $\geq 50\%$ of larvae reached the eyed larvae stage (morphological competence for metamorphosis) in the control tanks (non-exposed oysters derived from control parents). Each larval population was transferred to a PVC container with a 125- μm nylon mesh base (in triplicate per treatment), maintained in a flow-through system (9 L h^{-1} , $30\% \text{ h}^{-1}$ seawater renewal, 21°C , 35 PSU) and fed the Tiso/Chaeto diet as described previously. After 7 days (29 dpf), the number of remaining swimming larvae, dead larvae, and larvae settled on tank walls and sieve were counted (Fig. 1). The percentage of survival during the settlement step was evaluated as: total number of alive larvae at 29 dpf $\times 100/\text{total number of larvae initially stocked at 22 dpf}$. The larval settlement was evaluated as: number of settled larvae at 29 dpf $\times 100/\text{total number of alive larvae at 29 dpf}$.

2.12. Statistical analyses

Statistical analyses were performed using R version 3.2.2. All values are expressed as mean \pm standard error (SE). Differences were considered significant when $p < 0.05$. Differences in oyster algal consumption were evaluated using repeated measures ANOVA, where 'algal exposure' and 'day' were fixed factors (Huvet et al., 2015). Comparison for oyster gonadal maturation and occupation index between the two algal treatments were investigated for each sampling time using Mann-Whitney *U* test and *t*-test, respectively. Results of oyster condition index, gonadal maturation and occupation were pooled for the 3 tanks of each algal treatment, after verifying there were no statistical differences between tanks using one-way ANOVA, Fisher's exact test, and *t*-test, respectively. Number of oocytes, and gamete parameters were compared using *t*-test. Comparison for PST content in oyster digestive glands

between sampling times was assessed using a non-parametric Kruskal-Wallis test with Mann-Whitney pairwise as post hoc test, as homoscedasticity assumption was not met. To determine any significant differences between 'parental exposure', and 'larval exposure' on larvae, larval length, survival, and settlement were analyzed using a two-way ANOVA, where 'parental exposure' and 'larval exposure' were fixed and orthogonal factors. Levene's test was used to determine any heterogeneity of variances and data were transformed if significant. In case of a significant interaction between the two factors, a Tukey HSD was used to detect differences among means.

3. Results

3.1. Oyster algal consumption, gonad development and biochemical composition

No mortality was observed in adult oysters during the experiment. A significantly higher algal consumption (+10%, $F = 66.93$, $df = 1$, $p < 0.01$; two-way ANOVA) was observed over the whole experiment for oysters exposed to *A. minutum* ($3.32 \times 10^9 \pm 5.72 \times 10^7 \mu\text{m}^3$ of algae oyster $^{-1} \text{h}^{-1}$) compared to control oysters ($3.02 \times 10^9 \pm 5.87 \times 10^7 \mu\text{m}^3$ of algae oyster $^{-1} \text{h}^{-1}$) when averaged over the whole experiment. There was a significant effect of date ($F = 114.88$, $df = 47$, $p < 0.001$; two-way ANOVA), and a date-exposure interaction on algal consumption ($F = 4.92$, $df = 47$, $p < 0.001$; two-way ANOVA). No difference ($t = 0.39$, $df = 28$; t -test) in condition index was observed between exposed and control oysters at the end of the conditioning (10.8 ± 0.5 and 10.6 ± 0.4 , respectively).

Gonadal maturation was not different between exposed and control oysters during broodstock conditioning (Table S1). At T6, all oysters were ripe (stage 3) in both treatments and gonadal occupation index was not significantly different between exposed ($61.5 \pm 3.0\%$) and control ($55.1 \pm 3.1\%$) oysters (Table S1). The total number of oocytes collected by stripping at the time of breeding was not significantly different ($t = -1.06$, $df = 4$; t -test) in exposed ($5.4 \times 10^6 \pm 0.4$) and control ($6.5 \times 10^6 \pm 0.9$) females.

Gonad lipid content was higher (+55%, $t = 3.98$, $df = 4$, $p < 0.05$; t -test) in exposed males ($17.0 \pm 1.5\%$) than in controls ($10.9 \pm 0.4\%$), but protein and carbohydrate contents were not different (Table S2). No significant difference in oocyte biochemical composition was observed between both treatments (Table S2).

3.2. Gamete quality

Significantly lower percentage of motile spermatozoa (-36% , $t = -2.94$, $df = 9$, $p < 0.05$; t -test) was observed in exposed oysters ($30 \pm 5\%$) compared to control ($48 \pm 3\%$) oysters (Table S3). Spermatozoa velocity and ATP content were similar between the two treatments (Table S3). Oocyte diameter and circularity did not differ between treatments (Table S3). Oocyte and spermatozoa cellular characteristics measured by flow cytometry were similar in exposed and control oysters (Table S3).

3.3. Paralytic shellfish toxin content

The PST content in the digestive glands of exposed oysters was 10.9 ± 0.5 , 26.7 ± 1.6 , and $19.3 \pm 3.5 \mu\text{g STX } 100 \text{ g}^{-1}$ of DG after 2 (T2), 4 (T4), and 6 weeks of conditioning (T6), respectively. The PST content in the digestive glands of exposed oysters was higher ($p < 0.05$; Kruskal-Wallis) at T7 ($131.3 \pm 22.4 \mu\text{g STX } 100 \text{ g}^{-1}$ of DG) and T8 ($52.5 \pm 5.0 \mu\text{g STX } 100 \text{ g}^{-1}$ of DG) (i.e. after the mimicked *A. minutum* bloom at $10^3 \text{ cells mL}^{-1}$) than at T2, T4 and T6.

The PST content in exposed oyster gonads measured at T8 was $1.8 \pm 0.1 \mu\text{g STX } 100 \text{ g}^{-1}$ wet gonad weight ($n = 3$ pools of 4–6 oysters) for exposed males, and $0.3 \pm 0.1 \mu\text{g STX } 100 \text{ g}^{-1}$ wet oocytes weight ($n = 3$ pools of 9–10 oysters) for exposed females, corresponding to 3.3% and 0.6% of the PST content measured in the digestive glands, respectively.

3.4. Larval length, survival and settlement

Larval length was significantly affected by both parental and larval exposures to *A. minutum* (Fig. 2). Larval and parental exposures had significant independent ($F = 39.04$, $df = 1$, $p < 0.001$, and $F = 7.134$, $df = 1$, $p < 0.05$, respectively; two-way ANOVA) and interactive effects ($F = 15.22$, $df = 1$, $p < 0.01$; two-way ANOVA) upon larval length at 22 dpf (Fig. 3A). At 22 dpf, non-exposed larvae derived from exposed parents ($248.3 \pm 11.3 \mu\text{m}$) were significantly smaller (-15% , $p < 0.01$; Tukey HSD) compared to non-exposed larvae derived from control parents ($293.2 \pm 3.7 \mu\text{m}$).

Exposed larvae derived from control parents ($223.9 \pm 4.4 \mu\text{m}$) were smaller (-24% , $p < 0.001$; Tukey HSD) compared to non-exposed larvae derived from control parents, whereas the length of exposed larvae derived from exposed parents ($232.3 \pm 5.1 \mu\text{m}$) was not significantly different (-6%) from non-exposed larvae

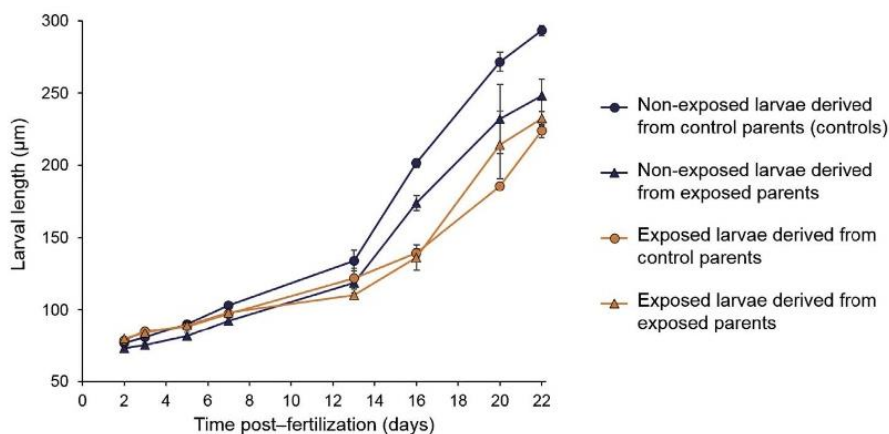


Fig. 2. Larval length from D-larvae up to metamorphosis of the controls (non-exposed larvae derived from control parents) and the three other combination of parental and larval exposures. Larval groups were obtained by crossing gametes collected from adult *Crassostrea gigas* exposed to the non-toxic *Heterocapsa triquetra* (control parents) and from oysters exposed to *Alexandrium minutum* (exposed parents), and then exposing the offspring continuously to *A. minutum* (exposed larvae) or not (non-exposed larvae). Mean \pm SE, $n = 3$.

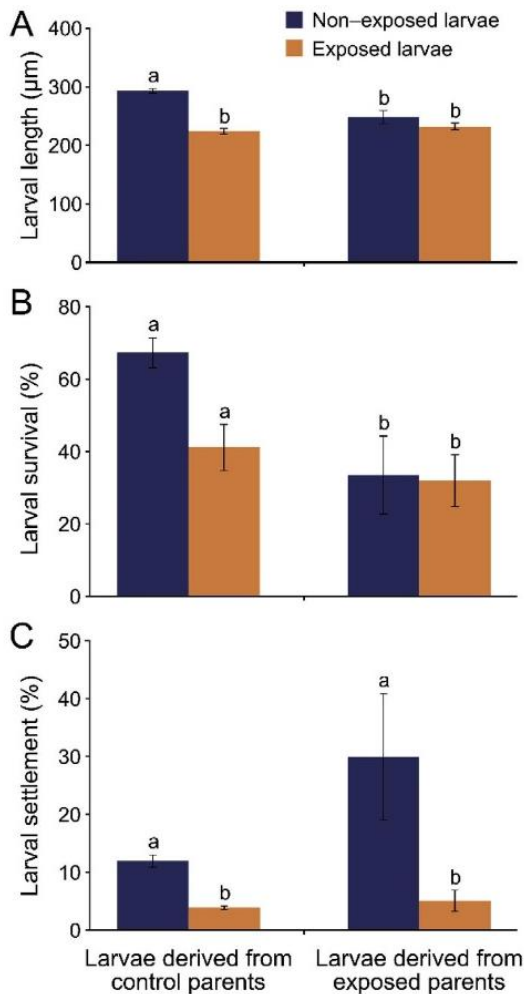


Fig. 3. Larval length 22 days post-fertilization (dpf) (A), survival (B) and settlement (C) of *C. gigas* larvae, non-exposed or exposed to *A. minutum*, derived from *A. minutum* exposed parents or control parents. Survival is estimated as the total number of alive larvae 29 dpf divided by number of larvae initially stocked at 22 dpf. Settlement is calculated as the number of settled larvae at 29 dpf divided by the total number of live larvae at 29 dpf. Mean \pm SE, $n = 3$. Letters denote significant groupings ($p < 0.05$; two-way ANOVA and Tukey HSD).

derived from exposed parents (Fig. 3A). The length of exposed larvae derived from exposed parents was, however, lower (-20% , $p < 0.01$; Tukey HSD) than non-exposed larvae derived from control parents (Fig. 3A).

Parental exposure, but not larval exposure, significantly affected larval survival during the settlement step (Fig. 3B, $F = 8.21$, $df = 1$, $p < 0.05$; two-way ANOVA). Larvae derived from exposed parents had reduced survival ($33.6 \pm 10.8\%$ and $32.0 \pm 7.1\%$ for non-exposed and exposed larvae, respectively) compared to larvae derived from control parents ($67.3 \pm 4.2\%$ and $41.2 \pm 6.3\%$ for non-exposed and exposed larvae, respectively; Fig. 3B).

Larval exposure, but not parental exposure, significantly reduced larval settlement ($F = 8.77$, $df = 1$, $p < 0.05$; two-way ANOVA, Fig. 3C). Settlement rates were $3.9\% \pm 0.3$ and $5.1\% \pm 1.8$ for exposed larvae, and $11.9\% \pm 1.1$ and $30.0\% \pm 10.9$ for non-exposed larvae derived from control and exposed parents, respectively (Fig. 3C).

Ingestion tests on control larvae indicated that eyed larvae fed on *A. minutum*, as these algal cells were observed in the intestine and feces of eyed larvae (Fig. 4C–E). Conversely, ingestion was not

observed with early umbonate larvae due to the relative large cell size of *A. minutum* ($23\text{--}29\ \mu\text{m}$) (Fig. 4A and B).

4. Discussion

Coastal areas regularly experience few days to several weeks of HAB during bivalve reproductive season (Guallar et al., 2017). This experiment was designed to assess the consequences of the toxic dinoflagellate *Alexandrium minutum* upon reproduction, early development, and settlement of *Crassostrea gigas* oysters, an environmentally and economic important species.

4.1. Presence of *A. minutum* modifies feeding of maturing oysters

Algal consumption was significantly higher for oysters exposed to *A. minutum* than for control oysters over the whole experiment. Pousse et al. (2019) applied a mechanistic model based on Dynamic Energy Budget theory to the data of the present study, coupling the kinetics of PST accumulation and bioenergetics in *C. gigas*. They evidenced that toxicant stress provoked by *A. minutum* affected the energy balance of oysters, more energy being needed for tissue damage repair and detoxification of toxic substances produced by *A. minutum*. Exposed adult oysters would therefore increase their food consumption to adjust energy intake. Such modification of feeding activity was observed in oysters exposed to polystyrene microspheres to compensate digestive interference caused by plastic particles (Sussarellu et al., 2016) and has been proposed for female copepods *Acartia (Acartiura) clausi* exposed to *A. minutum* (previously *A. lusitanicum*) (Dutz, 1998).

Under our experimental conditions of dual feeding with *A. minutum* and non-toxic Tiso/Chaeto algae, the higher feeding rate seemed to partly counterbalance the higher energy demand due to *A. minutum*. Coupled with active detoxification of toxins (Fabioux et al., 2015), this could explain the absence of major visible effects on gonadal maturation and reproductive effort. However, the lower percentage of motile spermatozoa in oysters exposed to *A. minutum* still suggests that this response might not be sufficient to overcome PST toxicity on broodstock.

4.2. Broodstock exposure to *A. minutum* affected quality of gametes

In male oysters, exposure to *A. minutum* decreased the percentage of motile spermatozoa. Haberkorn et al. (2010b) evidenced that a short acute exposure of mature oysters to *A. minutum* reduced spermatozoa motility and ATP content, and altered structural and reserve lipids of the digestive gland. In the present study, neither decreased ATP content nor sperm mortality can explain this reduced motility. Total lipid content in male gonads of exposed oysters increased maybe reflecting modifications in lipid metabolism. Lipids are key component of cellular membranes of spermatozoa and modifications in lipid metabolism could be associated with changes in gamete features. PST bind to voltage-gated Na^+ channels with high affinity and interact, to a lesser extent, with Ca^{2+} and K^+ channels, modifying ionic fluxes into cells (Llewellyn, 2006) and associated metabolic pathways (Mat et al., 2018). Spermatozoa motility in Pacific oysters is a key factor for reproduction and notably depends on concentrations of ions including K^+ , Ca^{2+} and Na^+ (Alavi et al., 2014). Indeed, the percentage of motile sperm is drastically reduced in Na^+ -free seawater (Boulais et al., 2018). In the present study, PST could be responsible for the decreased motility observed in spermatozoa through membrane alterations or ionic fluxes changes. In the field, the fertilization rate of oysters could be impaired by fewer motile spermatozoa with negative consequences on recruitment, as proposed for another free-spawning invertebrate, the sea urchin exposed to high doses of cadmium (Au et al., 2001).

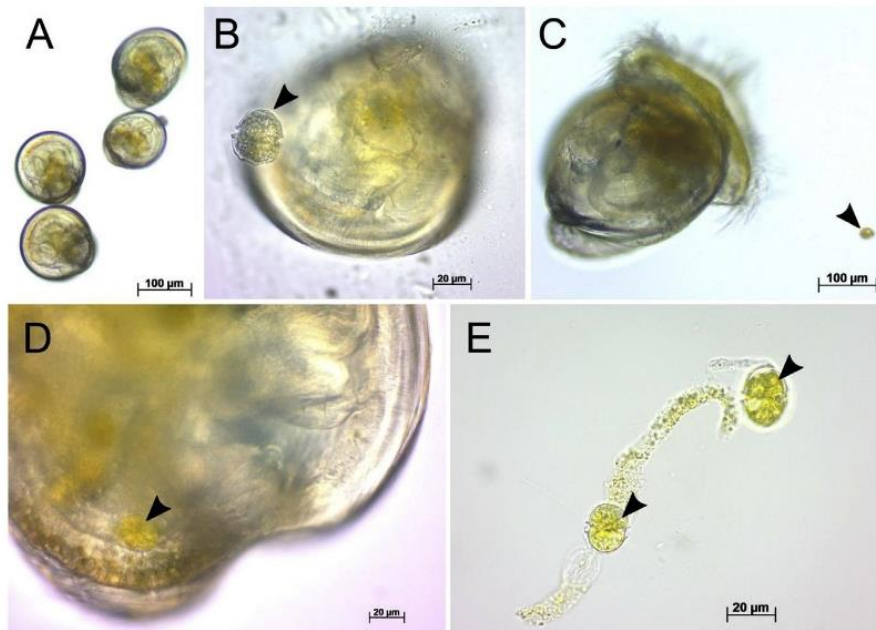


Fig. 4. Capacity of control *C. gigas* larvae to ingest *A. minutum*. Light micrographs. Umbonate larvae (A, B; mean shell length $150 \pm 22 \mu\text{m}$) were unable to ingest *A. minutum* cells due to their relative large size ($23\text{--}29 \mu\text{m}$), whereas eyed larvae (C–E; mean shell length $304 \pm 15 \mu\text{m}$) fed on *A. minutum*: algal cells were observed in the intestine (D) and fecal pellets (E). Black arrows indicate *A. minutum* cells.

The present experimental conditions probably hide this negative effect, as spermatozoa are put in excess compared to oocytes for *in vitro* fertilization, in a small limited volume, increasing pairing probability compared to natural conditions.

4.3. Broodstock exposure to *A. minutum* affected offspring growth and survival

Broodstock exposure to *A. minutum* decreased offspring growth and induced higher mortality in larvae during the settlement period. Intracellular PST initially accumulate in the digestive gland of bivalves following algal cell lysis and are then transferred into other organs, including gonads (Bricelj and Shumway, 1998). In this study, PST was detected in oocytes ($0.3 \pm 0.1 \mu\text{g STX } 100 \text{ g}^{-1}$ wet oocytes weight) and male gonad ($1.8 \pm 0.1 \mu\text{g STX } 100 \text{ g}^{-1}$ wet gonad weight), which is consistent with PST content observed in oocytes of mature oysters exposed to a natural *A. minutum* bloom (Hermabessiere et al., 2016). *Crassostrea virginica* larvae derived from oysters naturally exposed to *Karenia brevis* showed significantly higher mortalities and smaller length than larvae derived from non-exposed oysters, suggesting that these negative effects on larval development may be due to the presence of brevetoxins in oocytes (Rolton et al., 2018, 2016). In the present study, toxic effects on the next generation could thus originate from deleterious effect of PST transferred to offspring via gametes. Maternal effects in eggs influence embryogenesis and larval development (Bayne, 2017). PST may also have resulted in functional cell damage during the process of gametogenesis with consequences on development of offspring. Larval physiology could be affected even later during development and settlement like in the present study.

4.4. Larval growth and settlement are affected by direct exposure to *A. minutum*

A substantial alteration of larval growth was observed for larvae exposed to *A. minutum*, both derived from control and exposed

adult oysters. *Alexandrium* toxicity comes from intracellular PST but also from bioactive extracellular compounds (BECs) produced and excreted in the surrounding water by some *Alexandrium* strains, such as the *A. minutum* strain tested in this study (Borcier et al., 2017; Castrec et al., 2018; Long et al., 2018). These BECs can be allelopathic, cytotoxic, haemolytic, or ichthyotoxic (Arzul et al., 1999; Ford et al., 2008; Lelong et al., 2011; Mardones et al., 2015), however, their molecular structures remain largely unknown (Ma et al., 2011). This detrimental effect of *A. minutum* on larvae could be attributed sequentially to BEC and PST during development.

Toxic effects on small *C. gigas* larvae (D-larvae to larvae $< 150 \mu\text{m}$) which are unable to feed on *A. minutum* could not be related to PST toxicity arising from algal cell consumption, but rather arise from BECs. These bioactive substances produced by *Alexandrium minutum* mainly exert their action by direct contact with external tissues, e.g. the gills (Borcier et al., 2017; Castrec et al., 2018) or cells, e.g. gametes (Le Goïc et al., 2014). These BECs could be cytotoxic to the velum, the feeding and swimming organ of the larvae, thereby reducing energy uptake and subsequent larval growth. Similarly, the toxic effects of *A. minutum* and *A. ostenfeldii* on *M. edulis* larvae observed by De Rijcke et al. (2016) could mainly result from extracellular bioactive substances, as suggested by the authors. This hypothesis was also suggested by Banno et al. (2018) who identified some unknown bioactive compounds as responsible for the decrease of sperm motility and egg viability of oysters *P. fucata martensii* exposed to two *Alexandrium* species.

The PST probably become harmful from the moment larvae are able to ingest *A. minutum* cells. In this study, *C. gigas* eyed larvae (mean length \pm SD: $304 \pm 15 \mu\text{m}$) ingested *A. minutum* cells. Veliger larvae ($> 200 \mu\text{m}$) of the oyster *C. virginica* fed preferably on large food material ($22\text{--}30 \mu\text{m}$) in the presence of large cell dinoflagellate bloom (Baldwin, 1995), suggesting that oyster larvae ($> 200 \mu\text{m}$) likely fed on *A. minutum* in the present study. Thus, both the PST accumulated through *A. minutum* consumption and the BECs could have contributed to the adverse effects on growth at the end of the larval development. This hypothesis supports the

findings of Mu and Li (2013) who suggested that the reduced growth of early umbonate larvae of *C. gigas* following a 4-day exposure to 3×10^2 cells mL⁻¹ of *A. catenella* might relate to both PST and unknown toxins produced by *A. catenella*.

Larval exposure to *A. minutum* also altered the settlement of oyster larvae. Larval mortality does not explain the reduced settlement in exposed larvae. The decreased settlement could either result from the lagged growth observed after 22 days of exposure, as most exposed larvae did not reach the competence for settlement and metamorphosis, and/or from altered physiology caused by *A. minutum* toxins. Similarly, the activity of Japanese pearl oyster (*P. fucata martensii*) pre-settling larvae was decreased when exposed to *Alexandrium affine* and *A. catenella*, at 2.5×10^2 cells mL⁻¹ and 10 cells mL⁻¹, respectively (Basti et al., 2015). This effect was attributed to non-PST metabolites with potent lytic activity produced by the non-PST *A. affine* or to the PST produced by *A. catenella*, following ingestion of algal cells, leading to paralysis and/or altered cellular homeostasis (Basti et al., 2015).

4.5. Broodstock conditioning influenced larval response to *A. minutum* exposure

In the present study, growth of larvae derived from exposed parents was less affected by *A. minutum* exposure than growth of larvae derived from non-exposed oysters. This result suggests that parental exposure to *A. minutum* may have led to an improved capacity to cope with the stress caused by *A. minutum* exposure. Similarly, exposure of adult Sydney rock oysters to elevated pCO₂ improved the capacity of their offspring to regulate extracellular pH at elevated pCO₂ (Parker et al., 2012). Bouillot et al. (2018) revealed that the sensitivity of *C. gigas* nerves to saxitoxin was decreased when oysters had been previously exposed to PST-producing *A. minutum*. It can be hypothesized that larvae derived from PST-containing gametes produced by exposed parents may be less sensitive to PST during larval development.

5. Conclusions

Successful reproduction is essential for the sustainability of marine populations. This study demonstrates that long term exposure of adult oysters to *A. minutum* during gametogenesis affected spermatozoa motility, and reduced growth and survival of the subsequent offspring. The present laboratory experiment also evidenced that direct *A. minutum* exposure during oyster embryonic-larval development significantly altered growth and settlement of larvae. These effects of *A. minutum* blooms on oyster reproduction are likely to compromise recruitment of benthic post-larvae of *C. gigas* by slowing down growth, prolonging the time larvae remain in the seawater column, thus making them more vulnerable to predation. Further research is needed to investigate potential long term effects on marine bivalve populations by studying the consequences of recurrent *Alexandrium* blooms over multiple generations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2018.11.084>.

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Supplementary material

Table S1. Gonadal maturation and occupation index of adult oysters after 2, 4, and 6 weeks of conditioning (T2, T4, and T6, respectively) coupled with an exposure to *A. minutum* (Exposed) or to non-toxic *H. triquetra* (Control). Mean \pm SE, n in each group is indicated.

Sampling time	Gonadal maturation					Gonadal occupation index (%)				
	Control	n	Exposed	n	U-test	Control	n	Exposed	n	t-test
T2	2.2 \pm 0.1	15	2.1 \pm 0.1	15	ns	43.1 \pm 2.7	14	36.8 \pm 2.6	13	ns
T4	2.7 \pm 0.1	15	2.7 \pm 0.2	15	ns	42.4 \pm 3.5	11	43.0 \pm 4.7	15	ns
T6	3.0 \pm 0	15	3.0 \pm 0	15	ns	55.1 \pm 3.1	13	61.5 \pm 3.0	12	ns

ns: not significant.

Table S2. Male gonad and oocyte biochemical composition (percent of the male gonad or oocyte dry mass) of adult oysters after an 8-week conditioning coupled with an exposure to *A. minutum* (Exposed) or to non-toxic *H. triquetra* (Control). Mean \pm SE, n = 3 pools per treatment. Male gonad and oocyte biochemical composition were compared between control and exposed oysters by *t*-test.

Parameter	Oocytes			Male gonad		
	Control	Exposed	t-test	Control	Exposed	t-test
Proteins	65.4 \pm 1.4	61.2 \pm 4.3	ns	62.2 \pm 2.8	58.7 \pm 4.0	ns
Lipids	25.1 \pm 1.1	26.3 \pm 0.9	ns	10.9 \pm 0.4	17.0 \pm 1.5	*
Carbohydrates	4.5 \pm 0.1	4.6 \pm 0.1	ns	4.9 \pm 0.5	4.8 \pm 0.6	ns

ns: not significant,

* significant differences were indicated by $p < 0.05$.

Table S3. Gamete cellular variables of adult oysters after 7 weeks of conditioning coupled with an exposure to toxic *A. minutum* (Exposed) or to non-toxic *H. triquetra* (Control). Reactive oxygen species (ROS) production, percentage of dead cells, relative size and complexity, and mitochondrial membrane potential (MMP) were analyzed by flow cytometry. Oocyte Feret's diameter and circularity, spermatozoa velocity, and percentage of motile spermatozoa were analyzed by microscopy. Mean \pm SE, n = 6 oysters per treatment. a.u.: arbitrary units; n.a.: not analyzed.

Variables	Spermatozoa			Oocytes		
	Control	Exposed	<i>t</i> -test	Control	Exposed	<i>t</i> -test
ROS production (a.u.)	32.3 \pm 6.7	17.7 \pm 2.6	ns	60 \pm 26.7	56.7 \pm 21.7	ns
Dead cells (%)	1.8 \pm 0.3	1.3 \pm 0.2	ns	4.2 \pm 1.5	5.0 \pm 1.4	ns
Size (a.u.)	73.9 \pm 0.8	74.9 \pm 0.5	ns	365.2 \pm 9.4	349.6 \pm 8.6	ns
Complexity (a.u.)	36.8 \pm 1.6	33.3 \pm 0.8	ns	1592.4 \pm 38.3	1551.0 \pm 27.0	ns
MMP (a.u.)	2.8 \pm 0.2	2.9 \pm 0.2	ns	n.a.	n.a.	n.a.
Oocyte diameter (μ m)	n.a.	n.a.	n.a.	50.2 \pm 1.1	51.7 \pm 1.7	ns
Oocyte circularity	n.a.	n.a.	n.a.	0.9 \pm 0.1	0.9 \pm 0.1	ns
Spermatozoa velocity (μ m s ⁻¹)	79.1 \pm 6.7	67.5 \pm 3.1	ns	n.a.	n.a.	n.a.
Motile spermatozoa (%)	48 \pm 3 %	30 \pm 5 %	*	n.a.	n.a.	n.a.
ATP content (nmol 10 ⁻⁹ cells)	179.7 \pm 21.6	172.4 \pm 17.1	ns	n.a.	n.a.	n.a.

ns: not significant,

* significant differences were indicated by $p < 0.05$.

Analyses supplémentaires

Les ARNm maternels s'accumulent pendant la gamétogenèse sont les principales ressources pour la synthèse des protéines lors du développement précoce de l'embryon. L'épitranscriptome (m⁶A-ARN, méthylation des adénosines de l'ARN) émerge comme un régulateur épigénétique majeur du développement qui serait conservé chez l'huître. Une récente étude a suggéré l'implication des mécanismes épigénétiques dans l'impact d'une exposition parentale de *C. gigas* à un herbicide sur le transcriptome de la descendance (Bachère et al., 2017). Des mécanismes épigénétiques et notamment la méthylation des ARN (m⁶A-ARN) des ovocytes pourraient être également impliqués dans l'effet multigénérationnel d'*A. minutum*.

Afin de tester cette hypothèse, nous avons mesuré par immunoblot la méthylation globale des ARN totaux extraits des pools d'ovocytes (1 pool par bac d'huîtres) des huîtres exposées à *A. minutum* et des huîtres témoins, en utilisant un anticorps anti-m⁶A, comme décrit par Wang et al. (2016). Ce travail a été réalisé en collaboration avec Guillaume Rivière de l'UMR BOREA à Caen. La membrane d'hybridation (Hybond N+, GE Healthcare) a été hydratée, puis rincée avec une solution saline. Les ARN ont été dénaturés à 65°C pendant 10 min dans une solution de dénaturation, puis refroidis sur glace. Les échantillons d'ARN d'ovocytes ont été dilués dans une solution saline et déposés sur la membrane ($2,5 \times 10^3$ ng, 10^3 ng et 5×10^2 ng). Les contrôles (positif et négatifs) ont aussi été déposés sur la membrane (10^3 ng). Les contrôles négatifs étaient : un oligo siRNA synthétique donc non méthylé et de l'ADN génomique de larve trochophore de *C. gigas* qui ne possède pas de m⁶A. Le contrôle positif était de l'ARN de larve trochophore de *C. gigas* qui possède des m⁶-A (test préalable). Puis les ARN ont été fixés sur la membrane en chauffant à 70°C pendant deux heures. La détection des m⁶-A a ensuite été réalisée en utilisant un anticorps anti-m⁶-A (Diagenode) couplé à un anticorps secondaire igG Alexa Fluor 488 (Invitrogen) (Wang et al., 2016). Après rinçage, la membrane a été observée avec un scanner ProEXPRESS 2D Proteomic Imaging System (Perkin Elmer) à une longueur d'onde d'excitation de 480/30 et d'émission de (530/30). L'intensité de fluorescence de l'anticorps secondaire est proportionnelle au niveau de méthylation des ARN.

Les résultats de l'immunoblot mettent en évidence qu'il y a bien une fluorescence spécifique pour les échantillons d'ARN d'ovocytes d'huîtres, démontrant pour la première fois la présence de méthylation des adénosines de l'ARN des ovocytes de *C. gigas* (Figure 10). L'analyse d'image indique qu'il n'y a pas de différence quantitative de méthylation des ARN totaux des ovocytes entre les conditions, car l'intensité de la fluorescence n'est pas différente entre les

deux conditions (Figure 10). Néanmoins, le niveau de méthylation des divers types d'ARN (ARNm, ARNt, ARNrb, ARN non codant) pourrait être différents dans les ovocytes des deux conditions, influant de manière distincte le développement de la descendance.

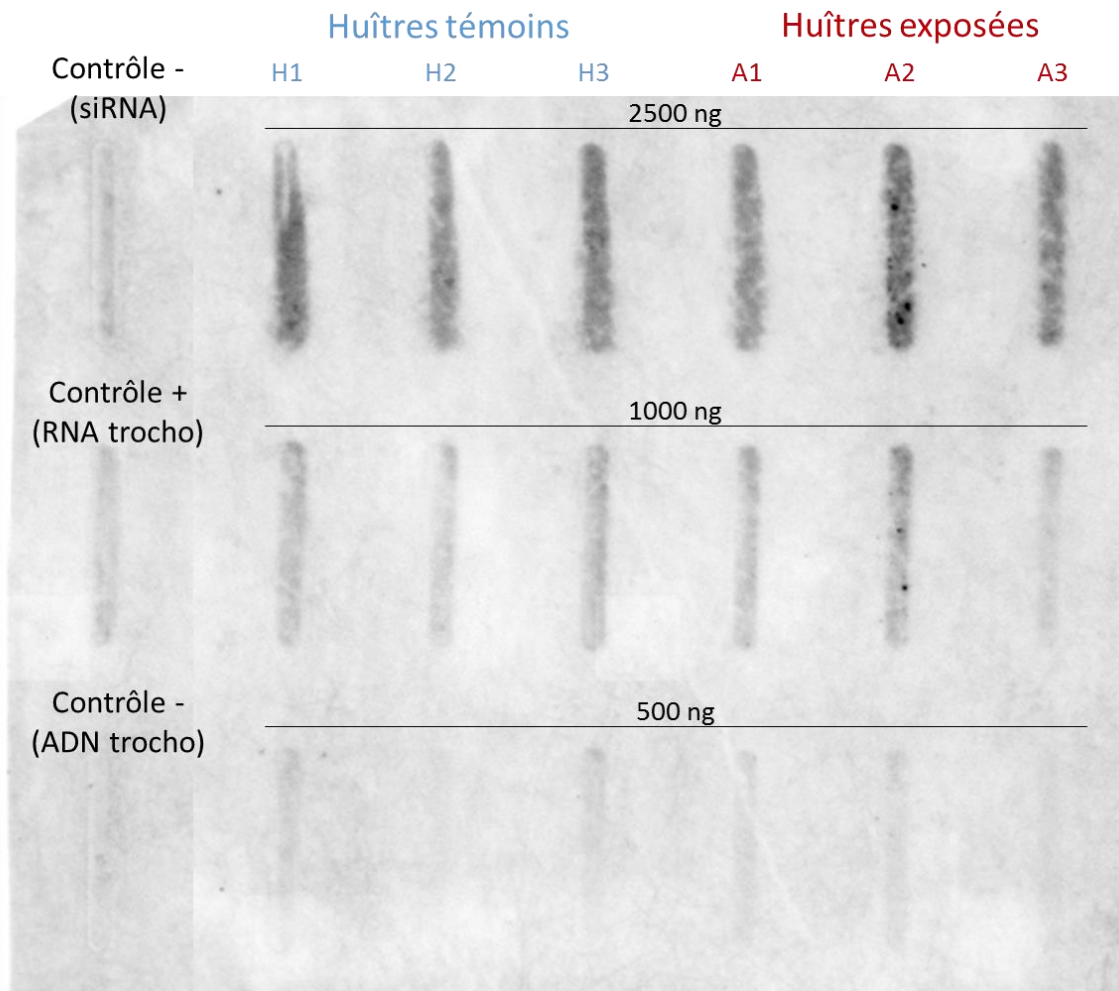


Figure 10. Détection du niveau de méthylation des ARN (m⁶A-ARN) des ovocytes d'huîtres *C. gigas* exposées à *A. minutum* et d'huîtres témoins. Trois pools d'ovocytes ont été testés pour chaque condition (H1, H2 et H3 pour les huîtres témoins ; A1, A2 et A3 pour les huîtres exposées). Différentes quantités d'ARN d'ovocytes a été déposées : 2500 ng (ligne du haut), 1000 ng (ligne du milieu) et 500 ng (ligne du bas). Les contrôles négatifs utilisés sont du siARN (1000 ng), de l'ADN de larve trochophore de *C. gigas* (1000 ng). Le contrôle positif est de l'ARN de trochophore de *C. gigas* (1000 ng).

Chapitre 3

Quels sont les effets respectifs des PST et des BEC produits par *A. minutum* sur la physiologie et le comportement des huîtres adultes ?

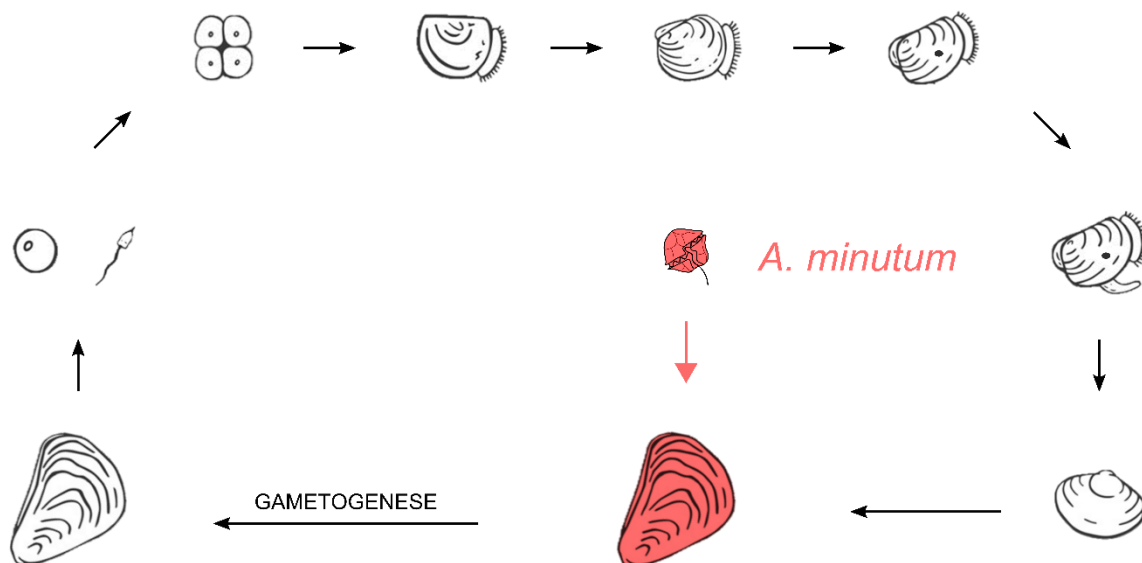
Dans le chapitre précédent, nous avons démontré qu'une exposition d'huîtres pendant deux mois à *A. minutum* altère la nutrition des huîtres et la mobilité des spermatozoïdes et affecte la croissance et la survie des larves. De plus, une exposition d'un mois des huîtres pendant le développement embryon-larvaire affecte la croissance et la fixation des larves.

Plusieurs études d'exposition en milieu contrôlé ont démontré que cette même souche d'*A. minutum* (souche AM89BM) affecte le comportement de *C. gigas* en induisant une augmentation de la durée d'ouverture valvaire, du nombre de micro-fermetures (fermetures partielles et rapides des valves) et une diminution de l'amplitude d'ouverture valvaire (Haberkorn et al., 2011; Tran et al., 2015). L'exposition à *A. minutum* perturbe les processus de nutrition de *C. gigas*, en réduisant l'activité de filtration (Lassus et al., 2004). Dans les branchies, une production accrue de mucus et une modification de l'expression de gènes impliqués dans le métabolisme oxydatif, l'horloge interne, l'immunité et les processus de détoxification ont été observées (Haberkorn et al., 2010b; Mat et al., 2013; Payton et al., 2017). L'exposition de *C. gigas* à *A. minutum* affecte aussi la composition lipidique de la glande digestive et induit dans cet organe une forte réponse inflammatoire, caractérisée par des infiltrations et des diapédèses d'hémocytes (Haberkorn et al., 2010b).

La souche AM89BM d'*A. minutum* utilisée dans toutes ces expériences est connue pour produire des toxines paralysantes (PST) mais aussi d'autres composés extracellulaires bioactifs (BEC) dont la nature chimique est encore inconnue. Jusqu'à récemment, les effets observés chez les huîtres exposées à cette souche étaient essentiellement attribués aux PST. Cependant ces deux types de molécules pourraient être responsables des effets délétères observés sur les huîtres adultes mais également sur les embryons et les larves. Ces deux types de molécules pourraient donc être responsables des effets délétères observés sur les huîtres adultes mais également sur les embryons et les larves.

Afin de mieux comprendre l'effet d'*A. minutum* sur la physiologie et la reproduction des huîtres, il paraît nécessaire de déterminer les effets distincts des PST et des BEC produits par cette micro-algue.

Dans ce contexte, nous avons analysé, en milieu contrôlé, les effets de différentes souches d'*A. minutum* sur le comportement valvaire et la physiologie de *C. gigas* hors de la période de gamétogenèse. Dans le but de discriminer les effets des PST et des BEC, nous avons sélectionné 3 souches d'*A. minutum* produisant soit des PST, soit des BEC, soit les deux à la fois.



Les hypothèses avancées étaient :

1. Les réponses physiologiques et comportementales des huîtres varient en fonction des souches d'*A. minutum* et donc potentiellement en fonction des toxines auxquelles les huîtres sont exposées,
2. La toxicité d'*A. minutum* sur la physiologie de l'huître adulte est liée à la présence des PST mais aussi des BEC.

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Bioactive extracellular compounds produced by the dinoflagellate *Alexandrium minutum* are highly detrimental for oysters

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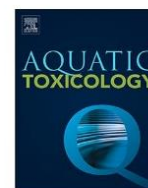
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Bioactive extracellular compounds produced by the dinoflagellate *Alexandrium minutum* are highly detrimental for oysters

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ABSTRACT

Blooms of the dinoflagellate *Alexandrium* spp., known as producers of paralytic shellfish toxins (PSTs), are regularly detected on the French coastline. PSTs accumulate into harvested shellfish species, such as the Pacific oyster *Crassostrea gigas*, and can cause strong disorders to consumers at high doses. The impacts of *Alexandrium minutum* on *C. gigas* have often been attributed to its production of PSTs without testing separately the effects of the bioactive extracellular compounds (BECs) with allelopathic, hemolytic, cytotoxic or ichthyotoxic properties, which can also be produced by these algae. The BECs, still uncharacterized, are excreted within the environment thereby impacting not only phytoplankton, zooplankton but also marine invertebrates and fishes, without implicating any PST. The aim of this work was to compare the effects of three strains of *A. minutum* producing either only PSTs, only BECs, or both PSTs and BECs, on the oyster *C. gigas*. Behavioral and physiological responses of oysters exposed during 4 days were monitored and showed contrasted behavioral and physiological responses in oysters supposedly depending on produced bioactive substances. The non-PST extracellular-compound-producing strain primarily strongly modified valve-activity behavior of *C. gigas* and induced hemocyte mobilization within the gills, whereas the PST-producing strain caused inflammatory responses within the digestive gland and disrupted the daily biological rhythm of valve activity behavior. BECs may therefore have a significant harmful effect on the gills, which is one of the first organ in contact with the extracellular substances released in the water by *A. minutum*. Conversely, the PSTs impact the digestive gland, where they are released and mainly accumulated, after degradation of algal cells during digestion process of bivalves. This study provides a better understanding of the toxicity of *A. minutum* on oyster and highlights the significant role of BECs in this toxicity calling for further chemical characterization of these substances.

1. Introduction

Harmful algal blooms (HABs) are present in freshwater, brackish and marine environments, as naturally occurring phenomena. For the last decades, there is clear evidence confirming the geographical extension of HABs worldwide (Anderson et al., 2012a). Factors contributing to this phenomenon are various: local hydrological events, global change through the selection of species according to their thermal preferences, N:P ratio (possible role of eutrophication), accidental transfer of marine species, and appearance of toxin-producing strains caused by genetic modification of harmless strains (Lassus et al., 2016). Those factors sometimes act in a complex interplay, and more

importantly, vary among geographical areas. Effects of HABs are expected to intensify even more as the use of coastal waters for aquaculture and shellfish farming is globally increasing (Lassus et al., 2016).

Among the microalgae responsible for HABs, the genus *Alexandrium* (Halim 1960) is one of the most important, in terms of diversity, distribution, and intensity (Anderson et al., 2012b). Blooms of *Alexandrium* are globally distributed, with species present in tropical, temperate and subarctic regions (Tillmann et al., 2016). Species of *Alexandrium* are known to produce Paralytic Shellfish Toxins (PSTs), composed of saxitoxin (STX) and approximately 50 derivatives of STX (Wiese et al., 2010). This group of toxins have a particularly acute toxicity (Kodama, 2010), causing neurotoxic syndromes. PSTs block the

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conduction of action potential by binding to the voltage-gated sodium channels that play an essential role in membrane excitability in nerve cells (Cestèle and Catterall, 2000). In human seafood consumers, paralytic shellfish poisoning (PSP) causes dizziness, tingling sensation of the mouth, headache, nausea, vomiting, diarrhea and in severe cases death by asphyxiation (Etheridge, 2010). Blooms of *Alexandrium* can also have ecological consequences by alteration of marine trophic structure, and death of marine fish, seabirds, and mammals, but also have dramatic economic effects, causing impairment of tourism and recreational activities, fishery closure and sell prohibition of shellfish (Anderson et al., 2012b). Filter-feeder bivalves including several commercial species, such as the Pacific oyster *Crassostrea gigas*, are known to accumulate PSTs by feeding on toxic algae (Bricelj and Shumway, 1998).

Many studies have also highlighted that *A. minutum* impacts oyster behavior and physiology. Laboratory experiments demonstrated that exposure to *A. minutum* deeply modified valve behavior (Tran et al., 2010; Haberkorn et al., 2011) and disrupted biological rhythms of oysters (Tran et al., 2015; Payton et al., 2017a). Functioning and structure of digestive organs (Lassus et al., 1999; Haberkorn et al., 2010a), and oyster defense responses were negatively affected (Haberkorn et al., 2010b; Hégaret et al., 2011; Mat et al., 2013; Mello et al., 2013).

From the above studies, relationships between *A. minutum* effects and PST microalgae content or PST bioaccumulation by oysters could not be established. *Alexandrium* species, however, were also shown to produce bioactive extracellular compounds (BECs) excreted in the surrounding seawater, independently from PSTs. The BECs produced by *Alexandrium* species can be allelopathic (Fistarol et al., 2004; Lelong et al., 2011; Zheng et al., 2016), cytotoxic (Ford et al., 2008; Flores et al., 2012), haemolytic (Arzul et al., 1999; Yang et al., 2010), or ichthyotoxic (Ogata and Kodama, 1986; Mardones et al., 2015). To date, knowledge on the chemical nature and the mode of action of the BECs produced by *Alexandrium* spp. is still scarce. Cytotoxic extracellular compounds produced by *Alexandrium* spp. were suggested to act primarily on cell membranes, with a high lysis potential (Emura et al., 2004; Tillmann et al., 2007, 2008). The lytic allelochemical activity of different strains of *Alexandrium* spp. studied on various protists appeared not related to the cellular PST content or composition (Tillmann and John, 2002; Tillmann et al., 2009).

An exposure of *Pecten maximus* juveniles to a non-PST BEC-producing strain of *A. minutum* (strain CCMI1002) resulted in a decrease of daily shell growth, tissues alterations and an increased reaction time when exposed to predators (Borcier et al., 2017). Similarly, this *A. minutum* non-PST strain induced dramatic effects on oyster hemocytes and spermatozoa, and strongly affected feeding behavior of oyster *C. gigas* and clam *Ruditapes philippinarum*, presumably because of BECs (Contreras, 2011; Lambert et al., 2013). These harmful effects of non-PST producing strains on marine organisms raise the question of the respective involvement of PSTs and BECs in the toxicity of *A. minutum*.

To discriminate the respective effects of PSTs and BECs produced by *A. minutum*, we analyzed, under controlled conditions, the effects of different strains of *A. minutum* on the oyster *C. gigas*. To address this question, we selected three *A. minutum* strains with very different toxicity in terms of both PSTs and BECs: the “PST” strain (Daoulas1257 strain, isolated from the Bay of Brest in France; Boullot et al., 2017) producing only PSTs; the “PST + BEC” strain (AM89BM strain, isolated from the Bay of Morlaix in France; Erard-Le Denn et al., 1990) producing both PSTs and BECs; and the “BEC” strain (CCMI1002 strain, isolated from Irish waters; Tillmann and John, 2002) producing only BECs. This work clarifies the respective effects of PSTs and BECs produced by *A. minutum* on valve behavior and physiology of the oyster.

2. Materials and methods

2.1. Oyster characteristics and general conditions

Diploid Pacific oysters, *Crassostrea gigas* (*Magallana* gen. nov.; Salvi and Mariottini, 2017), used in this study came from a single cohort produced in March 2014 in the Argenton Ifremer facilities (France). At the time the experiment began (January 2015), oysters were 10 months old, measured 56 ± 1 mm length (mean \pm SE), and weighed 25.1 ± 0.9 g (total wet weight; mean \pm SE). Three hundred and sixty oysters were randomly distributed into nine 30-L tanks, including 2 or 3 oysters equipped with valvometric electrodes per tank ($n = 6-7$ per condition) for valve activity measurements. Each tank was continuously supplied with 1- μ m filtered UV-treated seawater and algae at a total flow of 19 mL min^{-1} , allowing a renewal of the volume of the tank every day. Every tank was equipped with a circulation pump to homogenize microalgae and seawater. Experiments were carried out with a photoperiod of 10 h light (8 am to 6 pm) and 14 h dark (6 pm to 8 am) and seawater was maintained at a temperature of 16 ± 1 °C. Oysters were maintained in the same tanks during the experiment, in a blind room to minimize any external influences and disturbance due to handling on animal behavior.

2.2. Algal culture

The three strains of the dinoflagellate *Alexandrium minutum* (Daoulas1257, AM89BM and CCMI1002 strains) were cultured in 6-L batch culture using seawater filtered to 0.2 μ m supplemented with L1 medium. Cultures were maintained at 17 ± 1 °C with a dark:light cycle of 12:12 h. According to the bioassay developed by Long et al. (personal communication), the BEC strain is more cytotoxic than the PST + BEC strain. The PST + BEC strain is known to produced more PSTs ($10.6 \text{ fmol cell}^{-1}$ which corresponds to $1.3 \text{ pg STX eq. cell}^{-1}$; Haberkorn et al., 2010a) than the PST strain ($0.63 \text{ fmol cell}^{-1}$ which corresponds to $0.053 \text{ pg STX eq. cell}^{-1}$; Pousse et al., 2018), whereas no PSTs were detected in the BEC strain (Borcier et al., 2017). The dinoflagellate *Heterocapsa triquetra* (strain HT99PZ - Ehrenberg, 1840) was grown in 150-L cylinders containing 1- μ m filtered, UV-treated seawater enriched with L1 nutrients (Guillard and Hargraves, 1993) and cultures were maintained at 19 ± 1 °C with continuous light. Cultures of *A. minutum* and *H. triquetra* were not axenic, grown without antibiotics, and harvested in exponential growth phase for the experiment.

2.3. Experimental design of *A. minutum* exposure

Oysters were acclimated for 7 days with increasing *H. triquetra* densities from 1.65×10^3 to 3.3×10^3 cells mL^{-1} . The non-toxic *H. triquetra* was chosen for acclimation because of its similarity to *A. minutum* in terms of size and shape: *H. triquetra* cell size (19–28 μ m) is similar to *A. minutum* cell size (23–29 μ m). After the 7-day acclimation, oysters were fed continuously with the *A. minutum* strains for 4 days at 19 mL min^{-1} . Each *A. minutum* strain was distributed to three replicate tanks at a density of 3.1×10^3 cells mL^{-1} . This algal density and exposure time was environmentally realistic (Chapelle et al., 2015).

2.4. Measurement of *A. minutum* concentrations by flow cytometry

During the exposure phase, *A. minutum* cell concentration in the seawater was monitored from the inflow and outflow of the tanks from all conditions. Cell counts were performed using a FACScalibur (BD Sciences, San Jose, CA, USA) flow cytometer with a 488 nm argon blue laser. Threshold was set to FL3 (red fluorescence 670 nm Long pass

filter, i.e. > 670 nm) to detect only chlorophyll-containing cells. Concentrations of *A. minutum* were estimated using cell counts during 30 s of flow-cytometer acquisition and flow rate, measured according to Marie et al. (1999).

2.5. Oyster behavioral analysis

2.5.1. Valve activity measurement

Valve activity of *C. gigas* was studied using High Frequency Non Invasive valvometry (n = 2-3 oysters per tank). Lightweight electromagnets were glued on each valve of each animal and connected to a lab valvometer by flexible wires (Tran et al., 2003; Chambon et al., 2007). The sampling frequency for each individual was 0.2 Hz. Data were processed using LabView 8.0 software (National Instruments, Austin, TX, USA). The record of valve activity started 4 days before exposure to define oyster behavior under reference conditions (non-toxic *H. triquetra* feeding). Following initiation of experiments, valve activity was measured continuously during the 4 days of exposure to the *A. minutum* strains.

Several parameters of valve activity were analyzed. Hourly opening duration of each individual was expressed as the percentage of time oysters spent with their valves opened, and ranged from 100% (valves open for the entire hour) to 0% (valves closed throughout the hour). Additionally, hourly valve-opening amplitude and hourly number of valve micro-closures, which corresponds to the number of partial and fast valve closures, were measured for each individual. For each valve activity parameter, mean hourly values for the condition were calculated as the mean of hourly individual values of each condition.

2.5.2. Chronobiological analysis

Chronobiological analyses were made with the software Time Series Analysis Seriel Cosinor 6.3 (<http://www.euroestech.net/mainuk.php>). Different steps were performed to validate a biological rhythm in oysters (see Tran et al. (2011) and Mat et al. (2012)). Briefly, the quality of the dataset was checked by controlling the absence of randomness using the autocorrelation diagram and the absence of any stationary character by a partial autocorrelation function calculation. The periodicities in the recorded data were tested with the spectral method of the Lomb and Scargle periodogram. These methods give a threshold of probability ($p = 0.95$) defining the limit below which the signal can be regarded as “noise”. The confidence interval of the period was calculated with the method of Halberg (1969). The rhythmicity was then modeled with the Cosinor model, which uses a cosine function calculated by regression (Bingham et al., 1982). To validate the model two tests must be validated: the elliptic test must be rejected and the probability (p -value) for the null amplitude hypothesis must be lower than 0.05. Finally, the percent rhythm (PR), i.e., percentage of the cyclic behavior explained by the model, was calculated.

2.6. Oyster sampling

At the end of the exposure period, 12 oysters per tank were sampled and used as follows: for each tank, 7 oysters including oysters equipped with electrodes were analyzed for individual hemocyte variable measurements and condition index, and 5 oysters for histology. A 3-mm cross-section of soft tissues including digestive gland, gills, mantle, gonad, and a section of adductor muscle were taken for each of the 45 oysters sampled for histopathology. Hemolymph was withdrawn from the adductor muscle of each oyster (400 μ L minimum) with a syringe before oysters were shucked. Total and soft tissues weights were measured for condition index measurement. Digestive glands were dissected, weighted individually, and were ground with a “MM 400” mixer mill (Retsch) in liquid nitrogen and stored at -80°C for further toxin analyses. Condition index (CI) was determined for 62 individuals as follows (Bodoy et al., 1986): $\text{CI} = (\text{wet flesh weight}/\text{total weight}) \times 100$.

2.7. Histopathology

Dissected tissues were fixed immediately in modified Davidson's fixative (Latendresse et al., 2002) for 24 h at 4°C . Tissues were processed and observed as described by Hermabessiere et al. (2016). The histopathological figures observed per organ were classified as follows:

- Gills: (i) presence of mucus, (ii) hemocyte infiltration, (iii) hemocytes outside the gills;
- Digestive gland (stomach, intestine, digestive ducts and tubules): (i) hemocyte infiltration, (ii) hemocytes in diapedesis in the digestive epithelium, (iii) presence of *A. minutum* cells in the lumen.

Intensity of each histopathological observation was rated using a three-level semi-quantitative scale and the sum calculated for each organ. Each pathological condition was graded as stage 0 (absence or very light), stage 1 (light-moderate), and stage 2 (heavy). Based upon this scale, mean intensity of each histopathological figure were calculated for each specific tissue as follows:

$$\Sigma(\text{stage}_{\text{individual}1} + \text{stage}_{\text{individual}2} + \dots + \text{stage}_{\text{individual}n})/n$$

(where n is the number of diagnosed animals per condition).

2.8. Analyses of hemolymph variables

The quality of hemolymph sample (absence of contamination) was checked using microscope and then stored temporarily on ice before flow-cytometric analysis. Mortality (percentage of dead hemocytes) and characteristics determined in live circulating hemocytes, i.e. total and differential hemocyte counts (granulocytes, hyalinocytes and small agranulocytes) (in cell mL^{-1}), size, and internal complexity (in arbitrary units a.u.), were assessed following Haberkorn et al. (2010b) with an Easy-Cyte-Plus flow-cytometer (Guava-Millipore). Functional variables, i.e. production of Reactive Oxygen Species (ROS) (specifically H_2O_2 and $\text{O}_2^{\cdot-}$) by unstimulated hemocytes, mitochondrial membrane potential (MMP) and phagocytosis were determined as described in Lambert et al. (2003), Donaghy et al. (2012) and Haberkorn et al. (2010b), respectively. Hemocyte functional analyses were performed with a FACSVerse flow-cytometer (BD Biosciences, San Jose, C.A., USA) for ROS production and a FACScalibur (BD Biosciences) for MMP and phagocytosis.

2.9. Toxin quantification by liquid chromatography/fluorescence detection

The digestive glands of 14 oysters exposed to the PST strain and of 14 oysters exposed to the PST + BEC strain (2–6 per tank) were sampled for toxin content measurement. Toxins were also assayed in pooled digestive glands of 6 oysters (2 oysters per tank) exposed to the BEC strain to verify that the BEC strain did not produce PSTs. Acetic acid (1 mL of 0.1 N) was added to 100 mg of ground digestive gland and the sample was frozen at -20°C until extraction and analysis were performed. Toxin quantification were performed in triplicate for each sample using the liquid chromatography with fluorescence detection (LC/FD), as described by Boullot et al. (2017). The molar concentration ($\mu\text{mol L}^{-1}$) was converted to μg STX equivalent 100 g^{-1} of digestive gland wet weight using the conversion factors of EFSA (EFSA Journal, 2009).

2.10. Statistical analyses

Differences between microalgal treatments were investigated using One-Way ANOVA followed by Tukey HSD post hoc test, after checking assumptions (normality of data and equal variance tests). When assumptions were not validated, the non-parametric Kruskal-Wallis test (non-nested – comparison of three conditions categorized by algal

treatment) was applied, followed by the Nemenyi–Damico–Wolfe–Dunn (NDWD) post hoc test. For each condition and each valve activity parameter, Mann Whitney tests were used to compare the mean hourly values of the condition between the last 4 days of acclimation (n = 96 h) and the 4-day *A. minutum* exposure period (n = 96 h). Intensity of each pathological condition was compared statistically using the Kruskal-Wallis test. For the sum of binary pathological data (presence/absence), One-Way ANOVA was used. Differences were considered significant when $p < 0.05$. Statistical analyses were performed using R version 3.2.2 (R Core Team, 2012). All values are expressed as mean \pm standard error (SE).

3. Results

3.1. Paralytic shellfish toxin content in the digestive gland of *Crassostrea gigas*

The mean PST toxicity in the digestive glands of oysters after a 4-day exposure to *A. minutum* was 3.3-fold higher ($p < 0.01$, *t*-test) in oysters exposed to the PST strain ($174.2 \pm 40.3 \mu\text{g STX eq. } 100 \text{ g}^{-1}$ wet digestive gland, n = 14) compared to oysters exposed to the PST + BEC strain ($52.2 \pm 17.2 \mu\text{g STX eq. } 100 \text{ g}^{-1}$ wet digestive gland, n = 14). The mean total PST load in the digestive glands of oysters from the PST condition ($39.3 \pm 8.8 \mu\text{mol kg}^{-1}$ wet digestive gland, n = 13) was significantly higher (4.9-fold; $p < 0.001$, *t*-test) compared to oysters from the PST + BEC condition ($8.1 \pm 2.4 \mu\text{mol kg}^{-1}$ wet digestive gland, n = 14). No PSTs were detected in the digestive glands of oysters exposed to the BEC strain.

Toxin composition of oyster digestive glands were similar in oysters exposed to the PST or the PST + BEC strains: carbamates C2 and C1, N-sulfocarbamoyls GTX3 and GTX2, plus a minor quantity of decarbamoyls dc-GTX3 and dc-GTX2 (Fig. 1). Very low levels of saxitoxin (STX) and dc-STX were also detected in tissues of 4 oysters exposed to the PST strain ($\approx 0.2\%$) and only in one oyster exposed to the PST + BEC strain ($\approx 0.5\%$). The mean proportions of C1, GTX3, GTX2, dc-GTX3 and dc-GTX2 were significantly higher ($p < 0.01$, Mann-Whitney) and the proportion of C2 was significantly lower ($p < 0.001$, Mann-Whitney) in oysters exposed to the PST + BEC strain compared to oysters exposed to the PST strain (Fig. 1). The proportions of the

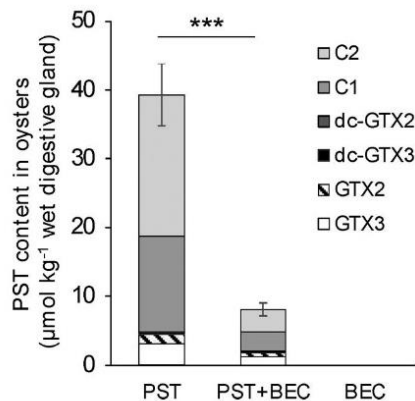


Fig. 1. Paralytic shellfish toxin accumulation (in $\mu\text{mol kg}^{-1}$ of wet digestive gland) in the digestive glands of *C. gigas* exposed for 4 days to the PST strain (n = 14 oysters), the PST + BEC strain (n = 14 oysters), or the BEC strain (n = 1 pool of 6 oysters) of *A. minutum*. Mean \pm SE. The values are given as the mean contribution (in percentage molar concentration) of each saxitoxin derivate to the global toxicity. Errorbars represent standard errors of total PST content. Significant difference in total PST content between PST + BEC and PST conditions is indicated by ***: $p < 0.001$ (*t*-test). STX and dc-STX were detected in digestive glands of oysters in both conditions but are not presented because they represented less than 1% of total toxicity. No PSTs were detected in the pool of 6 oysters from the BEC condition.

Table 1

Valve activity behavior modifications of *C. gigas* induced by exposure to different *A. minutum* strains: PST, PST + BEC and BEC strains. For each condition, Mann Whitney tests were used to compare the mean hourly values of valve activity parameter between acclimation (n = 96 h, i.e. 4 last days of acclimation) and *A. minutum* exposure (n = 96 h). For each condition, fold change of each valve activity parameter was calculated by dividing the mean hourly value of the parameter for the condition during *A. minutum* exposure by the mean hourly value during acclimation.

Condition	Valve-activity modifications between <i>A. minutum</i> exposure and acclimation					
	Opening duration		Opening amplitude		Micro-closure	
	Test	Fold change	Test	Fold change	Test	Fold change
PST	NS	1.06	NS	1.03	NS	0.88
PST + BEC	***	1.25	*	1.13	***	1.84
BEC	***	1.55	***	1.23	***	3.46

NS = non-significant.

* $p < 0.05$, *** $p < 0.001$.

other STX derivatives in digestive gland of oysters were not significantly different between the PST + BEC and PST conditions.

3.2. Oyster valve-activity behavior

Behavioral analyses were conducted on oysters during the 4 last days of acclimation and during the 4-day exposure to *A. minutum*. After acclimation period, valve behavior of oysters varied depending on the *A. minutum* strain they were exposed to (Table 1). During exposure to the PST strain, oysters showed no significant modifications of valve-activity behavior, in terms of hourly valve-opening duration, hourly valve-opening amplitude and hourly micro-closure activity (i.e. number of micro-closures per hour) as compared to the acclimation period (Table 1). On the contrary, oysters fed the PST + BEC or the BEC strains during the 4 days of *A. minutum* exposure were opened hourly for a significantly longer time ($p < 0.001$ in both conditions, Table 1) and with a higher amplitude ($p < 0.05$ and $p < 0.001$, respectively; Table 1) than during the acclimation period. Additionally, oysters from the PST + BEC and the BEC conditions exhibited significantly more valve micro-closures than during acclimation (1.84 and 3.46 fold higher, respectively; $p < 0.001$ in both conditions).

3.3. Daily rhythm of oyster behavior

Chronobiological analyses were performed on the mean valve hourly opening duration in each condition before and during the *A. minutum* exposure. Spectral analysis (Lomb and Scargle periodogram) allows the determination of significant rhythmic periodicity in valve behavior. The results showed a daily valve activity with a circadian period (range: 20–28 h) during the acclimation period with *H. triquetra* in the three groups (Fig. 2). All the significant periods were tested in the chronobiological model (Cosinor model), which validated the existence of a circadian rhythm. During the *A. minutum* exposure, valve activity of oysters exposed to the BEC strain still showed a significant circadian period ($p < 0.05$), whereas no significant period were found in oysters exposed to the PST + BEC or PST strains (Fig. 2).

3.4. *Alexandrium minutum* consumption

During the exposure, the percentage of *A. minutum* cells filtered by oysters from the PST condition was 93.6% the first day and then reached 100% during the 3 following days of exposure (Table 2). The percentage of *A. minutum* cells filtered by oysters ranged from 61.3% to 95.6% in the PST + BEC condition, while for the BEC condition this

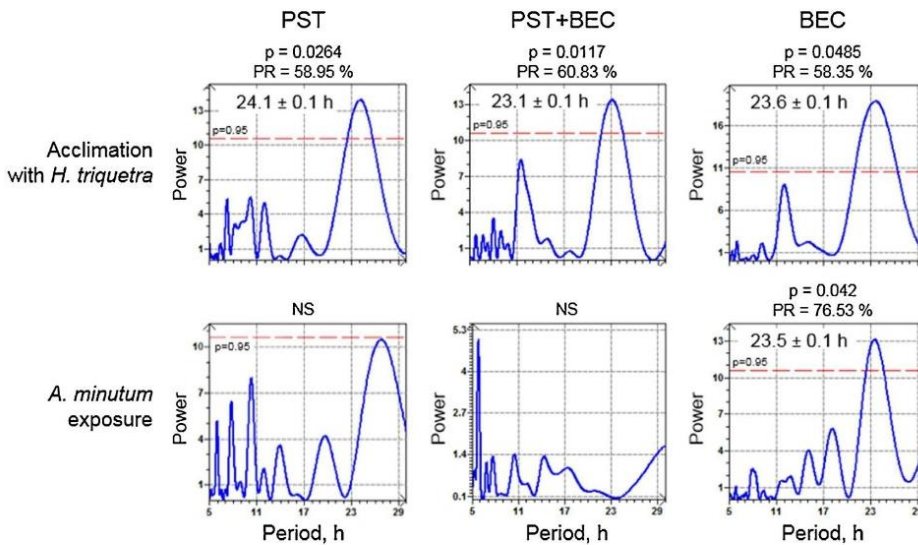


Fig. 2. Effect of three *A. minutum* strains producing PST, PST + BEC, or BEC, on the behavioral rhythm of *C. gigas*. Chronobiological analysis was made for each condition, during the 4 last days of the acclimation with non-toxic *H. triquetra* (top graphs), and during the 4-day exposure to *A. minutum* (bottom graphs). To reveal significant rhythm, spectral analysis by Lomb and Scargle periodogram was done first, the red dotted line corresponds to the significant threshold ($p = 0.95$), and the rhythmic activity is characterized by the period of the condition. Then, the rhythm is validated by the p -value and the percent rhythm (PR) of the Cosinor model. $n = 4$ -7 oysters per condition. NS: Non-significant at p -value = 0.05, i.e. no significant period.

Table 2
Percentage (%) of *A. minutum* cells filtered by oysters during the 4-day exposure period to the PST, PST + BEC and BEC strains. Mean \pm SE, $n = 3$ tanks per condition.

Condition	Day 1	Day 2	Day 3	Day 4
PST	93.6 \pm 3.7	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0
PST + BEC	61.3 \pm 2.0	62.5 \pm 2.3	85.0 \pm 0.5	95.6 \pm 2.6
BEC	50.3 \pm 1.7	14.8 \pm 2.4	41.8 \pm 2.7	53.2 \pm 4.6

percentage increased from 14.8% to 53.2% at the end of the exposure (Table 2).

3.5. Condition index

At the end of the exposure, wet weights of oyster digestive glands were not statistically different between the three conditions. Condition index in oysters exposed to the BEC strain (14.9 ± 0.5 , $n = 21$) was, however, significantly lower ($p < 0.05$ and $p < 0.001$, Tukey HSD test) compared to oysters of the two groups, exposed to the PST + BEC strain (17.1 ± 0.7 , $n = 21$) and to the PST strain (18.2 ± 0.6 , $n = 21$).

3.6. Histopathology

Histology revealed hemorrhages between the gills filaments in 53% and 27% of oysters exposed to the BEC (Fig. 3A) and PST + BEC (Fig. 3C) strains respectively (Table 3). Hemocytes migrated outside gill tissue via diapedesis (Fig. 3A) and aggregated around *A. minutum* cells (Fig. 3B). In the gills, hemocyte infiltration, diapedesis and hemorrhage were significantly more intense in oysters exposed to the BEC strain compared to oysters exposed to the PST strain (Table 3). Although gill tissue was not altered, mucus production was significantly higher in oysters from the BEC condition compared to oysters from the PST condition (Fig. 3C, Table 3). Intensity of histopathological observations in the gills of oysters from the PST + BEC condition was not significantly different from the other conditions, except for the intensity of hemocyte diapedesis which was intermediate between BEC and PST conditions (Table 3).

All oysters from the three conditions were actively feeding, as indicated by star-shaped digestive tubules (Fig. 3E). A relatively large number of intact *A. minutum* cells were observed in stomach and intestine lumen in oysters of the three conditions (Table 3). No *A.*

minutum cell was detected in digestive ducts or tubules, except in the tubules of one oyster of the PST condition. Pathological alterations observed in the digestive gland consisted of inflammatory responses characterized by intense hemocyte diapedesis, mainly through the epithelium of the stomach and digestive tubules, and hemocyte infiltrations detected mostly in digestive ducts and tubules (Fig. 3D, E). In the stomach, hemocyte diapedesis was significantly more intense in oysters exposed to the PST strain (1.7 ± 0.1) compared to oysters exposed to the BEC strain (1.1 ± 0.2 , Table 3). More rarely, hemocytes were observed in the lumen of the stomach, intestine and digestive ducts, in oysters exposed to the PST or the PST + BEC strain, but never in oysters exposed to the BEC strain.

The sum of mean intensities of pathological figures observed in the gills revealed that hemocyte responses were more intense in oysters exposed to the BEC strain (3.8) compared to oysters exposed to the PST strain (1.1), oysters from the PST + BEC condition being intermediate (2.3, Fig. 4A).

3.7. Hemocyte characteristics and functioning analyzed by flow cytometry

Total hemocyte count of oysters at the end of the experiment was significantly affected by algal exposure ($p < 0.05$, one-way ANOVA) (Fig. 5A). This effect was mainly attributable to variation in small agranulocyte counts between the conditions ($p < 0.01$, Kruskal-Wallis): small agranulocyte count of oysters from the PST condition ($1.1 \pm 0.1 \times 10^5$) was significantly lower compared to oysters exposed to the PST + BEC ($2.5 \pm 0.6 \times 10^5$) and BEC strains ($2.4 \pm 0.5 \times 10^5$). The composition of hemocyte subpopulations also appeared impacted: the percentage of small agranulocytes and granulocytes was significantly different in the three conditions ($p < 0.01$, Kruskal-Wallis, and $p < 0.01$, ANOVA) (Fig. 5B, C). Hemolymph from oysters exposed to the PST strain contained a significantly lower ($p < 0.01$, Kruskal-Wallis) percentage of small agranulocytes ($24.6 \pm 1.4\%$) compared to oysters from PST + BEC ($35.2 \pm 2.3\%$) and BEC condition ($34.0 \pm 2.8\%$), and a significantly higher ($p < 0.01$, ANOVA) percentage of granulocytes ($22.2 \pm 2.0\%$) compared to oysters from the PST + BEC condition ($14.1 \pm 1.4\%$) (Fig. 5B, C). Lastly, mortality of hemocytes (percentage of dead hemocytes) in oysters exposed to the PST strain was significantly higher ($9.6 \pm 2.5\%$) compared to oysters exposed the PST + BEC strain ($2.3 \pm 0.3\%$) and the BEC strain ($2.8 \pm 0.6\%$) (Fig. 5D). No significant difference in MMP, phagocytosis, ROS production, and morphological parameters was observed between the three conditions.

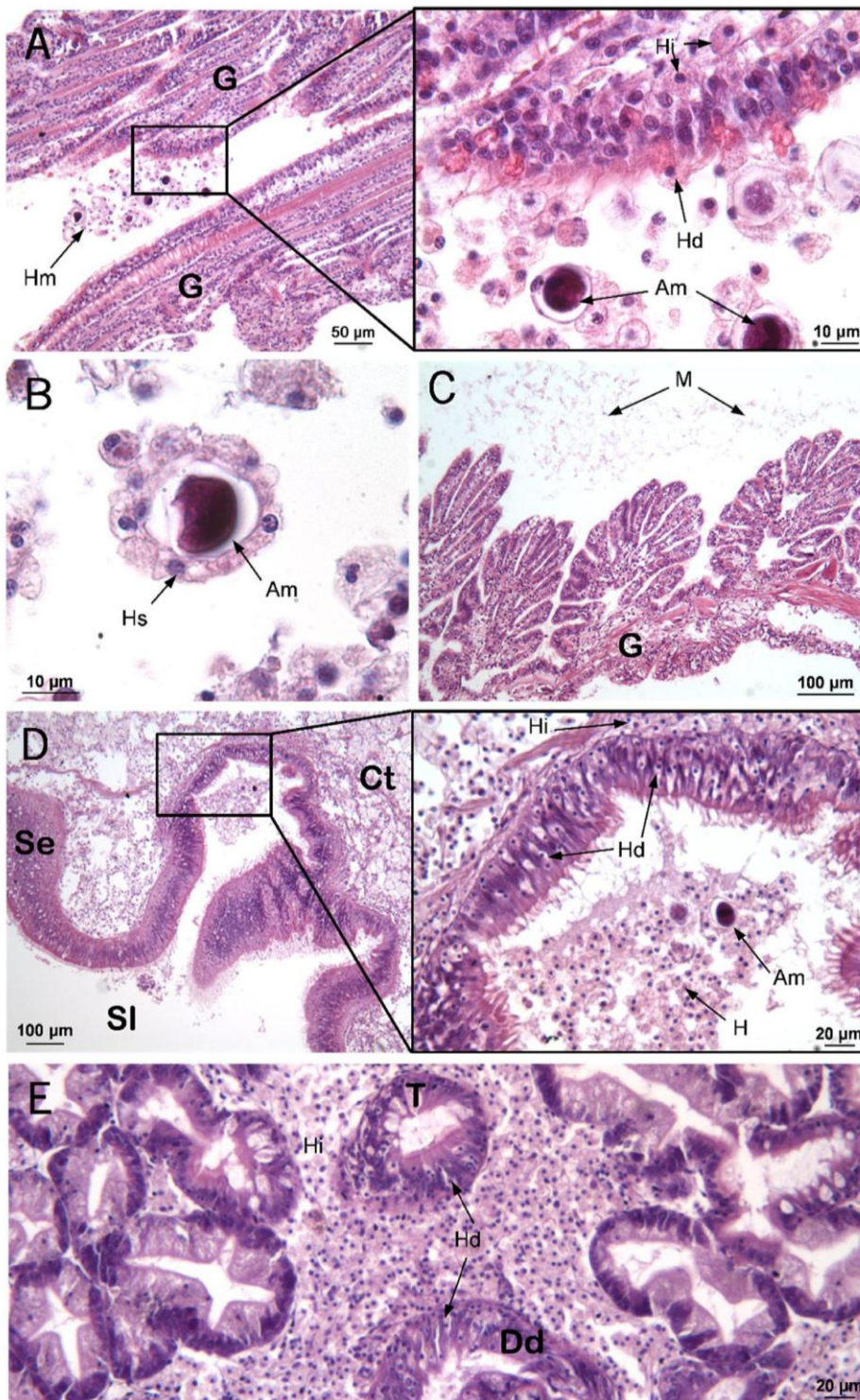


Fig. 3. Histological analysis (Hematoxylin-eosin stained paraffin sections) of gills of *C. gigas* exposed to the BEC strain of *A. minutum* (A, B) or to the PST + BEC strain (C) and digestive gland of oysters exposed to the PST strain (D, E). (A) Hemorrhage (Hm) between gill filaments (G), hemocyte diapedesis (Hd) and infiltration (Hi) in gill filament, and *A. minutum* cells (Am) outside the gills; (B) Detail of hemocytes surrounding (Hs) an *A. minutum* cell (Am); (C) Mucus (M) produced by gills (G); (D) Moderate inflammatory responses in the stomach characterized by infiltration of hemocytes (Hi) in the connective tissue (Ct) and intense hemocyte diapedesis (Hd) through stomach epithelium (Se) and lumen (Sl); (E) Diapedesis through the epithelium of digestive ducts (Dd) and tubules (T), and hemocyte infiltration (Hi) in the connective tissue.

4. Discussion

Although the effects of the toxic dinoflagellate *A. minutum* on bivalves have been well described, addressing specific oyster response to either paralytic shellfish toxins (PSTs) or bioactive extracellular compounds (BECs) is still challenging, since most studies either did not assess the production of BECs (Bricelj et al., 1993; Bricelj and Shumway, 1998) or often used strains producing both types of toxins

(Haberhorn et al., 2010a,b, 2011; Tran et al., 2010, 2015; Mat et al., 2013; Payton et al., 2017a). The present work investigated the effects of the different types of toxic compounds produced by the dinoflagellate *A. minutum*, the PSTs and the BECs, which chemical nature is still unknown, on the behavior and physiology of adult *C. gigas*. The most striking responses in this experiment were observed in oysters exposed to the BEC strain of *A. minutum*, on feeding activity, valve activity behavior, and on tissue and cellular gill parameters.

Table 3

Mean intensities of histological figures in *C. gigas* digestive gland and gills, after a 4-day exposure to the PST, PST + BEC or BEC strain of *A. minutum*. Results are expressed as mean of stage intensity ± SE (n = 15), with stage 0 = absence or very light, stage 1 = light-moderate, and stage 2 = heavy. Letters indicate significant differences between conditions (Kruskal-Wallis test followed by NDWD post-hoc test).

Organs	Pathologies	Conditions			Kruskal-Wallis test
		PST	PST + BEC	BEC	
Digestive gland					
Stomach	Hemocyte infiltration	0.6 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	NS
Intestine		0.9 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	NS
Digestive ducts		0.9 ± 0.2	0.7 ± 0.2	0.9 ± 0.1	NS
Digestive tubules		1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	NS
Stomach	Diapedesis	1.7 ± 0.1 ^a	1.2 ± 0.2 ^{ab}	1.1 ± 0.2 ^b	*
Intestine		0.9 ± 0.2	0.9 ± 0.1	0.5 ± 0.2	NS
Digestive ducts		0.3 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	NS
Digestive tubules		0.1 ± 0.1	0 ± 0	0 ± 0	NS
Stomach	Presence of <i>A. minutum</i> cells in the lumen	1.1 ± 0.2	0.7 ± 0.2	0.5 ± 0.2	NS
Intestine		1.6 ± 0.1	1.2 ± 0.2	1.0 ± 0.2	NS
Gills	Hemocyte infiltration	0.6 ± 0.2 ^a	0.9 ± 0.2 ^{ab}	1.1 ± 0.1 ^b	*
	Diapedesis	0.1 ± 0.1 ^a	0.3 ± 0.1 ^b	0.8 ± 0.1 ^c	***
	Hemorrhage	0 ± 0 ^a	0.3 ± 0.2 ^{ab}	0.8 ± 0.2 ^b	**
	Mucus production	0.5 ± 0.1 ^a	0.8 ± 0.1 ^{ab}	1.1 ± 0.2 ^b	*

NS = non-significant.

^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001.

4.1. BECs reduce algal consumption of oyster and thus PST accumulation

In this study, the PST + BEC *A. minutum* strain appeared more toxic than the PST strain regarding cellular PST content and toxicity. However, oysters exposed to the PST + BEC strain accumulated less PSTs in their digestive glands than oysters exposed to the PST strain. This could be the result of a reduced feeding activity. Reduced feeding activity is supported by the reduction of algal consumption observed for oysters of the PST + BEC condition compared to the PST condition, this reduction being even more drastic in the BEC condition. The BECs might be responsible for the reduction of feeding activity, as this response was only observed in oysters exposed to the BEC producing strains and more marked with the BEC strain, which is the most cytotoxic strain tested. Similarly, the BEC strain elicited a stronger decrease in clearance and ingestion rates, and a lower absorption rate than the PST + BEC strain in *C. gigas* and *R. philippinarum* (Contreras, 2011). Feces production also seemed to be lower in the BEC condition than in the PST and PST + BEC conditions, and a noticeable production of pseudofeces was observed in the BEC condition (data not shown). Pseudofeces has been described as a secondary regulatory mechanism of ingestion rate in marine bivalves and also a mechanism for pre-ingestive selection (Shumway and Cucci, 1987; Navarro and Widdows, 1997; Wildish et al., 1998). Previous studies showed that *C. gigas* reduced filtration, ingestion and biodeposition rates when exposed to different *Alexandrium* species (Bardouil et al., 1993; Lassus et al., 1999, 1996; Bougrier et al., 2003). Lassus et al. (2004) and Laabir et al.

(2007) reported partial inhibition of filtration rate in oysters exposed to the PST + BEC *A. minutum* strain compared to non-toxic *Skeletonema costatum*, related to a decreased feeding time activity and a lower biodeposit production rate. The decreased filtration rate coupled with pseudofeces production would be at the origin of the significantly-reduced condition index of oysters of the BEC condition, compared to oysters of the PST and PST + BEC conditions. This reduced feeding activity might consist in an avoidance response after direct contact between *Alexandrium* cells and oyster external organs.

4.2. BECs disturb valve-activity behavior

Valve activity behavior analysis showed increased hourly valve-opening duration and hourly micro-closure activity in oysters exposed to the BEC strain and, to a lesser extent in oysters exposed to the PST + BEC strain. Concerning the PST + BEC strain, similar results were already obtained by Tran et al. (2010, 2015), but without defining the respective effects of PSTs and BECs produced by the strain. These behavioral responses may be mostly related to the production of BECs by *A. minutum*. Indeed, the non-PST producing BEC strain caused more intense behavioral changes in oysters than the PST + BEC strain, whereas the PST strain did not induce any significant changes in oyster behavioral parameters. The frequency of micro-closures during a 2-day exposure to the PST + BEC strain of *A. minutum* was positively correlated to the *A. minutum* concentration in the ambient water and not to the PST content in the digestive gland of oysters (Haber Korn et al.,

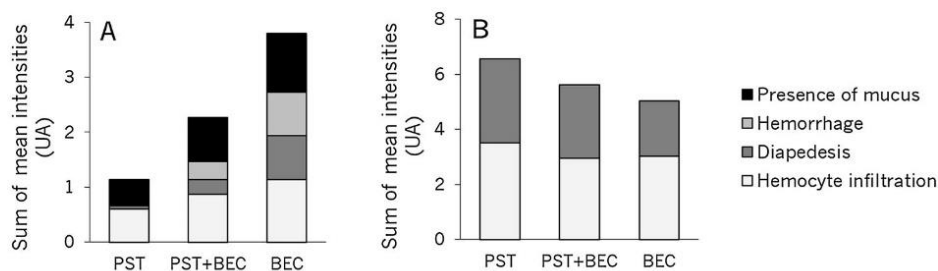


Fig. 4. Sum of mean intensities of each histopathological observations in the gills (A) and in the digestive gland (B) of oysters *C. gigas* after a 4-day exposure to the PST, PST + BEC or BEC strain of *A. minutum* (n = 15 in each condition).

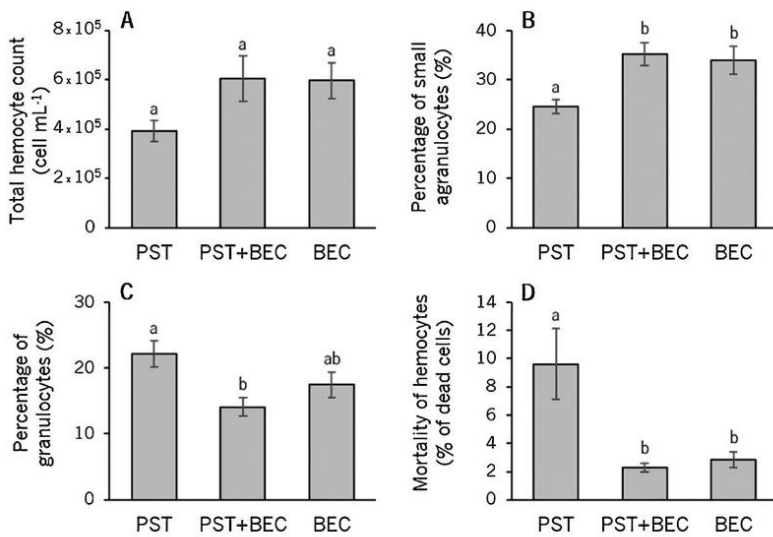


Fig. 5. Hemocyte variables (mean \pm SE) in the circulating hemolymph of oysters *C. gigas* after a 4-day exposure to different strains of *A. minutum*: the PST strain (n = 18 oysters), the PST + BEC strain (n = 18 oysters) or the BEC strain (n = 19 oysters). (A) Total hemocyte count (cells mL⁻¹); (B) Percentage of small agranulocytes; (C) Percentage of granulocytes; (D) Hemocyte mortality (percentage of dead hemocytes). Letters indicate significant differences between conditions (ANOVA followed by post-hoc Tukey HSD test).

2011). This supports our hypothesis, as a higher *A. minutum* concentration would imply a greater quantity of bioactive extracellular compounds in the water resulting in a higher impact on oyster micro-closure activity.

The BECs excreted in the water could activate chemoreceptors of the mantle edge or directly affect oyster gills when *A. minutum* cells come into contact with the tissues in the pallial cavity, responsible for the quick increase in frequency of valve micro-closures. Conversely, intracellular PSTs are mainly released in the digestive gland, once dinoflagellate cells have been digested by oysters. During the toxification phase, the digestive gland is considered as the initial repository of toxic cells following ingestion and is the organ where PST are initially accumulated in bivalves (Bricelj and Shumway, 1998). The hypothesis that behavioral changes might be caused by internal contamination by PSTs released after *A. minutum* cell digestion is not supported by our results, as behavioral changes were mostly observed in response to the BEC strain.

The increase in frequency of valve micro-closures is probably a protective behavior to increase intervalvar water renewal and minimize contact with BECs, potentially irritant to gill tissues. Increased closures and expulsions observed in *Pecten maximus* exposed to the PST + BEC strain was also hypothesized to serve as a reaction to expulse *A. minutum* cells from the mantle cavity (Coquereau et al., 2016). Exposing juvenile *Pecten maximus* to the BEC strain of *A. minutum* led to a less effective escape response after a contact with a predator supposedly due to direct tissue damage caused by BECs (Borcier et al., 2017). Given the noteworthy oyster behavioral changes in response to BEC-producing strains, *C. gigas* gills integrity could be impaired.

4.3. BECs induce hemorrhage and inflammation in gills

The BECs induced hemorrhage in gills and aggregation of hemocytes around *A. minutum* cells. These symptoms are indeed stronger with the BEC strain, whereas these pathologies were not observed in gills of oysters exposed to the PST strain, which had no detectable cytotoxic activity. Aggregation of hemocytes surrounding toxic algal cells had already been observed in *Argopecten irradians* and *Mercenaria mercenaria* respectively exposed to *Prorocentrum minimum* which also produce uncharacterized cytotoxic compounds (Wikfors and Smolowitz, 1993; Hégaret et al., 2011).

Besides, the intensity of hemocyte infiltration, diapedesis, and mucus production in the gills was significantly higher for oysters in the BEC condition than in the PST condition. The pathologies could be

interpreted as defensive inflammation responses against *A. minutum* cells and bioactive compounds released by those algal cells. Haberkorn et al. (2010a) previously reported an increased mucus production in *C. gigas* exposed to the PST + BEC strain of *A. minutum*, probably reflecting tissue irritation and/or defense reaction to BECs. Indeed, mucus contains lytic enzymes that play an important role in defense against a wide range of stressors (Fisher, 1992). Similarly, contact with the non-PST producing *Alexandrium ostenfeldii* led to edema in the mantle, associated with hemocyte infiltration in the gills of Manila clams (Lassudrie et al., 2014). This inflammatory response in external organs was hypothesized to be partly induced by uncharacterized extracellular compounds produced by *A. ostenfeldii* shown to be lytic to protists (Tillmann et al., 2007).

In the present study, the higher counts of hemocytes in circulating hemolymph may reflect hemocyte *de novo* production or mobilization from connective tissues to hemolymph to supply responses in specific tissues. Particularly, hemocyte participation in wound repair has been suggested based upon the observation of tissue lesions caused by toxic dinoflagellate exposure associated with hemocyte aggregates or infiltrations, a typical response to *Alexandrium* spp. exposure in bivalves.

4.4. PSTs disrupt biological rhythm

The two PST-producing strains disrupted the daily rhythm of oyster valve activity. Biological rhythms allow synchronization and anticipation of environmental cycles, allowing the temporal organization of biological processes and maximizing the fitness of organisms with their biotope (Yerushalmi and Green, 2009). Oyster behavior is known to follow circadian, ultradian, and infradian rhythms (Tran et al., 2011; Bernard et al., 2016). Their daily rhythm is driven by a circadian clock (Mat et al., 2012, 2013; Payton et al., 2017b), which genetic components, i.e. clock genes, have been recently identified (Perrigault and Tran, 2017). The clock genes are involved in the generation and/or synchronization of circadian rhythmic activities (Chaves et al., 2011). Additionally, *A. minutum* exposure (PST + BEC strain) can deeply modify temporal organization of gills transcriptome and induce a loss of daily cycle in oyster valve behavior, crystalline style length, and expression of genes involved in detoxification and oxidative stress (Tran et al., 2015; Payton et al., 2017a). These disruptions have been linked to the repression of the transcription of most of the clock genes in oysters exposed to *A. minutum* (Mat et al., 2013; Payton et al., 2017a).

PSTs are presumably responsible for the disappearance of the valve cyclic activity observed in the present study via down-regulation of

clock gene expression, as showed in Payton et al. (2017a). Indeed, saxitoxin and its derivatives have a mechanism of action close to tetrodotoxin, targeting the same voltage-gated sodium channels (Heggenes and Starkus, 1986); and tetrodotoxin is known to repress clock gene expression (Van Den Pol and Obrietan, 2002).

4.5. PSTs provoke inflammation in the digestive gland

Toxin accumulation measured in the digestive gland, the main storage organ for PSTs (Lassus et al., 2007; Guéguen et al., 2008), indicated that the PST-producing strains were ingested. Oysters exposed to the PST strain had higher PST burden in their digestive glands, where the strongest inflammatory response was also observed. Hemocyte infiltration and diapedesis across digestive epithelium were more intense in the PST condition than in the BEC condition, and intermediate in the PST + BEC condition. These typical hemocyte responses in bivalves exposed to *Alexandrium* spp. (Galimany et al., 2008; Lassudrie et al., 2014; Borcier et al., 2017) have been linked to the presence of PSTs (Haberkorn et al., 2010a). Saxitoxin and its derivatives could be directly released within the organ by lysis of *Alexandrium* cells, or assimilated and biotransformed within the tissues. Hemocytes could participate to toxin depuration by eliminating toxins through the lumen of the intestine and/or isolating *Alexandrium* cells.

Indeed, the percentage of dead circulating hemocytes was higher in the PST condition. Mortality of oyster hemocytes was already noticed *in vitro* in response to toxic *Alexandrium fundyense* and *A. minutum* (PST + BEC strain) (Hégaret et al., 2011). In the lion-paw scallop *Nodipecten subnodosus*, a lower count of hemocytes was attributed to the elicited caspase-dependent apoptosis of hemocytes (Estrada et al., 2010, 2014). Similarly, *in vitro* exposure of oyster hemocytes to different saxitoxin analogs (STX and C1/C2) caused apoptosis in hemocytes in a dose dependent manner, a process dependent on caspase activation (Abi-Khalil et al., 2017). Since these PST derivatives were detected in oysters of the PST condition, we suggest that the large amount of accumulated PSTs induced hemocyte apoptosis responsible of the higher mortality of circulating hemocytes.

5. Conclusions

In the present study, behavioral and physiological responses of oysters greatly differed depending on the *A. minutum* strain they were exposed to, and thus potentially on the type of bioactive substances produced by the strains. The results showed for the first time that the cytotoxic BECs produced by *A. minutum* could impair oyster feeding, by altering valve activity and gill tissues. These findings highlight the urgent need to characterize the bioactive extracellular compounds in toxic algae, as *Alexandrium* spp. The PSTs produced by *A. minutum* have disrupted the biological rhythm of oyster behavior, with significant impacts on the digestive gland. The potential genotoxic impacts of PSTs on clock genes, potentially leading to the loss of rhythmicity is to further investigate.

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Chapitre 4

Quelle est la sensibilité des gamètes libres de l'huître à *A. minutum* et à ses toxines ?

Dans le chapitre précédent, nous avons démontré que les toxines paralysantes (PST) et les composés extracellulaires bioactifs (BEC) produits par *A. minutum* entraînent des effets contrastés sur la physiologie et le comportement valvaire des huîtres adultes. Ce travail met en évidence que les perturbations comportementales, tissulaires et cellulaires les plus remarquables sont majoritairement provoquées par les BEC, plutôt que les PST. Les souches d'*A. minutum* produisant des PST perturbent le rythme biologique des huîtres et provoquent une inflammation de la glande digestive. Les souches produisant des BEC affectent la nutrition et l'activité valvaire des huîtres adultes, et induisent une mobilisation des hémocytes dans les branchies, un des premiers organes en contact avec les cellules d'*A. minutum* et les toxines extracellulaires. La souche BEC d'*A. minutum*, non productrice de PST, affecte négativement les hémocytes d'huîtres lors d'une exposition *in vitro* (Lambert et al., 2013). Ces résultats indiquent que les BEC ont des effets cytotoxiques sur les organes externes et les cellules qui sont en contact direct avec les cellules d'*A. minutum*.

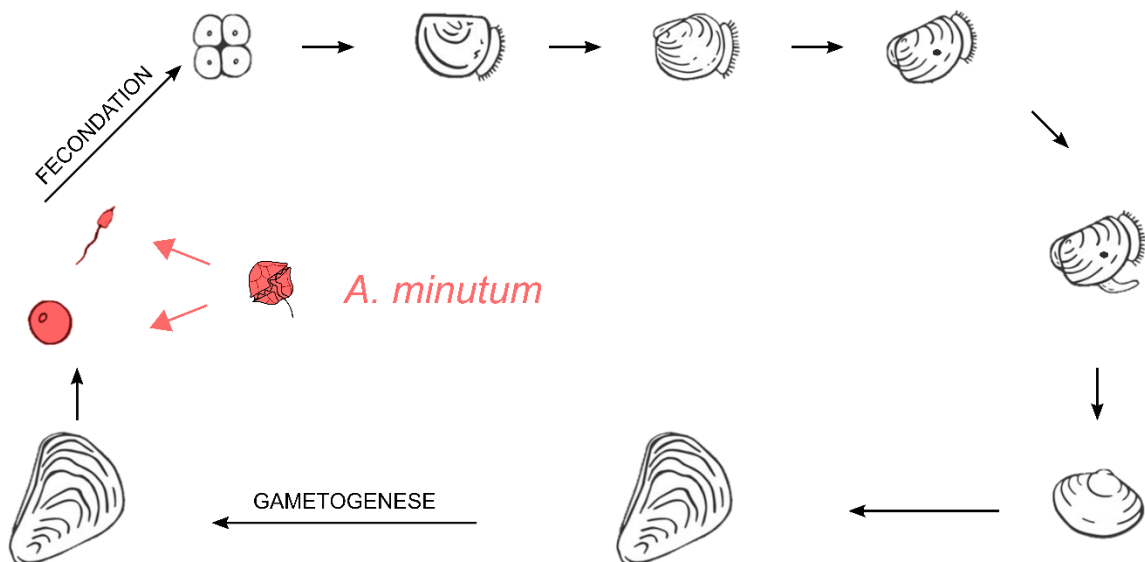
Dans le chapitre 2, nous avons observé une diminution du pourcentage de spermatozoïdes mobiles suite à l'exposition d'huîtres à *A. minutum* pendant la gamétogenèse. La qualité des gamètes peut donc être indirectement affectée par *A. minutum* via l'exposition des huîtres adultes, ce qui peut avoir des conséquences néfastes sur le développement de la descendance.

Lors de l'émission des gamètes des huîtres, les spermatozoïdes et les ovocytes sont émis dans l'eau de mer où la fécondation a lieu. Si l'émission des gamètes a lieu lors d'une efflorescence d'*A. minutum*, les gamètes se trouvent directement au contact des cellules de dinoflagellés et des composés bioactifs extracellulaires.

Quelques récentes études ont mis en évidence que certaines micro-algues responsables d'HAB ont des effets délétères sur les gamètes et la fécondation de bivalves marins, comme les huîtres *Crassostrea virginica* et *Pinctada fucata martensii* (Banno et al., 2018; Basti et al., 2013; Rolton et al., 2015). Banno et al. (2018) ont démontré qu'une souche d'*A. affine* non-productrice de PST affecte la vitesse de nage des spermatozoïdes de *P. fucata martensii* après

seulement quelques minutes d'exposition. Ces études suggèrent que des composés bioactifs produits par *Alexandrium* spp., indépendamment des PST, pourraient affecter les gamètes des bivalves marins.

Dans ce contexte, nous avons étudié les effets de différentes souches d'*A. minutum* sur les paramètres cellulaires des ovocytes et des spermatozoïdes de *C. gigas*, et les conséquences de cette exposition sur la fécondation. Comme dans le chapitre 3, nous avons testé les effets de 3 souches d'*A. minutum* produisant soit des PST, soit des BEC, soit des PST et des BEC, afin de décrire les effets distincts de ces composés toxiques sur la qualité des gamètes et leur capacité de fécondation. Différentes concentrations en *A. minutum* représentatives d'efflorescences dans le milieu naturel ont été testées afin de d'évaluer la sensibilité des gamètes.



Les hypothèses avancées étaient :

1. L'exposition *in vitro* des gamètes de *C. gigas* à *A. minutum* affecte la qualité et la viabilité des spermatozoïdes et des ovocytes,
2. Les composés extracellulaires bioactifs produits par *A. minutum* ont une activité cytotoxique sur les gamètes d'huîtres.

Ce chapitre fait l'objet d'un article en cours de préparation :

Castrec J., et al. (in prep-a) The toxic dinoflagellate *Alexandrium minutum* affects gametes and fertilization of the oyster, *Crassostrea gigas*.

The toxic dinoflagellate *Alexandrium minutum* affects gametes and fertilization of the oyster, *Crassostrea gigas*

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Abstract

Dinoflagellates from the globally distributed genus *Alexandrium* are known to produce both paralytic shellfish toxins (PST) and uncharacterized bioactive extracellular compounds (BEC) with allelopathic and cytotoxic activities. In France, *Alexandrium minutum* generally blooms during the spawning period of most bivalves, potentially affecting gametes and/or embryolarval development. This question is of real concern for recruitment sustainability of economic important species of bivalves, such as the Pacific oyster *Crassostrea gigas*.

The aim of this work was to test the effects of three strains of *Alexandrium minutum* producing either only PSTs, only BECs, or both PSTs and BECs upon oyster gametes and potential consequences on fertilization success. Oocytes and spermatozoa were exposed to a range of *A. minutum* concentrations (10 to 2.5×10^4 cells mL⁻¹) for 2 hours. Viability and reactive oxygen species (ROS) production were assessed by flow cytometry, spermatozoa motility was analysed by microscopy, and fertilization yield of exposed spermatozoa and oocytes were assessed.

Viability and fertilization capacity of spermatozoa and oocytes were drastically reduced following exposure to 2.5×10^4 cells mL⁻¹ and 2.5×10^3 cells mL⁻¹ of *A. minutum*, respectively. The BEC-producing strain decreased spermatozoa motility, increased ROS production of spermatozoa and oocytes, and inhibited fertilization at the highest concentration tested. This study highlights the significant toxicity of the BECs produced by *A. minutum* on oyster gametes. These results suggest that a direct exposure of oyster gametes to *A. minutum*, reducing gamete survival and “quality”, can affect oyster fertility and reproduction success.

Keywords: Harmful Algal Bloom (HAB), Paralytic Shellfish Toxins (PST), Bivalve, Bioactive Extracellular Compounds (BEC), gamete, Flow cytometry, Fertilization.

1. Introduction

The process of fertilization is essential for the reproduction success and sustainability of free spawning marine organisms (Marshall et al., 2002). Most marine bivalves release their gametes into the water where fertilization takes place. The chemical nature and “quality” of the seawater into which gametes are released therefore plays a key role in fertilization and larval development (Havenhand et al., 2008). Previous studies have shown that acute exposure of spermatozoa and oocytes to organic pollutants (Akcha et al., 2012; Mai et al., 2013; Vignier et al., 2017, 2015) or to copper (Fitzpatrick et al., 2008) could negatively affect fertilization success and embryo development of oysters.

Recent studies suggest that harmful algal blooms (HAB) can also affect gametes, development, growth, and survival of marine bivalve larvae (Basti et al., 2011; Binzer et al., 2018; Bricelj and MacQuarrie, 2007; De Rijcke et al., 2015; Rolton et al., 2018, 2014; Tang and Gobler, 2012), but the effects of HAB on fertilization of many bivalve species are largely unknown. HAB are annually recurring phenomena which threaten marine coastal areas by negatively affecting tourism, human health, fisheries, and aquatic ecosystems worldwide (Anderson et al., 2012a, 2008). A few recent studies have showed negative effects of HAB to marine bivalve gametes and fertilization. A 2-hour exposure of *Crassostrea virginica* gametes to the dinoflagellate *Karenia brevis* decreased spermatozoa viability and fertilization success (Rolton et al., 2015). The toxic *Heterocapsa circularisquama* decreased oocyte viability and fertilization of the Japanese pearl oyster *Pinctada fucata martensii* (Basti et al., 2013). Banno et al. (2018) reported reduction in the swimming velocity of spermatozoa of Japanese pearl oysters exposed to *Alexandrium catenella*, *A. affine*, *Heterosigma akashiwo* and *Chattonella marina*.

HAB species from the genus *Alexandrium* are known to produce paralytic shellfish toxins (PST) that can cause human poisoning through shellfish-mediated intoxications (Bricelj and Shumway, 1998). The consequences of *Alexandrium* blooms are both ecological, e.g. alteration of marine trophic structure, large-scale mortality of fish and shellfish, and economic, e.g. impairment of tourism and recreational activities, fishery closure and sell prohibition of shellfish (Anderson et al., 2012b). The early embryo development of *Mytilus edulis*, *C. gigas*, and *P. fucata martensii* was negatively affected by exposure of fertilized eggs to *A. ostensfeldii* and *A. minutum* (De Rijcke et al., 2016), *Alexandrium catenella* (Mu and Li, 2013), and *A. affine* and *A. catenella* (Basti et al., 2015), respectively. In France, blooms of *A. minutum*

usually occur from April to November (Pouvreau et al., 2016) overlapping with Pacific oyster breeding and spawning periods, thus gametes may come into direct contact with *A. minutum* cells. An understanding of the effects of *A. minutum* on gametes and early life stages of *C. gigas* is therefore critically important.

A toxic strain of *A. minutum* (AM89BM strain) affected oocytes exposed *in vitro* (Le Goïc et al., 2014) as well as spermatozoa derived from exposed oysters (Castrec et al., submitted; Haberkorn et al., 2010). This strain produces PST but also unidentified bioactive extracellular compounds (BEC) which are cytotoxic to other microalgae species (Lelong et al., 2011; Long et al., 2018). The deleterious effect of BEC have also been demonstrated upon the behavior of the scallop *Pecten maximus* (Borcier et al., 2017) and physiology of the oyster *C. gigas* (Castrec et al., 2018). Several species from the genus *Alexandrium* have been shown to produce such poorly uncharacterized bioactive compounds exerting allelopathic, hemolytic, ichthyotoxic or cytotoxic activities (Arzul et al., 1999; Ford et al., 2008; Lelong et al., 2011; Mardones et al., 2015). The respective involvement of PST and BEC in *A. minutum* toxicity toward *C. gigas* gametes is not yet clearly identified.

In the present study, we investigated the toxicity of *A. minutum* on oyster gametes by measuring their cellular characteristics by flow cytometry, the spermatozoa motility and swimming velocity and the fertilization success of exposed spermatozoa or oocytes by microscopy and image analysis. To determine the respective toxicity of BEC and PST, three *A. minutum* strains, either producing only PST, only BEC, or both PST and BEC were tested.

2. Materials and methods

2.1 Biological materials

Algal cultures. Three strains of *Alexandrium minutum* (Halim) were grown in filtered seawater (0.2 µm) supplemented with L1 medium (Guillard and Hargraves, 1993) and maintained at 17 ± 1 °C with a dark:light cycle of 12:12 h. These *A. minutum* strains were:

- the PST+BEC strain (strain AM89BM, isolated from a bloom in the Bay of Morlaix, France) produces 10.6 fmol PST cell⁻¹ (corresponding to 1.3 pg STX eq. cell⁻¹; Fabioux et al., 2015),
- the PST strain (strain Da1257, isolated from a bloom in the Bay of Daoulas, France) produces 0.63 fmol PST cell⁻¹ (corresponding to 0.053 pg STX eq. cell⁻¹; Pousse et al., 2018),

- the BEC strain (strain CCMI1002, isolated from Irish waters), for which no PST were detected (Borcier et al., 2017).

According to the bioassay developed by Long et al. (2018), the BEC strain is four times more cytotoxic than the PST+BEC strain, whereas the PST strain has a negligible cytotoxic potency. Cultures of *A. minutum* were not axenic, grown without antibiotics, and harvested in exponential growth phase for the experiment. Algal concentrations (cells mL⁻¹) were determined by counts using a FACScalibur (BD Sciences, San Jose, CA, USA) flow cytometer equipped with a blue laser (excitation 488 nm) according to Marie et al. (1999). For each experiment, *A. minutum* cultures were re-suspended in filtered seawater to reach final cell concentrations of 2×10^1 , 2×10^2 , 5×10^3 , and 5×10^4 cells mL⁻¹. These cultures were left to equilibrate for 24 h prior to the exposures.

Oysters. Mature adult Pacific oysters (n = 5 females and n = 4 males) were collected from Daoulas estuary in the Bay of Brest (North Brittany, France) for the first experiment studying cellular features of gametes exposed to *A. minutum*. Adult oysters used for the second experiment (n = 3 females and n = 3 males) assessing fertilization success originated from a cohort produced in 2017 according to Petton et al. (2015) and were deployed as spat in the bay of Brest. Oysters were collected during the natural reproduction period (June and July 2018) for *in vitro* experiments.

2.2 Collection of gametes

Oyster sex determination and rapid gamete quality check, i.e. round shape of oocytes and spermatozoa motility, were made by examination of 2 µl gonad samples under a light microscope (Leica MZ 125, ×10 objective). Oocytes and spermatozoa were collected by stripping the gonad of each oyster. Briefly, gametes were collected individually in 0.2-µm filtered sea water (FSW; pH 8.12, 21 °C) and sieved through 100 µm mesh. Cell concentrations were determined individually by flow cytometry (FCM) (duplicate, EasyCyte Plus cytometer, Guava Millipore) according to Le Goïc et al. (2014, 2013). The concentration of oocyte suspensions were adjusted to 1×10^5 cells mL⁻¹ with FSW for flow cytometry and fertilization experiments. Spermatozoa suspensions were diluted to 1×10^8 cells mL⁻¹ with FSW for movement measurements, and to 2×10^6 cells mL⁻¹ for flow cytometry and fertilization experiments.

2.3 Cellular characteristics of exposed gametes and movement of exposed spermatozoa

For each oyster, 2.5 mL of gamete suspension (oocytes or spermatozoa) were exposed to 2.5 mL of the *A. minutum* suspensions (3 *A. minutum* strains \times 4 concentrations) or to 2.5 mL of FSW (control treatment) in 10 mL glass vials for 2 hours for FCM analysis. Final *A. minutum* concentrations were 10, 10^2 , 2.5×10^3 , and 2.5×10^4 cells mL⁻¹. These concentrations were chosen to reflect the range of algal densities observed during natural blooms (Chapelle et al., 2015). In France, the alert threshold triggering weekly concentration monitoring for *A. minutum* blooms is 10 cells mL⁻¹ (Guallar et al., 2017). For spermatozoa motility analysis, 0.5 mL subsample of spermatozoa suspension from each male was transferred in 2 mL glass vials containing 0.5 mL of the *A. minutum* suspensions or 0.5 mL of SW (control treatment). After 2 hours of exposure, gametes were sampled for FCM analyses and spermatozoa were also sampled for motility measurements.

2.3.1 Characterization of relative cell size and complexity, viability and reactive oxygen species production

FCM measurements were performed using an EasyCyte Plus cytometer (Guava Millipore) equipped with standard optics and a 488 nm argon laser according to Le Goïc et al. (2013, 2014) for spermatozoa and oocytes.

2.3.2 Characterization of spermatozoa movement.

After spermatozoa suspension dilution in an activating solution (FSW, 5 g L⁻¹ bovine serum albumin, Tris 20 mM, pH 8.1, dilution rate 1:30), spermatozoa movement was analyzed under phase contrast microscope coupled to a video camera (Qicam Fast 1394) using a CASA plugin for ImageJ software. The percentage of motile spermatozoa and their velocity (VAP: Velocity of the Average Path) were assessed on a minimum of 30 spermatozoa for each sample, according to Boulais et al. (2015b).

2.4 Fertilization of exposed oocytes and spermatozoa

For each oyster, 2.5 mL of gamete suspension (oocytes or spermatozoa) were exposed for 2 hours to 2.5 mL of the *A. minutum* culture (3 *A. minutum* strains \times 4 concentrations) or to 2.5 mL of FSW (control treatment) in 10 mL glass vials.

To test fertilization success of exposed oocytes, fertilization was performed using a pool of control spermatozoa, at 5×10^7 cells mL⁻¹ prepared from the initial spermatozoa suspensions of 3 males (see section 2.2). To test fertilization success of exposed spermatozoa, fertilization was performed using a pool of control oocytes, at 5×10^4 cells mL⁻¹, prepared from initial oocyte suspensions of 3 female oysters (see section 2.2).

After incubation, exposed oocytes (5×10^3 oocytes) were transferred to 24-well microplates (NUNC®) filled with 2 mL of FSW and 10 µL of the non-exposed spermatozoa pool (100:1 spermatozoa:oocyte ratio). Similarly, exposed spermatozoa (5×10^5 spermatozoa) were transferred to 24-well microplates (NUNC®) filled with 1.4 mL of FSW and 100 µL of the non-exposed oocytes pool (100:1 spermatozoa:oocyte ratio). After 2 hours of contact between spermatozoa and oocytes, samples were fixed with a formaldehyde-seawater solution (1 % final) to estimate the fertilization yield using a light microscope (Leica DM-IRB). Numbers of fertilized (2-cell or 4-cell stages) and unfertilized (absence of segmentation) oocytes were counted, on at least 100 oocytes. Fertilization yield was defined as: number of fertilized oocytes $\times 100 /$ (number of unfertilized and fertilized oocytes). Each oocytes/spermatozoa fertilization assay was performed in triplicate, the mean of the triplicate fertilizations were used in statistical tests.

2.5 Statistical analyses

Statistical analyses and graphical representations were performed using R software (R Core Team, 2012). Differences were considered significant when $p < 0.05$. All values are expressed as mean \pm standard error (SE). Cellular characteristics measured by flow cytometry and fertilization yields were compared between the 13 treatments (3 *A. minutum* strains \times 4 concentrations, and the control) for oocytes and spermatozoa using repeated measures ANOVA, with 'treatment' as a fixed factor and 'oyster' as a random factor. The viability variable was transformed as arcsin(squareroot) before statistical analysis, but were presented as non-transformed data in figures. A repeated measures ANOVA was used to test the effect of *A. minutum* exposure on spermatozoa motility and velocity, accounting for oyster as a random factor. If the effect of treatment was significant, post-hoc analyses were performed using emmeans function. Normality and homogeneity of variance were verified by the Shapiro-Wilk and Levene methods, respectively.

3. Results

3.1 Effect of *A. minutum* exposure upon gamete cellular characteristics and spermatozoa movement

Exposure to the highest concentration of *A. minutum* (2.5×10^4 cells mL⁻¹) significantly affected the viability of oocytes ($F = 12.64$, $p < 10^{-10}$) and spermatozoa ($F = 17.28$, $p < 10^{-10}$). The percentages of viable spermatozoa exposed to the three *A. minutum* strains was lower than control spermatozoa (94.4 ± 1.4 %), the BEC strain having the strongest effect on spermatozoa viability (54.3 ± 9.3 %) compared to the PST+BEC (79.4 ± 5.1 %) and PST strains (78.5 ± 4.8 %) (Fig. 11A). The viability of oocytes exposed to 2.5×10^4 cells mL⁻¹ of the BEC strain (68.4 ± 5.3 %) or the PST+BEC strain (75.4 ± 5.2 %) was lower than control oocytes (91.3 ± 2.1 %) (Fig. 11B).

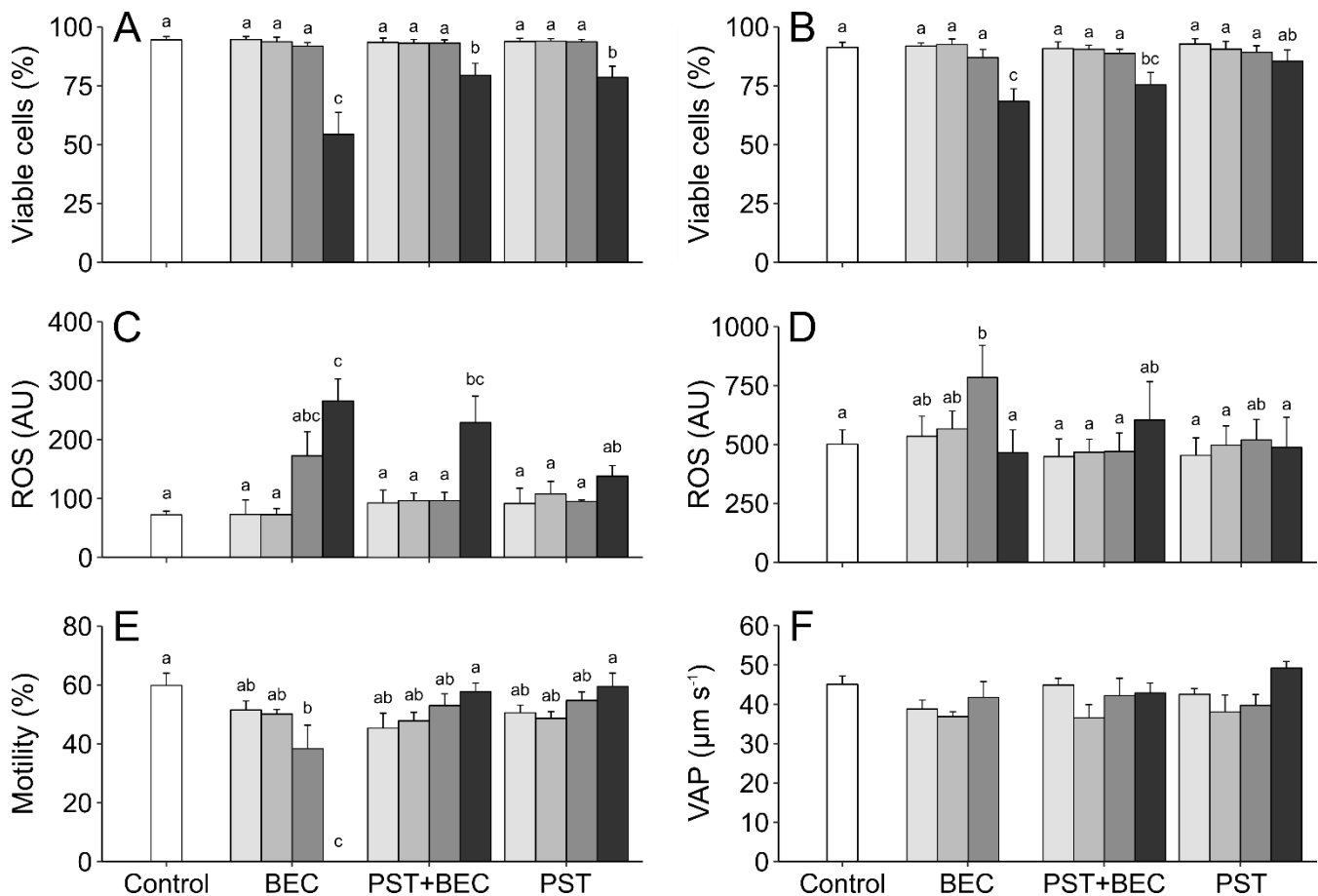


Fig. 11. Effects of three *A. minutum* strains producing BEC, PST+BEC, or PST, on cellular features of *C. gigas* gametes after a 2-hour exposure of spermatozoa ($n = 4$ males) or oocytes ($n = 5$ females) to 10 (\square), 10^2 (\blacksquare), 2.5×10^3 (\blacklozenge), and 2.5×10^4 (\blacksquare) cells mL⁻¹ of *A. minutum* or to seawater (\square) (control). Percentage of viable spermatozoa (A) and oocytes (B), reactive oxygen species (ROS) production of spermatozoa (C) and oocytes (D), spermatozoa motility (E) and velocity of the average path (VAP) (F). AU: arbitrary unit. Mean \pm SE. Letters denote significant groupings ($p < 0.05$; ANOVA).

Exposure to *A. minutum* strains had a significant effect upon production of reactive oxygen species (ROS) by spermatozoa ($F = 6.21, p < 10^{-5}$) and oocytes ($F = 2.70, p < 0.01$). The ROS production of spermatozoa exposed to 2.5×10^4 cells mL⁻¹ of the BEC strain (265.5 ± 37.5 AU) or the PST+BEC strain (228.6 ± 45.0 AU) was higher than control spermatozoa (72.4 ± 6.0 AU) (Fig. 11C). The ROS production of oocytes exposed to 2.5×10^3 cells mL⁻¹ of the BEC strain (783.9 ± 136.8 AU) was higher (+ 56 %) than control oocytes (500.8 ± 61.4 AU) (Fig. 11D).

Spermatozoa motility was significantly affected by *A. minutum* exposure ($F = 19.66, p < 10^{-11}$). Exposure to 2.5×10^4 cells mL⁻¹ of the BEC strain totally inhibited spermatozoa movement (Fig. 11E). The percentage of motile spermatozoa in spermatozoa exposed to 2.5×10^3 cells mL⁻¹ of the BEC strain (38.3 ± 8.0 %) was lower (-36 %) than in control spermatozoa (59.8 ± 4.1 %) (Fig. 11E). The motility of spermatozoa exposed to the PST+BEC and PST strains were similar to control spermatozoa (Fig. 11E). The spermatozoa velocity was not significantly affected by *A. minutum* exposure (Fig. 11F).

3.2 Fertilization of oyster gametes exposed to *A. minutum*

Prior exposure of oocytes or spermatozoa to *A. minutum* significantly affected fertilization yield (spermatozoa: $F = 10.78, p < 10^{-6}$; oocytes: $F = 29.60, p < 10^{-10}$). The fertilization yields of spermatozoa exposed to the highest concentration tested (2.5×10^4 cells mL⁻¹) of the BEC strain (1.4 ± 1.4 %), the PST+BEC strain (12.6 ± 10.8 %), or the PST strain (21.6 ± 14.4 %), or to 2.5×10^3 cells mL⁻¹ of the BEC strain (24.5 ± 18.2 %) were significantly lower than fertilization yield of control spermatozoa (92.3 ± 0.5 %) (Fig. 12A).

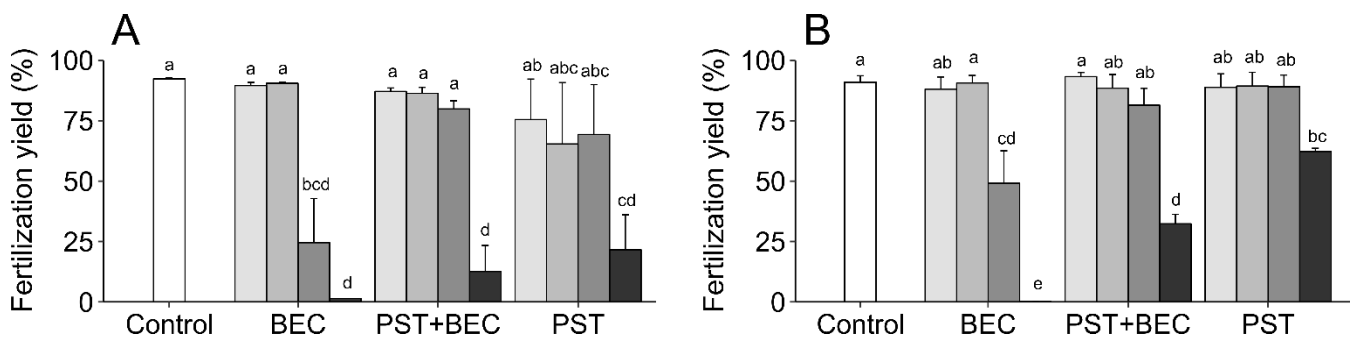


Fig. 12. Effects of three *A. minutum* strains producing BEC, PST+BEC, or PST, on *C. gigas* fertilization yield after a 2-hour exposure of spermatozoa (n = 3 males) or oocytes (n = 3 females) to 10 (□), 10² (▤), 2.5 × 10³ (▥), and 2.5 × 10⁴ (▦) cells mL⁻¹ of *A. minutum* or to seawater (□) (control). Fertilization yield of exposed spermatozoa (A) and exposed oocytes (B). Mean ± SE. Letters denote significant groupings (p < 0.05; ANOVA).

Exposure of oocytes to the highest concentration tested (2.5×10^4 cells mL⁻¹) of the BEC strain (0.1 ± 0.1 %), the PST+BEC strain (32.3 ± 3.9 %), and the PST strain (62.3 ± 1.3 %), or to 2.5×10^3 cells mL⁻¹ of the BEC strain (49.0 ± 13.5 %) also induced lower fertilization yields compared to control oocytes (90.9 ± 2.7 %) (Fig. 12B).

4. Discussion

The present study evidences that *A. minutum* induced sublethal defects and mortality on oyster gametes after two hours of exposure to the three stains tested at 2.5×10^4 cells mL⁻¹. These damages on spermatozoa or oocytes result in significant decreases in fertilization yields, the ultimate phenotype translating the quality of gametes. The consequences on fertilization were most severe with the BEC-producing *A. minutum* strain, the most cytotoxic strain tested (Long et al., 2018). The highest concentration of this strain results in almost total inhibition of fertilization after exposure of spermatozoa or oocytes and in decreases by 73 % and 49 % respectively, at 2.5×10^3 cells mL⁻¹. It is worth noting that *A. minutum* cells were still present during fertilization since oocytes and spermatozoa have not been filtrated before fertilization. Algal concentrations during fertilization were 4-fold and 20-fold lower than *A. minutum* concentrations during spermatozoa and oocyte exposures, respectively. Thus, *A. minutum* present during fertilization could have participated in reducing fertilization yield as demonstrated for the bay scallop *Chlamys farreri* (Yan et al., 2001), *C. gigas* (Mu and Li, 2013) and *P. fucata martensii* (Basti et al., 2015), exposed respectively to *A. tamarense*, *A. catenella* and *A. affine*. The highest concentrations tested in the present study for gamete exposures are in the range of values observed during severe *A. minutum* blooms in France, Spain, Egypt, and South Africa (Chapelle et al., 2015; Garcés et al., 2004; Labib and Halim, 1995; Pitcher et al., 2007) and demonstrate the potentiality of ecological consequences of *A. minutum* blooms for wild and cultivated bivalve populations.

For spermatozoa, the drastic decrease in fertilization yield could result from the significant loss of motility after 2 hours of exposure to 2.5×10^3 cells mL⁻¹ and the total movement inhibition at the highest concentration, since concentration of motile spermatozoa is a pivotal factor for successful fertilization in free-spawning marine invertebrates (Nice, 2005; Styán, 1998). Alteration of spermatozoa movement could result from membrane alteration or sub-cellular cytotoxic effect.

The increase of ROS observed in spermatozoa probably also results from BEC toxicity and could be one element to explain their reduced motility and viability, leading to reduced

fertilization yield. Indeed, fertilization success in oysters has been directly correlated to the viability of spermatozoa (Boulais et al., 2017). In human, oxidative stress is largely implicated in reproductive physiopathology. An excessive ROS production in spermatozoa was demonstrated to be deleterious for sperm membrane integrity, motility, and fertility (Agarwal et al., 2003).

The significant mortality of oocytes induced by exposure to 2.5×10^4 cells mL⁻¹ of *A. minutum*, BEC and PST+BEC strains could also be the result of BEC toxicity and likely contributed to fertilization success reduction. The increase in ROS production in oocytes exposed to 2.5×10^3 cells mL⁻¹ of the BEC strain could be associated with BEC as ROS generation in cells can be triggered by exposure to xenobiotic or toxins (Manduzio et al., 2005). As proposed by Le Goïc et al. (2014), ROS generation in oocytes exposed to *A. minutum* could lead to cellular damages and subsequently alter fertilization processes. The result of ROS production for oocytes exposed to 2.5×10^4 cells mL⁻¹ of the BEC strain or the PST+BEC strain cannot be properly interpreted since significant mortalities were observed in oocytes.

These results highlight the high toxicity of bioactive extracellular compounds of *A. minutum* to oyster gametes. More generally, extracellular toxic compounds produced by different dinoflagellate species appear highly toxic for early developmental stages of bivalves, not protected by a shell (Banno et al., 2018; Basti et al., 2015; Rolton et al., 2015). It is urgent to consider these toxins in impact studies of HAB on marine species, to characterize them, and to decipher the mechanisms underlying their toxicity.

Exposure of spermatozoa or oocytes to the PST-producing *A. minutum* strain at 2.5×10^4 cells mL⁻¹ altered fertilization and induced mortality in spermatozoa. Low levels of extracellular PST were measured in the culture media of some *Alexandrium* spp. isolates (Lefebvre et al., 2008). Extracellular PST might have been present in the *A. minutum* culture media of the PST strain and could be involved in the toxic effects observed. Saxitoxin, binding to Na⁺ and Ca²⁺ channels (Llewellyn, 2006), likely modifies fluxes of these ions in cells. Saxitoxin could therefore alter cellular processes involving Ca²⁺, such as oocyte maturation (Leclerc et al., 2000), fertilization (Togo and Morisawa, 1999) and spermatozoa motility (Boulais et al., 2017) in *C. gigas*. At this high concentration, repeated contacts between motile dinoflagellate cells and oyster gametes likely also contribute to alter oocytes and spermatozoa cellular integrity, reducing their fertilization capacity. Physical contact with algal cells seems to be an important mechanism involved in the toxicity of HAB species to bivalve gametes and embryos, and has been suggested for *Alexandrium* species such as *A. tamarense*, *A. taylori* and *A. affine* (Basti et al.,

2015; Matsuyama et al., 2001; Yan et al., 2001). Further studies should assess the effects of oyster gamete exposure to purified PST and the consequences on fertilization processes. The cellular integrity of gametes could be investigated by electronic microscopy.

In the present study, we exposed either oocytes or spermatozoa prior to fertilization. More drastic effects could be expected when both oocytes and spermatozoa are exposed, which is relevant to environmental conditions of spawning occurring during a HAB. Moreover, gametes were only exposed for 2 hours in the present study, while in the field duration of the exposure is likely longer. Oyster fertilization in the field might thus be sensitive to concentrations lower than 2.5×10^3 cells mL⁻¹. The impacts of *A. minutum* concentrations comprise between 10^2 and 2.5×10^3 cells mL⁻¹ upon oyster gametes and subsequent fertilization are to further investigate.

Toxin production is known to vary substantially within *Alexandrium* species and even among clones from the same species (Anderson et al., 2012b; Touzet et al., 2007). In the present study, different *A. minutum* strains have distinct effects on oyster gametes. As suggested for *A. ostenfeldii* (Brandenburg et al., 2018), this intraspecific trait variation could be an advantage for development and resilience of *A. minutum* blooms.

5. Conclusion

This experiment clearly highlights the potential negative effect of *A. minutum* on *C. gigas* gametes and their fertilization capacity. The most striking effects in this experiment were observed following exposure of oyster gametes to the non-PST-producing *A. minutum* strain, which decreased spermatozoa motility, increased gamete ROS production, and virtually inhibited the subsequent fertilization, thus confirming a PST-independent toxicity of *A. minutum* toward oyster gametes. Results suggest that negative effects of *A. minutum* on oyster gametes are strain specific and mainly caused by uncharacterized bioactive compounds, which are released as exudates and/or associated to algal cell membranes. Further research is necessary to determine the chemical identity of the bioactive compounds produced by *A. minutum* and the mechanism by which *Alexandrium* spp. toxins affect bivalve gametes and embryos. The results of this study suggest that *A. minutum* blooms affect gamete quality and fertilization success of *C. gigas*, and could have implications for the recruitment of oysters and other marine invertebrates.

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Chapitre 5

Quels sont les stades de développement de l'huître les plus sensibles à *A. minutum* ? Quels sont les effets respectifs des PST et des BEC produits par *A. minutum* sur le développement embryon-larvaire de l'huître ?

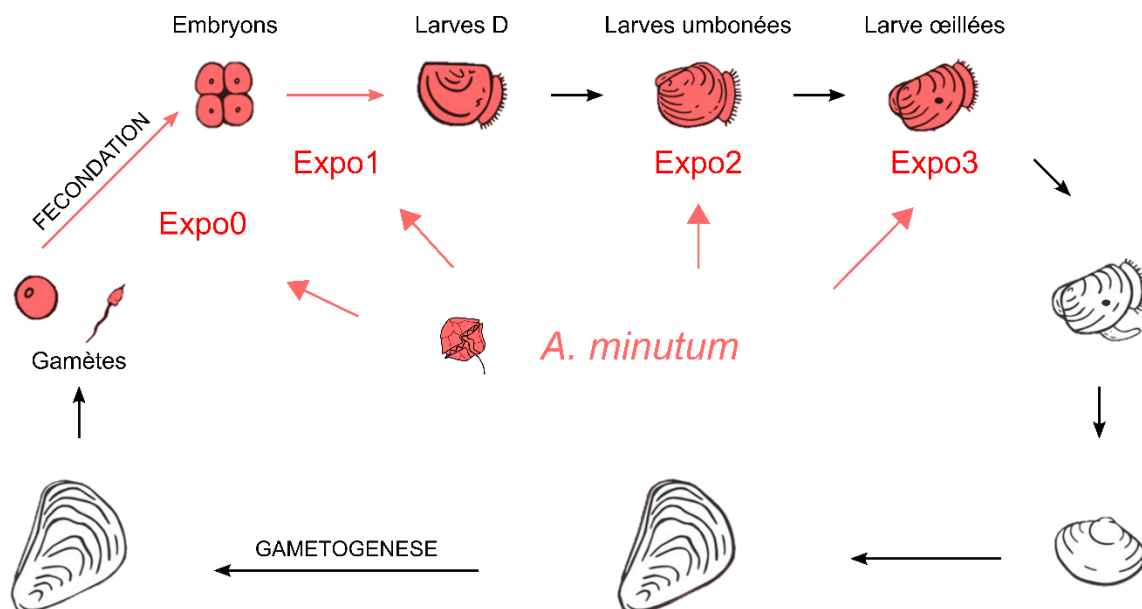
Dans le chapitre précédent, nous avons mis en évidence qu'*A. minutum* affecte considérablement la viabilité des gamètes de *C. gigas*, la mobilité des spermatozoïdes et la production d'espèces réactives de l'oxygène des ovocytes. Ces effets létaux et sublétaux sur les gamètes altèrent leur capacité de fécondation. Les effets les plus drastiques ont été observés après exposition des gamètes à la souche BEC (souche CCM1002) qui ne produit que des BEC, suggérant que les BEC sont en grande partie responsables de la cytotoxicité d'*A. minutum* sur les gamètes.

Dans le chapitre 3, une exposition continue des larves de *C. gigas* pendant un mois à *A. minutum* (souche AM89BM) réduit la croissance et la fixation des larves. La souche d'*A. minutum* utilisée dans cette expérience produit à la fois des PST et des BEC. Il est donc difficile d'attribuer les effets observés sur les larves à un type de molécules toxiques spécifiquement. De plus, l'exposition continue ne permettait pas de tester la sensibilité des différents stades de développement.

D'après les résultats des chapitres précédents, les BEC affectent les branchies des huîtres (chapitre 3) ainsi que les gamètes (chapitre 4), et pourraient donc aussi avoir un effet cytotoxique sur les embryons et premiers stades de développement de *C. gigas*. Contrairement aux larves, les embryons ne sont pas protégés par une coquille et pourraient être plus vulnérables face aux BEC. De plus, les embryons et les différents stades larvaires ont des modes de nutrition très distincts. Dans le chapitre 3, nous avons observé que les jeunes larves ne sont pas capables d'ingérer des cellules d'*A. minutum* à cause de leur taille relativement grande.

Ces résultats suggèrent qu'*A. minutum* entraîne des effets différents en fonction des stades de développement et de leur capacité à s'alimenter sur des cellules d'*A. minutum*. Il était donc intéressant de prendre en compte ces aspects en compte dans l'étude des impacts d'*A. minutum* sur le développement des huîtres.

Dans ce contexte, nous avons étudié la sensibilité de différents stades de développement de *C. gigas* à *A. minutum* : les gamètes pendant la fécondation (Expo0), les embryons (48 h d'exposition, Expo1), les larves umbonnées (48 h d'exposition, Expo2) et les larves œillées (24 h d'exposition, Expo3). Dans le but de discriminer les effets liés aux PST et ceux liés aux BEC, nous avons testé les effets de 3 souches d'*A. minutum* produisant soit des PST, soit des BEC, soit des PST et des BEC, comme dans le chapitre 3. La présence de malformations, l'activité de nutrition, et la croissance des larves ont été évaluées durant l'exposition pour déterminer la vulnérabilité des différents stades de développement. De plus, les potentielles conséquences de l'exposition à *A. minutum* sur le recrutement des huîtres ont été étudiées en mesurant la croissance, la survie et la fixation des larves à la suite de l'exposition.



Les hypothèses avancées étaient :

1. Une exposition à *A. minutum* affecte la fécondation et le développement embryonnaire,
2. Les embryons sont les stades les plus vulnérables aux BEC produits par *A. minutum*,
3. Les larves œillées accumulent des PST via l'ingestion de cellules d'*A. minutum*,
4. Les BEC produits par *A. minutum* affectent le développement larvaire de l'huître.
5. Une exposition à *A. minutum* impacte le recrutement des larves.

Ce chapitre fait l'objet d'un article en cours de préparation :

Castrec J., et al. (in prep-b) Stage-specific detrimental effects of the dinoflagellate *Alexandrium minutum* and its toxins on development of the oyster, *Crassostrea gigas*.

Stage-specific detrimental effects of the dinoflagellate *Alexandrium minutum* and its toxins on development of the oyster, *Crassostrea gigas*

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Abstract

The dinoflagellate genus *Alexandrium* comprises species that produce highly potent neurotoxins, paralytic shellfish toxins (PSTs), and bioactive extracellular compounds (BECs) of unknown structure and ecological significance. The toxic bloom-forming species *Alexandrium minutum* is distributed worldwide and adversely affects many bivalve species including the commercially and ecologically important Pacific oyster *Crassostrea gigas*. In France, the recurrent *A. minutum* blooms can co-occur with *C. gigas* spawning and larval development. Yet little is known about the effects of *A. minutum* upon embryo-larval development and recruitment of this species known to be highly variable among sites and years. The present study explores how *A. minutum* affects *C. gigas* fertilization and subsequent early life stages, *i.e.* embryos and larvae, by exposing them under controlled conditions to two strains of *A. minutum*, producing only BECs or both PSTs and BECs. The non-PST-producing *A. minutum* strain inhibited fertilization and hatching into D-larvae, disrupted larval swimming behaviour, feeding, and growth, and induced irreversible toxic effects in umbonate and eyed larvae, up to drastic decrease of survival and settlement. The embryos were the stage most susceptible to *A. minutum* toxicity, the BECs causing lysis of embryos. This work highlights the major role of BECs in *A. minutum* toxicity upon oyster development. It is also the first report of PST accumulation in oyster larvae suggesting its role in the decreased development and settlement of larvae. This study provides evidences that *A. minutum* blooms could hamper the recruitment of oysters and potentially other shellfish.

Keywords: Harmful Algal Bloom (HAB), Paralytic Shellfish Toxin (PST), *Crassostrea gigas*, Bioactive Extracellular Compounds (BEC), embryo, larvae

1. Introduction

Harmful algal blooms (HABs) are globally distributed and represent a real concern for marine environment (Kudela et al., 2015). Beside their toxicity to human through consumption of phycotoxin-contaminated shellfish or fishes (Grattan et al., 2016), HABs affect biology of marine organisms and ecology of marine coastal ecosystems (Hallegraeff, 2010; Hallegraeff et al., 2003). The dinoflagellate genus *Alexandrium* is remarkable among other genera responsible for HABs, owing to the acute and diverse consequences of its blooms worldwide, and its ability to colonize multiple habitats and to persist over large regions through time (Anderson et al., 2012). HABs frequently occur during bivalve reproduction period. Although it is not possible, at present, to prove that HABs are directly responsible for bivalve recruitment failures, this hypothesis was formulated according to indications from field observations (Erard-Le Denn et al., 1990; Pouvreau et al., 2015; Summerson and Peterson, 1990). Recent studies evidenced that *Alexandrium* species, *A. catenella*, *A. tamarense*, and *A. fundyense*, affect gamete quality and viability, embryo development, and decrease larval activity, survival, growth, and settlement of several bivalve species (Banno et al., 2018; Matsuyama et al., 2001; Mu and Li, 2013; Tang and Gobler, 2012; Yan et al., 2003, 2001). Development and survival of *Mytilus edulis* embryos appeared negatively affected by an exposure to *A. minutum* (De Rijcke et al., 2016).

Species from the dinoflagellate genus *Alexandrium* are known to produce paralytic shellfish toxins (PSTs), a group of more than 50 neurotoxic derivatives of saxitoxin (Wiese et al., 2010). PSTs can bioaccumulate in filter-feeding bivalves and other marine species, causing paralytic shellfish poisonings outbreaks (Bricelj and Shumway, 1998; Cembella et al., 2000) and other harmful effects (Landsberg, 2002; Shumway, 1990). These endogenous water-soluble alkaloids bind to the voltage-gated sodium channels and block conduction of action potential in excitable cells, eventually leading to paralysis (Cestèle and Catterall, 2000). Several species of *Alexandrium* also produce, independently from PST production (Tillmann and John, 2002), extracellular bioactive compounds (BECs) with allelopathic (Lelong et al., 2011; Long et al., 2018; Tillmann et al., 2008; Zheng et al., 2016), ichthyotoxic (Mardones et al., 2015; Ogata and Kodama, 1986), cytotoxic (Borcier et al., 2017; Castrec et al., 2018; Lush and Hallegraeff, 1996), and/or hemolytic activities (Eschbach et al., 2001; Yang et al., 2010). These compounds, whose molecular structures remain largely unknown (Ma et al., 2011), probably contribute to *Alexandrium* bloom toxicity. In particular, we demonstrated distinct negative effects of PST and BEC on adult *C. gigas* valve behaviour and cellular characteristics (Castrec et al., 2018).

Addressing specific reprotoxic effects of *Alexandrium* attributable to either PSTs or bioactive extracellular compounds (BECs) is still challenging, since most studies did not assess the production of PSTs and BECs of the tested strains (De Rijcke et al., 2016; Matsuyama et al., 2001; Tang and Gobler, 2012), and thus potentially used strains producing both types of toxins. Known non-PST-producing *Alexandrium* strains, *i.e.* *A. taylori*, *A. affine*, and *A. monilatum*, altered cleavage of embryos, hatching into D-larvae, and larval activity and survival of bivalve species (Basti et al., 2015; Matsuyama et al., 2001; May et al., 2010), suggesting that other bioactive compounds, non-related to PST, could contribute to the detrimental effects of *Alexandrium* upon bivalve embryo-larval development.

To evaluate the respective vulnerability of *C. gigas* early life stages to *A. minutum*, we analysed, under controlled conditions, the effects of different strains of *A. minutum* on various developmental stages: oyster gametes at fertilization, embryos, umbonate and eyed larvae. Two *A. minutum* strains with very different toxicity in terms of both PSTs and BECs (Castrec et al., 2018) were tested: the “PST+BEC” strain producing both PSTs and BECs, and the “BEC” strain producing only BECs. Concentration and durations of *A. minutum* exposures were chosen based on realistic densities and durations of natural blooms that have occurred in oyster production areas (Chapelle et al., 2015). Measurement of sub-lethal effects, such as morphological abnormalities, feeding activity or growth were assessed throughout the period of exposure. In addition, potential consequences on oyster recruitment were investigated by assessing larval growth, survival and settlement after exposure of embryos, umbonate or eyed larvae. Additionally, PST accumulation was measured in bivalve larvae.

2. Materials and methods

2.1 Biological materials

Algal cultures. *Tisochrysis lutea* (formerly *Isochrysis* sp., T. iso strain, CCAP 927/14) and *Chaetoceros* sp (formerly *Chaetoceros neogracile*, strain CCAP 1010-3) were used as the main non-toxic food for oyster larvae. They were cultured with continuous light (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in separated 300-L cylinders containing filtered seawater (1 μm) enriched with Conway medium (Walne, 1970) at 20°C, and with silica for *Chaetoceros* sp. Microalgae cultures were harvested at the late-logarithmic phase (after 3–4 days).

Two strains of *Alexandrium minutum* (Halim, PST+BEC and BEC strains) were grown in filtered seawater (0.2 μm) supplemented with L1 medium (Guillard and Hargraves, 1993) and

maintained at 17 ± 1 °C. The “PST+BEC” strain (AM89BM strain, isolated from a bloom in the Bay of Morlaix, France; (Erard-Le Denn et al., 1990) produces both bioactive extracellular compounds and paralytic shellfish toxins, whereas the “BEC” strain (CCMI1002 strain, isolated from a bloom in Gearhies, Bantry Bay, Ireland) produces only bioactive extracellular compounds, and is significantly more cytotoxic than the AM89BM strain (Long et al., 2018). The PST+BEC strain is known to produce $10.6 \text{ fmol PST cell}^{-1}$ which corresponds to $1.3 \text{ pg STX eq. cell}^{-1}$ (Fabioux et al., 2015), whereas no PSTs were detected in the BEC strain (Borcier et al., 2017). Cultures of *A. minutum* were harvested in exponential growth phase for the experiment.

Oyster. Adult oysters originated from a cohort of specific-pathogen free oysters produced and reared according to a standardized protocol (Petton et al., 2015). They were born in Ifremer experimental facilities (Argenton, France) and transferred as spat in the field in Marennes-Oléron, France. Oysters (18 mo, mean total weight 63.5 g) were then transferred to the Ifremer experimental facilities (Argenton, France) in June 2017.

Collection of gametes. Gender of mature oysters was determined by examination of $2 \mu\text{l}$ gonad samples under a light microscope (Leica MZ 125, $\times 4$ objective). Oocytes and sperm were collected from 15 females and 17 males, respectively, by stripping gonads individually (Boulais et al., 2015a, 2015b). Briefly, gametes were collected with UV-treated $1\text{-}\mu\text{m}$ filtered sea water (FSW; 21 °C, pH 8.3, salinity 36 PSU), and oocytes and sperm were sieved through $100 \mu\text{m}$ and $60 \mu\text{m}$ meshes, respectively to eliminate debris (Steele and Mulcahy, 1999). Gamete concentrations were determined individually by flow cytometry (duplicate, EasyCyte Plus cytometer, Guava Millipore) (Le Goïc et al., 2014, 2013). A pool of oocytes from the 15 females were realized by sampling 2.67×10^6 oocytes per oyster, and 4.17×10^8 spermatozoa per oyster were sampled for the pool of sperm from the 17 males.

2.2 Exposure of gametes during fertilization (Expo0)

Gametes (5×10^4 oocytes of the pool, $50 \text{ oocytes mL}^{-1}$; 100:1 spermatozoa:oocyte ratio) were placed at the same time in four replicate beakers filled with 1 L of FSW at $21^\circ\text{C} \pm 1^\circ\text{C}$, containing $10^3 \text{ cells mL}^{-1}$ of the BEC strain or the PST+BEC strain of *A. minutum* (“exposed” treatments) (Fig. 13). For the control, gametes were suspended in 1 L FSW. After 1.5 h of contact between spermatozoa and oocytes, a 10-mL subsample of each beaker was fixed with a formaldehyde-seawater solution (1% final) to estimate the fertilization success. Numbers of fertilized (2-cell or 4-cell stages) and unfertilized (absence of segmentation) oocytes were

counted using a light microscope (Olympus BX51). Mean fertilization yield (%) was determined by counting at least 30 oocytes for each sample. Beakers were filled up to 1.8 L with FSW after 3.5 h post-fertilization (hpf). D-larva yields were estimated 48 hpf under a light microscope (Olympus BX51), adding one drop of 4% formaldehyde solution into a 10 mL subsample of the beaker to prevent larval movement. D-larva yield was defined as: number of D-larvae/total number of oocytes used for fertilization.

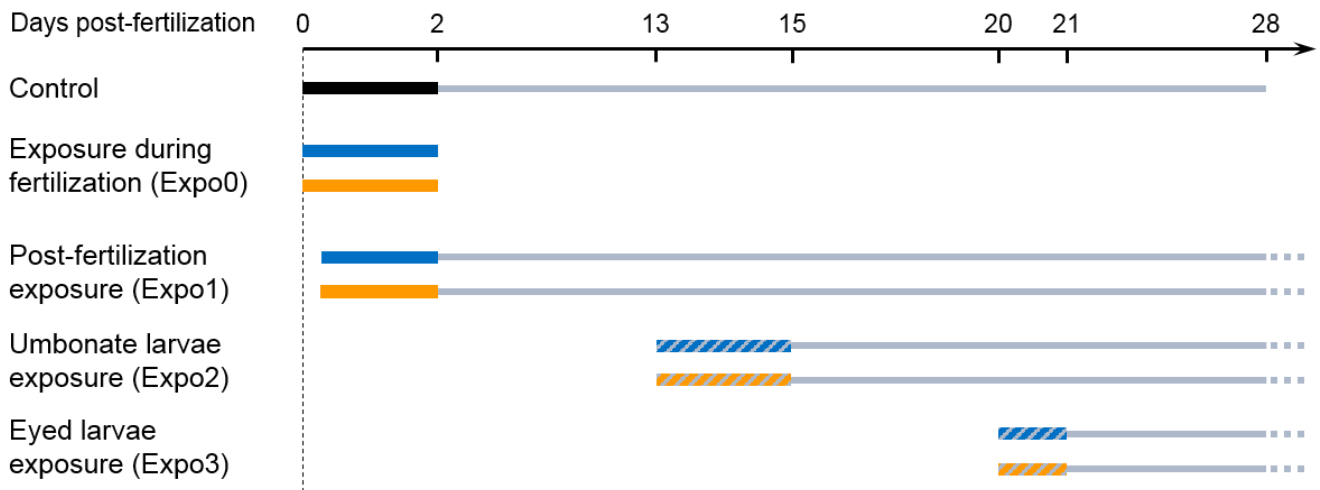


Fig. 13. Flow chart of the experiment. Different early life stages of oysters were exposed to two strains of *A. minutum*: 2-day exposure of gametes at fertilization (Expo0); 2-day exposure of embryos (Expo1); 2-day exposure of umbonate larvae (Expo2), and 1-day exposure of eyed larvae (Expo3). Gametes and embryos were exposed to 10^3 cells mL^{-1} of the PST+BEC strain (■) or the BEC strain (■) of *A. minutum*. In the control treatment, fertilization and embryo development were conducted in filtered sea water (■), and larvae were fed *ad libitum* with non-toxic algae (■, Tiso/Chaeto). During Expo2 and Expo3, larvae were fed *ad libitum* with Tiso/Chaeto, and exposed to 10^3 cells mL^{-1} of the PST+BEC strain (▨) or the BEC strain of *A. minutum* (▨).

2.3 Exposure of embryos and larvae

2.3.1 Experimental design

Four batches of 5×10^6 pooled oocytes (see section 2.1) were fertilized with a pool of 5×10^8 spermatozoa (see section 2.1) in beakers containing 5 L FSW. Embryos 1.5 hpf from each beaker were concentrated on a 20- μm mesh, rinsed and transferred into a 5-L cylindrical tank already containing the appropriate algal treatment in FSW at 21 °C (Fig. 13), at a final concentration of 100 embryos mL^{-1} . For each algal treatment, four replicate tanks were prepared corresponding to the 4 fertilization replicates described previously.

Each tank was sieved through 40- μm mesh 48 hpf and concentrated into a 100-mL graduated cylinder. D-larva yields were quantified as describe previously (see section 2.2), and

percentages of abnormal D-larvae were recorded by observing at least 100 larvae per replicate using a light microscope (Zeiss Axio Observer Z1). Larvae presenting shell, mantle and/or hinge malformations, or developmental arrest at the embryonic stage were considered as abnormal (Mottier et al., 2013). D-larvae were then transferred to 5-L cylindrical tanks at the density of 50 larvae mL⁻¹ for larval rearing. Larvae were maintained in a flow-through rearing system as described by Gonzalez Araya et al. (2012) (100% seawater renewal h⁻¹, FSW, 21 °C, 35 PSU, pH 8.4), and fed continuously with *T. lutea* and *Chaetoceros* sp. according to Asmani et al. (2016). Once ≥ 50% of larvae reached the eyed larvae stage, the replicate tanks were drained and larvae were collected on 60 µm mesh and counted. Larval survival was determined as the number of remaining larvae / 2.5 × 10⁵ larvae. A subsample of larvae (300-700 mg) was collected from each tank and stored in liquid nitrogen for subsequent PST quantification.

2.3.2 *A. minutum* exposures

Three exposures were run on three different oyster developmental stages using separate batches of embryos or larvae (Fig. 13). These stages were chosen because of their distinct feeding behaviors:

- “Expo1”: 2-day exposure from 1.5 hpf embryos to D-larval stage. The embryo development up to D-larvae involves only endogenous exchanges (Bayne, 2017), and D-larvae are unable to ingest *Alexandrium* cells due to their relatively large size (23–29 µm) (J. Castrec, pers. comm.);
- “Expo2”: 2-day exposure of 13-day-old umbonate larvae (shell height 163.7 ± 1.3 µm). These umbonate planktotrophic larvae (Rico-Villa et al., 2006) are not able to feed on *A. minutum* cells at a mean shell length of 160 µm (J. Castrec, pers. comm.);
- “Expo3”: 1-day exposure of 20-day-old eyed larvae exposed (shell height 295.2 ± 2.3 µm). These eyed competent larvae are also planktotrophic larvae with high phytoplankton consumption (Rico-Villa et al., 2006), and are able to feed on *Alexandrium* cells (J. Castrec, pers. comm.).

Three different algal treatments (control, BEC or PST+BEC strain) were run during the exposures. For the control treatment, embryos were grown in FSW and larvae were fed *ad libitum* with a mix diet of non-toxic microalgae *T. lutea* and *Chaetoceros* sp. (*T. lutea* + *C. muelleri*) (Fig. 13). During “Expo1”, embryos were incubated in FSW containing 10³ cells mL⁻¹ of the PST+BEC or the BEC strain of *A. minutum* (Fig. 13). For the “Expo2” and “Expo3”, larvae were exposed to 10³ cells mL⁻¹ of the PST+BEC strain or the PST strain and fed *ad*

libitum with *T. lutea* + *C. muelleri* (Fig. 13). When outside the exposure period, larvae were fed *ad libitum* with the *T. lutea* + *C. muelleri* diet used for control treatment.

2.3.3 Morphological measurements of larvae

Larvae were sampled every 1–3 d and stored in a 0.1% formaldehyde-seawater solution. Larval size was assessed by measuring shell height (greatest dorso-ventral dimension) using image analysis on at least 30 larvae per tank per day of sampling (WinImager 2.0 and ImageJ software for image capture and analysis, respectively). The eyed larvae yield was estimated on at least 30 larvae per tank per day of sampling, and expressed as the percentage of larvae developing an eyespot (morphological competence for metamorphosis).

2.3.4 Algal consumption

Once a day during larval rearing, seawater flow rate of each tank was measured and phytoplankton counts (cells mL⁻¹) were made using an electronic particle counter (Multisizer 3 equipped with a 100- μ m aperture tube) in inflow and outflow seawater (20 mL) sampled from each tank. Algal consumption, i.e. *T. lutea* + *C. muelleri*, was expressed in algal cell volume per larvae per day (μ m³ larva⁻¹ d⁻¹). A subsample of 1 mL was collected every 2-3 days from each larval tank replicate to determine survival by counting larvae using the light microscope.

2.3.5 Larval settlement

Once $\geq 50\%$ of larvae from one treatment reached the eyed larvae stage as described previously, larvae from the four replicate cylindrical tanks were transferred for settlement into 30-L tank with a 150- μ m sieve (5×10^4 larvae tank⁻¹, 4 replicate tanks per exposure and algal treatment), maintained in a flow-through system (9 L h⁻¹; 30 % h⁻¹ seawater renewal) and fed the bi-specific diet (*T. lutea* + *Chaetoceros* sp. as described previously). Larvae from each cylindrical tank were sampled for toxin quantification and stored in liquid nitrogen. After 7 days, the number of remaining swimming larvae, dead larvae (empty shells), and larvae settled on tank walls and sieve were counted. The mortality (%) during the settlement step was evaluated as: number of dead larvae $\times 100 / 5 \times 10^4$. The larval settlement (%) was evaluated as: number of settled larvae $\times 100 /$ total number of remaining alive larvae.

2.3.6 Toxin quantification

PST extraction was performed on one larvae sample per replicate: larvae were homogenized in HCl 0.1 M (1:1, w:v) using a FastPrep bead-grinder (MP Biomedicals), boiled for 5 min, and subsequently centrifuged (10 min, 4 °C, 3500 \times g). Toxin quantification of larvae exposed to

the PST+BEC or BEC strain and control larvae from Expo1, Expo2 and Expo3 was performed by spectrophotometry using the Abraxis ELISA PSP kit (Novakits, France) according to Lassudrie et al. (2015). Supernatants of the replicates were pooled for control larvae and larvae exposed to the BEC strain, for each exposure. Toxin load was expressed in ng STX g⁻¹ of wet larvae weight.

2.4 Statistical analyses

Statistical analyses and graphical representations were performed using R software (R Core Team, 2012). Differences were considered significant when $p < 0.05$. All values are expressed as mean \pm standard error (SE). Fertilization yield (Expo0), D-larvae yield (Expo0 and Expo1), abnormality percentage (Expo1), algal consumption (at 13, 14, and 15 dpf for Expo2; at 20 and 21 dpf for Expo3), and shell height (at 15 dpf for Expo2; at 21 dpf for Expo3) were compared among the three algal treatments using one-way ANOVA followed by Tukey's test. For Expo1, Expo2, and Expo3, comparisons for eyed larvae yield, larval survival at competence for metamorphosis, larval settlement (%), and mortality (%) during settlement period among the 7 treatments (3 exposures \times 2 algal treatments and the control treatment) were assessed using one-way ANOVA followed by Tukey HSD test for pairwise comparisons when differences were detected. Normality and homogeneity of variance were verified by the Shapiro-Wilk and Levene methods, respectively. Algal consumption data from Expo2 at 21 dpf was log transformed to conform to a normal distribution with equal variance.

3. Results

3.1 Exposure of gametes during fertilization to *A. minutum* strains (Expo0)

Fertilization yield of gametes exposed to the BEC strain (1.1 ± 0.5 %) was almost inexistent and significantly lower (Tukey HSD, $p < 0.0001$) than the control (96.0 ± 0.7 %). No D-larvae was observed at 2 dpf (Table 2). The fertilization yield (94.1 ± 1.4 %) and D-larval yield (54.9 ± 5.2 %) were not affected (Tukey HSD, $p > 0.05$) by exposure to the PST+BEC strain compared to control (fertilization yield = 96.0 ± 0.7 % and D-larval yield = 73.0 ± 11.3 %) (Table 2).

Table 2. Fertilization yield (%), D-larvae yield (%) and abnormality percentage of D-larvae (%) following 2-day *A. minutum* exposures of *Crassostrea gigas* gametes during fertilization, and embryos (1.5 h post-fertilization) during embryo development. Gametes and embryos were exposed to 10^3 cells mL⁻¹ of the PST+BEC or the BEC strain of *A. minutum*, whereas controls were placed in filtered sea water (Control). Letters indicate significant differences between conditions (ANOVA followed by Tukey HSD). Mean \pm SE, n = 4, n.a.: not analysed.

		Control	PST+BEC	BEC
Exposure of gametes during fertilization (Expo0)	Fertilization yield (%)	96.0 ± 0.7^a	94.1 ± 1.4^a	1.1 ± 0.5^b
	D-larvae yield (%)	73.0 ± 11.3^a	54.9 ± 5.2^a	0 ± 0^b
Exposure of embryos (Expo1)	D-larvae yield (%)	48.3 ± 5.8^a	47.3 ± 1.0^a	0 ± 0^b
	Abnormality (%)	7.0 ± 0.4^a	30.0 ± 0.8^b	n.a.

3.2 Exposure of embryos to *A. minutum* strains (Expo1)

The 2-day exposure of embryos to the BEC strain totally inhibited hatching into D-larvae (Table 2). The D-larvae yield of embryos exposed to the PST+BEC strain was similar (*t-test*, $p > 0.05$) to the control, however, their abnormality percentage (30.0 ± 0.8 %) was significantly higher (*t-test*, $p < 10^{-6}$) than in control larvae (7.0 ± 0.4 %) (Table 2). Exposure to the PST+BEC strain induced increased incidence of abnormal D-larvae with shell and mantle anomalies, and membrane swelling (Fig. S1).

At 21 dpf, larval height (300.4 ± 4.5 μ m) and eyed larvae yield (51.8 ± 4.1 %) of larvae (non-exposed) derived from embryos exposed to the PST+BEC strain were similar to larvae derived from control embryos (306.8 ± 4.0 μ m and 51.4 ± 2.9 %, respectively) (Fig. 14A, Fig. 15A). Survival up to competence for metamorphosis of larvae (68.7 ± 5.8 %), mortality during settlement (2.5 ± 0.6 %), and settlement (18.4 ± 1.0 %) of larvae derived from embryos exposed

to the PST+BEC strain were similar (*t*-tests, $p > 0.05$) to the control (67.9 ± 7.1 %, 2.1 ± 0.1 %, 16.6 ± 0.9 %, respectively) (Fig. 15B-D).

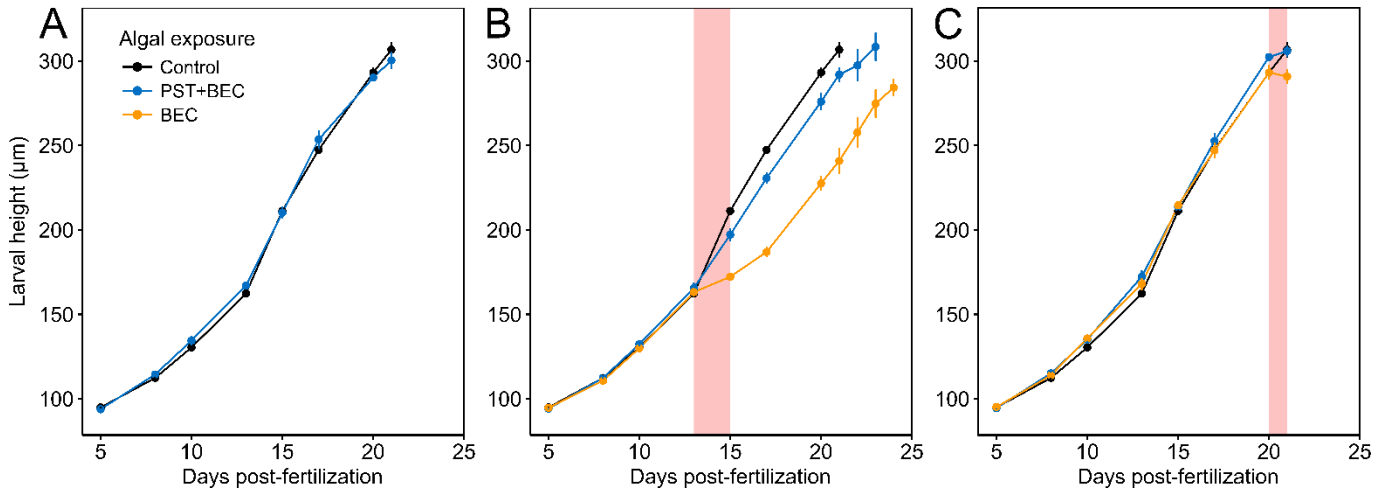


Fig. 14. Effects of *A. minutum* exposure on oyster larval growth. Larval height up to competence for metamorphosis (≥ 50 % of eyed larvae). A: Expo1, 2-day exposure of embryos; B: Expo2, 2-day exposure of umbonate larvae; C: Expo3, 1-day exposure of eyed larvae. Red bars represent the timing for *A. minutum* exposures. Black, blue, and orange lines represent control larvae only fed with the mix diet of *T. lutea* and *Chaetoceros* sp., larvae exposed to 10^3 cells mL^{-1} of the PST+BEC strain of *A. minutum*, and larvae exposed to 10^3 cells mL^{-1} the BEC strain of *A. minutum*, respectively. During the experiment, larvae from all treatments were fed *ad libitum* with the mix diet of *T. lutea* and *Chaetoceros* sp. Mean \pm SE; $n = 4$.

3.3 Exposure of umbonate larvae to *A. minutum* strains (Expo2)

Visual observations during the exposure indicated that the BEC strain modified larval behaviour, as most larvae stopped swimming and sank at the bottom of the tank (Fig. S2). Microscopic observation of larvae exposed to the BEC strain and sampled at the bottom of the tank showed that larvae were not closed, but rather shells remained slightly opened and the velum was rarely protruded (Fig. 16). On the contrary, control larvae and larvae exposed to the PST+BEC strain remained swimming in the tank (Fig. S2). Umbonate larvae exposed to the BEC strain showed several anomalies, including abnormal protrusion of the velum, abnormal masses in the velum, and loss of the velum cilia (Fig. 16).

Before exposure, algal consumption (*T. lutea* and *Chaetoceros* sp.) was similar (ANOVA, $p > 0.05$) for all umbonate larvae (Table 3). However, after 1 day of exposure, algal consumption of larvae exposed to the BEC strain was lower (Tukey HSD, $p < 0.01$ and $p < 0.001$) than in control larvae and larvae exposed to the PST+BEC strain (Table 3). After 2 days of exposure, larvae exposed to the BEC strain ($196.9 \pm 25.3 \mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$) had a further lower (Tukey HSD, $p < 10^{-6}$) algal consumption than larvae exposed to the PST+BEC strain (753.9 ± 33.7

$\mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$), which was itself lower (Tukey HSD, $p < 10^{-7}$) than control larvae ($940.9 \pm 21.0 \mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$) (Table 3).

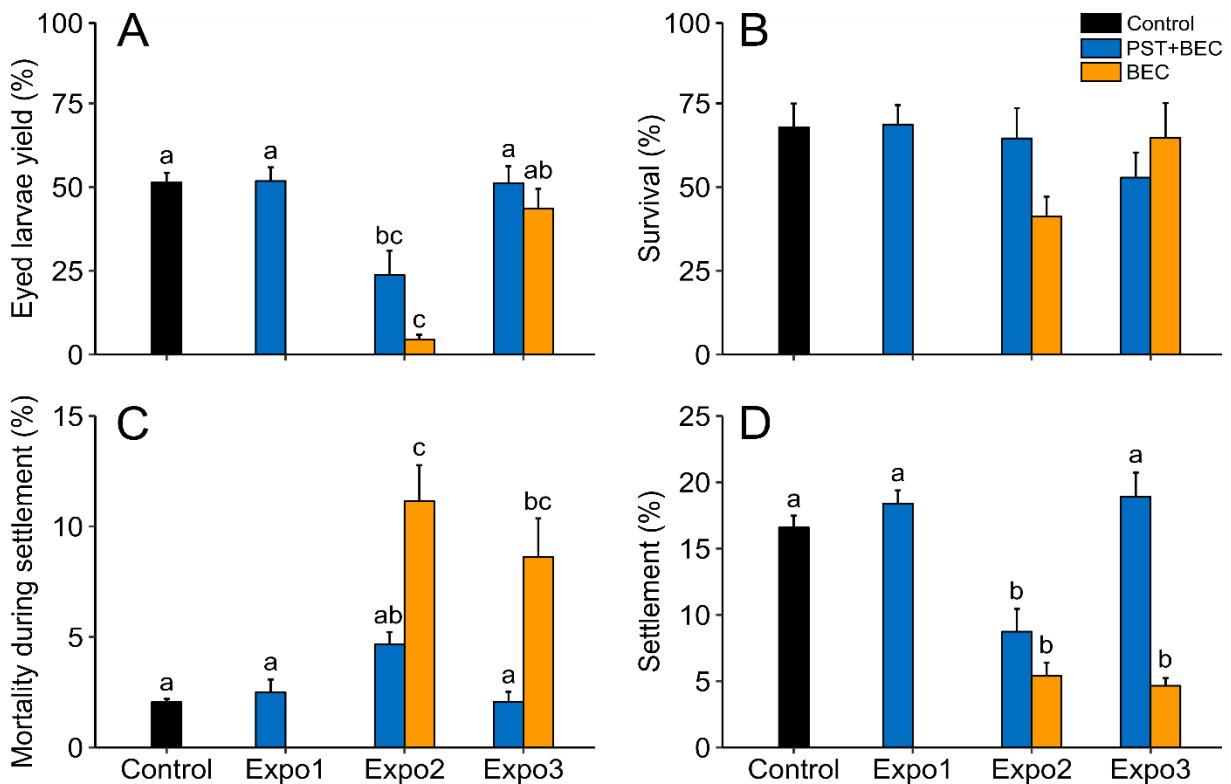


Fig. 15. Effects of *A. minutum* exposure on early life stages of *Crassostrea gigas*: 2-day exposure of embryos (Expo1), 2-day exposure of umbonate larvae (Expo2), and 1-day exposure of eyed larvae (Expo3). Black, blue, and orange bars respectively represent control larvae, larvae exposed to the PST+BEC strain of *A. minutum* ($10^3 \text{ cells mL}^{-1}$), and larvae exposed to the BEC strain of *A. minutum* ($10^3 \text{ cells mL}^{-1}$). A: eyed larvae yield (%) 21 days post-fertilization (dpf); B: survival (%) of larvae once $\geq 50\%$ of larvae reached the eyed larvae stage (21-24 dpf depending on the algal treatment); C: larval mortality (%) during the settlement period; D: larval settlement rate. Mean \pm SE; $n = 4$; letters denote significant groupings ($p < 0.05$; ANOVA and Tukey HSD).

The height of larvae was lower (ANOVA, $p < 10^{-5}$, $df = 2$, $F = 80.1$) after 2 days of exposure to both *Alexandrium* strains (Fig. 14B). At 15 dpf, larvae exposed to the PST+BEC strain ($197.2 \pm 3.3 \mu\text{m}$) or the BEC strain ($172.3 \pm 1.6 \mu\text{m}$) were smaller (Tukey HSD, $p < 0.01$ and $p < 10^{-5}$) than control larvae ($211.3 \pm 1.1 \mu\text{m}$); larvae exposed to the BEC strain being smaller (Tukey HSD, $p < 10^{-4}$) than those exposed to the PST+BEC strain (Fig. 14B).

At 21 dpf, eyed larvae yield of umbonate larvae exposed to the PST+BEC strain ($23.7 \pm 7.3\%$) or to the BEC strain ($4.5 \pm 1.4\%$) was lower (Tukey HSD, $p < 0.01$ and $p < 0.0001$) than that of control larvae (Fig. 15A). Competence in larvae exposed to the PST+BEC strain and the BEC strain was delayed by 2 (23 dpf) and 3 days (24 dpf), respectively, compared to control larvae (21 dpf). Size at competence for metamorphosis was similar in larvae exposed to the

PST+BEC strain ($308.4 \pm 8.1 \mu\text{m}$) and control larvae, however, larvae exposed to the BEC strain ($284.3 \pm 4.6 \mu\text{m}$) were significantly smaller (Tukey HSD, $p < 0.05$) than control larvae. Larval survival up to metamorphosis was not significantly affected by *A. minutum* exposure (ANOVA, $p > 0.05$) (Fig. 15B).

Larval mortality during settlement was higher (Tukey HSD, $p < 0.0001$) after exposure of umbonate larvae to the BEC strain ($11.1 \pm 1.6 \%$) compared to control larvae, whereas mortality after exposure to the PST+BEC strain ($4.7 \pm 0.6 \%$) was similar to the control (Fig. 15C). Settlement of larvae exposed to the PST+BEC ($8.7 \pm 1.7 \%$) and the BEC ($5.4 \pm 1.0 \%$) strains were significantly reduced (Tukey HSD, $p < 0.01$ and $p < 0.0001$) compared to the control (Fig. 15D).

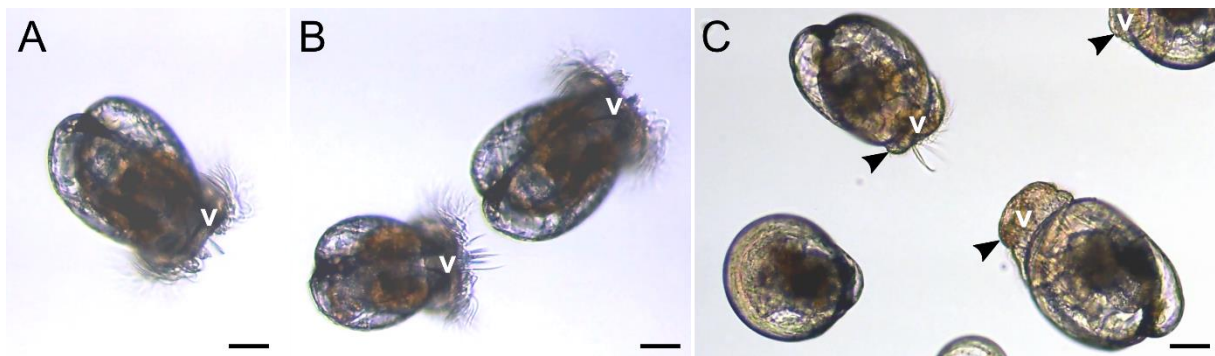


Fig. 16. Light micrographs of oyster umbonate larvae 15 days post-fertilization, after a 2-day exposure to 10^3 cells mL^{-1} of *A. minutum* (Expo2). Control larvae (A) and larvae exposed to the PST+BEC strain of *A. minutum* (B) were actively swimming with normal velum whereas larvae exposed to the BEC strain of *A. minutum* (C) were mostly immobile and some larvae showed abnormal masses in the velum (indicated by black arrows), and loss of the velum cilia. V: velum. Bar = $50 \mu\text{m}$.

3.4 Exposure of eyed larvae to *A. minutum* strains (Expo3)

Visual observations during the exposure indicated that the BEC strain of *A. minutum* modified the behaviour of eyed larvae, as described previously for umbonate larvae exposed to the same strain. Prior exposure, at 20 dpf, algal consumption was similar (ANOVA, $\text{df} = 2$, $F = 2.6$, $p = 0.127$) in the three subgroups, whereas after one day of exposure algal consumption was lower in larvae exposed to the BEC strain than in larvae exposed to the PST+BEC strain and control larvae (Table 3). After one day of *A. minutum* exposure, larval height was similar (Tukey HSD, $p > 0.05$) in larvae exposed to the PST+BEC strain ($305.9 \pm 2.9 \mu\text{m}$), larvae exposed to the BEC strain ($290 \pm 3.6 \mu\text{m}$), and control larvae ($306.8 \pm 4.0 \mu\text{m}$) (Fig. 14C). Eyed larvae yield at 21dpf and larval survival up to competence for metamorphosis of larvae exposed to both *A. minutum* strains were similar (Tukey HSD, $p > 0.05$) to control larvae (Fig. 15A, B).

Exposure of eyed larvae to the BEC strain increased (Tukey HSD, $p < 10^{-5}$) mortality during settlement (8.6 ± 1.8 %) and decreased (Tukey HSD, $p < 10^{-3}$, respectively) settlement rate (4.7 ± 0.6 %), as compared to control larvae (Fig. 15C, D). Exposure of eyed larvae to the PST+BEC strain had no significant effect (Tukey HSD, $p > 0.05$) on mortality during settlement (2.1 ± 0.5 %) and settlement (18.9 ± 1.8 %), compared to the control (Fig. 15C, D).

Table 3. Effects of *A. minutum* exposures of *Crassostrea gigas* larvae on algal consumption (μm^3 of *T. lutea* and *Chaetoceros* sp. larva⁻¹ d⁻¹). Umbonate larvae and eyed larvae were exposed to 10^3 cells mL⁻¹ of the PST+BEC or the BEC strain of *A. minutum*, for 2 days (Expo2) and 1 day (Expo3) respectively, whereas control larvae were only fed with *T. lutea* and *Chaetoceros* sp. Letters indicate significant differences between conditions (Tukey HSD after ANOVA). Mean \pm SE, n = 4, n.a.: not analysed. ***: $p < 0.001$.

Exposure	Days post fertilization	Algal consumption (μm^3 larva ⁻¹ d ⁻¹)			ANOVA		
		Control	PST+BEC	BEC	df	F	p
Exposure of umbonate larvae (Expo2)	13 (before exposure)	577.5 \pm 23.8	682.3 \pm 31.4	583.2 \pm 32.4	2	4.0	0.057
	14 (1 d exposure)	704.9 \pm 97.0 ^a	950.8 \pm 121.4 ^a	134.4 \pm 26.0 ^b	2	21.2	3.9 $\times 10^{-4}$ ***
	15 (2 d exposure)	940.9 \pm 21 ^a	753.7 \pm 33.7 ^b	196.9 \pm 25.3 ^c	2	202.6	3.3 $\times 10^{-8}$ ***
Exposure of eyed larvae (Expo3)	20 (before exposure)	2848.8 \pm 167.1	2674.1 \pm 284.8	2160.0 \pm 194.8	2	2.6	0.127
	21 (1 d exposure)	2627.2 \pm 217.5 ^a	3435.8 \pm 310.1 ^a	117.1 \pm 25.5 ^b	2	177.9	5.8 $\times 10^{-8}$ ***

3.5 Paralytic shellfish toxin content

Toxin quantification by the ELISA method indicated that PST content in larvae was 15.6 ± 1.1 ng STX g⁻¹ of wet larvae weight at 23 dpf (8 days after exposure), and 38.8 ± 5.0 ng STX g⁻¹ of wet larvae weight at 21 dpf (at the end of the exposure), following exposure to the PST+BEC strain at the umbonate stage (Expo2) and the eyed stage (Expo3), respectively.

No PST was detected in control larvae, in larvae following exposure to the PST+BEC strain at the embryo stage (Expo1), and in larvae following exposure to the BEC strain at the umbonate (Expo2) and the eyed-larvae stage (Expo3).

4. Discussion

4.1 The early life stages of *C. gigas* are highly vulnerable to the BECs produced by *A. minutum*

Our results suggest that the bioactive extracellular compounds produced by *A. minutum* are highly toxic on *C. gigas* early development and clearly more toxic than the PSTs. The most striking effects were observed on oyster fertilization, embryos survival and larvae phenotypes (feeding activity, growth and survival) following exposure to the *A. minutum* BEC strain. The fertilization yield of exposed oyster gametes appeared drastically reduced (-98% compared to the control). The BEC strain exerts a lytic activity toward fertilized eggs and dividing embryos. A cytotoxic activity was observed on the velum of umbonate and eyed larvae but among *C. gigas* early life stages, larvae may be considered less susceptible to the BECs produced by *A. minutum* compared to gametes and embryos. Larval soft tissues may have been protected from direct contact with BECs by closure of larval shells.

The BECs could be responsible for lytic activity by targeting membrane integrity, leading to disintegration of embryos. This supports the findings of Basti et al. (2015) and De Rijcke et al. (2016) who evidenced that non-PST producing *Alexandrium* strains, *A. affine* and *A. ostenfeldii*, drastically altered the development of bivalve embryos.

Even if effects were less drastic than those induced by the BEC strain, the PST+BEC strain induced sublethal effects on oyster embryos by increasing the incidence of D-larvae with mantle anomalies and membrane swelling. These effects on embryogenesis are likely not caused by PSTs since they are mainly intracellular and oysters start to feed at the D-larvae stage which is unable to ingest cells as large as *A. minutum*. Moreover, purified saxitoxin had no effect on hatching of fertilized oocytes of the scallop *Chlamys farreri* (Yan et al., 2001). The PST+BEC strain was characterized by a much lower cytotoxic activity than the BEC strain (Long et al., 2018), suggesting a lower production of BECs and/or a production of less potent BECs. That's probably why fertilization was not altered suggesting that gametes and fertilization processes were not impacted by PSTs and BECs of this strain. Similarly, the bioactive substances produced by the PST+BEC strain did not induce the lysis of embryos as observed with the BEC strain but were still responsible for D-larvae anomalies. Growth, percentage of eyed larvae, mortality and settlement percentages of larvae that had been exposed to the PST+BEC strain

were similar to control larvae, suggesting that larvae were able to recover when fed with non-toxic algae after the exposure.

4.2 Exposure of umbonate larvae to *A. minutum* reduces growth and delays competence for metamorphosis

Umbonate larvae exposed to both *A. minutum* strains at 10^3 cells mL^{-1} exhibited lower growth and delayed competence for metamorphosis. The drastic decrease in feeding activity of umbonate larvae likely explains their growth reduction and consequently, the delay to reach competence to metamorphosis. This may result from modified feeding behaviour due to the deterrent action of BECs produced by *A. minutum*, and/or physiological incapacitation related to velum degradation observed, i.e. cilia exfoliation and abnormal masses in the velum. As hypothesized for *A. catenella* effect on growth of oyster umbonate larvae (Mu and Li, 2013), these adverse effects might be related to non-PST uncharacterized toxins, since the BEC strain induced more severe phenotypes than the PST+BEC strain which produces less BECs.

The BEC strain radically modified the behaviour of umbonate and eyed larvae which stopped swimming and sank. In the present study, most larvae exposed to the BEC strain remained slightly opened, the velum kept inside the shells, even though ciliary movement of the velum was still active. Adult *C. gigas* exposed to the same *A. minutum* strain showed increased valve opening duration and micro-closure activity, reduced feeding activity, and inflammation responses in gills (Castrec et al., 2018). As hypothesized for adult oysters (Castrec et al., 2018), the BECs excreted in the water could activate chemoreceptors or directly affect larval tissues, particularly the velum which is in direct contact with the extracellular substances released in the water by *A. minutum*. These tissue damages did not result in increased larval mortality during exposure of umbonate or eyed larvae to the BEC strain, however, deleterious consequences were observed on the subsequent larval settlement.

4.3 The BECs decrease larval survival and settlement

The lower settlement and survival percentages of larvae following exposure to the BEC strain at the umbonate stage could result from a lower energy state due to starvation during *A. minutum* exposure, and/or disrupted feeding activity caused by BECs. The amount of food consumed during larval development is essential for successful settlement, as larvae must store sufficient reserves to meet the energy demands required during the metamorphosis (Holland and Spencer, 1973). Endogenous processes related to energy acquisition prior to metamorphosis have been

associated to greater survival during metamorphosis (Gallager and Mann, 1986; Plough, 2018). Larval size at competence for metamorphosis was lower in BEC-strain exposed umbonate larvae than in control larvae, suggesting that exposed larvae might have accumulated less reserves.

4.4 Oyster larvae accumulate PSTs

Intracellular PSTs are mainly released in oyster tissues following ingestion and lysis of toxic dinoflagellate cells (Bricelj and Shumway, 1998). The present study indicates that larvae accumulated PSTs following exposure to the PST+BEC strain at the umbonate and eyed stages, confirming ingestion of *A. minutum* cells by larvae during exposure. Previous laboratory study indicated that *C. gigas* eyed larvae (mean shell length 304 μm) fed on *A. minutum* (Castrec et al., in prep. a). Baldwin (1995) demonstrated that *C. virginica* larger than 200 μm fed preferably on large food material (22 to 30 μm) in the presence of large dinoflagellate cell bloom. The largest umbonate larvae may have ingested *A. minutum* cells, since mean larval height of exposed umbonate larvae was 210.3 μm after 2 days of exposure. PSTs were still detected at 23 dpf in larvae following exposure to the PST+BEC strain from 13 and 15 dpf (Expo2). The presence of PSTs in larvae 8 days after the end of the *A. minutum* exposure suggest a low detoxification of these toxins in oyster larvae.

The settlement of larvae following exposure to the PST+BEC strain at the eyed stage was impaired, even if their feeding activity during exposure was similar to control larvae. The reasons for this harmful effect on oyster settlement could be that PSTs produced by *A. minutum* could affect larvae, and/or that the BECs also affect oyster larval settlement by causing irreversible damages to larval tissues.

The role of PSTs in *Alexandrium* spp. toxic effects on bivalve larvae has been proposed in previous studies (Basti et al., 2015; Mu and Li, 2013; Yan et al., 2003), however, this study is the first report of PST accumulation in oyster larvae following *Alexandrium* exposure. Saxitoxin binds voltage-gated Na^+ channels with high affinity and interacts, to a lesser extent, with Ca^{2+} and K^+ channels, modifying ionic fluxes into cells (Llewellyn, 2006). PSTs could have played a role in the detrimental effects observed on exposed umbonate larvae especially on settlement, as the voltage-gated potassium channel may be implicated in the settlement of oyster larvae (Wang et al., 2015). Eyed larvae exposed to the PST+BEC also accumulated PSTs, however, their survival and settlement were similar to control larvae. These results suggest that eyed larvae were less sensitive to PSTs than umbonate larvae, or that the exposure of eyed

larvae (1 day for eyed larvae vs 2 days for umbonate larvae) was too short to induce visible effects attributable to both PSTs and BECs.

5. Conclusion

This study evidenced that the cytotoxic bioactive extracellular compounds produced by *Alexandrium minutum* are detrimental for oyster early life stages, affecting fertilization success, embryo development, and larval swimming behaviour, feeding activity, growth, survival and settlement, thus confirming a PST-independent toxicity of *A. minutum*. Our results showed that oyster embryos are more sensitive to *A. minutum* than larvae, BECs having highly potent lysis toxicity on embryos. The BECs also play a major role in *A. minutum* toxicity upon oyster umbonate and eyed larvae, causing irreversible sublethal effects on larval physiology, which impaired the subsequent settlement and survival. It can be expected that the change in larval swimming and feeding behaviour caused by *A. minutum* could influence larval dispersal and reduce growth, thereby increasing the susceptibility of larvae to predation in nature. The present study revealed for the first time that oyster larvae exposed to *A. minutum* accumulated PSTs, however, no effect on larvae could be clearly attributed to these toxins. The potential effects of PST on larval development are to further investigate. Given the fact that *A. minutum* form blooms overlapping with the spawning and larval development seasons of *C. gigas*, this study provides evidence that *A. minutum* could adversely affect the recruitment of *C. gigas* with potential consequences on the structure of wild and cultivated populations of oysters.

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Supplementary files

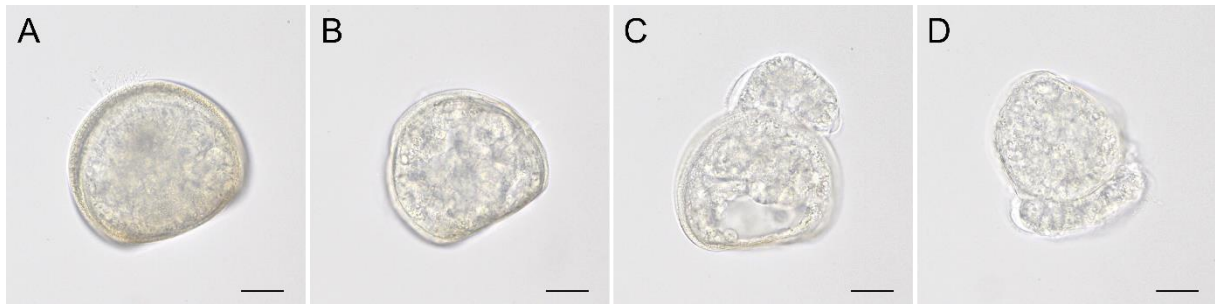


Fig. S1. Light micrographs of normal and abnormal *Crassostrea gigas* D-larvae 48 hours post fertilization. Embryos were non exposed (A, control) or exposed to 10^3 cells mL^{-1} of the PST+BEC strain of *A. minutum* (B,C,D) during two days (Expo1). (A) Normal control D-larvae; (B) Shell abnormality; (C) Mantle abnormality; (D) Developmental arrest. The BEC strain caused lysis and subsequent disintegration of embryos.

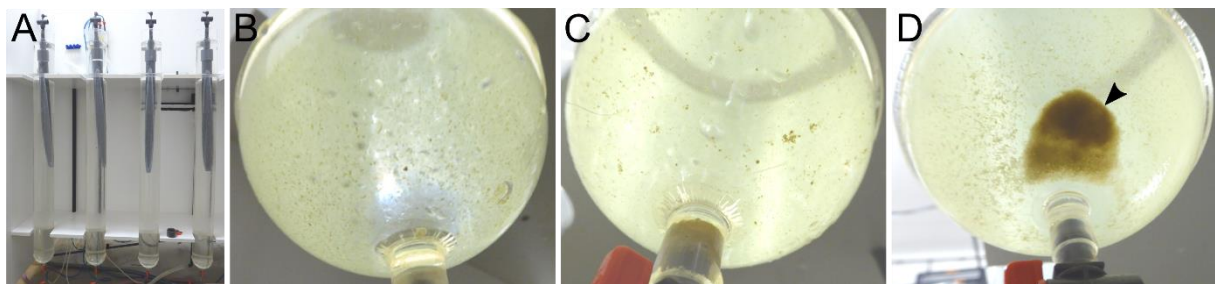


Fig. S2. Photographs illustrating behavior of *C. gigas* umbonate larvae in the experimental cylindrical tanks during Expo2 (A). Control larvae (B) and larvae exposed to the PST+BEC strain (10^3 cells mL^{-1}) (C) were swimming actively, whereas larvae exposed to the BEC strain (10^3 cells mL^{-1}) (D) sank to the bottom of the tanks (indicated by a black arrow) at 16 dpf (one day after the end of the exposure).

Chapitre 6

Discussion générale et perspectives

1. La reproduction et le développement *Crassostrea gigas* sont affectés par *Alexandrium minutum*

L'objectif de cette thèse était d'étudier les conséquences des efflorescences du dinoflagellé toxique *A. minutum* sur la reproduction, le développement et le recrutement de l'huître *C. gigas*, une espèce à l'importance économique majeure. Dans ce but, nous avons réalisé en laboratoire des expériences d'exposition de l'huître *C. gigas* à différents stades de vie (adultes, gamètes, embryons et larves) à des concentrations d'*A. minutum* qui peuvent être observées lors d'efflorescences naturelles. Afin d'évaluer le panel d'effets causés par *A. minutum* et ses différentes toxines, nous avons testé l'effet de trois souches d'*A. minutum* produisant soit des PST, soit des BEC, soit des PST et des BEC. Cette approche nous a également permis de déterminer l'implication relative de ces deux types de toxines dans la toxicité d'*A. minutum* sur la physiologie, la reproduction et le développement de l'huître.

1.1. *A. minutum* affecte la fécondation et le développement embryonnaire

Une exposition de deux jours des embryons de *C. gigas* à 10^3 cellules mL^{-1} d'*A. minutum* augmente le pourcentage de larves D anormales de 430 % par rapport aux embryons témoins et peut même induire la lyse des embryons, inhibant alors totalement le développement en larve D (chapitre 5). Cette concentration en *A. minutum* est régulièrement observée lors d'efflorescences naturelles (Chapelle et al., 2015; Garcés et al., 2004; Guallar et al., 2017), ce qui suggère que ces efflorescences pourraient altérer le développement embryonnaire des huîtres. Ces résultats sont soutenus par d'autres études qui ont mis en évidence la vulnérabilité des embryons de bivalves marins aux dinoflagellés toxiques (Basti et al., 2013, 2015; De Rijcke et al., 2015, 2016; Rolton et al., 2014; Stoecker et al., 2008). La concentration à laquelle *A. minutum* affecte le développement des embryons de *C. gigas* (10^3 cellules mL^{-1}) est du même ordre que celle rapportée pour *A. affine* (2.5×10^2 cellules mL^{-1}) et *A. catenella* (2×10^3 cellules mL^{-1}) qui provoquent la lyse des embryons de l'huître perlière *Pinctada fucata martensii* (Basti et al., 2015). Une exposition des gamètes libres de *C. gigas* à la micro-algue *A. minutum*, avant la mise en contact pour la fécondation, ou simultanément à cette mise en contact provoque une diminution significative du taux de fécondation, calculé sur la base du taux d'embryons formés (stade 2 ou 4 cellules). L'intensité d'inhibition de la fécondation est dépendante de la souche d'*A. minutum* utilisée. Pour les deux souches PST+BEC et BEC, les effets sont significatifs à $2,5 \times 10^4$ cellules mL^{-1} alors que la souche BEC se révèle être plus cytotoxique pour les gamètes de *C. gigas* puisqu'elle provoque une diminution significative de la fécondation dès 10^3 cellules

mL⁻¹ et l'inhibe complètement à 10⁴ cellules mL⁻¹. L'effet respectif des deux types de toxines sera discuté dans le paragraphe 2.

La sensibilité des gamètes de bivalves à des dinoflagellés toxiques et sa conséquence sur leur capacité de fécondation a déjà été rapportée (Rolton et al., 2015) mais à notre connaissance pas pour le couple *A. minutum* / *C. gigas*. Nous pouvons avancer plusieurs hypothèses concernant les modalités d'action des toxines d'*A. minutum* sur les gamètes. Les toxines pourraient induire une surproduction d'espèces réactives de l'oxygène (ROS) par les gamètes, induisant un stress oxydant néfaste pour l'intégrité des cellules (Le Goïc et al., 2014). Chez les mammifères et notamment chez l'homme, une production excessive de ROS par les gamètes a des effets délétères sur les membranes à cause de la peroxydation des lipides ainsi que sur la capacité de fécondation (Agarwal et al., 2003; Tamura et al., 2008). Les dommages à l'ADN induits par le stress oxydant peuvent aussi accélérer le processus d'apoptose (Agarwal et al., 2003). L'intégrité des membranes pourrait aussi être altérée directement par les toxines produites par *A. minutum* et/ou par une atteinte physique causée par les chocs répétés entre les gamètes et les cellules mobiles d'*A. minutum* recouverte d'une thèque cellulosique. Pour les spermatozoïdes, l'un des phénotypes marquant associé à l'atteinte des cellules est la diminution voir l'inhibition de la mobilité. Elle pourrait provenir d'une altération des organites cellulaires par une production accrue de ROS ou d'une atteinte de l'intégrité de la membrane par les toxines d'*A. minutum*. Les toxines pourraient également modifier les flux d'ions qui ont un rôle essentiel dans la motilité des spermatozoïdes de *C. gigas*. Afin de tester ces hypothèses, il faudrait, par exemple, étudier l'intégrité des membranes et des organites des gamètes par microscopie électronique à transmission, la peroxydation des lipides par le test des TBARS (thiobarbituric acid reactive substances) (Di Poi et al., 2016) et les dommages à l'ADN par le test des comètes (Akcha et al., 2012).

Dans le chapitre 2, nous avons mis en évidence qu'une exposition des huîtres à des concentrations modérées d'*A. minutum* (10² cellules mL⁻¹) pendant un mois lors de la gamétogenèse affecte la qualité des gamètes produits (Figure 17). Les spermatozoïdes produits par ces huîtres exposées présentent une mobilité réduite, sans que leur vitesse de nage et leur contenu en ATP ne soient modifiés. La mesure des paramètres cellulaires des gamètes par cytométrie en flux n'a pas mis en évidence d'effet significatif de l'exposition à *A. minutum*. Rolton et al. (2016) ont rapporté des résultats similaires sur les gamètes de *C. virginica* et de *M. mercenaria* suite à l'exposition en laboratoire à *K. brevis*. Dans le chapitre 2, la capacité de fécondation des spermatozoïdes issus d'huîtres exposées n'a pas été étudiée.

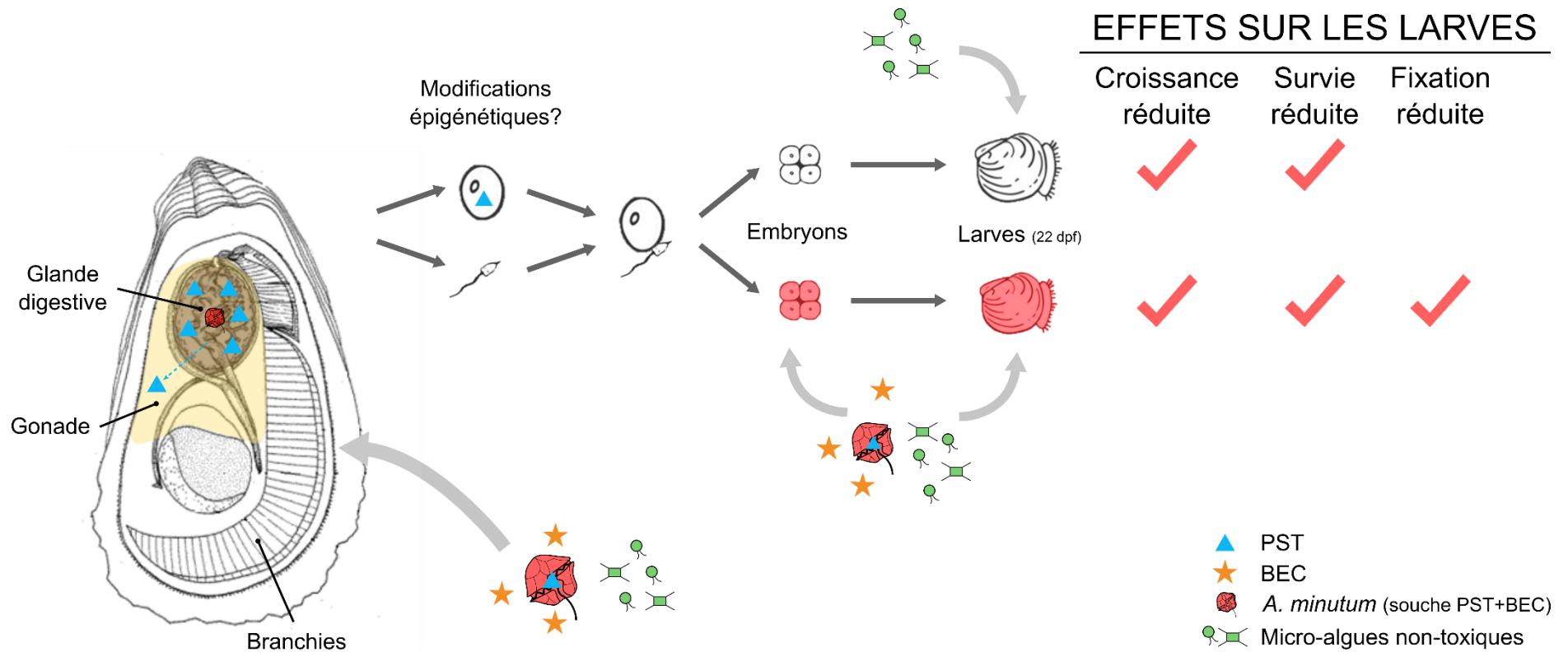


Figure 17. Effets néfastes d'une souche d'*A. minutum* produisant des PST et des BEC (souche PST+BEC) sur le développement larvaire de *C. gigas*, liés à l'exposition des géniteurs pendant deux mois lors de la gamétogenèse et à l'exposition directe de la progéniture pendant le développement embryo-larvaire. Ces résultats proviennent du chapitre 2 de cette thèse. PST : toxines paralysantes ; BEC : composés extracellulaires bioactifs ; Dpf : jours post-fécondation.

1.2. *A. minutum* affecte le développement et la survie des larves

La croissance et la survie des larves de *C. gigas* sont affectées indirectement par l'exposition des parents à une concentration modérée (10^2 cellules mL^{-1}) d'*A. minutum* pendant 2 mois lors de la gamétogenèse (chapitre 2) (Figure 17). Ces résultats confirment l'hypothèse d'Haberhorn et al. (2010b) qui suggérait qu'une exposition des géniteurs pourrait avoir des conséquences néfastes le développement larvaire *C. gigas*.

Les mécanismes moléculaires médiateurs de cet effet multigénérationnel sur le développement larvaire de *C. gigas* restent inconnus. Néanmoins, une perturbation de la balance énergétique chez *C. gigas* exposée à *A. minutum* a pu induire des effets néfastes sur les gamètes, perturbant en conséquence le développement de la descendance. En effet, Pousse et al. (2018b) ont démontré que le stress provoqué par l'exposition des huîtres adultes à *A. minutum* a augmenté leur demande énergétique, de l'énergie supplémentaire étant requise pour la réparation des tissus et la détoxification des composés toxiques produits par *A. minutum*. Ces résultats sont soutenus par Rolton et al. (2016) qui a suggéré que des huîtres *C. virginica* exposées à une efflorescence naturelle de *K. brevis* ont alloué moins d'énergie à la reproduction et augmenté l'allocation d'énergie à l'homéostasie et à la réparation des tissus.

Le stress provoqué par l'exposition des huîtres à *A. minutum* a pu conduire à des modifications épigénétiques dans les gamètes transmises à la descendance via les ARNm maternels. Ces ARNm s'accumulent pendant la gamétogenèse et sont les principales ressources pour la synthèse des protéines lors du développement précoce de l'embryon. L'épitranscriptome (m^6A -ARN, méthylation des adénosines de l'ARN) émerge comme un régulateur épigénétique majeur du développement qui serait conservé chez l'huître. Une récente étude a suggéré l'implication des mécanismes épigénétiques dans l'impact d'une exposition parentale de *C. gigas* à un herbicide sur le transcriptome de la descendance (Bachère et al., 2017). Des mécanismes épigénétiques et notamment la méthylation des ARN des ovocytes pourraient être également impliqués dans l'effet multigénérationnel d'*A. minutum*. Dans le chapitre 2, nous avons mis en évidence la présence d'un épitranscriptome dans les ovocytes de *C. gigas* par immunoblot. Ces résultats n'ont pas mis en évidence de différences quantitatives de méthylation des ARN entre les ovocytes provenant d'huîtres exposées à *A. minutum* et ceux issus d'huîtres témoins. Néanmoins, le niveau de méthylation des divers types d'ARN (ARNm, ARNt, ARNrb, ARN non codant) pourrait être différent dans les ovocytes des deux conditions, influant de manière distincte le développement de la descendance. Pour creuser cette hypothèse, les ARN méthylés pourraient être identifiés par séquençage haut-débit après immunoprécipitation. Ensuite,

l'épitranscriptome serait analysé en regard du transcriptome associé, déterminé après séquençage des ARN des ovocytes par RNAseq.

Lors de ce travail, nous avons également démontré que les larves sont sensibles à une exposition directe à *A. minutum* (Tableau 4). Les résultats du chapitre 4 ont mis en évidence qu'une exposition de *C. gigas* à partir du stade embryon pendant vingt-deux jours à 10^2 cellules mL^{-1} d'*A. minutum* réduit la croissance et la fixation des larves. Une courte exposition de deux jours des larves umbonées à une plus forte concentration (10^3 cellules mL^{-1}) diminue la filtration, la croissance, la survie et la fixation des larves (chapitre 5). Le stade de larve œillée semble moins vulnérable à la souche PST+BEC, bien que la durée d'exposition plus courte par rapport à celle des larves umbonées pourrait expliquer ces différences. Néanmoins, une exposition d'un jour des larves œillées à la souche BEC diminue la filtration, la survie et la fixation des larves. L'exposition à *A. minutum* pourrait diminuer la filtration des larves en altérant l'intégrité du vélum et/ou en activant, par l'intermédiaire de toxines, les chémorécepteurs présents dans le manteau de la larve, modifiant alors leur comportement de filtration et de nage. Les toxines produites par *A. minutum* pourraient également affecter la digestion et l'intégrité des tissus de la glande digestive. L'inhibition de la filtration et la perturbation de la digestion pourraient diminuer les apports énergétiques et donc affecter la croissance et la fixation des larves. En effet, la quantité de nourriture consommée pendant le développement des larves est essentielle au succès de la fixation, car les larves doivent disposer de réserves suffisantes pour répondre aux besoins en énergie requis pour de la métamorphose (Holland and Spencer, 1973). Les toxines produites par *A. minutum* pourraient directement affecter la physiologie des larves et causer un stress qui pourrait conduire à une mortalité. Les effets respectifs des deux types de toxines sur les larves seront discutés dans le paragraphe 2.

Le recrutement des invertébrés benthiques dépend du nombre de larves produites, de leur survie jusqu'à la fixation et la métamorphose mais aussi de la survie des post-larves (Osman, 1994). Les résultats de ce travail démontrent que des durées d'exposition et des concentrations environnementales d'*A. minutum* altèrent la survie, la croissance et la fixation des larves. Les efflorescences d'*A. minutum* concomitantes au développement embryon-larvaire pourraient donc affecter le recrutement de *C. gigas*.

Toutes les expériences menées dans le cadre de cette thèse ont mis en œuvre des moyens techniques pour garantir un traitement approprié des effluents contenant des cellules d'*A. minutum*. Ces moyens n'étaient pas disponibles pour le dispositif expérimental utilisé lors de la fixation des larves. Nous n'avons donc pas pu exposer directement les larves pédivéligères à *A.*

minutum lors de la fixation. Compte tenu de la modification drastique du comportement des larves lors de l'exposition à *A. minutum*, il serait intéressant d'étudier le comportement de nage et de crawling de larves de bivalves exposées à différentes concentrations d'*A. minutum* et d'évaluer les conséquences sur la fixation. Le comportement de nage des larves pourrait être étudié en milieu contrôlé par video-tracking, comme cela a été fait par Meyer et al. (2018).

Tableau 4. Résumé des effets d'*A. minutum* sur la filtration, la croissance, la survie et la fixation des larves de *C. gigas*, lors des expériences menées dans les chapitres 2 et 5.

		EXPOSITION			EFFETS SUR LES LARVES			
	Stade exposé	<i>A. minutum</i>		Durée exposition (jours)	Filtration réduite	Croissance réduite	Survie réduite	Fixation réduite
		Souche	Concentration (cellules mL ⁻¹)					
Chapitre 2	Embryons jusqu'au stade de larve œillée	PST+BEC	10 ²	22	n.a.	✓		✓
Chapitre 5	Larves umbonées (7 dpf)	PST+BEC	10 ³	2	✓	✓		✓
Chapitre 5	Larves umbonées (7 dpf)	BEC	10 ³	2	✓	✓	✓	✓
Chapitre 5	Larves œillées (20 dpf)	PST+BEC	10 ³	1				
Chapitre 5	Larves œillées (20 dpf)	BEC	10 ³	1	✓		✓	✓

Ce travail de thèse révèle que la reproduction et le développement embryo-larvaire de *C. gigas* sont sensibles à l'exposition à *A. minutum*. Il serait aussi intéressant d'étudier la sensibilité d'autres bivalves marins à *A. minutum*, par exemple l'huître plate *Ostrea edulis*. Cette espèce exploitée présente un fort intérêt économique mais aussi écologique car c'est une espèce-ingénieur de l'écosystème (Pouvreau et al., 2018). L'huître plate figure dans la liste des espèces menacées de la convention OSPAR (Lallias et al., 2010) et est aussi régulièrement exposée aux efflorescences d'*A. minutum* sur les côtes françaises. La fécondation de l'huître plate a lieu dans la cavité palléale de la femelle, puis les larves sont incubées pendant plusieurs jours avant d'être expulsées dans la colonne d'eau (Suquet et al., 2018). Les modalités d'exposition des premiers stades de vie d'*Ostrea edulis* à *A. minutum* seraient donc différentes de celles de *C. gigas* et pourraient affecter différemment le développement de cette espèce.

2. Implication des composés extracellulaires bioactifs et des toxines paralysantes produits par *A. minutum* dans sa toxicité

Les effets d'*A. minutum* sur la physiologie des bivalves ont été bien décrits. Cependant, attribuer les réponses spécifiques des huîtres aux PST ou aux BEC reste difficile, car la plupart des études n'ont pas évalué la production de BEC (Bricelj et al., 1993; Bricelj and Shumway, 1998; Landsberg, 2002) ou ont utilisé des souches produisant les deux types de toxines (Haberkorn et al., 2010b, 2010a; Le Goïc et al., 2014; Mat et al., 2013; Tran et al., 2010, 2015). De récentes études ont mis en évidence que des souches d'*Alexandrium* non-productrices de PST, *A. taylori*, *A. affine*, and *A. monilatum*, altèrent le clivage des embryons, leur développement en larves D ainsi que l'activité et la survie des larves de plusieurs espèces de bivalves marins (Basti et al., 2015; Matsuyama et al., 2001; May et al., 2010). Ces auteurs ont suggéré que les composés bioactifs produits par *Alexandrium* spp., indépendamment des PST, contribuent aux effets néfastes de ces micro-algues sur le développement embryo-larvaire des bivalves.

Dans le présent travail, les effets respectifs des PST et des BEC produits par *A. minutum* ont été étudiés afin de mieux comprendre comment *A. minutum* affecte la physiologie, la reproduction et le développement de *C. gigas*. Pour répondre à cet objectif, les effets de trois souches d'*A. minutum* présentant des toxicités distinctes en termes de PST et de BEC ont été examinés en exposant des huîtres adultes (chapitre 3), des gamètes (chapitre 4), des embryons et des larves (chapitre 5).

2.1. Toxicité des BEC

Les réponses des huîtres adultes, gamètes, embryons et larves à la souche BEC d'*A. minutum*, non productrice de PST, confirment que les composés extracellulaires produits par cette souche sont néfastes pour la physiologie et le développement précoce de *C. gigas*. A notre connaissance, la seule étude s'intéressant aux effets de l'exposition de larves de bivalves marins à *A. minutum* a mis en évidence que cette micro-algue affecte la viabilité et le développement des larves de *M. edulis* (De Rijcke et al., 2016). De Rijcke et al. (2016) ont émis l'hypothèse que l'effet délétère d'*A. minutum* sur les larves de *M. edulis* est causé par des composés bioactifs non caractérisés, de manière indépendante des PST.

Les gamètes et embryons sont particulièrement affectés par les BEC produits par *A. minutum* et la souche BEC provoque même la lyse des embryons (chapitres 4 et 5). Ces résultats sont soutenus par d'autres études qui suggèrent un effet toxique des BEC produits par le genre

Alexandrium sur l'embryogenèse de bivalves marins (Basti et al., 2015; Mu and Li, 2013; Yan et al., 2001).

Nos résultats indiquant que les embryons sont très vulnérables face à la toxicité des BEC produits *A. minutum* sont soutenus par des études qui suggèrent que les BEC produits par *Alexandrium* spp. agissent directement sur les membranes externes des cellules cibles (Ma et al., 2009), comme suggéré pour l'effet d'*A. minutum* sur une autre espèce de micro-algue (Long et al., 2018b). Bien que la nature chimique des BEC soit encore inconnue, des études suggèrent que l'activité lytique d'*Alexandrium* serait liée à une atteinte de l'intégrité de la membrane externe de la cellule par une induction de la peroxydation des lipides (Flores et al., 2012) ou par une augmentation de la perméabilité des ions Ca^{2+} (Ma et al., 2011). L'implication de ces mécanismes dans la toxicité d'*A. minutum* sur les gamètes et les embryons de *C. gigas* pourrait être étudiée afin de mieux comprendre le mode d'action des composés bioactifs. L'intégrité des membranes des gamètes et des embryons d'huître exposés à *A. minutum* pourrait être étudiée par microscopie électronique à transmission et la peroxydation lipidique pourrait être mesurée par le test des TBARS (thiobarbituric acid reactive substances) (Di Poi et al., 2016).

Afin de pouvoir mesurer et comparer rapidement l'activité de différentes souches d'*Alexandrium* ou d'extraits purifiés, un bioessai utilisant des embryons de *C. gigas* pourrait être développé en se basant sur la procédure AFNOR (AFNOR XP-T-90-382). Ce bioessai consisterait à exposer des embryons à différentes concentrations de filtrats de culture et à quantifier leur taux de développement. De manière similaire au bioessai développé par De Rijcke et al. (2016), cette technique expérimentale permettrait de déterminer une valeur d'EC50 qui peut être comparée de manière statistique entre des filtrats provenant de différentes souches d'*Alexandrium* spp. Ce bioessai compléterait celui conçu par Long et al. (2018a) qui évalue l'activité allélopathique des BEC en mesurant l'inhibition du photosystème de la diatomée *Chaetoceros muelleri* exposée au filtrat de culture d'*A. minutum*. Ceci permettrait d'évaluer si une même fraction purifiée présente une activité allélopathique sur un modèle algue et/ou une activité cytotoxique sur un modèle bivalve.

L'exposition à la souche BEC provoque aussi une réduction drastique de l'activité de filtration des larves (chapitre 5) et des huîtres adultes (chapitre 2). Cette inhibition de la filtration pourrait résulter d'une altération des organes permettant la nutrition par une activité cytotoxique des BEC sur le velum des larves et des branchies des huîtres adultes. Les BEC pourraient également avoir une activité allélopathique qui modifie le comportement des larves : les larves se ferment et arrêtent de nager, ce qui diminue par conséquent leur filtration. Des études supplémentaires

sont nécessaires pour mieux comprendre comment les BEC réduisent la filtration des larves d'huîtres. Les lésions causées par les BEC sur le vélum des larves pourraient être étudiées plus en détail par histologie et microscopie électronique à transmission.

2.2. Toxicité des PST

Les réponses observées chez les huîtres adultes exposées aux souches d'*A. minutum* productrices de PST au cours de ce travail de thèse suggèrent que l'accumulation de PST perturbe les rythmes biologiques des huîtres (chapitre 2). Ces résultats sont en accord avec ceux de Payton et al. (2017) qui ont suggéré que les PST perturbent le rythme d'activité valvaire de *C. gigas* en diminuant la transcription des gènes de l'horloge impliqués dans les rythmes biologiques. Cette perturbation résulterait potentiellement d'un effet génotoxique des PST, dont le mode d'action reste à élucider. Les rythmes biologiques permettent la synchronisation et l'anticipation des cycles environnementaux, permettant l'organisation temporelle des processus biologiques comme la reproduction en adéquation avec leur biotope (Yerushalmi and Green, 2009). L'accumulation de PST par les bivalves pourrait altérer la reproduction en perturbant les rythmes biologiques.

Les PST pourraient aussi être responsables de la perturbation de la balance énergétique observée chez les huîtres adultes exposées à *A. minutum* lors de la gamétogenèse (chapitre 2), en modifiant le métabolisme et le transport des glucides. Cette hypothèse est soutenue par Mat et al. (2018) qui ont mis en évidence une modification de l'expression des gènes impliqués dans la glycogénèse, la glycolyse et la glycolyse dans la glande digestive d'huîtres ayant fortement accumulé des PST. La diminution de la mobilité des spermatozoïdes d'huîtres exposées à *A. minutum* (chapitre 2) pourrait également être causée par la présence de PST dans la gonade, en modifiant les flux d'ions qui jouent un rôle important dans la mobilité des spermatozoïdes (Alavi et al., 2014; Boulais et al., 2018). Les PST, en se fixant aux canaux Na^+ , Ca^{2+} et K^+ , pourraient modifier les flux d'ions (Llewellyn, 2006) et les voies métaboliques associées dont la contraction musculaire, la communication nerveuse et les processus de digestion (Mat et al., 2018).

Dans le chapitre 2, nous avons mis en évidence un impact multigénérationnel de l'exposition des huîtres à *A. minutum*. Par rapport à des huîtres témoins, les huîtres exposées à la souche PST+BEC d'*A. minutum* lors de la gamétogenèse ont produit des spermatozoïdes moins mobiles ainsi que des larves présentant une croissance et une survie plus faibles. De même, des larves issues d'huîtres *C. virginica* exposées à une efflorescence naturelle du dinoflagellé

toxique *Karenia brevis*, producteur de brevétocines, présentent une survie et une taille plus faible que des larves issues d'huîtres non-exposées (Rolton et al., 2016). Rolton et al. (2016) suggèrent que les effets délétères observés chez les larves sont liés à la présence de brevétocines dans les ovocytes des huîtres *C. virginica* exposées à *K. brevis*. Dans cette thèse, un transfert des PST de la glande digestive vers la gonade mâle et les ovocytes a été observé chez les huîtres exposées à *A. minutum*, suggérant que les conséquences délétères sur la descendance pourraient provenir des effets toxiques des PST via les gamètes. Ces travaux de thèse suggèrent qu'*A. minutum* comme d'autres dinoflagellés producteurs de PST, comme *A. tamarense*, *A. catenella* mais aussi *Gymnodinium catenatum* et *Pyrodinium bahamense* var. *compressum* pourraient également affecter la reproduction et le développement de *C. gigas* et potentiellement d'autres bivalves marins. L'effet délétère d'*A. minutum* via les PST sur la reproduction pourrait varier en fonction de la capacité d'accumulation et de détoxification des PST ainsi que de la sensibilité aux PST des espèces de bivalves (Bougrier et al., 2003). Par exemple, la cinétique de détoxification des coquilles Saint-Jacques *Pecten maximus* est beaucoup plus faible que celle de *C. gigas* et conduit à une période de toxicité plus longue chez *P. maximus* (Bougrier et al., 2001). De plus, une accumulation sélective de certains PST a été observée dans la gonade de *P. maximus* (Lassus et al., 1992). Dans ce contexte, il semble nécessaire d'étudier l'impact des efflorescences d'*A. minutum* sur la reproduction de *P. maximus*, bivalve à forte valeur commerciale.

L'accumulation de PST par les bivalves adultes après ingestion de cellules d'*A. minutum* a été largement étudiée (Bricelj and Shumway, 1998; Guéguen et al., 2008; Haberkorn et al., 2010b; Pousse et al., 2018a, 2018b). Néanmoins, ces travaux de thèse ont mis en évidence pour la première fois une accumulation de PST par les larves umbonées et œillées de *C. gigas* exposées à *A. minutum*. Ces résultats soulèvent des interrogations quant aux conséquences de cette accumulation sur la physiologie des larves et sur la cinétique de détoxification de ces toxines chez ces jeunes stades. Les processus de détoxification sont beaucoup moins connus chez les larves que chez les adultes de *C. gigas*. Néanmoins, des gènes potentiellement impliqués dans la biotransformation des xénobiotiques ont été retrouvés après analyse du transcriptome de larves de *C. gigas* et pourraient participer à la détoxification des PST (Nogueira et al., 2017). L'exposition des larves œillées pendant 2 jours à la souche PST+BEC d'*A. minutum* conduit à une réduction du pourcentage de fixation des larves. Les PST toujours présentes dans les larves au stade de larve œillée pourraient perturber la fixation des larves en agissant sur les canaux potassium voltage-dépendant qui sont potentiellement impliqués dans le processus de fixation

(Wang et al., 2015). Les PST pourraient également se fixer sur les canaux sodium voltage dépendant (Cestèle and Catterall, 2000) et ainsi perturber la communication neuromusculaire des larves. La présence des canaux sodium voltage-dépendant est attestée chez les adultes de *C. gigas* (Boullot et al., 2017) mais reste inconnue chez les larves. Afin de vérifier ces hypothèses, la présence de ces canaux chez les larves de bivalves doit être démontrée, ce qui permettra ensuite d'étudier l'effet de PST purifiées sur ces canaux.

3. Les efflorescences d'*A. minutum* dans le milieu naturel : quelles conséquences sur le recrutement de *C. gigas* ?

Les expériences menées en laboratoire dans le cadre de cette thèse mettent clairement en évidence que la reproduction et le développement de l'huître *C. gigas* sont affectés par l'exposition à *A. minutum* et que les efflorescences d'*A. minutum* représentent donc un risque réel pour la reproduction et le recrutement de *C. gigas* en milieu naturel. Néanmoins, il est délicat d'extrapoler directement nos résultats à l'impact réel de ces efflorescences sur la reproduction et le recrutement des huîtres en milieu naturel. En fonction de l'occurrence et de la durée de l'efflorescence, plusieurs stades de vie *C. gigas*, qui se reproduit pendant l'été sur les côtes françaises (Pouvreau et al., 2016; Suquet et al., 2016; Ubertini et al., 2017), peuvent être exposés lors d'un même cycle de reproduction. L'exposition directe des jeunes stades de l'huître à des concentrations fortes (10^3 cellules mL^{-1}), même pour une courte durée (1-2 jours), peut causer des effets cumulés en réduisant la viabilité des gamètes, le succès de fécondation ainsi que la croissance et la survie des larves. Ces impacts létaux et sublétaux sur les stades de développement pourraient s'additionner aux effets délétères liés à l'exposition antérieure des adultes. De plus, un très grand nombre de facteurs environnementaux peuvent moduler la réponse des huîtres et la toxicité des efflorescences d'*A. minutum*.

3.1. Variabilité des caractéristiques des efflorescences d'*A. minutum*

La dynamique des populations de dinoflagellés toxiques et leur production de toxines (PST et BEC) sont influencées par de nombreux facteurs environnementaux, comme la salinité, la température, l'irradiation solaire et la pression partielle en dioxyde de carbone (pCO_2) (Li et al., 2018). Les caractéristiques des efflorescences d'*A. minutum* sont ainsi variables en fonction des zones géographiques et en fonction des années (Anderson et al., 2012a; Touzet et al., 2007). Par exemple, Guallar et al. (2017) ont étudié la phénologie et les caractéristiques des efflorescences d'*A. minutum* sur les côtes françaises, en Manche et Atlantique Nord-Est, en

analysant les séries temporelles couvrant plusieurs sites sur près de 25 ans. La température de l'eau de mer et le débit des rivières sont les principaux facteurs influençant la magnitude, le taux de croissance et la durée des efflorescences d'*A. minutum* sur ces côtes françaises (Andrieux-Loyer et al., 2008; Guallar et al., 2017). L'intensité des efflorescences d'*A. minutum* dans ces régions varie d'un facteur 100 entre différentes stations avec des abondances maximales comprises entre 1.02×10^4 cellules L^{-1} à 4.46×10^7 cellules L^{-1} . La durée des efflorescences varie de 2 semaines à 6 mois, cette valeur maximale ayant été observée en rade de Brest en 2014 (Guallar et al., 2017). Ces observations sont cohérentes avec des efflorescences d'*A. minutum* observées dans d'autres régions du globe, notamment en Espagne (Bravo et al., 2010) et en Irlande (Cosgrove et al., 2014). La période des efflorescences d'*A. minutum* est aussi fortement variable. En Espagne, les cellules végétatives peuvent se développer en continu tout au long de l'année (Bravo et al., 2008). Sur les côtes françaises, la date du début des efflorescences s'étend d'avril à août, et la date de fin des efflorescences est comprise entre juin et octobre, avec des concentrations maximales atteintes entre mai et septembre (Guallar et al., 2017).

Une forte diversité génétique spatiale et temporelle a aussi été mise en évidence dans les efflorescences d'*A. minutum*, mais aussi au sein même d'une efflorescence (Dia et al., 2014). Cette diversité serait le reflet de la présence successive ou simultanée de différentes populations génétiquement distinctes lors d'une efflorescence d'*A. minutum* (Guallar et al., 2017). On peut supposer que ces différentes populations d'*A. minutum* présentent des phénotypes différents, notamment en termes de production de PST et de BEC.

La variabilité intraspécifique de la teneur et la composition en PST est bien connue pour *Alexandrium* spp. (Cembella et al., 1987; Maranda et al., 1985; Ogata et al., 1987). Ces études indiquent que la toxicité en PST d'*A. minutum* est variable : dans l'espace et dans le temps lors d'une efflorescence, au sein d'une même population et pour une même souche. La quantité de PST produits par une souche d'*A. minutum* est influencée par de nombreux paramètres environnementaux, dont la salinité (Hwang and Lu, 2000) et la concentration en nutriments (Lippemeier et al., 2003).

La production de BEC par *A. minutum* varie aussi en fonction des souches. Quelques études ont mis en évidence la variabilité intraspécifique de l'activité lytique d'*A. tamarense* et *A. minutum* (Alpermann et al., 2010; Borcier et al., 2017; Tillmann et al., 2009) et de l'activité allélopathique d'*A. minutum* (Long et al., 2018a). L'augmentation de l'activité allélopathique

d'*A. minutum* en présence de fortes concentrations en cuivre suggère que cette activité est aussi dépendante de facteurs environnementaux (Long et al., 2019).

Les travaux de cette thèse ont démontré que les BEC ainsi que les PST sont toxiques pour les adultes et les jeunes stades de vie de l'huître. L'intensité des effets des efflorescences d'*A. minutum* sur la reproduction de l'huître dépend donc probablement de la concentration algale et de la toxicité de ces phénomènes en termes de PST et de BEC et est donc difficilement prévisible. Il serait pertinent à plus long terme de développer des modèles populationnels qui permettraient de prédire les conséquences des efflorescences d'*A. minutum* sur la reproduction et le recrutement de *C. gigas*, en implémentant les modèles avec les données biologiques issues de cette thèse et les informations sur la toxicité des différentes souches. Il faudrait également intégrer à ces modèles les paramètres environnementaux qui peuvent influencer la cinétique et l'intensité de la reproduction de *C. gigas*, la phénologie des efflorescences mais aussi les paramètres extérieurs qui pourraient moduler les interactions entre *A. minutum* et *C. gigas*.

3.2. Importance relative des HAB par rapport à d'autres facteurs environnementaux

Un nombre croissant d'ostréiculteurs pratiquent avec succès le captage de *C. gigas* dans la rade de Brest, néanmoins, des irrégularités dans le recrutement ont été observées en fonction des années (Pouvreau et al., 2015). La variabilité du recrutement pourrait être liée à la présence d'efflorescences d'*A. minutum* quasi récurrentes depuis 2012 en rade de Brest.

Plusieurs défauts de recrutement ont pu être observés en milieu naturel au moment d'efflorescences de micro-algues toxiques, et même s'il n'est, à l'heure actuelle pas encore possible de prouver que ces efflorescences en sont à l'origine, cette hypothèse a été plusieurs fois proposée (Bricelj and MacQuarrie, 2007; Erard-Le Denn et al., 1990; Leverone et al., 2006; Summerson and Peterson, 1990; Tang and Gobler, 2009). En effet, le lien de cause à effet entre efflorescences toxiques et défaut de recrutement est difficile à établir car le recrutement dépend du succès de nombreuses étapes dont la gamétogenèse, l'émission des gamètes, la fécondation, le développement embryonnaire et larvaire ainsi que la métamorphose et la fixation des larves (Bernard, 2011). Les variations de recrutement des bivalves marins s'expliquent par un ensemble de facteurs intervenant, souvent conjointement, pour favoriser ou perturber le recrutement (Houde, 2008). La quantité et la qualité du phytoplancton joue un rôle crucial sur le recrutement de *C. gigas*, en influant sur la gamétogenèse des adultes, ainsi que sur la croissance et la survie des larves en été (Pouvreau et al., 2008) (Figure 18).

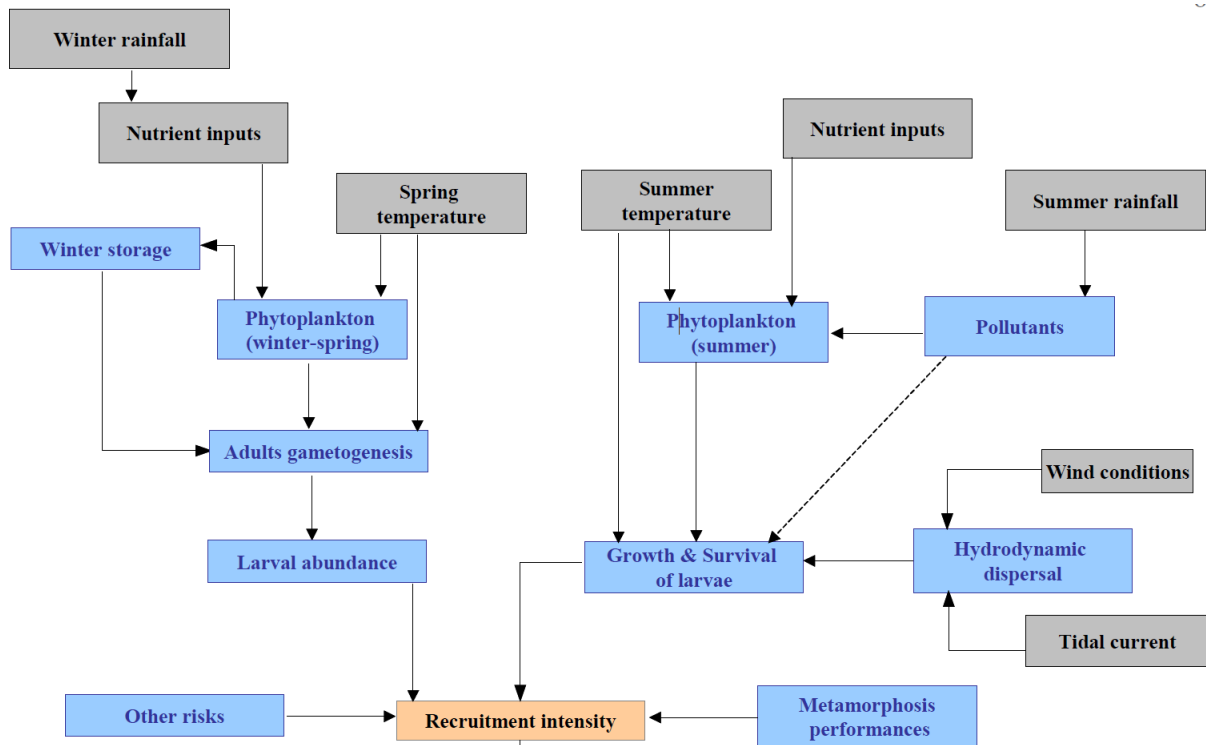


Figure 18. Implications des facteurs hydro-climatiques dans le succès du recrutement de *Crassostrea gigas*, d’après Pouvreau et al. (2008). Les facteurs climatiques marqués en gris influent indirectement sur la reproduction de *C. gigas*.

Afin de déterminer l’influence des efflorescences toxiques sur le recrutement des huîtres, il serait intéressant d’analyser conjointement la présence de ces efflorescences, la reproduction de l’huître et les autres paramètres environnementaux (température, marée, vent). Ce travail pourrait être mené pour la rade de Brest et l’huître *C. gigas* grâce aux données des séries temporelles du réseau REPHY de l’IFREMER pour les données de phytoplancton (Bourlès et al., 2009) et le réseau VELYGER pour les données sur la reproduction et le recrutement de *C. gigas* (Pouvreau et al., 2016).

4. Des interactions biotiques à intégrer dans un environnement global, en évolution

4.1. Interaction entre HAB, contaminants et pathogènes

En plus des HAB, les eaux côtières sont exposées à la présence de nombreux contaminants, comme les métaux lourds, les pesticides, les plastiques mais aussi les pathogènes (Rossini, 2014). L’évaluation des conséquences de l’exposition naturelle aux HAB et leurs toxines sur la reproduction des bivalves marins soulève donc également des questions sur les effets confondants ou synergiques de ces autres facteurs environnementaux.

Certains éléments traces métalliques, comme le cadmium et le cuivre, diminuent l'accumulation de PST dans les tissus de *C. gigas* exposées à *A. minutum* (Haberhorn et al., 2014). Parmi les contaminants émergents, les microplastiques sont néfastes pour la reproduction de *C. gigas* (Sussarellu et al., 2016) et de l'huître perlière *Pinctada margaritifera* (Gardon et al., 2018). Les nanoplastiques altèrent aussi la fécondation et le développement embryonnaire de *C. gigas* (Tallec et al., 2018). La présence concomitante de ces contaminants pourrait aggraver les conséquences des HAB sur la reproduction des bivalves marins et doit donc être considérée avec attention.

Les rares études d'interactions bivalve – pathogène – *Alexandrium* spp. démontrent que ces interactions sont complexes et dépendent des espèces d'*Alexandrium* et des pathogènes considérés. L'exposition de juvéniles de *C. gigas* à *A. catenella* diminue l'infection par l'herpesvirus (Lassudrie et al., 2015a), contrairement à une souche d'*A. tamarense* non-productrice de PST, qui augmente considérablement la susceptibilité des juvéniles à la bactérie *Vibrio tasmaniensis* (Abi-Khalil et al., 2016). L'exposition à *A. fundyense* augmente la sensibilité de l'huître *C. virginica* au parasite *Perkinsus marinus* (Lassudrie et al., 2015b). De plus, l'exposition à des pathogènes interfère avec le processus d'accumulation des PST de *C. gigas* exposées à *A. catenella* (Lassudrie et al., 2015a, 2016).

Les larves de bivalves dont *C. gigas* sont également sensibles aux bactéries du genre *Vibrio* (Kesarcodi- Watson et al., 2009) et à l'herpesvirus (Dégremont et al., 2016; Le Deuff et al., 1996; Nicolas et al., 1992). Néanmoins, les interactions bivalve – pathogène – HAB sont beaucoup moins connues chez les larves de bivalves marins. De Rijcke et al. (2016) ont démontré que des dinoflagellés toxiques (*A. minutum*, *A. ostenfeldii*, *Karenia mikimotoi* et *Protoceratium reticulatum*) et la bactérie *Vibrio splendidus* affectent la viabilité des larves de la moule *M. edulis* mais n'ont pas observé d'interaction entre HAB et pathogènes lors d'une exposition simultanée. Cette absence d'interaction pourrait être due à un timing différent des effets des HAB et des pathogènes. Les travaux de cette thèse démontrent que les larves de bivalves sont particulièrement vulnérables à l'exposition à *A. minutum* et que de futures études devraient s'intéresser aux interactions larves de bivalves – pathogène – HAB. Par exemple, une exposition de larves de *C. gigas* à *Alexandrium* spp. pourrait être combinée à une infection par un pathogène, comme l'herpesvirus OsHV-1, pour évaluer si la présence de dinoflagellé toxique peut modifier l'interaction hôte-pathogène et potentiellement rendre les larves plus sensibles aux pathogènes.

4.2. HAB et changement climatique

La prédiction de l'impact du changement climatique sur les HAB marins est hasardeuse car de complexes interactions entre différents facteurs existent. Néanmoins, il est fort probable que le changement climatique conduira à i) un changement de la répartition des espèces thermophiles et psychrophiles, ii) des changements d'abondance et de saisonnalité des espèces responsables de HAB, iii) une apparition précoce du pic de concentration de certaines espèces de phytoplancton et à iv) des effets secondaires sur les chaînes trophiques marines (Hallegraeff, 2010).

L'augmentation des épisodes d'HAB a déjà été reliée aux changements globaux dans certaines zones (Gobler et al., 2017; Wells et al., 2015), pour des espèces de microalgues nuisibles pour les bivalves comme *A. fundyense* (Galimany et al., 2008) et *Dinophysis acuminata* (Mello et al., 2010). Des études suggèrent que les micro-algues nuisibles et les HAB risquent de s'étendre au cours du prochain siècle dans le nord-ouest de l'Europe, en partie à cause d'une modification de la charge en nutriments (Glibert et al., 2014; Townhill et al., 2018).

L'augmentation de la température de l'eau de mer et l'acidification des océans liées au changement climatique pourraient aussi modifier les processus d'accumulation et de dépuración des PST par les bivalves marins. Une expérience en laboratoire a étudié les effets simples ou combinés de l'acidification et de l'augmentation de la température sur l'accumulation de PST par des moules *Mytilus galloprovincialis* exposées à *Gymnodinium catenatum* pendant 5 jours, puis pendant une phase de dépuración où les moules étaient nourries avec des micro-algues non toxiques (Braga et al., 2018). Les moules acclimatées à une température plus élevée ont une accumulation et élimination plus faibles, alors que les moules acclimatées à pH plus faible présentent un plus fort potentiel pour accumuler plus de PST, mais présentent un taux d'élimination plus élevé, ce qui les empêche d'atteindre les toxicités élevées observées dans les moules maintenues en condition contrôle. L'effet conjoint du réchauffement et de l'acidification pourraient ainsi conduire à des toxicités des coquillages moins intenses mais plus prolongées dans le temps (Braga et al., 2018).

Les résultats du chapitre 2 suggèrent que l'exposition de *C. gigas* pendant la gamétogenèse à *A. minutum* affecte la balance énergétique des huîtres, plus d'énergie étant nécessaire pour la détoxification des toxines et le maintien de l'intégrité des tissus. Cette demande d'énergie accrue semblait en partie compensée par une augmentation de l'activité de filtration observée chez ces huîtres. Les HAB et l'acidification des océans pourraient interagir pour augmenter les besoins

énergétiques des organismes marins, dont les huîtres. L'étude de Scanes et al. (2018) illustrent cette hypothèse en démontrant un effet synergique de l'exposition au cuivre et à l'acidification sur la demande énergétique de l'huître *Saccostrea glomerata*, qui entraîne une réduction de la taille et de la teneur en lipides des ovocytes.

Dans le futur, les populations de bivalves confrontées à l'acidification durant leur gamétogenèse seront probablement plus vulnérables à d'autres stress environnementaux comme les HAB (Griffith and Gobler, 2017). Inversement, des larves issues d'huîtres exposées à des HAB lors de la gamétogenèse pourraient être plus sensibles à l'acidification des océans. Dans ce contexte de changement global, des études s'intéressant à plusieurs générations permettraient de mieux appréhender les effets potentiels à long terme des HAB sur les organismes marins.

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Castrec J.*, Soudant P., Tran D., Payton L., Miner P., Lambert C., Le Goïc N., Huvet A., Quillien V., Mat A., Boullot F., Amzil Z., Hégaret H., Fabioux C. Behavioural and physiological responses of the oyster *Crassostrea gigas* exposed to three strains of *Alexandrium minutum* producing different toxin types. Phycotox National Workshop, Villefranche-Sur-Mer, France, 15-16 March 2016.

Castrec J.*, Soudant P., Tran D., Payton L., Miner P., Lambert C., Le Goïc N., Huvet A., Quillien V., Mat A., Boullot F., Amzil Z., Hégaret H., Fabioux C. Étude comparative des réponses comportementales et physiologiques d'huîtres *Crassostrea gigas* exposées à trois souches d'*Alexandrium minutum* produisant différents types de molécules toxiques. Réunion annuelle du projet ACCUTOX (ANR-13-CESA-0019), IUEM, Brest, 28-29 janvier 2016.

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Castrec J.*, Hégaret H., Boulais M., Le Goïc N., Séchet V., Raimbault V., Fabioux C. An *in vitro* approach to assess the impact of *Dinophysis* sp. on oyster *Crassostrea gigas* reproduction. Physiomar, The Physiology of Aquatic Invertebrates in a Changing World, Cambridge, Royaume-Uni, 18-21 September 2017.

Castrec J.*, Soudant P., Tran D., Payton L., Miner P., Lambert C., Le Goïc N., Huvet A., Quillien V., Mat A., Boullot F., Amzil Z., Hégaret H., Fabioux C. Behavioural and physiological responses of the oyster *Crassostrea gigas* exposed to three strains of *Alexandrium minutum* producing different toxin types. SETAC Europe 26th Annual Meeting, Nantes, France, 22-26 May 2016.

Castrec J.*, Hégaret H., Soudant P., Tran D., Payton L., Miner P., Lambert C., Le Goïc N., Huvet A., Quillien V., Mat A., Amzil Z., Boullot F., Fabioux C. Comparative study of the responses of *Crassostrea gigas* exposed to three strains of *Alexandrium minutum* producing different toxin types. Phycotox/Cyano National Workshop, Plouzané, France, 31 March-2 April 2015.

Titre : Impacts des efflorescences du dinoflagellé toxique *Alexandrium minutum* sur la reproduction et le développement de l'huître *Crassostrea gigas*.

Mots clés : Efflorescences toxiques ; Toxines paralysantes ; Composés extracellulaires bioactifs ; Huître ; Reproduction ; Larves.

Résumé : Les dernières décennies ont été marquées par l'intensification et l'expansion des efflorescences de micro-algues toxiques (HAB). Connues pour perturber les écosystèmes côtiers et pour leur toxicité sur les organismes marins, les HAB sont suspectées d'être à l'origine de défauts de recrutement de bivalves. Cette thèse avait pour objectif d'étudier les conséquences des efflorescences du dinoflagellé toxique *Alexandrium minutum*, producteur de toxines paralysantes (PST) et des composés bioactifs extracellulaires (BEC), sur la reproduction, le développement et le recrutement de l'huître *Crassostrea gigas*, une espèce à l'importance économique majeure. Les gamètes libres et les jeunes stades de développement se révèlent être les plus sensibles, en particulier aux BEC produits par

A. minutum qui inhibent la fécondation et l'embryogenèse. *A. minutum* modifie le comportement des larves véligères, provoque une diminution de leur filtration, de leur croissance et du taux de fixation. Une exposition des adultes, pendant la gamétogenèse, affecte le développement des descendants, traduisant des altérations du contenu gamétique et/ou un transfert vertical des PST. Les modalités d'action des PST et des BEC devront être précisées. Nos expérimentations, réalisées à des concentrations de micro-algues rencontrées dans l'environnement, suggèrent que des efflorescences récurrentes d'*A. minutum* lors des périodes de reproduction et de développement larvaire pourraient, sur le long terme, affecter la structure des populations naturelles et cultivées de *C. gigas*.

Title : Effects of the toxic dinoflagellate *Alexandrium minutum* on the reproduction and development of the oyster *Crassostrea gigas*

Keywords: Harmful algal bloom; Paralytic shellfish toxins; Bioactive extracellular compounds; Reproduction; Bivalves; Larvae.

Abstract: Recent decades have witnessed the intensification and spread of harmful algal blooms (HAB). HAB are known to disrupt coastal ecosystems and to be toxic for marine organisms. These phenomena are also suspected to be responsible for recruitment failures of bivalves. The aim of this PhD was to study the consequences of blooms of toxic dinoflagellate *Alexandrium minutum* on the reproduction, development and recruitment of the oyster *Crassostrea gigas*, a species of major economic importance. *A. minutum* is known to produce paralytic shellfish toxins (PST) and bioactive extracellular compounds (BEC). Gametes and early life stages were the most sensitive, particularly to the bioactive extracellular compounds (BEC) produced by

A. minutum, which inhibited fertilization and embryogenesis. *A. minutum* modified the behaviour of veliger larvae, decreased their filtration, growth and settlement. Exposure of adult oysters during gametogenesis affected the development of offspring, reflecting alterations in gamete content and/or vertical transfer of PST. Mode of action of PST and BEC are to further investigate. These oyster exposures, conducted at environmentally relevant concentrations of microalgae, suggest that recurrent blooms of *A. minutum* during oyster spawning and larval development could have long-term consequences on the structure of wild and cultured populations of *C. gigas*.