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Malwenn Lassudrie


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Préparée au Laboratoire des Sciences de l'Environnement Marin, Institut Universitaire Européen de la Mer

# Effets combinés des dinoflagellés toxiques du genre *Alexandrium* et d'agents pathogènes sur la physiologie des bivalves

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# Résumé

Les populations de bivalves subissent régulièrement des épizooties qui affaiblissent ou déciment les stocks exploités et limitent ainsi l'aquaculture. Ces maladies, principalement dues à des virus, des bactéries ou des parasites, se développent particulièrement au printemps et en été. Cette période de l'année offre également des conditions propices à l'apparition d'efflorescences de dinoflagellés toxiques, dont des espèces du genre *Alexandrium*. Ainsi, le risque de co-occurrences d'efflorescences d'*Alexandrium* sp. et de maladies infectieuses chez les bivalves est élevé. Or, ces micro-algues synthétisent et excrètent des toxines et des composés cytotoxiques responsables d'altérations physiologiques chez les bivalves et pouvant mener à un état immunodéprimé.

L'objectif de cette thèse est d'évaluer les effets combinés d'une exposition à un dinoflagellé toxique, *Alexandrium* sp., et d'une infection par des agents pathogènes sur la physiologie des bivalves, à travers l'étude de différentes interactions tripartites bivalve – pathogène – *Alexandrium* sp..

Les résultats de ce travail de thèse soulignent le caractère espèce-spécifique de ces impacts. Ainsi, l'exposition à *Alexandrium catenella* diminue l'infection de naissain d'huîtres *Crassostrea gigas* par l'herpesvirus, alors que le dinoflagellé *A. fundyense* augmente la susceptibilité d'huîtres *C. virginica* au parasite *Perkinsus marinus*, probablement via une immunodépression, comme le suggère la répression des réponses hémocytaires observée.

De plus, l'effet d'une efflorescence toxique sur la susceptibilité à des maladies opportunistes chez des huîtres exposées à un nouvel environnement microbien (simulant un transfert) a été évalué. Les réponses hémocytaires au changement d'environnement microbien ont été réprimées par l'exposition à *A. catenella*, bien qu'aucune nouvelle infection bactérienne n'ait été détectée.

Enfin, l'exposition à des agents pathogènes et à un nouvel environnement microbien interfère avec le processus d'accumulation de toxines algales chez des huitres exposées à *A. catenella*, illustrant la complexité de ces interactions.

Ces résultats apportent ainsi une meilleure compréhension de l'implication des efflorescences toxiques dans le développement des maladies touchant les bivalves d'intérêt commercial, mais également de l'implication de l'environnement biotique des bivalves sur l'accumulation de phycotoxines réglementées.

# Abstract

Bivalve populations undergo regular epidemics that weaken or decimate exploited stocks and thus limit aquaculture. These diseases are caused mainly by viruses, bacteria or parasites, and occur primarily during spring and summer. This period of the year also provides favorable conditions for toxic dinoflagellate blooms, including species of the genus *Alexandrium*. Thus, the risk of *Alexandrium* sp. blooms and infectious diseases co-occurring in bivalves is high. However, these micro-algae synthesize and excrete toxins and cytotoxic compounds responsible for physiological changes in bivalves and could lead to an immuno-compromised status.

The objective of this thesis is to evaluate the combined effects on bivalve physiology of exposure to the toxic dinoflagellate, *Alexandrium* sp., and infection by pathogens, through the study of different bivalve - pathogen - *Alexandrium* sp. tripartite interactions.

The results of this work highlight the species-specific nature of these impacts. Thus, exposure to *Alexandrium catenella* reduces the herpesviruses infection in oyster *Crassostrea gigas*, whereas the dinoflagellate *A. fundyense* increases the susceptibility of *C. virginica* oyster to the parasite *Perkinsus marinus*, probably via immuno-suppression, as suggested by the partial inhibition of hemocyte responses.

Additionally, the effect of a toxic algal bloom on oyster susceptibility to opportunistic diseases when exposed to a new microbial environment (simulating a transfer) was evaluated. Hemocyte responses to a changing microbial environment were suppressed by exposure to *A. catenella*, although no new bacterial infection was detected.

Finally, exposure to pathogens or to a new microbial environment interferes with the processes by which oysters exposed to *A. catenella* accumulate algal toxins, illustrating the complexity of these interactions.

These results provide a better understanding of the involvement of toxic algal blooms in the development of diseases affecting commercial bivalve species, but also of the involvement of the bivalve biotic environment in the accumulation of regulated toxins.

# Liste des figures (Hors publications)

**Figure 1.** Anatomie d'une huître *Crassostrea virginica* dans sa valve gauche. Les axes d'orientation de l'animal sont indiqués en italique (adapté par Delaporte, 2005, d'après Galtsoff, 1964).

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# Liste des tables (Hors publications)

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**Tableau 2.** Espèces de dinoflagellés toxiques étudiées dans cette thèse. Toxines produites et mode d'action identifié chez les mammifères ; zone géographique concernée par ces travaux de thèse.

**Tableau 3.** Récapitulatif des interactions étudiées dans chaque chapitre et article.

# Liste des abréviations (Hors publications)

A

**ADN :** Acide désoxyribonucléique

**AMP :** Peptide antimicrobien ou « Antimicrobial Peptide »

B

**BMD :** Maladie du muscle marron ou « Brown Muscle Disease »

**BRD :** Maladie de l'anneau brun ou « Brown Ring Disease »

C

**CAT:** Catalase

D

**DEB :** Dynamic Energy Budget

E

**ERO:** Espèce Réactive de l'Oxygène = Reactive Oxygen Species (voir ROS)

F

**FAO :** Organisation des Nations unies pour l'alimentation et l'agriculture ou « Food and Agriculture Organization of the United Nation »

G

**GPX:** Glutathion péroxydase

**GR:** Glutathion réductase

**GST:** Glutathion S-transférase

**GTX :** Gonyautoxine

H

**HAB :** Efflorescence de micro-algues toxiques ou « Harmful Agal Bloom »

I

**IL :** InterLeukines

**IUEM :** Institut Universitaire Européen de la Mer

J

**JAK-STAT :** Janus kinase - Signal Transducer and Activator of Transcription

L

**LEMAR :** Laboratoire des Sciences de l'Environnement Marin

M

**MAPK :** Mitogen-activated protein kinase

**MSX :** Multinucleate Sphere X

N

**$Na^+$ :** ion sodium

**NF-kB :** Nuclear factor kappa-light-chain-enhancer of activated B cells

O

**OsHV-1** : ostreid herpesvirus-1

P

**PAMP :** Pathogen-Associated Molecular Patterns »

**PGRP :** protéine de reconnaissance des peptidoglycanes ou « Peptidoglycan Recognition Protein »

**PO :** Phénoloxydase

**proPO :** proPhénoloxydase

**PRR :** Pattern-Recognition Receptor

**PST :** Toxine paralysante ou « Paralytic Shellfish Toxin »

Q

**QPX :** Quahog Parasite X

R

**REPAMO :** Réseau de Pathologie des Mollusques

**RESCO** : Réseau d'Observations Conchylicoles

**ROS :** Reactive Oxygen Species = Espèce Réactive de l'Oxygène (voir ERO)

S

**SOD :** Superoxyde Dismutase

**SPX :** Spirolide

**STX :** Saxitoxine

**SSO :** Sea Side Organism

T

**THC :** Concentration totale en hemocytes circulants ou « Total Hemocyte Count »

**TNF :** Tumor Necrosis Factor

# Contexte scientifique

## 1 Bivalves

La production de mollusques bivalves, issue de la pêche et de l'aquaculture, représente une importante activité économique au niveau mondial. Les bivalves peuvent également fournir des services écologiques (restauration d'habitats, bio-extraction,…) (Beck et al., 2011; Fulford et al., 2010).

Au cours de cette thèse, trois espèces de bivalves d'intérêt commercial ont été étudiées :

**- La palourde japonaise, *Ruditapes* (=*Venerupis*) *philippinarum***, est le bivalve le plus exploité au monde, principalement en Asie, Europe et Amérique du Nord. Sa production globale atteignait 3,7 $10^6$ tonnes en 2011. La Chine est largement en tête en contribuant à hauteur de 97% de la production globale. La France se classe 8$^{ème}$, avec 6,0 $10^2$ tonnes produites en 2011 (FAO, 2014).

**- L'huître creuse du Pacifique, *Crassostrea gigas***, est la 2$^{ème}$ espèce la plus exploitée au monde, avec 6,7 $10^5$ tonnes en 2011. En revanche, cette espèce domine la production de bivalves issus de l'aquaculture, puisqu'elle représente 93% de sa production globale. Les principales régions productrices sont l'Asie, l'Europe et l'Amérique du Nord, mais *C. gigas* est exploitée sur les côtes de tous les continents du monde. La France se classe 3$^{ème}$ avec 9,5 $10^4$ tonnes produites en 2011, derrière la République de Corée et le Japon (FAO, 2014).

- **L'huître américaine *Crassotrea virginica***, dont la production globale s'élevait à 1,9 $10^5$ tonnes en 2011. Elle est principalement exploitée le long de la côte est des Etats-Unis, qui contribuait à hauteur de 75% de la production globale en 2011. L'espèce est également exploitée le long de la côte Est du Mexique et du Canada. Elle constitue l'espèce d'huître la plus exploitée aux Etats-Unis (FAO, 2014).

### Biologie

Les mollusques bivalves sont des organismes sessiles et ectothermes, soumis aux conditions du milieu, qui peuplent des zones intertidales à infratidales. Alors que les huîtres sont des organismes épigés, les palourdes sont des organismes endogés. Ces bivalves, suspensivores, filtrent l'eau environnante afin d'en soustraire le dioxygène nécessaire à leur respiration mais aussi d'en retenir les particules nutritives. Ils peuvent se nourrir de micro-algues pélagiques (ou phytoplancton), mais aussi de microphytobenthos (micro-algues benthiques), de matériel détritique, de nanoplancton, de bactéries, de zooplancton ou encore de débris de macro-algues (Birkbeck and McHenery, 1982; Gosling, 2003).

## Anatomie

Les bivalves sont des animaux à corps mou renfermé dans une coquille à deux valves asymétriques articulées autour d'une charnière et maintenues par un muscle adducteur puissant (Figure 1). La palourde possède également un pied musculeux qui lui permet de s'enfouir dans le sédiment. Les parties molles du corps sont enveloppées dans un manteau qui délimite la cavité palléale. Une circulation d'eau de mer est assurée par le mouvement des cils branchiaux, apportant oxygène et particules alimentaires. Chez la palourde, l'eau est conduite aux branchies par un siphon inhalant. Les branchies ont une double fonction respiratoire et alimentaire, puisqu'ils retiennent et conduisent les particules aux palpes labiaux, où ils elles sont triées. Une partie est rejetée par les pseudofaeces, et l'autre est ingérée et conduite vers l'estomac via l'œsophage. Le stylet cristallin situé dans l'estomac participe à la dégradation mécanique et enzymatique des particules alimentaire. Les éléments assimilables sont dirigés vers les diverticules digestifs connectés à l'estomac. Les éléments non assimilables sont conduits par l'intestin et le rectum, et rejetés par l'anus sous forme de faeces.
Les bivalves possèdent un système circulatoire semi-ouvert. L'hémolymphe (fluide circulant), constituée de plasma (portion fluide) et d'hémocytes (cellules circulantes), baigne les organes et circule via les vaisseaux vers le cœur, qui est constitué d'un ventricule et de deux oreillettes.

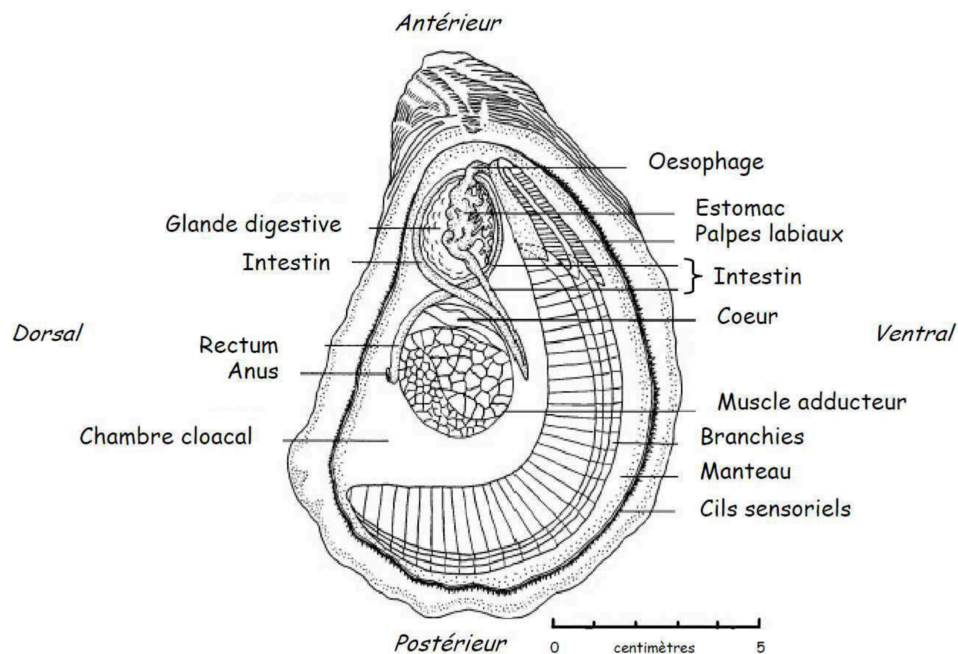


**Figure 1.** Anatomie d'une huître *Crassostrea virginica* dans sa valve gauche. Les axes d'orientation de l'animal sont indiqués en italique (adapté par Delaporte, 2005, d'après Galtsoff, 1964).

# 2 Système immunitaire

Chez les bivalves, la défense contre les pathogènes s'effectue via un système immunitaire qualifié d'inné, c'est-à-dire non spécifique, non-adaptatif et sans phénomène de mémoire, contrairement à l'immunité acquise rencontrée chez les vertébrés.

La reconnaissance du non-soi est la première étape de l'activation des réponses immunitaires. L'hôte possède des récepteurs spécialisés, solubles ou fixés sur la membrane des hémocytes, nommés « Pattern-Recognition Receptors » (PRRs), qui reconnaissent des composants microbiens, les « Pathogen-Associated Molecular Patterns » (PAMPs). Les lipopolysaccharides des bactéries à Gram-négatif (tels que les vibrions), ou des glycanes particuliers portés par un parasite de la palourde, *Perkinsus olseni*, constituent ainsi des PAMPs. Les PAMPs sont reconnus par des PRRs, tels que les protéines de reconnaissance des peptidoglycanes (« Peptidoglycan Recognition Protein », PGRPs), des lectines de type C, des galectines ou des récepteurs de type Toll. Les PRRs activent ainsi des voies de signalisation telles que la NF-kB, la MAPK, la JAK-STAT ou la voie des récepteurs Toll-like (Song et al., 2010).

Ainsi, lorsqu'une particule du non-soi (agent pathogène notamment) est reconnue, ces cascades de réactions aboutissent à l'activation de la réponse immunitaire, via des facteurs humoraux et des mécanismes cellulaires assurés par les hémocytes.

**Facteur humoraux**

La défense humorale repose sur des molécules solubles présentes dans le plasma. Les facteurs humoraux peuvent être constitutifs ou inductibles, et une partie d'entre eux sont produits par les hémocytes.

Les lectines sont des protéines qui peuvent être produites par les hémocytes, et qui interviennent dans le chimiotactisme et l'attraction des pathogènes grâce à des fonctions d'opsonines et d'agglutinines (Pruzzo et al., 2005). Les lectines immobilisent et agglutinent les bactéries, activent et facilitent l'attachement entre les hémocytes et les pathogènes pendant la phagocytose en reconnaissant les composés glucidiques des pathogènes et en s'y liant (Chu, 2000). Cette reconnaissance de motifs moléculaires caractéristiques des pathogènes (PAMPs) participe au déclenchement de la réponse immunitaire innée (Medzhitov and Janeway, 2000). Récemment, Soudant et al. (2013) ont recensé les dernières lectines découvertes chez les bivalves et leurs caractéristiques.

Les peptides antimicrobiens (« antimicrobial peptides », AMPs), dont font partie les défensines, sont en général produits et stockés dans les hémocytes, et excrétés en réponse à la présence de pathogènes (Bachère et al., 2004; Mitta et al., 1999b; Zhao et al., 2007). Leur action entraîne la destruction de la membrane des micro-organismes ainsi que la lyse cellulaire (Li et al., 2009). La production d'AMPs a été documentée

chez différents bivalves marins, dont les moules *Mytilus edulis* et *M. galloprovincialis* (Charlet et al., 1996; Mitta et al., 1999a, 1999b), l'huître *C. gigas* (Gonzalez et al., 2007; Gueguen et al., 2006), le pétoncle *Argopecten irradians* (Zhao et al., 2007), et la palourde *R. philippinarum* (Adhya et al., 2012).

Les lysosymes sont des enzymes qui interviennent dans la défense immunitaire mais aussi dans la digestion grâce à leur capacité à dégrader la paroi cellulaire des bactéries (Itoh et al., 2007; McHenery et al., 1979; Xue et al., 2004; Zhao et al., 2010).

Des protéines majeures du plasma, présentant une homologie de séquences importante d'une espèce de bivalve à l'autre seraient des protéines multifonctionnelles ayant un rôle dans la défense immunitaire (Gonzalez et al., 2005; Green et al., 2009; Huvet et al., 2004; Simonian et al., 2009; Tanguy et al., 2004; Taris et al., 2009). Par exemple, la dominine, isolée chez *C. virginica*, pourrait contribuer à la défense contre des pathogènes comme les parasites *Perkinsus* spp. en limitant la disponibilité en fer (Itoh et al., 2011), lequel est indispensable à leur propagation *in vitro* (Gauthier and Vasta, 2002, 1994).

Les inhibiteurs de protéases participeraient également à la défense immunitaire chez les bivalves en inactivant les protéases des pathogènes, comme le suggèrent la caractérisation et l'expression de gènes codant pour ces protéines (Montagnani et al., 2001; Wang et al., 2008; Xue et al., 2006; Zhu et al., 2006).

Les cytokines, comme les interleukines (IL) et les « Tumor Necrosis Factor » (TNF), sont des molécules de régulation agissant sur l'activité et la fonction d'autres cellules, comme la motilité, le chimiotactisme, la phagocytose ou encore la cytotoxicité (Beschin et al., 2001; Hughes et al., 1990; Ottaviani et al., 2004; Roberts et al., 2008).

Le système des phénoloxydases (POs) est constitué d'enzymes stockées sous forme inactive (prophénoloxydases, ProPOs) dans les hémocytes et sont activées lorsqu'elles sont libérées dans le plasma (Johansson and Söderhäll, 1985). Elles jouent un rôle dans la destruction des agents pathogènes de par la toxicité et les propriétés bactéricides, fongicides et antivirales de leurs produits d'oxydation, ainsi que dans la reconnaissance du non-soi, l'encapsulation et la réparation tissulaires (Söderhäll and Cerenius, 1998; Söderhäll et al., 1994). La caractérisation et le rôle de ces enzymes dans les fonctions immunitaires des bivalves marins ont fait l'objet d'études récentes (Butt and Raftos, 2008; Hellio et al., 2007; Le Bris et al., 2014, 2013; Luna-Acosta et al., 2011, 2010) ont montré que chez certains bivalves, le système PO pouvait aussi être actif dans les hémocytes, et être impliqué dans la phagocytose.

### Facteurs cellulaires

Bien que les hémocytes soient également impliqués dans plusieurs autres fonctions (nutrition, biominéralisation, réparation tissulaire…), ils assurent l'immunité cellulaire. Les hémocytes sont chémotactiques, mobiles, phagocytiques et capables de s'agréger

(Canesi et al., 2002; Hine, 1999). Ils peuvent proliférer et pourraient se différencier (Hine, 1999; Rebelo et al., 2013).

Trois sous-populations d'hémocytes sont habituellement distingués d'après des critères morphologiques : les cellules de type souche ou « blast-like cells » qui sont de petite taille et non granuleux, les hyalinocytes, pas ou peu granuleux et les granulocytes, fortement granuleux (Figure 2) (Cheng, 1996; Hine, 1999). Ces différences morphologiques traduisent des différences fonctionnelles. Les granulocytes, sont davantage impliqués dans l'immunité, du fait d'une plus grande capacité de phagocytose et à un contenu important en enzymes lysosomales (Cajaraville and Pal, 1995; Chu, 2000). Les hyalinocytes, en revanche, seraient plus spécialisés dans la réparation tissulaire et l'agrégation (Chu, 2000; Ruddell, 1971). Ces différences fonctionnelles associées à chaque type cellulaire suggèrent un lien entre morphologie et fonction. Les hémocytes sont capables d'infiltrer les tissus afin de se rendre sur les sites infectés ou endommagés. De par leur rôle dans la réparation des tissus, les hémocytes peuvent aussi s'y infiltrer en réponse à des lésions.

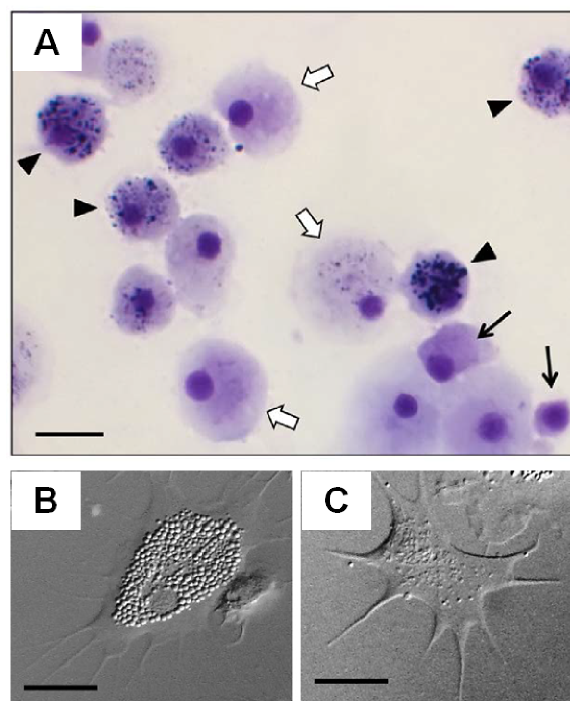


**Figure 2.** Microphotographie d'hemocytes de bivalves. (A) exemple chez l'huître *Crassostrea rhizophorae* après coloration de May-Grünwald Giemsa. Les cellules de type souche (« blast-like cells ») sont indiquées par des flèches noires, les hyalinocytes par des flèches blanches, et les granulocytes par des pointe de flèches noires (d'après Rebelo et al., 2013) ; (B) granulocytes et (C) hyalinocytes observés en microscopie à contraste d'interférence différentielle (d'après Aladaileh et al., 2007). Barre d'échelle : 10 µm.

**Mécanismes de défense immunitaire**

La phagocytose, considérée comme le mécanisme principal de l'immunité cellulaire innée (Feng, 1988), consiste en quatre grandes étapes qui font intervenir les facteurs humoraux et cellulaires (Figure 3) : (1) le chimiotactisme et l'attraction; (2) la reconnaissance et l'adhésion, qui est facilitée par l'opsonisation des particules étrangères, (i.e. la sécrétion par les hémocytes d'opsonines qui vont se fixer sur les particules étrangères afin de faciliter l'adhésion du phagocyte); (3) l'internalisation, au cours de laquelle l'hémocyte se transforme, en remaniant son cytosquelette, pour créer des pseudopodes afin d'englober la particule étrangère dans une vacuole : le phagosome (Cheng, 1981); et enfin (4) la destruction intracellulaire via l'action des lysosomes et des espèces réactives de l'oxygène (ou « Reactive Oxygen Species », ROS) (Cheng, 1996; Janeway and Medzhitov, 2002; Song et al., 2010; Soudant et al., 2013).

Des réactions d'encapsulation peuvent aussi être observées en réponse aux agents pathogènes trop volumineux pour être phagocytés. Elles consistent, après les deux premières étapes communes à la phagocytose décrites précédemment, en (3') leur isolement par de multiples couches d'hémocytes formant une capsule, aboutissant (4') à la dégradation extracellulaire des pathogènes (Figure 3) (Cheng, 1981; Feng, 1988; Fisher, 1988, 1986; Soudant et al., 2013).

Les ROS produites par les hémocytes lors de la destruction intra- (phagocytose) ou extra-cellulaire (encapsulation) sont des molécules oxygénées (radicalaires pour la plupart) très instables et réactives, au pouvoir hautement microbicide (Bogdan et al., 2000). Bien qu'essentiels car impliqués dans de nombreux processus cellulaires, les ROS en concentration élevée sont également toxiques pour les cellules de l'hôte (Bartosz, 2009; Stowe and Camara, 2009). Afin de se protéger du stress oxydant (i.e. des dommages que les ROS pourraient occasionner) l'hôte possède des systèmes antioxydants, non-enzymatiques tels que le glutathion, les vitamines E et C, les caroténoïdes, l'acide urique (Cadenas, 1989), et enzymatiques, tels que la superoxyde dismutase (SOD), la glutathion peroxydase (GPX), la catalase (CAT), la glutathion réductase (GR) et la glutathion S-transférase (GST) (Bogdan et al., 2000) ou encore d'autres protéines telles que les transferrines, les ferritines, les métallothionéines, qui se complexent aux métaux afin d'en limiter la disponibilité lors du processus de génération des radicaux libres (Regoli and Giuliani, 2014). De plus, la production d'espèces réactives de l'azote, très instables, comme l'oxyde nitrique, peut également participer à la destruction des particules étrangères grâce à leurs propriétés microbicides (Bogdan et al., 2000; Rivero, 2006).

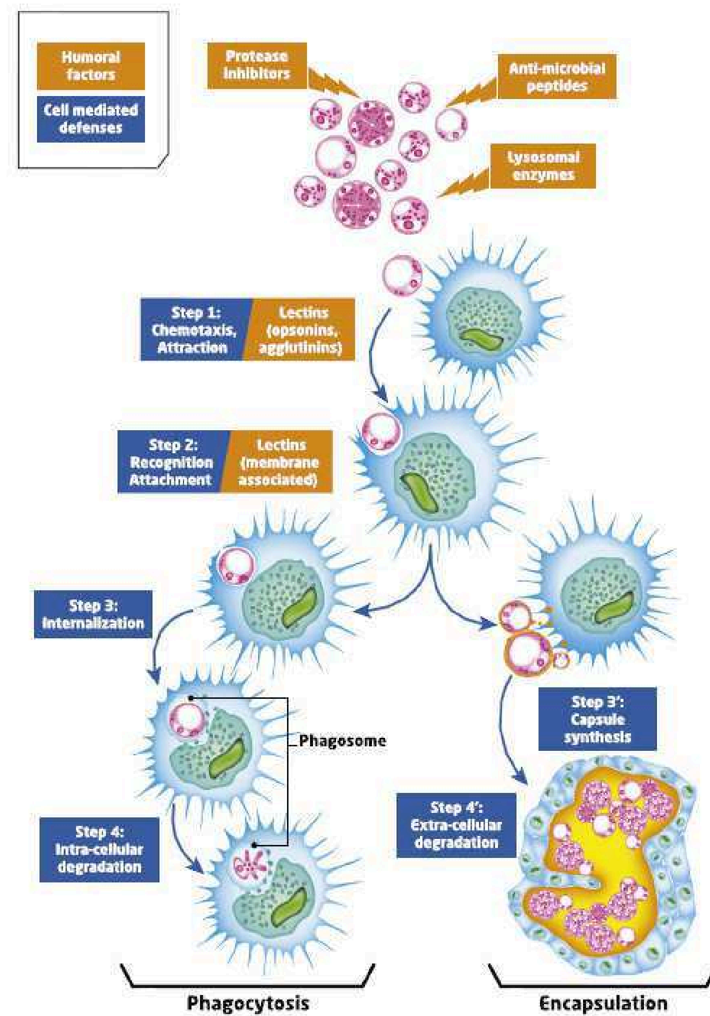


**Figure 3.** Représentation schématique des réponses humorales et cellulaires impliquées dans les mécanismes de défense lors de l'infection par des micro-organismes (d'après Soudant et al., 2013).

# 3 Maladies

Les populations de bivalves subissent régulièrement des épizooties qui déciment ou affaiblissent les stocks exploités et limitent donc l'aquaculture. L'importance des échanges commerciaux entre les différentes régions du monde contribue à l'apparition et la propagation de maladies infectieuses. Ces maladies sont principalement dues à des virus, des bactéries ou des parasites.

Par exemple, en France, entre 1966 et 1973, la « maladie des branchies », associée à un iridovirus, a totalement décimé les populations d'huîtres creuses portugaises *Crassostrea angulata* (Comps, 1988), ce qui a motivé l'introduction des huîtres du Pacifique *C. gigas*, dont la résistance à ce virus a permis de relancer l'économie ostréicole. Des mortalités d'huîtres plates *Ostrea edulis*, engendrées par la marteiliose et la bonamiose, deux maladies parasitaires causées respectivement par les protozoaires *Marteilia refringens* et *Bonamia ostrea*, ont également affaibli la production ostréicole française (Renault, 1996). Au début des années 1990, l'huître creuse *C. gigas* a été touchée par des épisodes de surmortalités estivales associées à une maladie multifactorielle, fruit d'une interaction complexe entre la température, le statut physiologique de l'huître (période de reproduction), la présence d'agents infectieux opportunistes (ostreid herpesvirus-1 OsHV-1 et bactéries du genre *Vibrio*, notamment *V. aesturianus* et *V. splendidus*), et la génétique de l'hôte (Samain and McCombie, 2007).

Plus récemment, depuis 2008, le phénomène annuel de mortalités massives s'est amplifié et décime le naissain et les juvéniles d'huîtres *C. gigas* au printemps et en été, lorsque la température de l'eau excède 16°C, touchant 40 à 100% des lots français selon les localisations (Cochennec-Laureau et al., 2011; EFSA Panel on Animal Health and Welfare, 2010; Fleury and Bédier, 2013; Fleury, 2014). Ce phénomène a été associé à un microvariant de l'herpesvirus, OsHV-1µVar (Segarra et al., 2010). D'autres pays, notamment en Europe (Irlande, Royaume-Uni) et en Océanie (Australie), observent ce phénomène de mortalités massives du naissain et de juvéniles, associé à OsHV-1 µVar (EFSA Panel on Animal Health and Welfare, 2010; Jenkins et al., 2013; Paul-Pont et al., 2014; Renault et al., 2012; Segarra et al., 2010). Bien que OsHV-1µVar soit impliqué dans ces mortalités (Schikorski et al., 2011a, 2011b), d'autres agents pathogènes, notamment des souches bactériennes du genre *Vibrio*, pourraient également jouer un rôle (EFSA Panel on Animal Health and Welfare, 2010; Garnier et al., 2007; Gay et al., 2004). Les suivis opérés par le REPAMO (REseau de PAthologie des Mollusques), qui assure la surveillance de l'état de santé des mollusques du littoral français métropolitain, suggère l'implication récente de *Vibrio aesturianus* dans les mortalités de *C. gigas*. Entre 2001 et 2011, *V. aesturianus* fut détecté dans près de 15% des cas de mortalité, généralement chez les adultes. Cette fréquence de détection a augmenté à 30% en 2011, 60% en 2012 et a atteint 77% en 2013 et incluait de plus en plus de naissain et juvéniles (Garcia et al., 2014).

En France, les palourdes *R. philippinarum* sont touchées par plusieurs maladies létales. La « maladie de l'anneau brun » (« Brown Ring Disease » - BRD), causée par la bactérie *Vibrio tapetis*, entraîne un dépôt de conchioline sur la face interne des valves, formant ainsi un anneau brun symptomatique de la maladie. Après son entrée dans l'organisme, *V. tapetis* peut, en cas de stades avancés de la maladie, pénétrer dans le système circulatoire et dans les tissus et finalement engendrer la mort par septicémie (Allam et al., 2002). D'autre part, une maladie émergente a récemment été décrite dans le bassin d'Arcachon : la « maladie du muscle marron » ou « Brown Muscle Disease » (BMD), dont l'agent étiologique, potentiellement un virus, n'a pas encore été caractérisé (Dang et al., 2008). Enfin, la prévalence de *Perkinsus olseni*, un parasite protozoaire responsable de la perkinsose, est élevée dans les populations de palourdes en France, atteignant régulièrement 100% dans certains sites comme le bassin d'Arcachon (Dang et al., 2010). La prolifération du parasite, en revanche, reste limitée à des intensités sub-létales dans les populations françaises (Binias et al., 2014, 2013; da Silva et al., 2008; Dang et al., 2013; de Montaudouin et al., 2010; Hégaret et al., 2007a), sans présenter de variation saisonnière (Binias et al., 2014; Dang et al., 2013, 2010). Cependant, l'infection par *P. olseni* des populations de palourdes *R. philippinarum* en Corée du Sud provoque des mortalités massives, en partie dues à des conditions environnementales plus favorables pour le parasite (Choi and Park, 2010, 1997; Park and Choi, 2001).

Le long de la côte est des Etats-Unis, les parasites protozoaires, *Perkinsus marinus*, *Haplosporidium nelsoni* et *Haplosporidium costale* entraînent les maladies les plus conséquentes sur l'industrie des huîtres américaines *C. virginica* (Ford and Tripp, 1996). La principale cause de mortalité des huîtres *C. virginica* cultivées le long de la côte Est l'infection par *P. marinus* (Ford and Smolowitz, 2007), couramment désignée par l'appellation « Dermo » en référence à *Dermocystidium marinum*, nom donné au parasite lors de sa première identification (Mackin, 1951). Les mortalités associées à ce parasite ont d'abord été caractérisées dans le Golfe du Mexique (Mackin, 1951), mais depuis une vingtaine d'années, la maladie a progressé vers le nord, infectant désormais les populations d'huîtres du nord-est des Etats-Unis (Ford and Smolowitz, 2007). La prévalence et la charge en *P. marinus* dans les populations de *C. virginica* du nord-est des Etats-Unis présente une dynamique saisonnière plus ou moins marquée selon les sites, qui est fortement liée à la température. Les maxima de prévalence et d'intensité sont atteints en fin d'été – début d'automne, et les minima en fin d'hiver – début de printemps (Ford and Smolowitz, 2007). Ces variations sont moins marquées au sud-est des Etats-Unis (Ford and Tripp, 1996). Malgré ces fluctuations, le parasite persiste tout au long de l'année dans les populations (Ford and Smolowitz, 2007).

Les parasites *H. nelsoni* et *H. costale* sont les agents étiologiques des maladies désignées « MSX » (« Multinucleate Sphere X ») ou « Delaware Bay disease » et SSO (« Seaside organism »). Alors que *H. nelsoni* a été rapporté dans les populations d'huîtres américaines distribuées de la Nouvelle-Ecosse (Canada) à la Floride (Etats-Unis), *H. costale* possède une aire de répartition plus restreinte qui s'étend de la

Nouvelle-Ecosse (Canada) à la Virginie (Etats-Unis) (Couch and Rosenfield, 1968; Ford and Tripp, 1996).

D'autres parasites, comme des trématodes digènes du genre *Bucephalus* peuvent impacter la reproduction de *C. viriginica*, sans pour autant engendrer de mortalités notables (Cheng and Burton, 1965; Tripp, 1973). Les bivalves sont des hôtes intermédiaires pour ces parasites, dont les hôtes définitifs sont des poissons. Les larves de *Bucephalus* sp. observées dans les tissus d'huîtres peuvent se présenter sous les stades de sporocystes et métacercaires.

Les travaux présentés dans cette thèse portent sur certaines de ces relations hôte – pathogène, dont les caractéristiques sont présentées dans le Tableau 1.

**L'interaction de naissain de *C. gigas* et herpesvirus**, agent étiologique principal associé aux mortalités estivales, a été étudiée sur le mécanisme de primo-infection, qui a été induit par cohabitation avec des huîtres porteuses et entraîne une infection détectable en quelques jours (Petton et al., 2013).

**L'interaction *R. philippinarum* – *P. olseni*** a été étudiée chez des palourdes prélevées dans le bassin d'Arcachon, naturellement infectées par *P. olseni* avec une prévalence de 100%.

**L'interaction *C. virginica* – *P. marinus* & *Bucephalus* sp.** a été étudiée chez une population d'huîtres au sein de laquelle l'infection présente des fluctuations saisonnières faibles (Milford, CT ; Ford and Smolowitz, 2007).

**Tableau 1.** Bivalves, appellation ou nom de la maladie, agents étiologiques identifiés ou suspectés, sévérité et dynamique saisonnière de la maladie, et zones géographiques concernées par ces travaux de thèse.

| **Bivalves** | **Appellation / maladie** | **Agents pathogènes associés** | **Sévérité et dynamique saisonnière** | **Zone d'intérêt** |
|---|---|---|---|---|
| ***Crassostrea gigas*** | Mortalités massives de naissains | OsHV-1 µVar<br>(et éventuellement *Vibrio aesturianus* et *V. splendidus*) | Létale<br>Un pic pendant un mois au printemps-été | France |
| ***Crassostrea virginica*** | Dermo | Parasite protozoaire *Perkinsus marinus* | Létale à sub-létale<br>Maximum : été –automne<br>Minimum : hiver –printemps<br>Saisonnalité peu marquée dans la population étudiée | Nord-est des Etats-Unis |
| | Bucéphalose | Parasite trématode digénique *Bucephalus* sp. | Sub-létale | |
| ***Ruditapes philippinarum*** | Perkinsose | Parasite protozoaire *Perkinsus olseni* | Sub-létale<br>Pas de saisonnalité | France, bassin d'Arcachon |

# 4 Efflorescences de dinoflagellés toxiques

Les bivalves, en tant qu'organismes filtreurs suspensivores sessiles, peuvent être exposés à des efflorescences de micro-algues toxiques qui se produisent régulièrement le long des côtes littorales, et qui sont à l'origine de problèmes socio-économiques et écologiques majeurs (Burkholder, 1998; Landsberg, 2002). En accumulant les toxines produites par ces algues, les bivalves deviennent impropres à la consommation humaine. En outre, ces efflorescences toxiques affectent également les populations naturelles ou cultivées. En effet, l'exposition à certaines de ces micro-algues et aux composés toxiques qu'elles produisent peut avoir des effets toxiques chez l'Homme (pouvant être létaux), mais peuvent aussi affecter la survie et la physiologie des organismes marins, à différents stades de vie (Landsberg, 2002; Shumway, 1990). Or, la fréquence de ces efflorescences toxiques apparait en augmentation au cours de ces dernières décennies (Figure 5) et semblerait en partie liée à l'augmentation des apports d'origine anthropique (Anderson et al., 2008).

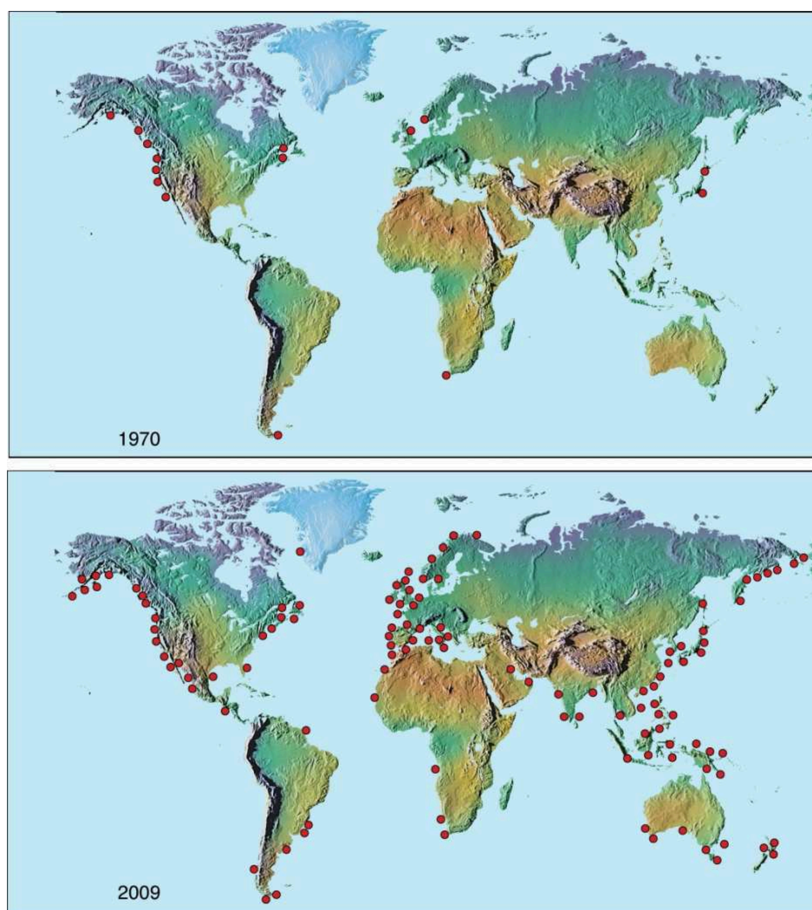


**Figure 5.** Répartition géographique des zones où la présence de PSTs avait été enregistrée en 1970 et 2009 (WHOI / US National Office for Harmful Algal Blooms).

Parmi ces micro-algues toxiques, les dinoflagellés (Dinophyceae) représentent 75 à 80% des espèces toxiques (Hallegraeff et al., 2003; Smayda, 1997). Ces algues unicellulaires sont protégées par une thèque formée par des plaques cellulosiques rigides. Le cycle de vie des dinoflagellés inclue un stade végétatif haploïde, au cours duquel la cellule peut se diviser et être mobile dans la colonne d'eau grâce à des flagelles ; et un stade diploïde associé à la reproduction sexuée (Figure 6).

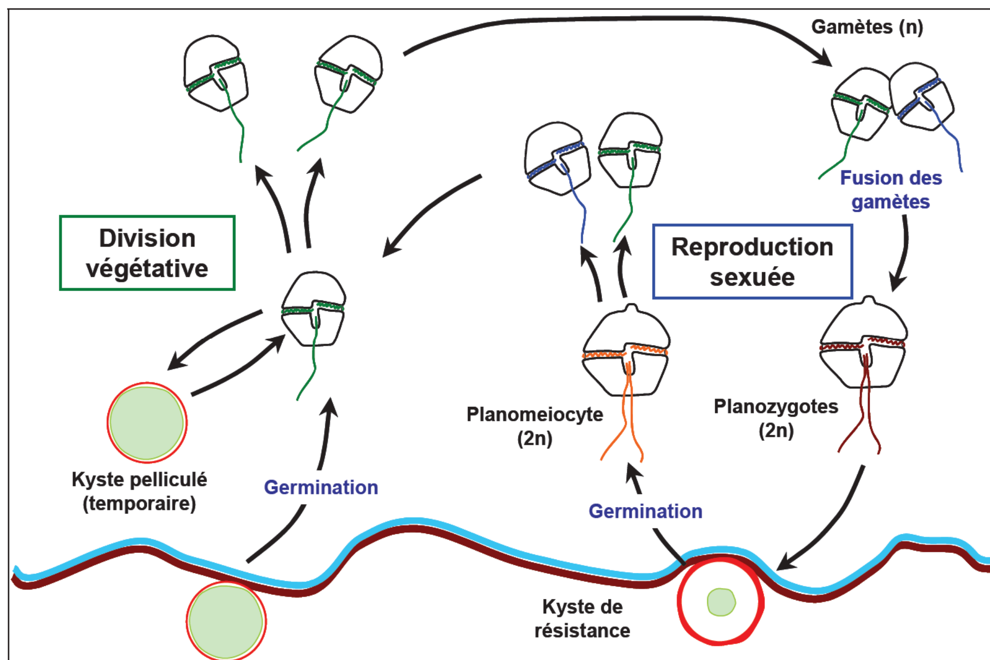


**Figure 6.** Cycle de vie d'*Alexandrium* sp. (d'après Haberkorn, 2009).

Les efflorescences de dinoflagellés toxiques en zone tempérée sont généralement des phénomènes ponctuels et saisonniers. Leur apparition est majoritairement influencée par les conditions physico-chimiques du milieu (Sellner et al., 2003), bien que des facteurs biotiques puissent aussi réguler la dynamique des efflorescences (e.g. parasites ou prédateurs, Chambouvet et al., 2008; Turner and Granéli, 2006). Certaines espèces de dinoflagellés comme *Alexandrium* sp. ont adopté une stratégie d'enkystement qui leur confère une résistance aux conditions environnementales défavorables. Les kystes, qui ont une morphologie différente des cellules végétatives, perdent leur mobilité et sédimentent. Deux types de kystes se distinguent : le kyste de résistance et le kyste temporaire. Les kystes de résistance sont associés à la reproduction sexuée, suite à la fusion de deux gamètes, notamment à la fin d'une efflorescence. Ces kystes correspondent à une phase de dormance, et permettent aux dinoflagellés de résister aux conditions hivernales. La présence de ces kystes dans les sédiments (« banques » de kystes), favorise la réapparition des efflorescences l'année suivante lorsque les conditions environnementales redeviennent favorables. L'enkystement temporaire (ou pelliculé) peut survenir en quelques minutes en cas de stress (carence en nutriments,

choc thermique , etc) (Doucette et al., 1989; Grzebyk and Berland, 1995). L'ingestion de cellules végétatives par des bivalves, ou le passage dans le tractus digestif, entraîne en grande partie la formation de kystes temporaires, qui résistent à la digestion. Ceux-ci ont notamment été observés intacts et viables dans les faeces de bivalves (Hégaret et al., 2008; Laabir et al., 2007). L'enkystement provoque aussi, des changements de composition toxinique (Persson et al., 2012, 2006; Smith et al., 2011). Ainsi, Persson et al. (2006) ont observé chez *A. fundyense* une toxicité totale environ deux fois plus faible dans les kystes temporaires que dans les cellules végétatives.

Les dinoflagellés du genre *Alexandrium,* qui font partie des plus importants en termes de toxicité, de diversité, et de distribution, comptent plus de 30 espèces connues, dont près de la moitié produisent des toxines (Anderson et al., 2012). Au cours de ce travail de thèse, trois espèces du genre *Alexandrium*, qui interagissent régulièrement avec des bivalves d'intérêt commercial, ont été étudiées (Tableau 2). En France, les palourdes japonaises *Ruditapes philippinarum,* sont régulièrement exposées à des efflorescences d'*Alexandrium ostenfeldii* (REPHY). Les huîtres *Crassostrea gigas* qui sont produites le long de toutes les côtes françaises sont exposées à des efflorescences d'*Alexandrium minutum* et d'*Alexandrium catenella,* respectivement sur les côtes Atlantique et Méditerranée (REPHY). La côte est des Etats-Unis, où l'huître américaine *Crassostrea virginica* est fortement exploitée, est régulièrement soumise à des efflorescences d'*Alexandrium fundyense* (Anderson et al., 2005; Hattenrath et al., 2010; Lopez et al., 2014).

Les dinoflagellés *Alexandrium catenella* et *A. fundyense* produisent des PSTs, comme d'autres espèces du genre *Alexandrium,* mais également des genres *Pyrodinium* et *Gymnodinium* (Van Dolah, 2000). La quantité de PSTs dans la chair des bivalves destinés à l'alimentation humaine est soumise à réglementation (800 µg éq. STX $kg^{-1}$ de chair) du fait de leurs caractères neurotoxiques avérés pour l'Homme. Ces toxines hydrosolubles sont composées de saxitoxine (STX) et de dérivés de la saxitoxine : carbamates (STX, NéoSTX, les GTX1-GTX4), N-sulfocarbamoyles (B1, B2, C1-C4) et dérivés décarbomoyl (dcGTX1-dcGTX4). Le mode d'action des PSTs consiste à bloquer la conduction de l'influx nerveux en se fixant sur les canaux sodium voltage dépendants ; le flux de $Na^+$, qui génère le potentiel dans les nerfs et fibres musculaires, est inhibé, entraînant la paralysie neuromusculaire. Chez les bivalves, les PSTs peuvent aussi se fixer sur les canaux sodium voltage dépendants (Catterall, 2000, 1980).

Les dinoflagellés *Alexandrium ostenfeldii* peuvent produire des PSTs et des spirolides. Les spirolides (SPXs), produits également par *A. peruvianum* (Touzet et al., 2008), sont des molécules lipophiles du groupe des imines cycliques composées de plusieurs analogues. Les principaux analogues accumulés par les bivalves sont les spirolides 13, 19-didesmethyl-C, 13-desmethyl-C et 13-desmethyl-D. Leur quantité dans la chair de bivalve n'est pas réglementée car leur caractère toxique pour l'Homme n'est à ce jour

pas avéré. Elles sont en revanche surveillées, car elles entraînent la mort rapide de souris injectées par voie intra-péritonéale, d'où leur qualification de « Fast Acting Toxins » (Munday et al., 2012). Des études sur cultures cellulaires ont montré une action antagoniste des spirolides sur les récepteurs cholinergiques nicotiniques (Bourne et al., 2010; Gill et al., 2003; Hauser et al., 2012; Wandscheer et al., 2010), leur conférant des propriétés neurotoxiques (Gill et al., 2003; Munday et al., 2012; Otero et al., 2012; Richard et al., 2001).

En plus de ces toxines, produites intracellulairement et contenues dans les cellules algales mais également en partie excrétées (Lefebvre et al., 2008; Persson et al., 2012), d'autres composés extracellulaires de nature inconnue, toxiques pour différents types cellulaires de par leurs propriétés allélopathiques, hémolytiques, ichtyotoxiques et oxydatives peuvent être produites par différentes espèces de dinoflagellés (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011).

**Tableau 2.** Espèces de dinoflagellés toxiques étudiées dans cette thèse. Toxines produites et mode d'action identifié chez les mammifères ; zone géographique concernée par ces travaux de thèse.

<table>
<tr><th>Espèce</th><th>Toxines et mode action</th><th>Zone géographique d'intérêt</th><th>Saison</th></tr>
<tr><td>Alexandrium catenella</td><td rowspan="2">PSTs (STX et dérivées) : Blocage des canaux $Na^+$ voltage dépendants (neurotoxique)</td><td>France, côte méditerranéenne</td><td>Printemps -été</td></tr>
<tr><td>Alexandrium fundyense</td><td>Nord-est des Etats-Unis</td><td>Printemps -été</td></tr>
<tr><td>Alexandrium ostenfeldii*</td><td>PSTs (STX et dérivées) : Blocage des canaux $Na^+$ voltage dépendants (neurotoxique)<br>*SPXs : Antagoniste des récepteurs cholinergique nicotiniques (neurotoxique)</td><td>France, Bassin d'Arcachon</td><td>Printemps -été</td></tr>
</table>

* La souche d'*A. ostenfeldii* CCMP1773 utilisée au cours de cette thèse ne produit que des spirolides.

# 5 Réponses des bivalves aux dinoflagellés toxiques

Les bivalves peuvent être exposés aux toxines extracellulaires (PSTs ou spirolides et autres composés) d'*Alexandrium* sp. lors de la filtration dès la mise en contact avec le manteau, les branchies, les palpes labiaux et les siphons (si présents). La première ligne de défense des bivalves face à une perturbation extérieure consiste à **fermer leurs valves afin d'éviter le contact avec ces micro-algues**, comme cela est observé chez certains bivalves, telles la mye *Mya arenaria*, la moule *Mytilus edulis* et l'huître *Geukensia demissa,* exposés à *Alexandrium tamarense* (Shumway and Cucci, 1987).

Une autre façon, pour le bivalve, de limiter le contact avec l'algue est d**e réduire le taux de filtration**, c'est-à-dire le volume d'eau filtrée, **le taux de rétention**, c'est-à-dire le nombre de cellules retenues par le mucus branchial, ou le taux d'ingestion, c'est-à-dire la fraction ingérée après le tri opéré au niveau des palpes labiaux et **pendant lequel sont formés les pseudofaeces qui seront rejetés vers le milieu extérieur**. La réduction de la filtration et des taux de rétention et d'ingestion constituent une réponse classiquement observée chez plusieurs espèces de bivalves (moules, huîtres, myes,…) exposées à différentes espèces du genre *Alexandrium* (Cucci et al., 1985; Lassus et al., 1999; Shumway and Cucci, 1987). De plus, Haberkorn et al. (2011) et Tran et al. (2010) ont observé l'augmentation de la fréquence de micro-fermetures valvaires de *C. gigas* exposée à *A. minutum.*

Cependant, des compromis existent entre la limitation du contact avec *Alexandrium* sp. et ses toxines et les besoins en oxygène et en nourriture. Ainsi, **une partie de ces algues au moins est le plus souvent ingérée**. Les cellules algales sont alors observables en coupes histologiques dans la lumière de l'intestin. Le passage dans le tube digestif où le contact avec les enzymes digestives du bivalve peut entraîner **l'enkystement des cellules algales pour augmenter leur résistance à la digestion**. Elles peuvent ainsi être éliminées intactes dans le milieu via les faeces (Hégaret et al., 2008; Laabir et al., 2007; Persson et al., 2006).

**Lorsque la digestion entraîne la lyse des cellules** pour l'absorption des nutriments, **le contenu toxinique intracellulaire est libéré et accumulé dans les tissus,** majoritairement dans la glande digestive (Bricelj and Shumway, 1998; Guéguen et al., 2008; Lassus et al., 2007; Medhioub et al., 2012).

L'observation d'hémocytes en diapédèse (i.e. migrant entre les cellules de l'épithélium) mais aussi autour des cellules algales dans la lumière de l'intestin et dans les faeces a soulevé l'hypothèse d'un **mécanisme similaire à l'encapsulation afin de protéger les organes internes en isolant les algues et leurs composés toxiques** (Galimany et al., 2008b). Ces hémocytes infiltrés dans les tissus digestifs et observés en diapédèse pourraient aussi avoir **une fonction de détoxication,** via un transport des toxines ou de leur produits vers la lumière du tractus digestif afin de les éliminer, comme cela a été suggéré par Galimany et al. (2008b) et Haberkorn et al. (2010).

En effet, l'exposition à *Alexandrium* sp. est fréquemment associée à des **lésions des tissus digestifs** (Haberkorn et al., 2010b; Medhioub et al., 2012) qui traduisent le caractère toxique pour les bivalves des composés produits par *Alexandrium* sp. Ces lésions peuvent dès lors être responsables d'altération des fonctions digestives (Fernández-Reiriz et al., 2008; Li et al., 2002).

Des infiltrations hémocytaires associées à ces lésions pourraient également indiquer un **rôle des hémocytes dans la réparation tissulaire** (Estrada et al., 2007; Galimany et al., 2008a, 2008b, 2008c; Haberkorn et al., 2010b). Des modifications hématologiques dans l'hémolymphe (concentration en hémocytes circulants, différenciations morphologiques…) suggèrent effectivement des **réponses fonctionnelles des hémocytes** (da Silva et al., 2008; Galimany et al., 2008c; Haberkorn et al., 2010a; Hégaret and Wikfors, 2005; Hégaret et al., 2007a).

**Les modifications des variables hémocytaires pourraient aussi résulter d'effets cytotoxiques** des composés produits par les dinoflagellés. En effet, les études de Hégaret et al. (2007b) et Mello et al. (2012) font état d'une augmentation du pourcentage d'hémocytes circulants morts, ou encore de la réduction de la capacité de phagocytose, associée à l'exposition ou aux PSTs accumulées. Ces effets, suggèrent des **altérations fonctionnelles des hémocytes**, qui pourraient avoir des conséquences sur la défense immunitaire des bivalves.

**Finalement, bien que le système immunitaire semble modulé par l'exposition, il est difficile d'en estimer des effets immuno-stimulants ou immuno-suppresseurs ; d'une part, en raison de la grande spécificité des effets d'exposition en fonction du couple bivalve – dinoflagellé ; et d'autre part, en raison des effets observés sur les autres variables physiologiques** qui peuvent également impliquer les hémocytes (de par leurs multiples fonctions), ou rendre l'organisme plus vulnérable à des infections par des agents pathogènes.

# 6 Interactions bivalve – pathogène – dinoflagellé toxique

**Les impacts des dinoflagellés toxiques sur les hémocytes et plus généralement sur la physiologie des bivalves suggèrent que les efflorescences d'*Alexandrium* sp. pourraient augmenter la susceptibilité aux maladies,** comme l'avait suggéré Harvell et al. (1999). Ainsi, leurs effets pourraient interférer avec les fonctions immunitaires, et donc **moduler les interactions hôtes-pathogènes**. Lorsque l'on considère également les atteintes physiologiques induites par les dinoflagellés toxiques, de nouvelles questions se posent : **la combinaison de l'exposition aux dinoflagellés toxiques et de l'infection par des pathogènes entraîne-t-elle chez le bivalve des dommages physiologiques synergiques, c'est-à-dire propres à leur interaction ? Ces interactions peuvent-elles conduire à l'émergence de maladies** ?

Conceptuellement, les effets combinés des dinoflagellés toxiques et des agents pathogènes sur l'état physiologique du bivalve, autour duquel cette étude est centrée, pourraient aboutir à trois types de scénarios différents :

- « **Négatif** » : les effets combinés du dinoflagellé toxique et de l'agent pathogène sont davantage délétères pour le bivalve que l'effet de l'un ou l'autre des facteurs individuellement.

- « **Positif** » : les effets combinés du dinoflagellé toxique et de l'agent pathogène ont des conséquences moins délétères pour le bivalve que l'un ou l'autre des facteurs individuellement.

- « **Neutre** » : les effets combinés du dinoflagellé toxique et de l'agent pathogène n'entraînent pas d'effet spécifique, par rapport à l'effet individuel de chacun des facteurs.

A ce jour, peu d'études ont porté sur les effets de l'interaction tripartite bivalve – micro-algue toxique – agent pathogène. Cependant, **des effets potentiels de leur interaction sur l'immunité** du bivalve ont été mis en évidence. Ainsi, une **réduction de la capacité de phagocytose** des hemocytes a été observée chez la palourde américaine *Mercenaria mercenaria* infectée par le parasite QPX (« Quahog Parasite X »), et chez la palourde japonaise *R. philippinarum* infectée par *P. olseni*, lors d'une exposition aux dinoflagellés toxiques *Prorocentrum minimum* et *Karenia selliformis*, respectivement (Hégaret et al., 2010, 2007a).

D'autre part, l'exposition de la palourde *R. philippinarum* au dinoflagellé *P. minimum* et au parasite *P. olseni* a provoqué des **effets synergiques altérant la structure musculaire du pied et provoquant des dégénérescences des ovocytes** (Hégaret et al., 2009).

En outre, l'exposition à *Alexandrium tamarense* chez des palourdes juvéniles *R. philippinarum* infectées expérimentalement par la bactérie *V. tapetis* (l'agent

étiologique de la BRD) a **réduit l'évolution de la BRD** (i.e. l'intensité du dépôt de conchyoline), **mais augmenté le taux de mortalité** (Bricelj et al., 2011). Les auteurs de cette étude proposaient trois scénarios possibles. Le premier scénario consistait en une diminution de la défense contre *V. tapetis* par une inhibition de la capacité à produire la conchyoline, dont le rôle serait d'isoler les bactéries. Le deuxième scénario consistait en une immuno-activation via l'augmentation de la capacité phagocytaire (décrite dans cette même étude chez des adultes exposés à la même algue) qui aurait au contraire amélioré la lutte contre la bactérie. La dernière hypothèse reposait sur d'éventuels effets toxiques d'*A. tamarense* sur la bactérie.

En effet, ce type d'interaction directe entre agent pathogène et dinoflagellé toxique peut moduler l'interaction hôte – pathogène. Ainsi, une diminution de la prévalence et de l'intensité de la perkinsose a été rapportée chez des palourdes *R. philippinarum* exposées pendant trois semaines au dinoflagellé toxique *K. selliformis* (da Silva et al., 2008). Cette réduction de la charge parasitaire dans l'organisme a été attribuée aux **effets toxiques des composés extracellulaires produits par cette algue envers le parasite *P. olseni*** mesurés *in vitro* (da Silva et al., 2008).

Ces différentes études soulignent la complexité de ces interactions et l'importance de leurs conséquences sur les bivalves, ainsi que la nécessité d'étudier chaque cas individuellement étant donné leur caractère espèce-spécifique.

Les efflorescences d'*Alexandrium* sp. se produisent principalement au printemps et en été, dans les régions tempérées. Cette période de l'année offre également des conditions propices à l'infection et la prolifération d'agents pathogènes chez les bivalves. Ainsi, le risque de co-occurrences d'efflorescences toxiques et de maladies est élevé.

En France, sur la côte méditerranéenne, les épisodes de **mortalités massives de naissain d'huîtres creuses *C. gigas*,** associées principalement à **l'herpesvirus OsHV-1µVar**, peuvent se produire aux mêmes périodes que des efflorescences d'***A. catenella***.

Dans le bassin d'Arcachon, les populations de **palourdes**, **naturellement infectées par le parasite *P. olseni*¸** sont soumises régulièrement à des efflorescences d'***A. ostenfeldii***.

Le long de la côte nord-est des Etats-Unis, des efflorescences d'***A. fundyense*** se produisent régulièrement, exposant les huîtres ***C. virginica*** qui sont **naturellement infectées par des trématodes *Bucephalus* sp. et par le protozoaire *P. marinus*.**

Les conséquences de ces interactions potentielles pour les bivalves n'ont pourtant jamais été étudiées.

# Objectifs

**L'objectif de ce travail de thèse est d'évaluer les effets combinés d'une exposition à un dinoflagellé toxique, *Alexandrium* sp., et d'une infection par des agents pathogènes sur la physiologie des bivalves** (Figure 6).

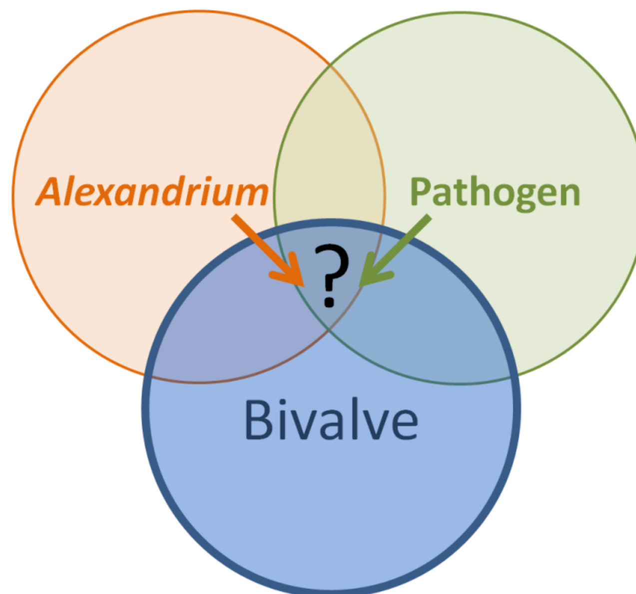


**Figure 6.** Schéma conceptuel de l'interaction tripartite *Alexandrium* sp. – pathogène – bivalve, centré sur le compartiment « bivalve ».

Trois axes ont été suivis afin de répondre à cet objectif : (i) déterminer les effets d'une efflorescence toxique sur l'interaction hôte – pathogène, mais également (ii) les effets du pathogène sur l'interaction dinoflagellé toxique – bivalve, et (iii) évaluer l'effet de ces interactions sur l'état physiologique du bivalve à travers une approche intégrative à différents niveaux organisationnels, tissulaires, cellulaires et subcellulaires. Des bivalves ont ainsi été exposées à des efflorescences d'*Alexandrium* sp., combinées soit à l'infection par des agents pathogènes spécifiques connus pour être impliqués dans des épizooties, soit à un changement d'environnement microbien, afin d'évaluer l'émergence de maladies opportunistes (Tableau 3).

Ce travail de thèse est structuré en trois chapitres visant à répondre à chacune de ces questions :

**- Une exposition à *Alexandrium* peut-elle moduler l'interaction hôte – pathogène, et à l'inverse, un agent pathogène peut-il moduler l'interaction bivalve – *Alexandrium* sp.?**
(Chapitre 1, Article 1 : Lassudrie et al., subm.).

**- Quelles sont les effets combinés d'une exposition à *Alexandrium* sp. et de parasites sur des bivalves naturellement infectés, ainsi que leurs implications physiologiques ?**
(Chapitre 2, Article 2 : Lassudrie et al., 2014; Article 3 : Lassudrie et al., in rev.).

**- Les effets d'*Alexandrium* sp. sur la physiologie du bivalve peuvent-ils favoriser des infections opportunistes lors de l'exposition du bivalve à un nouvel environnement microbien ?**
(Chapitre 3, Article 4 : Lassudrie et al., subm.).

**Tableau 3.** Récapitulatif des interactions étudiées dans chaque chapitre et article.

| Chapitre | Article | Interaction |
|---|---|---|
| Chapitre 1 | Article 1 | *C. gigas* – OsHV-1 – *A. catenella* |
| Chapitre 2 | Article 2 | *R. philippinarum* –*P. olseni* – *A. ostenfeldii* |
| | Article 3 | *C. virginica* – *P. marinus* & *Bucephalus* sp. – *A. fundyense* |
| Chapitre 3 | Article 4 | *C. gigas* – nouvel environnement microbien – *A. catenella* |

# Chapitre 1

## Une exposition à *Alexandrium* peut-elle moduler l'interaction hôte – pathogène, et à l'inverse, un agent pathogène peut-il moduler l'interaction bivalve – *Alexandrium* sp.?

**Article 1:** *Soumis à Harmful Algae.*

Lassudrie, M., Soudant, P., Nicolas, J.L., Fabioux, C., Lambert, C., Miner, P., Le Grand, J., Petton, B., Hégaret, H. Interaction between toxic dinoflagellate *Alexandrium catenella* exposure and disease associated with herpesvirus OsHV-1 in Pacific oysters *Crassostrea gigas*. Submitted.

# Article 1

## Interaction between toxic dinoflagellate *Alexandrium catenella* exposure and disease associated with herpesvirus OsHV-1 in Pacific oyster spat *Crassostrea gigas*

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**Abstract**

In France, blooms of toxic dinoflagellates can co-occur with mass mortality events associated with herpesvirus OsHV-1µVar infection that have been decimating Pacific oyster *Crassostrea gigas* spat and juveniles every summer since 2008. This study investigated the possible effect of a harmful dinoflagellate, *Alexandrium catenella,* a producer of Paralytic Shellfish Toxins (PSTs), upon the oyster spat – herpesvirus interaction. Oyster spat from a hatchery were challenged by cohabitation with oysters contaminated in the field with OsHV-1 and possibly other pathogens. Simultaneously, they were exposed to cultured *A. catenella*. Infection with OsHV-1 and PST accumulation were measured after 4 days of experimental exposure.

Exposure to *A. catenella* modified the host-pathogen interaction by reducing prevalence of OsHV-1 infection, probably by inhibiting viral transmission or replication processes. In addition, oysters challenged with OsHV-1 and possibly other pathogens accumulated smaller amounts of PSTs than unchallenged oysters. Possible interactions between *A. catenella* and herpesvirus or associated pathogens could reduce oyster consumption and digestion of toxic algae, as well as viral transmission. Finally, hemocyte activation by *A. catenella* could enhance defense efficiency against OsHV-1 infection. These findings suggest further research on relationships between OsHV-1 and toxic dinoflagellates and their combined effects upon disease transmission and proliferation processes, as well as on oyster physiological and immunological involvement in this complex, tripartite interaction.

**Keywords** : *Crassostrea gigas*; OsHV-1; harmful algal blooms; *Alexandrium*; host-pathogen interaction.

## 1 Introduction

Since 2008, French oyster production has been diminished by massive mortality events that have decimated *Crassostrea gigas* spat (<12 months old) and juveniles (12-18 months old), when seawater temperature exceeds 16°C. These mortality events decimate 40 to 100% of the spat, depending upon location (Cochennec-Laureau et al., 2011; EFSA Panel on Animal Health and Welfare, 2010; Fleury and Bédier, 2013; Fleury, 2014). Other countries, such as Ireland, the UK, New Zealand, and Australia, also recently experienced massive spat and juvenile oyster mortality events associated with microvariants of ostreid herpesvirus 1 (OsHV-1) (EFSA Panel on Animal Health and Welfare, 2010; Jenkins et al., 2013; Martenot et al., 2011; Paul-Pont et al., 2014; Renault et al., 2012). The microvariant genotype detected in France, designated as OsHV-1µVar, was found also in Ireland, the UK, and Australia (EFSA Panel on Animal Health and Welfare, 2010; Jenkins et al., 2013; Renault et al., 2012; Segarra et al., 2010). A causal relationship between OsHV-1µVar and mortality events has been postulated (Schikorski et al., 2011a, 2011b), although bacterial strains from *Vibrio splendidus* clade and *Vibrio aesturianus* also could be involved (EFSA Panel on Animal Health and Welfare, 2010; Garnier et al., 2007; Gay et al., 2004), and environmental factors and oyster physiological condition may play roles in disease severity and oyster susceptibility to the disease (Pernet et al., 2014, 2012; Petton et al., 2013).

Concurrent with these mortality events, during spring and summer, toxic dinoflagellates *Alexandrium minutum* and *Alexandrium catenella* recurrently bloom along the French coasts (Chapelle et al., 2013; Lilly et al., 2002; REPHY). Temperature thresholds above which blooms usually are reported reach 15-18°C (Chapelle et al., 2013, 2007; Collos et al., 2009; Laabir et al., 2011). These *Alexandrium* species can produce Paralytic Shellfish Toxins (PSTs). Bivalves, as filter-feeders, can accumulate PSTs, leading to toxicity for human shellfish consumers and thus resulting in temporary shellfish harvest prohibitions. Species of the genus *Alexandrium* also produce extracellular compounds with allelopathic, hemolytic, ichtyotoxic, and oxidative properties (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011). Interactions between bivalves and *Alexandrium* sp. – and thus PSTs and extracellular compounds – have been examined in many studies. Alterations of physiological processes and tissue integrity have been reported in *Alexandrium* sp.-exposed bivalves (Cucci et al., 1985; Gainey and Shumway, 1988a, 1988b; Galimany et al., 2008; Haberkorn et al., 2010b; Landsberg, 2002; Lassudrie et al., 2014; Medhioub et al., 2012; Shumway, 1990). Hemocytes,

which are involved in many functions including immunity, also are affected by *Alexandrium* sp. exposure (Galimany et al., 2008; Haberkorn et al., 2010a; Hégaret et al., 2007). By altering cell functions involved in immune response, physiological processes and tissue integrity in bivalves, *Alexandrium* sp. may modify shellfish susceptibility to pathogens. Indeed, Lassudrie et al. (in rev.) reported that an exposure to *Alexandrium fundyense* (a PST producer) resulted in modified immune functions associated with higher susceptibility to infection with the protozoan parasite *Perkinsus marinus* in trematode-infested Eastern oysters. On the contrary, da Silva et al. (2008) reported a decrease in intensity of *Perkinsus olseni* infection in Manila clams exposed to the toxic dinoflagellate *Karenia selliformis*, which was shown to result from direct toxicity of algae to *P. olseni*. Similarly, Hégaret et al. (2010) suggested a toxic effect of the dinoflagellate *Prorocentrum minimum* upon the protistan Quahog Parasite Unknown (QPX) in northern quahog clams. Thus, toxic algal exposure may modify the host-pathogen interaction, either by affecting host physiological or immune status, or through direct toxicity to the pathogen.

Despite the importance of OsHV-1μVar in oyster mortality events in France and the repeated co-occurrence with harmful algal blooms, to the best of our knowledge, the effect of toxic algal exposure upon the oyster spat – herpesvirus interaction has not been described.

The aim of this study was, thus, to assess if and how exposure to the toxic dinoflagellate, *A. catenella* could modulate the host-pathogen interaction, i.e. oyster spat – herpesvirus interaction. For this purpose, oyster spat naïve from OsHV-1μVar (Specific Pathogen-Free, SPF) were simultaneously exposed to cultured *A. catenella* (at realistic concentrations compared to the field) and challenged with herpesvirus and possible other pathogenic agents associated with mortality events (condition 1). To discriminate the effects of each factor, three other conditions were used: *A. catenella* exposure and unchallenged oysters (condition 2); non-toxic algal exposure and pathogenic challenged oysters (condition 3); non-toxic algal exposure and unchallenged oysters (condition 4). After four days, herpevirus DNA quantification and oyster PST concentrations were compared between conditions, and condition index was determined.

## 2 Material and methods

### *2.1 Algal cultures*

*Tisochrysis lutea* (Bendif & Probert) (T-*Iso*) was used as diet during acclimation and maintenance stages at $5 \times 10^5$ cells $mL^{-1}$. T-*Iso* was cultured in 300-L cylinders containing seawater enriched with Conway medium (Walne, 1966) at 20°C with continuous light (200 µmol photons $m^{-2}$ $s^{-1}$). T-*Iso* was harvested after 3 to 5 days of growth, at a cell density approaching $1 \times 10^7$ cells $mL^{-1}$.

The dinoflagellate *Alexandrium catenella* (Whedon & Kofoid) strain VGO676, a PST producer (Lassus et al., 2007) isolated in 2003 from Thau lagoon (France), was used for toxic algal exposure, and *Heterocapsa triquetra* (Ehrenberg) Stein, strain HT99PZ (isolated from Penzé Bay, France in 1999) was used as a control, non-toxic dinoflagellate. Both strains were provided by the Phycotoxin laboratory, Ifremer, Nantes (France). Both dinoflagellate cultures were grown in L1 medium (Guillard and Hargraves, 1993) at 17°C with a light:dark cycle of 12:12h and were harvested during exponential growth phase at a cell density approaching $5 \times 10^4$ cells $mL^{-1}$.

Algal cell densities were determined by counts using Malassez and Nageotte cells under a light microscope.

### *2.2 Specific Pathogen-Free (SPF) oysters*

The Pacific oysters, *Crassostrea gigas* (Thunberg), used in this study came from a single cohort produced in March 2012 in the Argenton Ifremer facilities (France) following a standardized process to obtain OsHV-1-free diploid-oysters described by Petton et al. (2013). Screening for OsHV-1 DNA (i.e. OsHV-1 reference as well as OsHV-1µVar) was conducted by qPCR (following the standard procedure described in Pépin et al., 2008) once during D-larval stage and again at 3 months of age following thermal challenge; all results were negative for this cohort (analyses by IDHESA, Quimper, France). At the time the experiment began (July 2012), oysters were 4 months old, measured 23.1 ± 0.2 mm length (mean ± SE), and weighed 1.45 ± 0.3 g (total wet weight; mean ± SE).

*2.3 OsHV-1 contamination by field-exposure*

It should be noted that the OsHV-1 reference genotype has been replaced by OsHV-1μVar since 2009 in seeds of French rearing sites suffering mass mortality (including Bay of Brest) (François et al., 2010; Petton et al., 2013; Renault et al., 2012). Thus, the OsHV-1 DNA detected in this study (section 2.5 OsHV-1 DNA quantification) was assumed to belong to OsHV-1μVar genotype, however we will refer to the general denomination OsHV-1.

On June 18$^{th}$, 2012, a subsample of SPF oyster spat (n=1000) was transferred into the Bay of Brest, at Pointe du Chateau (48° 20' 06.19" N, 4° 19' 06.37" W), in a farming area with recurrent mass mortality of oysters (Fleury and Bédier, 2013; Fleury, 2014; François et al., 2010). This time corresponded with the highest peak of spat mortality in this location in 2012 (30-34% instantaneous mortality) (RESCO, http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-nationaux/Resultats-nationaux-2012/Mortalite-par-site-et-par-classe-d-age). After 3 weeks in the field (i.e. July 2$^{nd}$, 2012), detection of OsHV-1 DNA in experimental oysters was confirmed by qPCR (following the procedure described below), and oysters were transferred to the experimental facilities. Oysters were held for three days before the beginning of the experiment in two tanks supplied with an open flow of 1-µm-filtered seawater treated with UV, at 20°C, and fed continuously with T-*Iso* at 3-5 × $10^5$ cell mL$^{-1}$. One hundred and eighty field-exposed oysters then were used to challenge SPF oysters by cohabitation, as described below, and were also analyzed (designated as "field-exposed oysters"). The same number of SPF oysters was maintained in two other tanks in the same conditions, and were used for cohabitation with unchallenged oysters.

*2.4 Experimental design*

Experimental design is summarized in Figure 1.

On June 25$^{th}$, 2012, SPF oysters were distributed in 15-L tanks (30 oysters per tank, 12 tanks) and acclimated for 10 days being fed T-*Iso*. At the end of this acclimation period, on July 5$^{th}$ ($T_0$), 30 field-exposed oysters per tank, held in a net, were added to six of these tanks. The SPF oysters that were thus maintained in cohabitation with these field-exposed oysters were

designated as “challenged”. In the six other tanks, 30 other SPF oysters per tank, held in a net, were added to establish the same number of oysters in each tank. The oysters in these tanks were designated as “unchallenged”. In addition, 3 “challenged” tanks and 3 “unchallenged” tanks were exposed continuously to $3.5 \times 10^2$ cells $mL^{-1}$ of the toxic dinoflagellate *A. catenella*; whereas, the other tanks were exposed to the same concentration of the control, non-toxic dinoflagellate, *H. triquetra.*

Similar cohabitation design previously has been demonstrated to successfully infect SPF oysters with the pathogenic agents responsible for mass mortality, including OsHV-1µVar (Petton et al., 2013).

During the entire experiment, 1-µm-filtered and UV-sterilized seawater was supplied to the tanks (10-15 mL $min^{-1}$, i.e. one tank renewal every 24h) with aeration at 20°C, a temperature favorable for OsHV-1µVar transmission (Petton et al., 2013).

*2.5 Sampling*

Oysters were sampled at the end of the acclimation period, before the beginning of the experiment ($T_0$), and after four days ($T_4$).

At $T_{0,}$ 12 field exposed-oysters, 12 SPF oysters from each “unchallenged” tank (2 per tank), and 2 SPF oysters from each “challenged” tank (2 per tank) were sampled.

At $T_4$, 72 oysters from “unchallenged” tanks (12 per tank), 72 oysters from “challenged” tanks (12 per tank), and 72 field-exposed oysters from “challenged” tank (12 per tank) were sampled.

For condition index, soft tissues and shells were weighed after the excess of water was removed. Mantle and digestive gland were dissected for herpesvirus and toxin quantification, respectively, and tissue samples were frozen immediately in liquid nitrogen before being stored at -80°C prior to quantification. Oyster mortality was checked daily. Dead oysters were removed from the tanks, and mantle and digestive gland were dissected, when the degradation stage of the tissue allowed it, and stored at -80°C for further analyses. Not all oysters sampled were used for each analysis (see sections below).

OsHV-1 data for field-exposed oysters were provided only for oysters that died during the experiment. Indeed, it should be noted that oyster deployment in the field unintentionally co-occurred with a bloom of *Alexandrium minutum* (a PST producer), undetected as this time (Chapelle et al., 2013), rendering complicated the interpretation of PST and OsHV-1 quantification from field-exposed oysters in the context of this study.

### *2.6 OsHV-1 DNA quantification*

OsHV-1 DNA was quantified in the mantle at the end of the acclimation period ($T_0$) (before algal exposure and cohabitation challenge) in two SPF oysters per tank, which were designated to be challenged (i.e. 12 oysters) and one oyster per tank designated to remain unchallenged (i.e., 6 oysters), as well as in 11 field-exposed oysters. After four days of the experiment, 34 and 36 challenged oysters exposed to *H. trique*tra and *A. catenella*, respectively (11-12 oyster per tank), as well as 3 unchallenged oysters per algal condition (one per tank), were analyzed. Analysis also was performed for 25 of the 27 field-exposed oysters that died during the experiment, and for all the 5 challenged oysters which died over the course of the experiment.

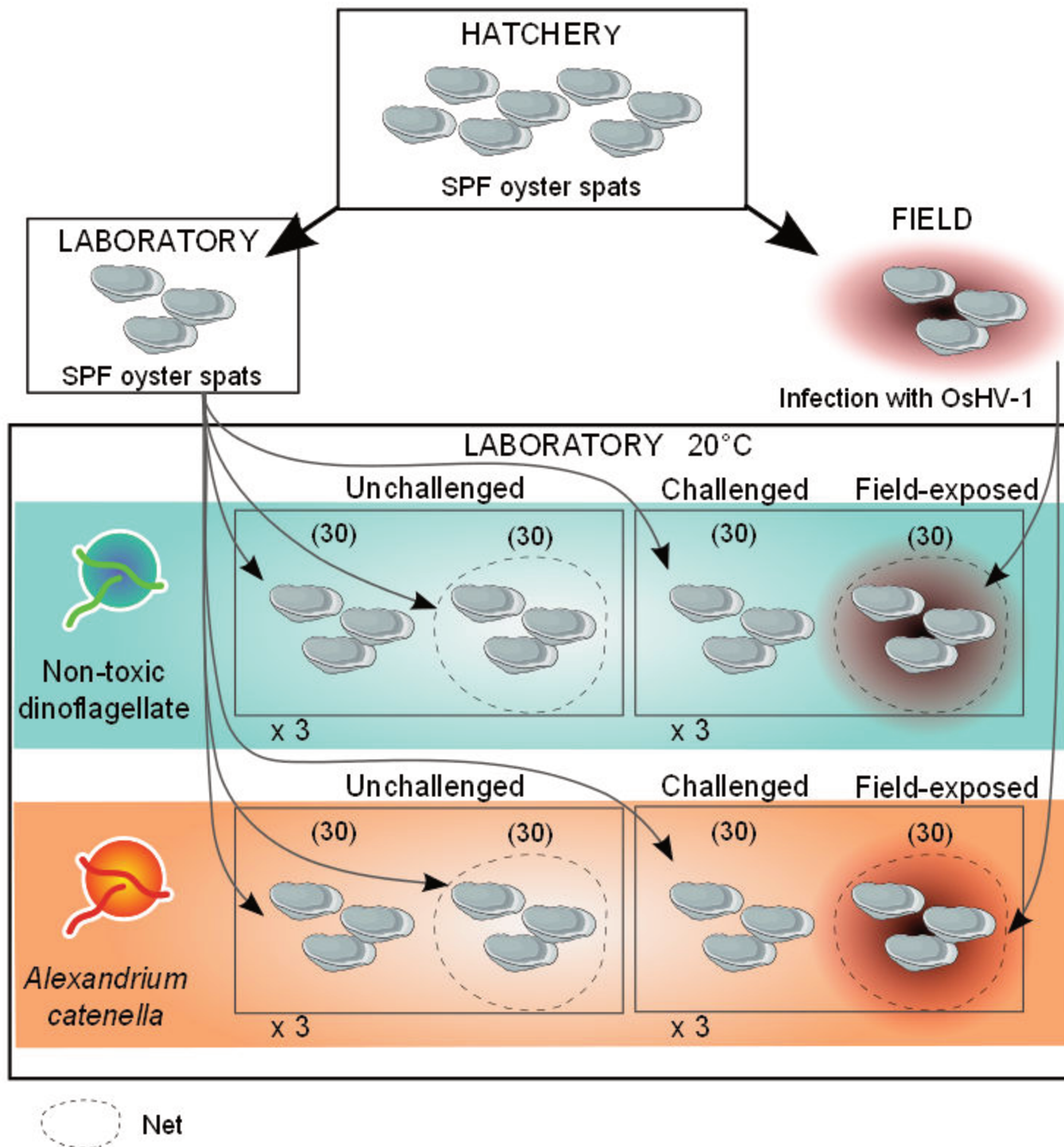


Figure 1. Scheme of the experimental design. All oysters used were produced in hatchery in conditions naive from OsHV-1 and were designated as Specific Pathogen-Free (SPF). SPF and field-exposed oysters were sampled prior to exposure and cohabitation ($T_0$); challenged and unchallenged oysters were sampled after 4 days of algal exposure and cohabitation with field-exposed oysters ($T_4$).

DNA extraction was performed with the QIAamp DNA Mini Kit (QIAgen), analyzing 20 mg of wet mantle, following the manufacturer instructions. 100 µL of molecular biology grade water (DNase-free) were used for elution. Nucleic acid concentration was measured immediately with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (conversion factor: 1 OD = 50 µg $mL^{-1}$ DNA) at 260 nm and adjusted at 3 ng $\mu L^{-1}$ after purity was checked using the 260 / 230 nm and 260 / 280 nm ratios.

A standard protocol was followed to quantify OsHV-1 DNA (OsHV-1 reference as well as OsHV-1µVar) using qPCR (Pépin, 2013; Pépin et al., 2008) with the HVDP-F – HVDP-R primer pair (forward, HVDP-F 5'-ATT-GAT-GAT-GTG-GAT-AAT-CTG-TG-3', reverse, HVDP-R 5'-GGT-AAA-TAC-CAT-TGG-TCT-TGT-TCC-3'), targeting the OsHV-1 polymerase sequence. Each reaction was performed in triplicate and carried out in a final volume of 15 µL containing HVDP-F and HVDP-R primers at final concentrations of 5 µM, 7.5 µL of IQ SYBR Green Supermix and 5µL of DNA samples diluted at 3 ng $\mu L^{-1}$. Each run included a no-template control (water), a positive control (DNA from OsHV-1-infected oyster), and six standards (from $10^5$ to $10^0$ OsHV-1 copies $\mu L^{-1}$), prepared by successive, ten-fold dilutions of a stock solution of OsHV-1 genomic DNA at $5 \times 10^6$ copies $\mu L^{-1}$ extracted from purified virus particles (Le Deuff and Renault, 1999). The standard curve obtained was used to calculate the percentage of amplification efficiency (% E) described in (1), which was contained between 90% and 110%, and quantification of the samples was determined by comparing Ct values.

(1) $\% E = (10^{-1/a} - 1) \times 100$, with $a$ = slope of the linear regression line calculated from Ct = f($\log_{10}$ of dilution).

An MyIQ2 Thermocycler (Biorad) was used with the following thermal profile: 1 cycle of enzyme activation (95°C, 3min.), 40 cycles of amplification/detection (95°C, 30s; 60°C, 1min.; 72°C, 1min), and a final step for melting temperature curve analysis (80 cycles, 95°C to 55°C, decreasing the temperature by 0.5°C after each cycle, 10s). The specificity of the PCR products was checked systematically with the melting temperature (Tm) value calculated from the dissociation curve.

Results were expressed as number of OsHV-1 DNA copies $ng^{-1}$ total DNA. In addition, quantification expressed as OsHV-1 DNA copies $mg^{-1}$ of wet mantle was determined.

Correlation between both ways of expressing OsHV1 DNA quantification was verified (Pearson product moment correlation, $p<0.001$, correlation coefficient = 0.98, n=269).

The following criteria were used to consider OsHV-1 detection as positive: Ct<38 and a Tm corresponding to OsHV-1 DNA amplicon (77.5°C). When OsHV-1 DNA concentration in samples considered as positive were below the detection limit (i.e. $Ct_{sample}>Ct_{standards}$), a numerical value of 0 was given for quantification.

### *2.7 Toxin accumulation*

Three oysters exposed to the non-toxic *H. triquetra* were tested to ensure that no PST was detected. Toxin quantification in *A. catenella*-exposed oysters was performed for 23 challenged and 23 unchallenged-oysters with the PSP ELISA kit (Abraxis). The three oysters that had died during the experiment that were unexposed to the field and exposed to *A. catenella* also were analyzed. PST extraction was performed following manufacturer instructions: digestive gland tissue was homogeneized in HCl 0.1 M (1:1, w:v) using a Precellys®24 bead-grinder and then boiled for 5 min. A dilution of this homogenate was used in the ELISA assay. Toxicity was expressed as µg STX $kg^{-1}$ of wet digestive gland.

### *2.8 Condition index (CI) and digestive gland index (DGI)*

Condition index (CI) was calculated in all live oysters sampled at $T_0$ and $T_4$, with a formula adapted from Bodoy et al. (1986):

$$CI = \frac{\text{wet soft tissue weight}}{\text{wet soft tissue weight} + \text{shell weight}} \times 100$$

Calculation of digestive gland index (DGI) was adapted from Thompson et al. (1974):

$$DGI = \frac{\text{wet digestive weight}}{\text{wet soft tissue weight} + \text{shell weight}} \times 100$$

*2.9 Statistical analyses*

To compare cumulative mortality curves (Kaplan Meier method) between conditions, a Log-rank (Mantel-Cox) test followed by multiple comparison tests with Holm-Bonferroni correction was used.

Difference between OsHV-1 prevalence in live oysters according to algal exposure was tested with a Chi-square test.

In live oysters, effect of algal treatment (*A. catenella* or *H. triquetra*) was tested upon OsHV-1 DNA quantification with a *t*-test. OsHV-1 DNA data were $\log_{10}$ (X + 1) transformed to obtain normality of residuals and homogeneity of variances. A $\log_{10}$ scale was used in graphical representation of OsHV-1 DNA data.

Differences in PST accumulation in digestive glands of challenged compared to unchallenged oysters were tested with a Mann-Whitney test.

For condition index and digestive gland index, 4-way ANOVAs were used to examine the effect of algal treatment, challenge condition, field-exposure, and sampling time ($T_0$ vs. $T_4$).

Differences were considered significant when $p<0.05$. Statistical analyses were performed using Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA) and GraphPad Prism. All values were expressed as mean ± standard error.

## 3 Results

### *3.1 Mortality*

Cumulative mortality (Figure 2) accounted for 16.7% and 13.3% of field-exposed oysters after 4 days of exposure to *H. triquetra* and *A. catenella*, respectively. After 4 days of the experiment, 3.3% and 2.2% mortality was found in challenged oysters exposed to *H. triquetra* and *A. catenella*, respectively; whereas 2.8% and 0.6% mortality were found in unchallenged oysters exposed to *H. triquetra* and *A. catenella*, respectively. Cumulative mortality was significantly higher ($p<0.05$) in field-exposed oysters compared to unchallenged oysters fed both *A. catenella* and *H. triquetra*, and challenged-oysters fed *A. catenella*. Cumulative mortality in field-exposed oysters, however, did not differ significantly from cumulative mortality in challenged oysters fed *H. triquetra*.

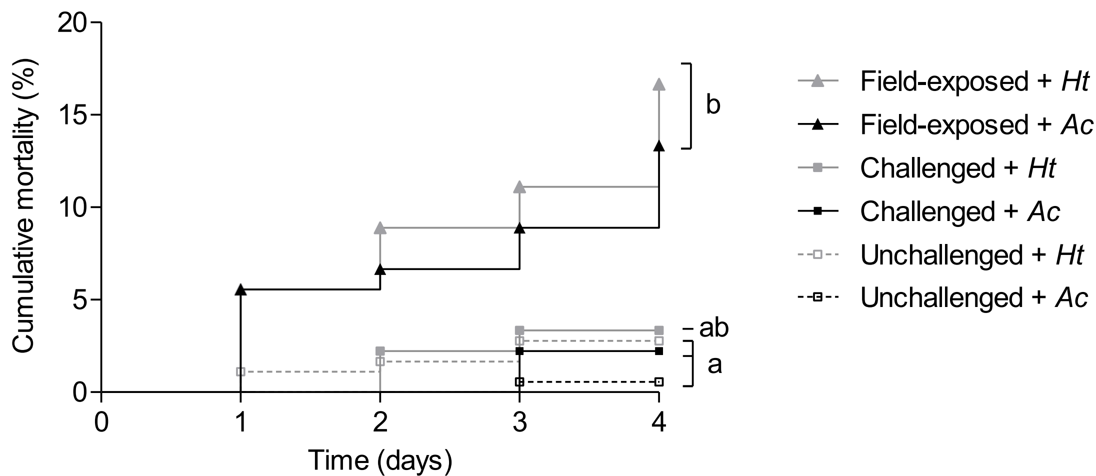


Figure 2. Kaplan-Meier cumulative mortality curves in oyster spat according to field-exposure, challenged (cohabitation with field-exposed oysters) or unchallenged, and algal exposure (*Ht*: *Heterocapsa triquetra* or *Ac*: *Alexandrium catenella*). Significant differences between mortality curves are indicated by letters (Log-rank test adjusted with Holm-Bonferroni correction for multiple comparisons; $p<0.05$). At $T_0$, N =90 field-exposed oysters per algal treatment, 90 challenged oysters per algal treatment and 180 unchallenged oysters per algal treatment.

*3.2 OsHV-1 DNA detection and quantification*

OsHV-1 DNA was not detected in the SPF oysters sampled at the end of the acclimation period (n=12) or in live, unchallenged oysters sampled after 4 days of experimental treatment (n=6). OsHV-1 DNA was detected in all field-exposed oysters sampled at $T_0$ (n=11), with a mean of $4.9 \pm 3.0\ 10^5$ copies $ng^{-1}$ total DNA in mantle ($1.2 \pm 0.8\ 10^9$ copies $mg^{-1}$ of wet mantle).

OsHV-1 DNA was detected in 100% of the dead oysters, either previously exposed to the field ($1.1 \pm 0.2 \times 10^6$ copies $ng^{-1}$ total DNA, equivalent to $2.5 \pm 0.5 \times 10^9$ copies $mg^{-1}$ of wet mantle, n=25) or challenged ($1.0 \pm 1.0 \times 10^5$ copies $ng^{-1}$ total DNA, equivalent to $2.0 \pm 2.0 \times 10^8$ copies $mg^{-1}$ of wet mantle, n=5). In dead, unchallenged oysters, OsHV-1 was detected in 3 of 4 oysters, but only at low intensity levels (between $6.6 \times 10^0$ and $2.9 \times 10^2$ copies $ng^{-1}$ total DNA).

After 4 days of the experiment, OsHV-1 prevalence was significantly lower ($p<0.05$) in challenged oysters exposed to *A. catenella* (29%) compared to challenged oysters exposed to *H. triquetra* (58%, Figure 3A). OsHV-1 infection intensity (i.e. considering only oysters detected positive to OsHV-1) tended to be lower in *A. catenella*-exposed oysters with $7.0 \pm 5.6 \times 10^2$ copies $ng^{-1}$ total DNA (or $1.9 \pm 1.5 \times 10^6$ copies $mg^{-1}$ of wet mantle, n=10), compared to $2.8 \pm 2.5 \times 10^4$ copies $ng^{-1}$ total DNA (or $6.4 \pm 5.6 \times 10^7$ copies $mg^{-1}$ of wet mantle, n=21) in oysters exposed to *H. triquetra*, although no significant difference was detected (Figure 3B). Finally, a significantly lower OsHV-1 weighted prevalence (i.e. considering all live-challenged oysters) was detected in *A. catenella*-exposed oysters ($p<0.001$) ($2.0 \pm 1.6 \times 10^2$ copies $ng^{-1}$ total DNA or $5.4 \pm 4.5 \times 10^5$ copies $mg^{-1}$ of wet mantle, n=35) compared to oysters exposed to the non-toxic *H. triquetra* ($1.6 \pm 1.5 \times 10^4$ copies $ng^{-1}$ total DNA or $3.7 \pm 3.3 \times 10^7$ copies $mg^{-1}$ of wet mantle, n=36) (Figure 3B).

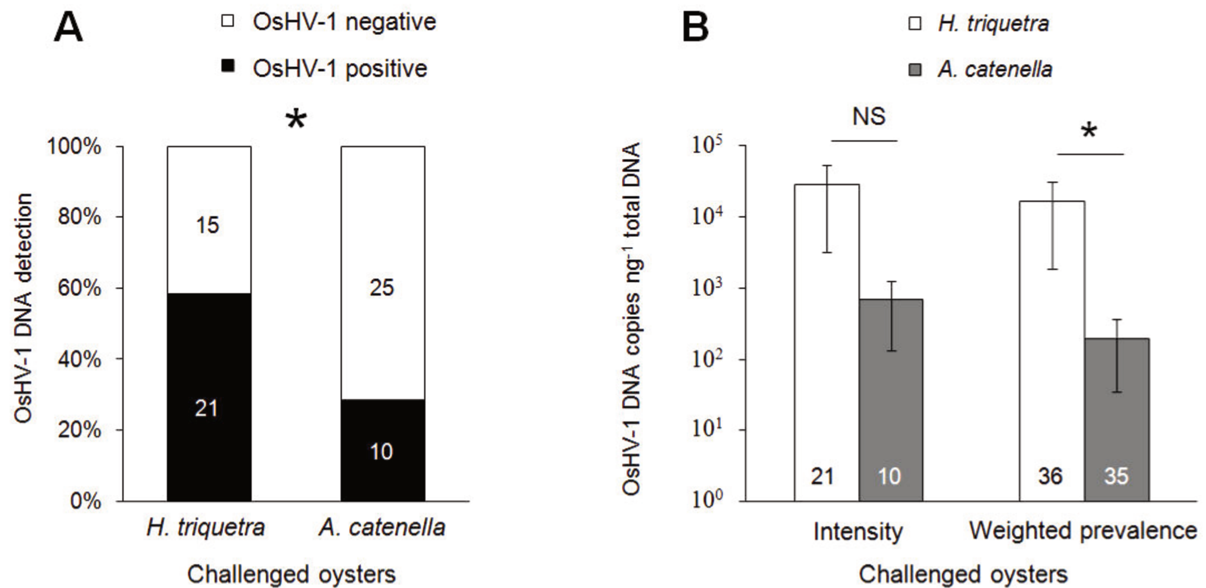


Figure 3. (A) OsHV-1 prevalence in challenged oyster spat, after 4 days exposure to the non-toxic *Heterocapsa triquetra* or the toxic *Alexandrium catenella*. * indicates a statistical difference between conditions (Chi-square test; $p<0.05$). N is indicated in each bar. (B) OsHV-1 infection intensity (considering only challenged oysters detected positive to OsHV-1) and weighted prevalence (considering all challenged oysters), quantified in the mantle after 4 days exposure to the non-toxic *H. triquetra* or the toxic *A. catenella*. * indicates a statistical difference (*t*-test; $p<0.05$). Mean ± SE. N is indicated in each bar.

*3.3 Toxin accumulation*

No PST was detected in digestive glands of SPF oysters sampled at $T_0$ and after 4 days exposure to the non toxic dinoflagellate *H. triquetra*. PSTs were not detected in the 3 dead oysters exposed to *A. catenella* (2 challenged and 1 unchallenged, dead after 3 days of experiment) that were analyzed.

After 4 days of *A. catenella* exposure, significantly ($p<0.05$) lower PST accumulation was detected in digestive gland of challenged oysters ($3.6 \pm 0.6 \times 10^2$ µg STX kg$^{-1}$) compared to unchallenged oysters ($6.9 \pm 1.1 \times 10^2$ µg STX kg$^{-1}$) (Figure 4).

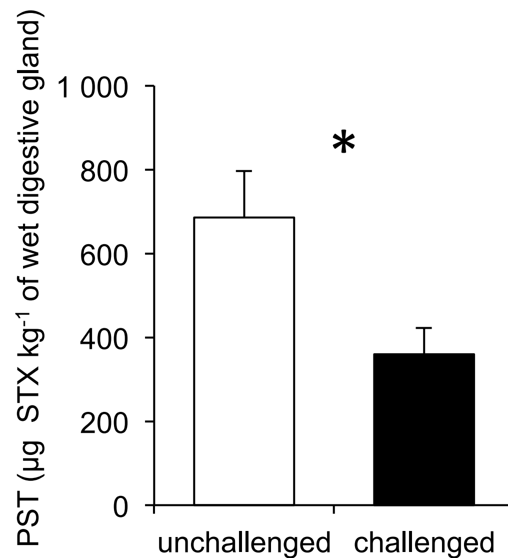


Figure 4. Paralytic Shellfish Toxin (PST) accumulation after 4 days of exposure to *Alexandrium catenella* in the digestive glands of live, unchallenged or challenged oyster spat. * indicates a significant difference between conditions (Mann-Whitney test; $p<0.05$). Mean ± SE. N=23 per condition.

*3.4 Condition index (CI) and digestive gland index (DGI)*

Field-exposed oysters had a significantly lower CI than other experimental oysters unexposed to the field (i.e., SPF at $T_0$, challenged and unchallenged oysters at $T_4$) over the course of the experiment ($CI_{field\text{-}exposed} = 22.7 \pm 0.3$; n=84 and $CI_{unexposed\ to\ field} = 26.7 \pm 0.3$; n=168, $p<0.001$). Algal treatment, challenge condition, field-exposure, and sampling time did not affect DGI.

**4 Discussion**

Exposure to *A. catenella* was shown for the first time to reduce herpesvirus infection; furthermore, PST accumulation decreased in oysters challenged with the herpesvirus and possibly also other pathogenic agents associated with mortality. These interactions constitute an antagonistic relationship between the virus and the toxic alga when they co-occur.

The low cumulative mortality (from 2.2% to 16.7% depending upon experimental treatment) observed after 4 days of experimental treatment at 20°C was in a similar range, although slightly lower, compared to those observed by Petton et al. (2013) at 17.5°C after 4 days of cohabitation (no mortality) or maintenance in the laboratory after field-exposure (~8%). Cumulative mortality observed in the present study, however, was lower than that observed at 21.9°C by Petton et al. (2013), reaching approximately 20% in challenged oysters and 40% in field-exposed oysters. These authors also observed higher mortality in oyster spat previously held in the field during mass mortality events than in challenged oysters, which is consistent with our study. All oysters exposed to the field or challenged by cohabitation with field-exposed oysters that died during the experiment were infected with OsHV-1. In some dead, unchallenged oysters, OsHV-1 DNA was detected, but at a very low intensity (maximum of $2.9 \times 10^2$ copies $ng^{-1}$ total DNA in dead, unchallenged oysters, compared with $10^5$ to $10^6$ copies $ng^{-1}$ total DNA in dead, field-exposed or challenged oysters), suggesting that death was unlikely related to OsHV-1 infection.

The relatively low infective biomass (field-exposed oysters) used, and the short cohabitation time limited mortality in challenged oysters (for more information about influence of infective biomass and duration of cohabitation see Petton et al., submitted). These conditions were implemented to allow the study of live oysters containing sub-lethal levels of OsHV-1, based upon results reported by Petton et al. (2013). These authors detected OsHV-1 DNA in 50% of oysters challenged for 3 days at 21.9°C, a result consistent with the 58% detection found in the present study in challenged oysters exposed to the non-toxic dinoflagellate for 4 days, at 20°C.

Exposure to the toxic dinoflagellate *A. catenella*, however, significantly reduced the prevalence of OsHV-1 from 58% to 29%. Exposure to *A. catenella* also induced a non significant decrease of the infection intensity in challenged oysters that tested positive with OsHV-1. When considering the entire oyster batch (both positive and negative for OsHV-1), OsHV-1 weighted prevalence was significantly lower upon *A. catenella* exposure. These results suggest a direct and / or indirect effect of *A. catenella:* (i) upon proliferation and virulence mechanisms of OsHV-1, or (ii) upon horizontal transmission of OsHV-1.

Proliferation of OsHV-1 may have been inhibited by activation of the host immune responses induced by *A. catenella* exposure. Although immune defense against herpesvirus is not well documented, hemocyte-mediated responses appear to be involved, as shown by hemocyte infiltrations (Jenkins et al., 2013) and differentially expressed genes related to hemocyte functions in virus-infected oysters (Green et al., 2014; Jouaux et al., 2013; Renault et al., 2011). Previous studies showed an inflammatory response in bivalves exposed to *Alexandrium* sp., which increased circulating hemocyte concentration, as well as hemocyte infiltration and diapedesis, particularly in the digestive gland but also in the gills and the mantle (Galimany et al., 2008; Haberkorn et al., 2010a, 2010b; Lassudrie et al., 2014). More numerous hemocytes in these organs that are also targeted by OsHV-1 (Corbeil et al., 2014; Schikorski et al., 2011a), could result in a faster, more efficient defense and accelerate elimination of herpesvirus, possibly through release of humoral factors. For example, α2-macroglobulin molecules are protease inhibitors expressed in hemocytes that play a role in the immune defense (Gueguen et al., 2003; Vaseeharan et al., 2007), and are upregulated with herpesvirus infection in *C. gigas* (Jouaux et al., 2013). Increase in hemocyte apoptosis induced by *A. catenella* (Medhioub et al., 2013) also may play a role, as apoptosis is an important component of immune defense against intra-cellular pathogens by limiting their proliferation (Sokolova, 2009), and appears to be involved in response to OsHV-1 (Jouaux et al., 2013).

Virulence mechanisms of OsHV-1 could be altered by PSTs accumulated in oyster tissues. Saxitoxin (STX), from which PSTs are derived, has been reported to affect prokaryotic organisms, for example modulating metabolic activity of bacteria (Pomati et al., 2003). PSTs could also affect entry mechanisms of the viral particle in host cells or inhibit the viral lytic cycle stage during which massive replication of the viral DNA occurs (Boehmer and Nimonkar, 2003; Honess and Roizman, 1974). As a result, latent stage, i.e. asymptotic phase

during which OsHV-1 DNA can be undetectable (Petton et al., 2013), would be prolonged. Additionally, cells of *Alexandrium* spp. have been reported to produce extracellular compounds with allelopathic, hemolytic, ichtyotoxic, and oxidative properties, that can have deleterious effects upon different target cells (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011). These compounds have been shown to affect hemocytes of bivalves (Ford et al., 2008), a cell type targeted by OsHV-1 (Hine and Thorne, 1997; Renault et al., 2011; Schikorski et al., 2011a), which could thus be affected indirectly. Toxic effects of these extracellular compounds upon pathogens have been reported to modify host-pathogen interactions. For example, the extracellular products of the dinoflagellate *Karenia selliformis* induced mortality and morphological changes in the parasite *Perkinsus olseni*, likely explaining the lower intensity of infection detected in Manila clams exposed to this alga (da Silva et al., 2008).

Toxic effects of extracellular compounds of *A. catenella* toward OsHV-1 particles also could affect transmission process directly in the water column. In fact, Paul-Pont et al. (2013) and Evans et al. (2014) hypothesized that horizontal transmission of OsHV-1 would be promoted by planktonic cells carrying the virus. Lytic or oxidative effects of extracellular compounds upon the OsHV-1 particle-lipid-containing envelope (Mettenleiter, 2002) thus could have decreased the viral particle load carried by algal cells and consequently reduced the viral load entering the host organism.

Another hypothesis to explain a lower transmission of OsHV-1 particles in oysters exposed to *A. catenella,* is based upon the effects of *Alexandrium* spp. exposure upon bivalve feeding activities. Indeed, filtration, clearance, and ingestion rates were reported to be reduced during exposure to toxigenic algae (Cucci et al., 1985; Lassus et al., 1999; Shumway and Cucci, 1987). Such effects could limit the introduction of viral particles into the organism. A similar, hypothetic relationship between filtration rate and herpesvirus load has already been proposed by Schikorski et al. (2011a).

Challenge of Specific Pathogen-Free (SPF) oysters by cohabitation with field-exposed oysters led to a lower PST accumulation after exposure to *A. catenella*, compared with unchallenged oysters. A higher feeding rate of field-exposed oysters is not likely the cause of this result, as digestive gland index did not vary either during field-exposure or over the course of the

experiment. The difference in PST accumulation suggests that exposure to an environment with pathogens either: (i) affected filtration, clearance, or ingestion rates of the oysters, or the digestive processes resulting in different PST load; or (ii) reduced the availability and / or the digestibility of the *A. catenella* cells.

The first hypothesis involves nutrition and digestion processes. Recent results of an experimental study suggested that OsHV-1 can affect food ingestion and absorption, as lower sterol contents were observed in oysters exposed to pathogens agents associated with mortality events (Tamayo et al., 2014). These authors also described oyster responses that mimicked food deprivation associated with the disease, as indicated by the utilization of proteins for energetic requirements following glycogen depletion. Depleted energetic reserves, coincident with mass mortality events in the field (Pernet et al., 2014), could be a consequence of stimulation of glycolysis and lipolysis by OsHV-1 infection, as suggested by a recent proteomic study (Corporeau et al., 2014). Additionally, ulcerative lesions of mantle and gill epithelium were observed in OsHV-1-infected oysters *C. gigas*, in Australia (Jenkins et al., 2013), possibly interfering with filtration and sorting involved in feeding. Altogether, findings from the present and recent studies could indicate alteration of feeding processes and digestive functions by viral or bacterial infections affecting *A. catenella* consumption by challenged oysters.

Finally, as mentioned previously, the interaction of *A. catenella* cells with micro-organisms carried by-field exposed oysters could have reduced availability or digestibility of the algal cells prior to filtration. In fact, bacteria are known to produce bio-active compounds that can have algicidal properties and can thus degrade harmful algal physiology (see review of Doucette, 1995). In addition, when subjected to a stress, dinoflagellates are able to rapidly form temporary cysts, which can still be ingested by filter feeders; however, impenetrable cyst walls protect them from being digested and thus limit toxin release (Hégaret et al., 2008; Laabir et al., 2007; Persson et al., 2006).

## 5 Conclusions

This study revealed that *A. catenella* can significantly impact the *C. gigas* – herpesvirus interaction. Additionally, the herpes virus infection, and / or putative associated microbiota, can change interactions between oysters and *A. catenella* and decrease oyster PST accumulation, demonstrating the complexity of such tripartite interaction. Responses of oysters may result from direct interactions between OsHV-1 and *A. catenella* and their toxic compounds, or may be the consequence of immune and physiological responses to the virus and / or the microalga.

The results show that *A. catenella* could partially inhibit transmission or proliferation of herpesvirus if a bloom occurs at the initial stage of the infection. The effect upon mortality events, however, remains to be investigated *in situ*, although these results suggest that an *A. catenella* bloom could delay the mortality outbreaks.

Further research focusing on direct interactions between toxic dinoflagellates and OsHV-1, as well as on oyster immune- and physiologically-related processes would be needed to better understand the mechanisms involved in this tripartite interaction and better project consequences upon oyster industry.

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# Chapitre 2

## Quelles sont les effets combinés d'une exposition à *Alexandrium* sp. et de parasites sur des bivalves naturellement infectés, ainsi que leurs implications physiologiques ?

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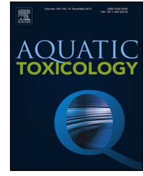

# Physiological responses of Manila clams *Venerupis* (=*Ruditapes*) *philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure

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ABSTRACT

Manila clam stock from Arcachon Bay, France, is declining, as is commercial harvest. To understand the role of environmental biotic interactions in this decrease, effects of a toxic dinoflagellate, *Alexandrium ostenfeldii*, which blooms regularly in Arcachon bay, and the interaction with perkinsosis on clam physiology were investigated. Manila clams from Arcachon Bay, with variable natural levels of perkinsosis, were exposed for seven days to a mix of the nutritious microalga T-*Iso* and the toxic dinoflagellate *A. ostenfeldii*, a producer of spirolides, followed by seven days of depuration fed only T-*Iso.* Following sacrifice and quantification of protozoan parasite *Perkinsus olseni* burden, clams were divided into two groups according to intensity of the infection ("Light–Moderate" and "Moderate–Heavy"). Hemocyte and plasma responses, digestive enzyme activities, antioxidant enzyme activities in gills, and histopathological responses were analyzed. Reactive oxygen species (ROS) production in hemocytes and catalase (CAT) activity in gills increased with *P. olseni* intensity of infection in control clams fed T-*Iso*, but did not vary among *A. ostenfeldii*-exposed clams. Exposure to *A. ostenfeldii* caused tissue alterations associated with an inflammatory response and modifications in hemocyte morphology. In the gills, superoxide dismutase (SOD) activity decreased, and an increase in brown cell occurrence was seen, suggesting oxidative stress. Observations of hemocytes and brown cells in tissues during exposure and depuration suggest involvement of both cell types in detoxication processes. Results suggest that exposure to *A. ostenfeldii* disrupted the pro-/anti-oxidant response of clams to heavy *P. olseni* intensity. In addition, depressed mitochondrial membrane potential (MMP) in hemocytes of clams exposed to *A. ostenfeldii* suggests that mitochondrial functions are regulated to maintain homeostasis of digestive enzyme activity and condition index.
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## 1. Introduction

The Manila clam *Venerupis* (=*Ruditapes*) *philippinarum* is one of the most exploited bivalves in the world. Endemic to Indo-Pacific waters, it was introduced to the coast of France in the early 1970s for commercial purpose (Flassch and Leborgne, 1992). The Manila clam population from Arcachon Bay ranked first in France until 2012 (among the surveyed stocks) in terms of biomass (7200 t) and exploitable stock (720 t) (Sanchez et al., 2013). The exploitable stock, however, is declining (1159 t in 2008, 916 t in 2010), associated with decreasing harvests (Sanchez et al., 2013), causing a crisis for commercial fishermen. In this context, studies were conducted to understand the reason for this decrease and improve

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stock management. Although fishing is partially responsible (Dang et al., 2010b), effects of environmental factors remain to be investigated.

This population of clams is infected by the protozoan parasite *Perkinsus olseni*, with prevalences reaching high values (>90%) (Dang et al., 2010a). The parasite *P. olseni* resides in the clam connective tissue and usually induces hemocytic infiltration and parasite encapsulation that may lead to milky-white cysts or nodules in heavily-infected clams (Choi et al., 2002; Lee et al., 2001). Immune responses of clams *V. philippinarum* to *P. olseni* infection involve changes in humoral and hemocyte characteristics (Soudant et al., 2013). Although *P. olseni* infection was associated with massive mortalities in cultured and wild Manila clams in South Korea (Choi and Park, 2010, 1997; Park and Choi, 2001), this parasite was not reported to affect clam populations along the French Atlantic coast severely (Dang, 2009).

Concurrently, Arcachon Bay is a site of recurring Harmful Algal Blooms (HABs). The "French Phytoplankton and Phycotoxin Monitoring Network" (REPHY, Ifremer) detects the repeated presence of *Dinophysis* sp., *Pseudo-nitzschia* sp. and *Alexandrium* sp., associated to DSP (Diarrheic Shellfish Poison), ASP (Amnesic Shellfish Poison), and PSP (Paralytic Shellfish Poison) toxins, respectively. For the first time in 2005, spirolides, a group of macrocyclic imine toxins, were detected in shellfish from Arcachon Bay (Amzil et al., 2007). These toxins were coincident with *Alexandrium ostenfeldii* blooms (data from REPHY). Despite the absence of toxic effects of spirolides reported to date in humans, these toxins are considered to be "fast-acting toxins" because of acute toxicity to mice following intra-peritoneal injection (Hu et al., 1995). Although mechanisms of spirolide action are not fully elucidated, studies focusing mainly on 13-desmethyl-C spirolide suggested that these compounds are antagonists of cholinergic nicotinic receptors in mammalian systems (Bourne et al., 2010; Gill et al., 2003; Hauser et al., 2012; Wandscheer et al., 2010), causing neurotoxic symptoms (Gill et al., 2003; Munday et al., 2012; Otero et al., 2012; Richard et al., 2001). To date, only one study documented the effect of *A. ostenfeldii* exposure on bivalves, showing that ingestion of this dinoflagellate by the Pacific oyster *Crassostrea gigas* caused an inflammatory response in the digestive gland, which is the main organ accumulating spirolides (Medhioub et al., 2012). Effects of other HAB species upon bivalves have been reported at different biological levels. Various effects upon general physiological processes (Cucci et al., 1985; Gainey and Shumway, 1988a,b; Landsberg, 2002; Leverone et al., 2007; Shumway, 1990), on behavior (Gainey and Shumway, 1988b; Haberkorn et al., 2011; Hégaret et al., 2012; Shumway and Cucci, 1987; Tran et al., 2010), at tissue and cellular levels (da Silva et al., 2008; Galimany et al., 2008a,b,c; Haberkorn et al., 2010a,b; Hégaret et al., 2007a,b,c), and more recently at the molecular level (Estrada et al., 2007; Fabioux et al., submitted; Mello et al., 2013, 2012; Romero-Geraldo et al., 2012) have been observed. The first possible defense of bivalves against toxic or noxious particles in water is to close the valves and reduce filtration to minimize contact between microalgae and tissues (Gainey and Shumway, 1988b). There is, however, a balance between this protective response and the need for respiration and nutrition; consequently shell closure can be but a temporary response. The next line of defense in molluscan shellfish to noxious, harmful, or pathogenic agents is the immune system, involving humoral and cellular hemocyte responses which mediate internal defense mechanisms (Cheng, 1996; Song et al., 2010). Effects of HABs upon hemocyte morphology and functions have been reported (Hégaret and Wikfors, 2005a,b; Hégaret et al., 2007a,c; da Silva et al., 2008; Galimany et al., 2008a,b,c; Haberkorn et al., 2010a). Only few studies, however, have reported the effects of simultaneous exposures to HABs and pathogens upon bivalve physiology, although this phenomenon is common in the natural environment. Modifications of the host–pathogen interaction were observed after exposure of parasitized bivalves to toxic dinoflagellates (da Silva et al., 2008; Hégaret et al., 2009; Bricelj et al., 2011). In addition, the interaction of HABs and pathogens could lead to synergistic outcomes in bivalve physiology, affecting hemocyte functions (Bricelj et al., 2011; Hégaret et al., 2009, 2007a), but also inducing higher bivalve mortality rate, suggesting defeat of the defense system (Bricelj et al., 2011).

Manila clams from Arcachon Bay are likely to be subjected simultaneously to both perkinsosis and *A. ostenfeldii* exposure. This situation raises three questions: (i) What are the effects of different perkinsosis intensities? (ii) How does exposure to *A. ostenfeldii* affect clam physiology? (iii) Does exposure to *A. ostenfeldii* modifiy the host–pathogen interaction? To answer these questions, the effects upon different physiological responses of Manila clams caused by (i) varying natural *P. olseni* infection levels; (ii) experimental exposure of clams to *A. ostenfeldii*; and (iii) the combined effects of both biotic factors, were investigated. To describe the physiological responses of clams and identify the mechanisms underlying these responses, different biological levels and functions were studied, from individual to gene, with specific focus on digestive, immune, and mitochondrial functions, and pro-/anti-oxidative mechanisms.

## 2. Materials and methods

### 2.1. Experimental clams

Manila clams, *V. philippinarum* of 32.7 ± 0.3 mm shell length and 8.8 ± 0.2 g live weight (mean ± SE), were collected from "Estey de Tessillat" station in Arcachon Bay, France, where high prevalence of *P. olseni* was recorded previously (Dang, 2009). Manila clams were collected on May 17th, 2010 and transferred the next day to IUEM, Plouzané, France, where the experiment was performed.

### 2.2. Algal cultures

The microalga *Tisochrysis lutea* (El M. Bendif and I. Probert) (T-*Iso*), commonly provided as an aquaculture food for bivalves, was obtained from the Argenton hatchery (Ifremer, France) and was used as a control and a complementary diet for the experiment. T-*Iso* was cultured in f/2 medium (Guillard and Ryther, 1962) in 300-L open tanks, at 18 °C with 24-h light. Batch cultures of T-*Iso* were harvested after 3 to 5 days of growth, usually at a cell density approaching $5 \times 10^6$ cells mL$^{-1}$.

The Phycotoxin laboratory, Ifremer, Nantes (France), provided the toxic dinoflagellate *Alexandrium ostenfeldii*, strain CCMP1773 (isolated from Denmark), which is known to produce exclusively one type of toxin: spirolides (Otero et al., 2010). This strain was grown in L1 medium with soil extract (Guillard and Hargraves, 1993), and cultures were maintained at 16 °C with 12-h light in 20-L carboys. Cells were harvested in stationary phase, usually at a cell density approaching 5–7 $\times 10^3$ cells mL$^{-1}$.

Algal cell densities were determined by counts using Nageotte cells under a light microscope.

### 2.3. Experimental design and sampling

One-hundred and twenty-eight clams were distributed randomly in eight 20-L tanks (16 clams per tank) containing a 7-cm sediment layer of ultra-pure SILAQ silica sand (granulometry from 0.8 to 1.4 mm diameter) to allow clams to burrow. Before starting the experiment, clams were acclimated for one week, fed T-*Iso* ($5 \times 10^5$ cells mL$^{-1}$), in the 1-μm filtered, flow-through seawater system used for the experiment (15 L per 24 h per tank, *i.e.*, 10 mL min$^{-1}$), at 16 ± 1 °C, a temperature compatible with the

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transmission and development of infection by *P. olseni* (Villalba et al., 2004).

Acclimation was followed by seven days of exposure to two different treatments with four replicates (*i.e.*, 4 tanks) per treatment: (i) control clams, fed only T-*Iso*, at a concentration of $5 \times 10^5$ cells $mL^{-1}$; (ii) HAB-exposed clams fed T-*Iso* at the same concentration with an addition of *A. ostenfeldii* at $10^3$ cells $mL^{-1}$. Following this, a depuration period of seven days was applied during which clams were fed only T-*Iso* at $5 \times 10^5$ cells $mL^{-1}$. Six clams per tank were sampled (i) at the end of exposure ($T = 14$); and (ii) at the end of depuration ($T = 21$).

Hemocyte and plasma variables, condition index, histopathological condition, infection with *P. olseni*, activity of antioxidant and digestive enzymes, and toxin accumulation were analyzed at each sampling time.

### 2.4. Analysis of hemocyte and plasma variables

Hemolymph was withdrawn from the adductor muscle of each clam using a needle and a 1-mL syringe, filtered through an 80-μm mesh, and stored temporarily in an Eppendorf microcentrifuge tube held on ice.

Hemocyte morphology and functions were analyzed on hemolymph extracted from each clam. Procedures for characterization of clam hemocytes, concentration (=total hemocyte counts – THC, cells $mL^{-1}$), size, internal complexity and mortality of circulating hemocytes, as well as for functional responses, such as phagocytosis, unstimulated hemocyte production of reactive oxygen species (ROS) (specifically $O_2^{\bullet-}$ and $H_2O_2$), and mitochondrial membrane potential (MMP) were adapted from Delaporte et al. (2003), Soudant et al. (2004) and Donaghy et al. (2012). Analyses of hemocytes were performed with a FACScalibur flow-cytometer (BD Biosciences, San Jose, CA).

In addition, remaining hemolymph was centrifuged (800 × *g*, 5 min, 4 °C) to be separated into two fractions: plasma (supernatant) and hemocytes (pellet), prior to freezing (−20 °C). Plasma was used for agglutination and hemolysis tests with horse red blood cells, following the protocol of Barracco et al. (1999), and expressed as the log (base 2) of the reciprocal of the highest dilution showing a positive pattern of agglutination or hemolysis of erythrocytes, respectively.

### 2.5. Condition index (CI)

Condition index (CI) of the Manila clams was calculated using the wet flesh weight (WFW) in relation to dry shell weight (DSW): CI = (WFW/DSW) × 100 (adapted from Bodoy et al., 1986).

### 2.6. Histopathological observations

For histology, each clam was shucked, and two diagonally-slanted, 5-mm sections of soft tissue, including gills, mantle, digestive gland, intestine, and gonad, were excised. Additionally, a section of adductor muscle was sampled. Tissues were fixed immediately in Davidson's solution (Shaw and Battle, 1957) for 24 h. Tissues then were transferred into 70% ethanol, dehydrated in ascending ethanol solutions, cleared with Claral®, and embedded in paraffin wax. Five-μm sections were stained with Harris' hematoxylin and eosin (Howard et al., 2004) and observed under a light microscope.

A six-level, semi-quantitative scale was established to assess intensity of each histopathological condition observed, from 0 to 2.5, as described in Table 1.

**Table 1**
Semi-quantitative scale categorizing intensity of histopathological conditions observed.

| Level intensity | Occurrence of the pathological condition in the examined tissue area |
|---|---|
| 0 | Absence |
| 0.5 | Very low (1–5 total occurrence) |
| 1 | Low (>5 occurrence/presence in all fields at magnification 10×) |
| 1.5 | Moderate (presence in all fields at magnification 20×/covering about 10% of the tissue) |
| 2 | High (presence in all fields at magnification 40×/covering about 20% of the tissue) |
| 2.5 | Very high (presence in all fields at magnification 60×/covering about 30% or above of the tissue) |

### 2.7. Detection and quantification of infection with P. olseni in V. philippinarum

From each clam, after removing the section for histological studies, a portion of gills was excised and weighed. Gills were incubated in Ray's fluid-thioglycollate medium (RFTM, Ray, 1966) for 7 days in the dark at room temperature and prepared to assess the parasite burden according to the protocol developed by Choi et al. (1989), as described in Hégaret et al. (2009). Briefly, gills were centrifuged (800 × *g*, 10 min) to remove medium and digested with NaOH (2 N, 1 h at 60 °C, twice), followed by two washes in phosphate-buffered saline (PBS) (0.1 M). Finally, the pellet was re-suspended in 1 mL of PBS. Ten microlitres of Lugol's solution was added, and the number of *P. olseni* hypnospores was counted using a Nageotte chamber under a light microscope.

The intensity of infection with *P. olseni* was calculated as the number of hypnospores per gram of wet weight of gill. To run multifactor statistical tests, the level of infection with *P. olseni* was divided into two equally-abundant classes: "Very light–Light" infection class ($<4.2 \times 10^4$ *P. olseni* cells $g^{-1}$ wet gill) and "Moderate–High" infection class ($\geq 4.2 \times 10^4$ *P. olseni* cells $g^{-1}$ wet gills).

### 2.8. Activity of antioxidant enzymes

The rest of the gills were removed and frozen immediately in liquid nitrogen after dissection, and stored at −80 °C. Gill tissues were ground using a "Dangoumeau"-type ball grinder into liquid nitrogen.

#### 2.8.1. Protein extraction

One fraction of 50 mg of ground gills was homogenized, using an ultra-turrax, in 250 μL of cold lysis buffer prepared following Guévélou et al. (2013) [NaCl 150 mM, Tris 10 mM, pH 7.4, EDTA 1 mM, EGTA 1 mM, Phosphatase inhibitor cocktail II (Sigma-Aldrich) 0.01%, Triton X-100 1% v/v, CA-630 Igepal (Sigma-Aldrich) 0.5%, 1 tablet of complete EDTA-free protease inhibitor cocktail (Roche) were added extemporaneously per 25 mL buffer; pH 8.8 at 4 °C] and centrifuged at 10,000*g* for 45 min at 4 °C. The supernatant then was stored at −80 °C until further analysis.

#### 2.8.2. Protein assay

Total protein content in gills was determined using the Bio-Rad DC™ Protein Assay, based upon the method of Lowry et al. (1951) and appropriate for protein extracts containing detergents such as Triton X-100. Bovine serum albumin (Sigma-Aldrich) was used as protein standard. Absorbance at 750 nm was determined after 15 min of incubation at room temperature.

#### 2.8.3. Activity of total superoxide dismutase (SOD), Mn SOD and Cu/Zn SOD

Total superoxide dismutase (SOD; EC 1.15.1.1) activity in gills was measured using an SOD Assay Kit (Sigma-Aldrich) according to manufacturer's instructions. Protein extracts were diluted 40 times

and absorbance at 440 nm was recorded after 20 min incubation at 25 °C. A standard inhibition curve was constructed using SOD from bovine erythrocytes (Sigma-Aldrich).

Mn SOD (=mitochondrial SOD) activity in gills was determined using the SOD Assay Kit and KCN (5 mM final concentration), a known inhibitor of the Cu/Zn SOD (=cytoplasmic SOD) (Fridovich, 1975; Manduzio et al., 2005). Protein extracts were diluted 5 times, and absorbance at 440 nm was recorded after 20 min of incubation at 25 °C.

Cu/Zn SOD activity was evaluated as the difference between total SOD activity and Mn SOD activity.

Total SOD, Mn SOD and Cu/Zn SOD specific activities were expressed in U $mg^{-1}$ total proteins, one unit being defined as the amount of enzyme inhibiting by 50% the xanthine/xanthine oxidase complex.

#### 2.8.4. Activity of catalase (CAT)

Catalase (CAT; EC 1.11.1.6) activity in gills was determined following the method of Aebi (1984). Decrease in absorbance at 240 nm attributable to the decomposition of $H_2O_2$ (10 mM) was recorded at 9-s intervals for 90 s. Protein extracts were diluted 200 times and CAT-specific activities were expressed in U $mg^{-1}$ total proteins, one unit of CAT being defined as the amount of enzyme catalyzing 1 μmol of $H_2O_2$ $min^{-1}$.

### 2.9. Activity of digestive enzymes

#### 2.9.1. Protein extraction

Digestive gland stored at −80 °C immediately after sampling was homogenized using a Potter in phosphate buffer 0.02 M (0.02 M $NaH_2PO_4$/0.02 M $Na_2HPO_4$) (100 mg wet weight $mL^{-1}$), and centrifuged at 4000 × g for 30 min. Supernatant was stored at −80 °C until further analyses.

#### 2.9.2. Protein assay

To assess specific activity of digestive enzymes, protein extracts were diluted at 1/20 in phosphate buffer 0.02 M, and total protein content was determined with a Bio-Rad Protein Assay, based on the Bradford (1976) method using Bovine Serum Albumin as a protein standard.

#### 2.9.3. Activity of amylase, cellulase and laminarinase

Activities of digestive enzymes were assessed using the method of Bernfeld (1955) slightly modified. Activities of amylase, cellulase and laminarinase were measured, by incubating diluted protein supernatants at 37 °C (dilution in phosphate buffer 0.02 M: 1/20 for amylase, 1/2 for cellulase, 1/4 for laminarinase) with the sugar they reduce: starch (30 min of incubation), cellulose (1 h), and laminarin (1 h), respectively. After the reaction was stopped by adding 250 μL of 3,5-dinitrosalicylic acid (3,5-DNS), samples were boiled for 15 min to enable the reduction of 3,5-DNS in 3-amino-5-nitrosalicylic acid, producing a coloration proportional to the quantity of sugar reduced, and absorbance was read spectrophotometrically at 540 nm. Specific activity was determined using maltose standards ranging from 0.2 to 1.0 mg $mL^{-1}$ and expressed in mg of maltose liberated $mg^{-1}$ proteins $h^{-1}$.

### 2.10. Toxin accumulation

Accumulation of spirolides was measured at each sampling time on 8 exposed individuals (2 per tank) and 2 control individuals in two fractions of soft tissue: digestive gland and remaining tissues (including siphon, foot, gills, adductor muscle and mantle), that had been frozen in liquid nitrogen immediately after sampling, then stored at −80 °C. Lipophilic toxins were extracted in methanol/water (95/5) and analyzed by LC–MS/MS at the EMP/PHYC laboratory of Ifremer, Nantes, France, as described in Amzil et al. (2007) and Medhioub et al. (2012).

### 2.11. Statistical analyses

*T*-test ($\alpha = 0.05$) was used to compare total spirolide accumulation between clams from algal treatments (T-*Iso vs. A. ostenfeldii*), sampling times (exposure *vs.* depuration), or tissues (digestive gland *vs.* remaining tissues), and one-way ANOVA followed by Tukey HSD post-hoc test ($\alpha = 0.05$) was used to assess differences in accumulation between the three spirolide analogues.

To assess the effect of time of experiment (exposure or depuration), algal treatment, and level of infection with *P. olseni* and their interactions on hemocyte variables and enzyme activities, a 3-way ANOVA (type 3 Sum of Squares) was performed, in which above factors were independents. After determining that the effect of the time during experiment was not significant, data from algal treatments and perkinsosis categories were analyzed using values from all sampling times of the experiment combined. In addition, to identify specific responses upon exposure or depuration, data were analyzed separately at each sampling time, using a 2-way ANOVA (type 3 Sum of Squares), in which algal treatment and level of infection with *P. olseni* were independent factors. When the effect of the interaction was significant, the Tukey HSD post-hoc test was used to identify differences between treatment means. Effects attributable to *P. olseni* burden were combined from the duration of the experiment.

When needed, data were transformed (log or 1/*X*) prior to analysis to meet homoscedasticity and normality assumptions.

For histopathology results, a unilateral Mann–Whitney *U* test ($\alpha = 0.1$) was used to assess the effect of *P. olseni* burden over the duration of the experiment, and to assess the effect of algal treatment over the entire time of experiment, and separately after exposure and after depuration period.

Statistics were performed with Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA).

## 3. Results

### 3.1. *Perkinsus olseni* burden in clam gills

Prevalence of perkinsosis in experimental clams was 100%, and mean intensity of infection was $7.7 \pm 0.9 \times 10^4$ cells $g^{-1}$ wet gills (mean ± SE). The mean intensity of infection of each class, "Very light–Light" (46 clams) and "Moderate–High" (45 clams), was $1.3 \pm 0.2 \times 10^4$ and $14 \pm 1 \times 10^4$ *P. olseni* cells $g^{-1}$ wet gills (mean ± SE), respectively.

*P. olseni* burden was not significantly affected by exposure to *A. ostenfeldii* but showed a decreasing trend. The mean intensity of infection of all clams sampled was $6.6 \pm 1.0 \times 10^4$ cells $g^{-1}$ wet gills (47 clams) in *A. ostenfeldii* exposed individuals, and was $8.8 \pm 1.6 \times 10^4$ cells $g^{-1}$ wet gills (44 clams) in control clams (mean ± SE).

### 3.2. Toxin quantification

Three spirolide analogues were identified and quantified within the tissues: 13,19-didesmethyl-C (13,19-didesMeC), 13-desmethyl-C (13-desMeC) and 13-desmethyl-D (13-desMeD). Mean accumulation of total spirolides was significantly higher in the digestive gland than in the remaining tissues at both sampling times ($p < 0.001$). In both digestive gland and remaining tissues, and at both sampling times, accumulation of total spirolides was significantly higher in dinoflagellate-exposed individuals ($4.0 \pm 0.7 \times 10^2$ μg 13-desMeC eq. $kg^{-1}$ in digestive gland

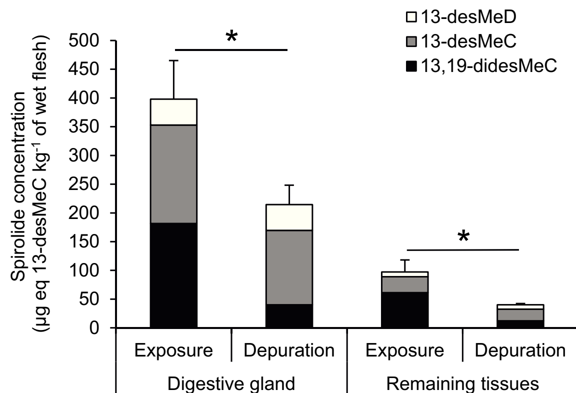


**Fig. 1.** Spirolide analogue content in digestive gland and in the remaing tissues of Manila clams *V. philippinarum* exposed to a mix of T-*Iso* and *A. ostenfeldii* for 7 days ("Exposure"), followed by 7 days of depuration fed T-*Iso* ("Depuration"). Error bars represent standard errors of total spirolide content. Significant differences in total spirolide content between exposure *vs.* depuration in each tissue are indicated by *: $p < 0.05$ (*t*-test). $N = 8$ in each group.

and $2.1 \pm 0.3 \times 10^2$ µg 13-desMeC eq. $kg^{-1}$ in remaining tissues) compared to controls, in which only 13-desMeC was detected ($2.9 \pm 0.1 \times 10^1$ µg 13-desMeC eq. $kg^{-1}$ in digestive gland and $2.6 \pm 0.2 \times 10^1$ µg 13-desMeC eq. $kg^{-1}$ in remaining tissues) ($p < 0.05$ in digestive gland at both sampling times; $p < 0.01$ after exposure in remaining tissues; $p < 0.001$ after depuration in remaining tissues). Among spirolide analogues, only the 13,19-didesMeC decreased significantly after seven days of depuration in both digestive gland and remaining tissues of *A. ostenfeldii*-exposed clams ($p < 0.001$ in digestive gland; $p < 0.01$ in remaining tissues), leading to a significant decrease in total spirolide content in both digestive gland and remaining tissues ($p < 0.05$) (Fig. 1).

### 3.3. Condition index, hemocyte and plasma variables, digestive and antioxidant enzyme specific activities

Results of statistical analyses are summarized in Table 2. Effects of algal treatment are presented over the entire experiment (both sampling times combined), after 7 days exposure, and after 7 days of depuration. Effects attributable to *P. olseni* burden are presented over the entire experiment as well as effects of algal treatment and *P. olseni* burden interaction (because no significant effect was detected at each sampling time).

#### 3.3.1. Condition index

Condition index of experimental clams did not vary with algal treatment or intensity of *P. olseni* infection.

#### 3.3.2. Hemocyte and plasma analyses

Although granulocytes and hyalinocytes have already been described by cytochemical assays in Manila clams (Cima et al., 2000), morphological distinction between clam hemocyte types using flow cytometry is not always successful (Donaghy et al., 2009). Consequently, in this study, granulocytes and hyalinocytes were analyzed together.

Total hemocyte count (THC), internal complexity of hemocytes, phagocytosis, hemocyte mortality, agglutination and hemolysis titer did not vary significantly between the different experimental treatments.

Exposure to *A. ostenfeldii* led to an increase in the mean size of hemocytes, which was significant after exposure and over the duration of the experiment (Fig. 2A). Mean size of hemocytes also increased significantly with *P. olseni* burden, from 133 ± 1 a.u. (arbitrary units) in "Very lightly–Lightly" infected clams to 137 ± 1 a.u. in "Moderately–Highly" infected clams (mean ± SE).

Mitochondrial membrane potential (MMP) of hemocytes decreased significantly in clams exposed for 7 days to *A. ostenfeldii* (Fig. 2B).

A significant interaction of both algal treatment and *P. olseni* burden was detected throughout the experiment for ROS production in hemocytes. In individuals exposed to the non-toxic T-*Iso*, ROS production in "Very lightly–Lightly" infected clams was significantly lower than in "Moderately–Highly" infected clams (Fig. 2C).

#### 3.3.3. Digestive enzyme specific activities

No significant effect was observed on specific activities of amylase, cellulase or laminarinase for algal treatment or intensity of *P. olseni* infection.

#### 3.3.4. Antioxidant enzyme activities in gills

Total SOD specific activity was significantly lower in gills of clams exposed to *A. ostenfeldii* over the entire experiment, mostly attributable to the significant difference detected after depuration between control and exposed animals (Fig. 3A). The specific activites of both SOD isoforms Cu/Zn and Mn SOD, comprised in total SOD, were also significantly lower in gills of clams exposed to *A. ostenfeldii* throughout the experiment.

A significant effect of the interaction of *P. olseni* burden and algal treatment was identified (Fig. 3B) upon specific activity of CAT in gills. After exposure to T-*Iso*, CAT specific activity was higher in "Moderately–Highly" infected clams than in "Very lightly–Lightly" infected ones, but this response was not observed in *A. ostenfeldii*-exposed clams.

### 3.4. Histopathological effects

Effects of algal treatment over the entire experiment (both sampling times combined), after seven days of exposure and after seven days of depuration, and effects of *P. olseni* burden upon histological features of experimental clams are reported in Table 3.

To identify effects of *A. ostenfeldii* exposure upon clam tissues, intensities of pathologies first were compared between all clams exposed to *A. ostenfeldii* and all control clams sampled throughout the experiment. Exposure to *A. ostenfeldii* caused a decrease in hemocyte infiltration into the digestive gland ($p < 0.05$), as well as an increase in edema in the mantle ($p < 0.05$), vacuolation in the gills ($p < 0.1$) (Fig. 4A and B), hemocyte diapedesis into the intestine ($p < 0.05$) (Fig. 4D and E), and epithelium sloughing into the stomach lumen ($p < 0.1$). These pathologies were associated with observations of *A. ostenfeldii* cells within the intestinal lumen (Fig. 4E) after both exposure and depuration in exposed clams.

After seven days of exposure to *A. ostenfeldii*, hemocyte infiltration in gills ($p < 0.05$) (Fig. 5A), edema in the mantle ($p < 0.05$) (Fig. 5B), and epithelium sloughing into the intestinal lumen increased ($p < 0.05$) (Fig. 5C), while hemocyte infiltration into the digestive gland decreased ($p < 0.01$). In addition, *A. ostenfeldii* cells were observed in digestive tubules of one individual after seven days of exposure (Fig. 4F). After seven days of depuration, an increased intensity of cell vacuolation in gill filaments ($p < 0.1$) (Figs. 4A and B and 5D) and of brown cells ($p < 0.1$) (Figs. 4A and C and 5E), as well as an increased number of hemocytes in the intestinal lumen (Fig. 5F), were observed in *A. ostenfeldii*-exposed clams.

Histopathological observations showed that clams with "Moderate–Heavy" *P. olseni* burden, had more intense vacuolation in digestive tubules ($p < 0.1$) (Fig. 4G and H) compared to "Very lightly–Lightly" infected individuals, as well as more hemocytic encapsulation bodies in gonadal tissue ($p < 0.1$) and in the digestive gland ($p < 0.1$) (Fig. 4I). These pathologies were associated with a

**Table 2**
Comparison of physiological variables measured in *V. philippinarum* (i) over the entire experiment, (ii) after 7 days of exposure to *A. ostenfeldii* or to control T-*Iso*, and (iii) after 7 days of depuration (fed only T-*Iso*). NS: no significant difference; * Significant difference indicated by $p < 0.05$; ** Significant difference indicated by $p < 0.01$ (2-way ANOVA); "–": no analysis over the entire experiment because there was a significant effect of time of sampling (exposure *vs.* depuration); between brackets: significant effect after exposure and after depuration are indicated separately because of the absence of analysis over the entire experiment since there was a significant effect of time of sampling (exposure *vs.* depuration).

| Physiological parameters | Over the entire experiment | | | | | | After seven days of exposure | | After seven days of depuration | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Factor algal treatment | | Factor *P. olseni* burden | | Interaction | | Factor algal treatment | | Factor algal treatment | |
| | *p* | *N* | *p* | *N* | *p* | *n* | *p* | *n* | *p* | *N* |
| Condition index | NS | 44 | NS | 44 | NS | 21–23 | NS | 23–24 | NS | 20–21 |
| Hemocyte and plasma parameters | | | | | | | | | | |
| Phagocytosis (% of phagocyting hemocytes) | – | – | (NS; NS) | (18–27; 16–26) | (NS; NS) | (8–16; 8–13) | NS | 21–24 | NS | 21 |
| Mortality of hemocytes | – | – | (NS; NS) | (16–27; 16–27) | (NS; NS) | (8–16; 8–15) | NS | 19–24 | NS | 20–23 |
| Hemocyte ROS production | NS | 31–47 | * | 38–40 | * | 17–24 | NS | 15–24 | NS | 16–23 |
| Hemocyte MMP | – | – | (NS; NS) | (18–29; 16–28) | (NS; NS) | (8–16; 8–15) | ** | 23–24 | NS | 21–23 |
| THC | NS | 40–47 | NS | 43–44 | NS | 19–24 | NS | 20–24 | NS | 20–23 |
| Size of hemocytes | ** | 44–47 | * | 45–46 | NS | 21–24 | * | 23–24 | NS | 21–23 |
| Complexity of hemocytes | NS | 44–47 | NS | 45–46 | NS | 21–24 | NS | 23–24 | NS | 21–23 |
| Agglutination titer | NS | 32–43 | NS | 37–38 | NS | 15–23 | NS | 17–23 | NS | 15–20 |
| Hemolysis titer | – | – | (NS; NS) | (16–24; 14–21) | (NS; NS) | (8–16; 6–12) | NS | 17–23 | NS | 15–20 |
| Antioxidant enzymes specific activity (gills) | | | | | | | | | | |
| CAT | NS | 20 | NS | 20 | * | 9–11 | NS | 9–10 | NS | 10–11 |
| Total SOD | ** | 19–21 | NS | 19–21 | NS | 9–12 | NS | 9–10 | * | 10–11 |
| Cu/Zn SOD | * | 17–20 | NS | 17–20 | NS | 8–11 | NS | 8–9 | NS | 9–11 |
| Mn SOD | ** | 17–20 | NS | 17–20 | NS | 8–11 | NS | 8–9 | * | 9–11 |
| Digestive enzyme specific activity (digestive gland) | | | | | | | | | | |
| Amylase | NS | 12–14 | NS | 11–15 | NS | 5–9 | NS | 6–7 | NS | 8–5 |
| Cellulase | NS | 12–14 | NS | 11–15 | NS | 5–9 | NS | 6–7 | NS | 8–5 |
| Laminarinase | NS | 12–14 | NS | 11–15 | NS | 5–9 | NS | 6–7 | NS | 8–5 |

ROS: reactive oxygen species; MMP: mitochondrial membrane potential; THC: total hemocyte count; CAT: catalase; SOD: superoxide dismutase.

higher number of *Perkinsus* cells observed in the digestive gland ($p < 0.05$) (Fig. 4I).

Exposure to *A. ostenfeldii* resulted in a decrease in *Perkinsus* cells observed in clam tissues. Indeed, significantly fewer *Perkinsus* cells were observed in gills of clams throughout the experiment ($p < 0.1$), in mantle ($p < 0.01$) after seven days of exposure, and in adductor muscle ($p < 0.1$) after the depuration period, compared to clams fed to T-*Iso*.

## 4. Discussion

This study revealed physiological and histological consequences of variable parasite, *Perkinsus olseni*, infestation intensities upon Manila clams, *Venerupis philippinarum*, effects of exposure to the toxic dinoflagellate *Alexandrium ostenfeldii* upon the clams, and interactive effects as well. Consideration of two microbiological interactions in individual clams highlights the fact that these interactions occur in nature, although they may not be considered in controlled experiments that are designed to eliminate potentially confounding independent variables. Considering parasite and harmful algal effects individually and combined provided a better indication of the compound challenges that confront clams in nature, but this consideration also makes untangling causes and effects a complex task. Accordingly, we proceed by discussing individual responses of clams to parasitism or to harmful-algal-exposure, finally interpreting interactive responses.

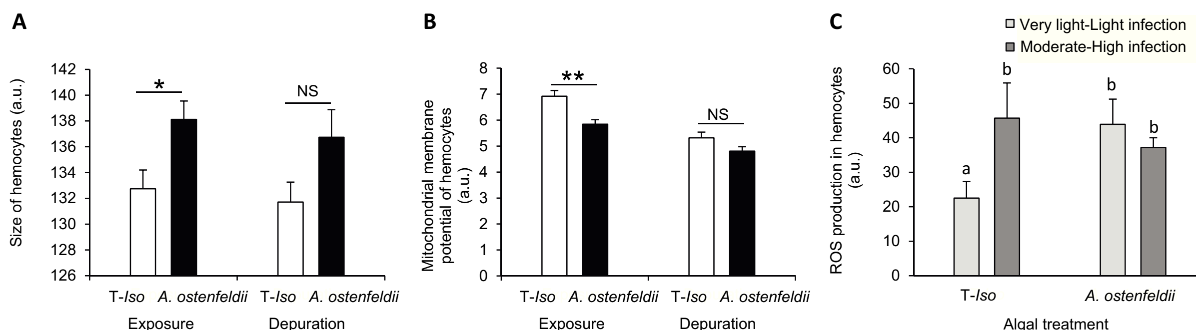


**Fig. 2.** Circulating hemocyte variables of Manila clams *V. philippinarum* exposed to T-*Iso* (control) or to a mix of T-*Iso* and *A. ostenfeldii*. (A) Size of hemocytes ($n = 21–24$ per group) and (B) mitochondrial membrane potential (MMP; $n = 8–16$ per group) after 7 days of dinoflagellate exposure ("Exposure") and after 7 days of depuration with the diet T-*Iso* ("Depuration"); (C) hemocyte ROS production according to algal treatment and to *P. olseni* burden ("Very light–Light" or "Moderate–Heavy"), over 7 days of dinoflagellate exposure followed by 7 days of depuration with the diet T-*Iso* ($n = 17–24$ per group). Error bars represent standard errors. * And letters indicate significant difference between groups, NS indicates no significant difference (ANOVA followed by post-hoc Tukey HSD test, $\alpha = 0.05$). a.u.: Arbitrary units.

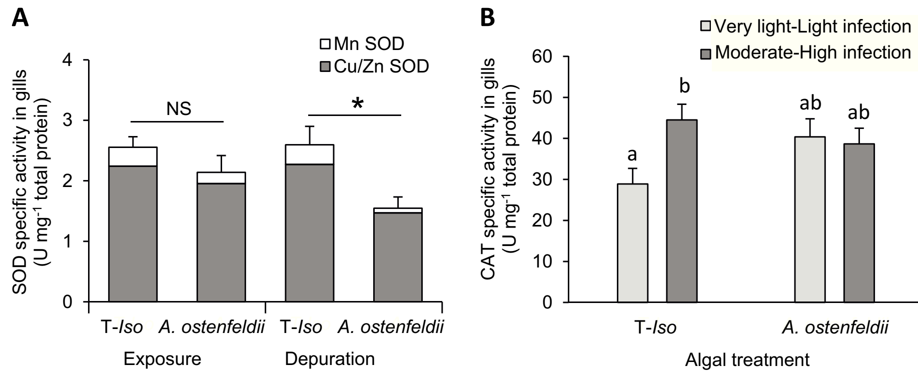


**Fig. 3.** Specific activity of antioxidant enzymes of Manila clams *V. philippinarum* exposed to T-*Iso* (control) or to a mix of T-*Iso* and *A. ostenfeldii*. (A) SOD specific activity after exposure and after depuration ($n = 8$–11 per group); (B) CAT specific activity according to algal treatment and *P. olseni* burden ("Very light–Light" or "Moderate–Heavy"), over 7 days of algal exposure followed by 7 days of depuration with T-*Iso* ($n = 9$–11 per group); Error bars represent standard errors. * And letters indicate significant difference between groups, NS indicates no significant difference (ANOVA followed by post-hoc Tukey HSD test, $\alpha = 0.05$).

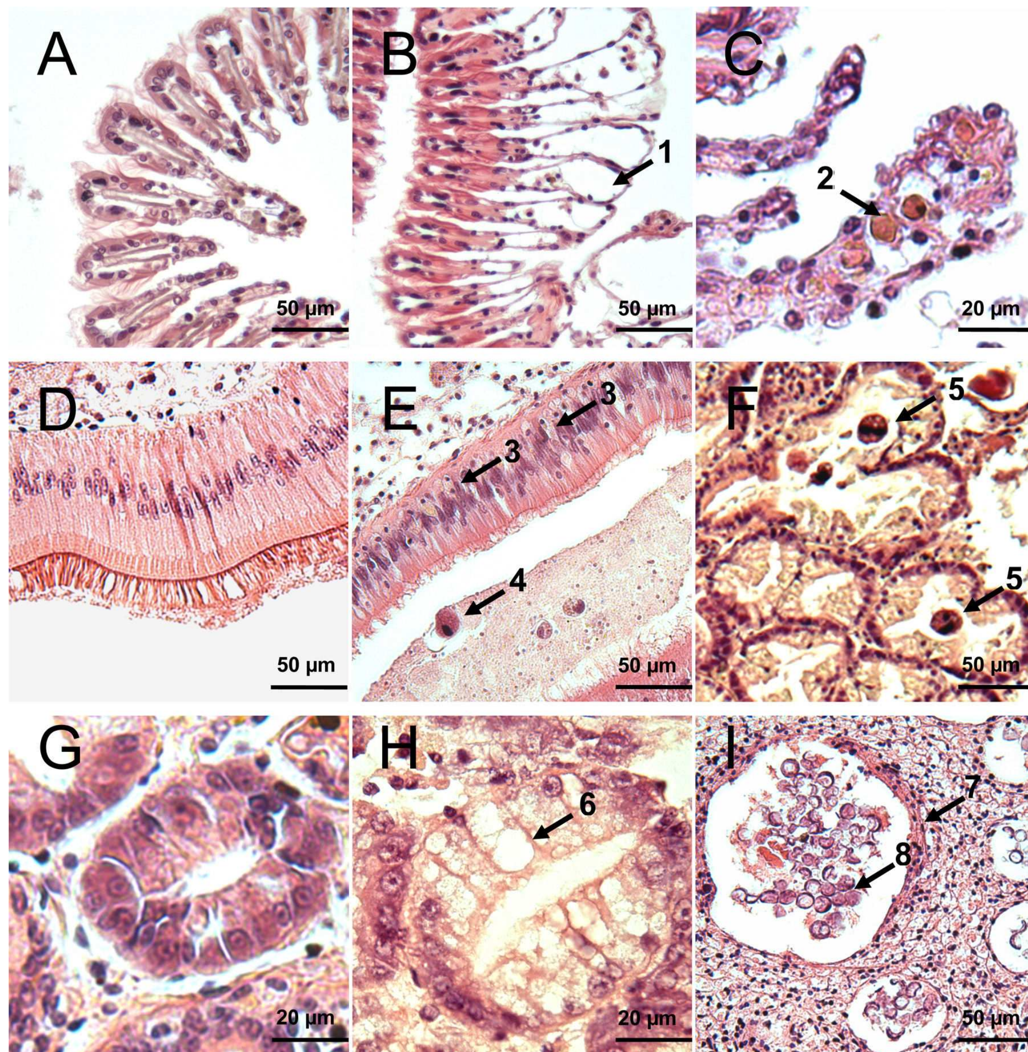


**Fig. 4.** Histopathological conditions. (A) Gills of control clam; (B) vacuolation (1) of gill filaments in *A. ostenfeldii*-exposed clam; (C) brown cells (2) in gills of *A. ostenfeldii*-exposed clam; (D) intestinal epithelium of control clam; (E) diapedesis of hemocytes (3) in intestinal epithelium and *A. ostenfeldii* cell (4) in intestinal lumen of *A. ostenfeldii*-exposed clam; (F) *A. ostenfeldii* cells (5) in digestive tubule lumen of one *A. ostenfeldii*-exposed clam; (G) digestive tubule of a control clam; (H) vacuolation (6) in the digestive tubule of "Moderately–Heavily" *P. olseni*-infected clam; (I) hemocytic encapsulation (7) of *P. olseni* trophozoites (8) in connective tissue of the digestive gland.

**Table 3**

Comparison of histological features in experimental clams, according to *P. olseni* burden categories ("Very light–Light" *vs* "Moderate–High"), and upon algal treatment (mix of T-*Iso* and *A. ostenfeldii vs* control T-*Iso*) after 7 days of exposure ("Exposure"), after 7 days of depuration (fed only T-*Iso*) ("Depuration"), and during the entire course of the experiment (Exposure + Depuration). NS: no significant difference. Significant differences indicated by #: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$ (Mann–Whitney test). †: A single clam showed this characteristic. NA: not available (not enough data). "–": No analysis over the entire course of the experiment because there was a significant effect of time of sampling (exposure *vs.* depuration).

| Histological features | Differences between *P. olseni* burdens | | Differences between algal treatments | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Exposure | | Depuration | | Over the entire experiment | |
| | *p* | *n* | *p* | *n* | *p* | *n* | *p* | *n* |
| Hemocyte infiltration: | | | | | | | | |
| Gills | NS | 12 | * | 5–7 | NS | 5–7 | – | – |
| Mantle | NS | 11 | NS | 6 | NS | 4–6 | – | – |
| Digestive gland | NS | 13 | # | 5–7 | NS | 6–8 | * | 11–15 |
| Digestive epithelium | NS | 12–14 | NS | 5–7 | NS | 6–8 | NS | 11–15 |
| Gonadal tissue | NS | 13–14 | NS | 6–7 | NS | 6–8 | NS | 12–15 |
| Adductor muscle | NS | 6–7 | NA | | NS | 3–6 | NS | 5–8 |
| Hemocyte encapsulation: | | | | | NS | | | |
| Gills | NS | 11–13 | NS | 6 | NS | 5–7 | NS | 11–13 |
| Mantle | NS | 9–11 | NS | 5 | NS | 4–6 | NS | 9–11 |
| Digestive gland | # | 12–14 | NS | 5–7 | NS | 5–8 | NS | 11–15 |
| Digestive epithelium | NS | 13 | NS | 6–7 | NS | 5–8 | NS | 11–15 |
| Gonadal tissue | # | 13 | NS | 6–7 | NS | 5–8 | NS | 11–15 |
| Adductor muscle | NS | 8–10 | NS | 3–5 | NA | | NS | 8–10 |
| Mantle: edema | NS | 11–12 | * | 6 | NS | 5–6 | * | 11–12 |
| Gills: | | | | | | | | |
| Edema | NS | 12–13 | NS | 6–7 | NS | 5–7 | NS | 11–14 |
| Brown cells | NS | 12–13 | NS | 6–7 | # | 5–7 | NS | 11–14 |
| Vacuolation | NS | 12–13 | NS | 6–7 | # | 5–7 | # | 11–14 |
| Stomach: epithelium sloughing into the lumen | NS | 13 | NS | 5–7 | NS | 6–8 | # | 11–15 |
| Intestine: | | | | | | | | |
| Hemocyte diapedesis | NS | 12–13 | NS | 4–7 | NS | 6–8 | * | 10–15 |
| Brown cells through the epithelium | NS | 12–13 | NS | 4–7 | NS | 6–8 | – | – |
| *A. ostenfeldii* cells in the lumen | NS | 12–13 | * | 4–7 | # | 6–8 | ** | 10–15 |
| T-*Iso* cells in the lumen | NS | 12–13 | NS | 4–7 | NS | 6–8 | NS | 10–15 |
| Brown cells in the lumen | NS | 12–13 | NS | 4–7 | NS | 6–8 | – | – |
| Epithelium sloughing into the lumen | NS | 12–13 | * | 4–7 | NS | 6–8 | – | – |
| Hemocytes in the lumen | NS | 12–13 | NS | 4–7 | # | 6–8 | NS | 10–15 |
| Digestive tubules: | | | | | | | | |
| Hemocyte diapedesis | NS | 10–11 | NS | 4–5 | NS | 5–7 | NS | 9–12 |
| Brown cells through the epithelium | NS | 10–11 | NS | 4–5 | NS | 5–7 | – | – |
| *A. ostenfeldii* cells in lumen | NS | 10–11 | NS † | 4–5 | NS | 5–7 | NS † | 9–12 |
| Brown cells in the lumen | NS | 10–11 | NS | 4–5 | NS | 5–7 | – | – |
| Epithelium sloughing into the lumen | NS | 10–11 | NS | 4–5 | NS | 5–7 | NS | 9–12 |
| Hemocytes in the lumen | NS | 10–11 | NS | 4–5 | NS | 5–7 | NS | 9–12 |
| Vesicles | NS | 10–11 | NS | 4–5 | NS | 5–7 | NS | 9–12 |
| Vacuolation | # | 10–11 | NS | 4–5 | NS | 5–7 | NS | 9–12 |
| *P. olseni* cells: | | | | | | | | |
| Gills | NS | 12–13 | NS | 6–7 | NS | 5–7 | # | 11–14 |
| Mantle | NS | 11–12 | ** | 6 | NS | 4–7 | – | – |
| Digestive gland | * | 13–14 | NS | 6–7 | NS | 6–8 | NS | 12–15 |
| Gonadal tissue | NS | 13–14 | NS | 6–7 | NS | 6–8 | NS | 12–15 |
| Adductor muscle | NS | 6–7 | NA | | # | 3–6 | # | 5–8 |

### 4.1. Effects of *P. olseni*

Overall, effects of higher *P. olseni* infections were relatively light and did not affect important physiological functions of clams. Although the digestive gland showed evidence of tissue damage, *i.e.*, vacuolation in digestive tubules, digestive functions were maintained, as shown by the representative digestive enzyme activities analyzed. Furthermore, presence of encapsulation bodies highlighted the effectiveness of the immune response, allowing other cellular and humoral immune responses, such as phagocytosis, hemolytic activity or agglutination titer, to occur. Encapsulation is indeed believed to be an important mechanism by which clams defend against *P. olseni* (Chagot et al., 1987; Montes et al., 1996, 1995; Navas et al., 1992; Ordás et al., 2001; Park and Choi, 2001; Sagrista et al., 1995). The increase in size of circulating hemocytes detected in our study could reflect cell differentiation for this immune response. These light effects can be attributed to the relatively low intensity of *P. olseni* infection compared to other studies. Waki and Yoshinaga (2013) and Waki et al. (2012) suggested that *P. olseni* pathogenicity to the Manila clam was considerable at a level of $\sim 10^6$ cells $g^{-1}$ soft tissues, above which mortality occurred. Although such intensity levels are frequent in Manila clam populations in Asia (Choi and Park, 2010), mean *P. olseni* burdens reported in French populations usually do not exceed $10^5$ cells $g^{-1}$ of soft tissue (Binias et al., 2014, 2013; da Silva et al., 2008; Dang et al., 2013; De Montaudouin et al., 2010; Hégaret et al., 2007a), with no seasonal variation (Binias et al., 2014; Dang et al., 2013, 2010a), in contrast to other locations such as Gomso Bay, Korea (Yang et al., 2012) or Galicia, Spain (Villalba et al., 2005). Accordingly, Hégaret et al. (2007a, 2009) and da Silva et al. (2008), did not observe significant effects of *P. olseni* upon most of the hemocytic variables they measured in French Manila clams. For this intensity of infection, an explanation suggested by Hégaret et al. (2007a) is that, once the host hemocytes have accomplished isolation of the parasite, the remaining circulating hemocytes resume the main functions. No effects of parasite burden upon condition index or

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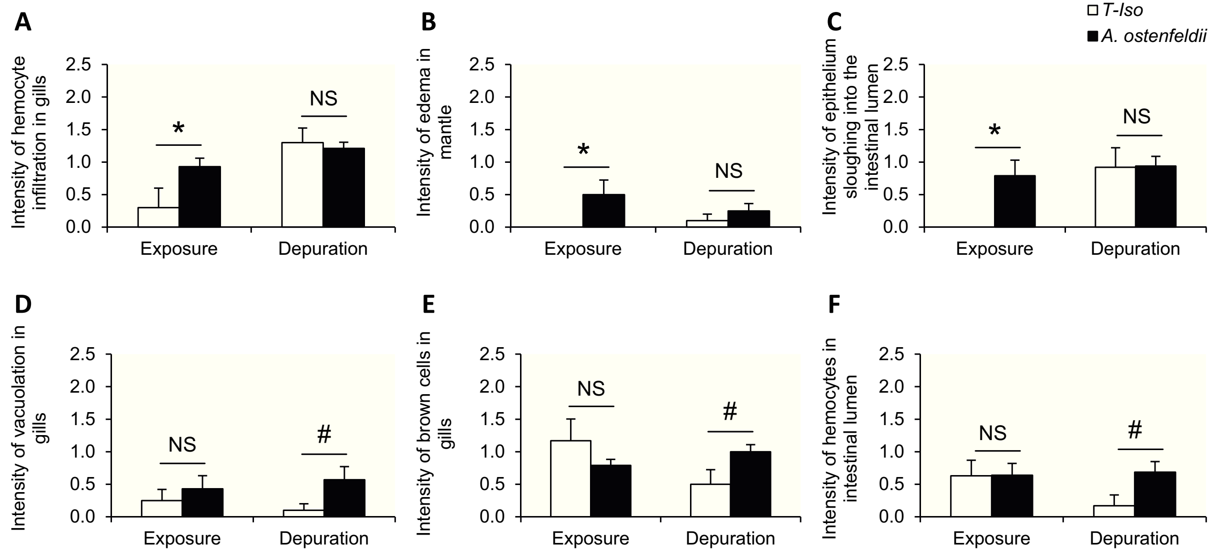


**Fig. 5.** Intensity of histopathological features (semi-quantitative scale) in tissues of Manila clams *V. philippinarum*, from two different algal treatments, T-*Iso* (control) or a mix of T-*Iso* and *A. ostenfeldii*, after 7 days of exposure and after 7 more days of depuration. Error bars represent standard errors. Significant differences between control ("T-*Iso*") and exposed clams ("*A. ostenfeldii*") are indicated by #: $p < 0.1$ and *: $p < 0.05$, and NS indicates no significant difference (Mann–Whitney test). $N = 4$–8 per group. (A) Hemocyte infiltration in gills; (B) edema in mantle; (C) epithelium sloughing into the intestinal lumen; (D) vacuolation in gill filaments; (E) brown cells in gills; (F) hemocytes in intestinal lumen.

mortality of clams were observed, thus confirming that the global host-pathogen interaction remained in equilibrium.

### 4.2. Effects of *A. ostenfeldii* exposure

After exposure of experimental Manila clams to the toxic dinoflagellate *A. ostenfeldii*, spirolide analogues accumulated preferentially in the digestive gland, as reported in oysters by Medhioub et al. (2012), but also in the remaining tissues including siphon, foot, gills, muscle and mantle. In addition, the 13-desmethyl-C spirolide was detected at very low levels in control clams, showing that these clams had been exposed to *A. ostenfeldii* in the field. Indeed, since 2005, spirolides have been detected recurrently by the "French Phytoplankton and Phycotoxin monitoring Network" (REPHY) in tissues of oysters and mussels from Arcachon Bay and were attributed to *A. ostenfeldii* blooms.

After seven days exposure, overall effects of exposure to *A. ostenfeldii* on clam "external" (mantle and gills) and "internal" organs (digestive gland) were identified. As clams are filter feeders, the first contact of microalgae with tissues occurs through siphons (not investigated in this study), mantle, and gills. Contact with *A. ostenfeldii* led to edema in the mantle, associated with hemocyte infiltration in the gills, indicating an inflammatory response. We suggest that the increase in hemocyte size is attributed to a differentiation process associated to this inflammatory response. These effects may be induced partly by the spirolides released after lysis of *A. ostenfeldii* cells during digestion and accumulated in tissues. As mantle and gills were in contact with living *A. ostenfeldii* cells, however, these tissues also may have been exposed to algal extracellular compounds. Indeed, independently from spirolides, *A. ostenfeldii* is able to produce extracellular compounds known to provoke immobilization and cell lysis of protists (Tillmann et al., 2007). Other *Alexandrium* species were reported to produce extracellular compounds with allelopathic, hemolytic, and ichthyotoxic properties (Arzul et al., 1999; Lelong et al., 2011; Ogata and Kodama, 1986; Simonsen et al., 1995) or oxidative properties (Flores et al., 2012). These compounds also can be deleterious to bivalves, as demonstrated by Ford et al. (2008) upon hemocyte functions. Ingestion of *A. ostenfeldii* cells, as observed on histological slides, was followed by deleterious effects upon internal organs, such as digestive epithelium sloughing into the intestinal lumen. In addition, the presence of intact *A. ostenfeldii* cells in the digestive tubules, where at least partially digested particles normally are found, and observation of *A. ostenfeldii* within the intestinal lumen after seven days of depuration, suggest partial dysfunction of digestive processes. Medhioub et al. (2012) also observed failure to digest *A. ostenfeldii* cells by oysters *C. gigas* and hemocyte diapedesis through the digestive epithelium. A role of hemocyte diapedesis after harmful algal exposure was suggested by other authors as a mechanism to eliminate toxin through the lumen of the intestine and/or to isolate *Alexandrium* cells (Galimany et al., 2008c; Haberkorn et al., 2010b; Hégaret et al., 2009). The detection of yessotoxin (another phycotoxin) in hemocytes of contaminated mussels by Franchini et al. (2003) strengthens the hypothesis that these cells could transport toxins out of the tissues as a detoxication process.

After a seven-day depuration time, spirolide content in digestive glands and remaining tissues of *A. ostenfeldii*-exposed clams decreased. Toxins, mainly 13-desmethyl-C spirolide, however, still were present in tissues, which could explain observations again of hemocytes undergoing diapedesis into the intestine and present in the lumen, consistent with our hypothesis that hemocytes participate in the detoxication process. In clam gill filaments, the harmful-algal exposure followed by a depuration period induced cell-vacuolation that probably affected gill functions, reflecting a persistent deleterious effect of *A. ostenfeldii* toxic compounds, extracellular compounds or spirolides. Alterations in gills were associated with higher amounts of brown cells (also referred to as ceroid bodies), suggesting oxidative stress, as these cells contain lipofuscin-like pigments constituted of non-degradable material, resulting mainly from the accumulation of oxidized proteins and lipids (Yin, 1996). Other authors also observed induction of brown cells in bivalve tissues following harmful-algal exposure (Galimany et al., 2008b), but also in response to organic pollutant exposure

(Lehmann et al., 2007; Morado and Mooney, 1997; Smolowitz and Leavitt, 1996). In addition, brown cells from the "red gland" or pericardial gland of bivalves have been shown to play a role in accumulation, detoxication, and degradation of pollutants (Zaroogian and Anderson, 1995; Zaroogian and Yevich, 1994; Zaroogian et al., 1989). In the present study, we suggest that the brown cells observed within gills indicated oxidative stress, and these cells may in addition participate in detoxication. Another indicator of possible oxidative stress in gills was the depression of total SOD specific activity, an antioxidant enzyme, as reported by Estrada et al. (2007) in gills of *Nodipecten subnodosus* exposed to the toxic dinoflagellate *Gymnodinium catenatum* and associated with lipid peroxidation. Exposure to *A. minutum* also presumably provoked oxidative stress in *C. gigas* gills, detected by measurement of antioxidant enzyme transcript levels (Fabioux et al., submitted). In this study, the decrease in mitochondrial SOD (Mn SOD) activity measured in gills of *A. ostenfeldii*-exposed clams may indicate mitochondrial metabolic adjustments to maintain homeostasis, as suggested by Romero-Geraldo et al. (2012). These authors observed an overall repression in antioxidant enzyme transcript levels, including SOD, in *C. gigas* for the first 14 days of exposure to toxic dinoflagellate *Prorocentrum lima*. The decrease of MMP in hemocytes in our study also supports the involvement of mitochondrial adjustments.

### 4.3. Modulation of *P. olseni* burden responses in clam pro-/anti-oxidative mechanisms by exposure to *A. ostenfeldii*

Exposure to *A. ostenfeldii* led to higher ROS production in hemocytes of "Very lightly–Lightly" infected clams. In addition, ROS production also increased in hemocytes of clams fed T-*Iso* as a response to the higher intensity of *P. olseni* infection. Changes in CAT activity within gills were concomitant with changes in circulating hemocyte ROS production, suggesting a protective response against higher production of ROS. These ROS compounds measured in unstimulated hemocytes probably originate from mitochondria, as shown in unstimulated hemocytes of *C. gigas* (Donaghy et al., 2012). ROS produced by mitochondria are involved in the modulation of many cellular processes such as cell signaling, immune responses, apoptosis, or ion-channel functions (Bartosz, 2009; Stowe and Camara, 2009). The increase in ROS production observed in our experiment may thus either play a role in the response to *P. olseni* infection and/or to *A. ostenfeldii* exposure by inducing modifications in cellular processes or it may be a consequence of physiologically-related mitochondrial changes (Stowe and Camara, 2009) induced by the parasitic infection and/or the algal exposure. No variation in ROS production, however, was observed in hemocytes of *A. ostenfeldii*-exposed clams with higher *P. olseni* infestation, suggesting that ROS production reached a threshold or that exposure to *A. ostenfeldii* is able to neutralize the increase in ROS stimulated by *P. olseni*.

### 4.4. Effect of *A. ostenfeldii* exposure on *P. olseni* burden in tissues of Manila clams

Although exposure to *A. ostenfeldii* did not modify *P. olseni* gill burden in experimental clams, a decreasing trend was observed, which also was confirmed by histological observations. *P. olseni* observations significantly decreased in gills, mantle and adductor muscle, suggesting a deleterious effect of *A. ostenfeldii* upon the parasite. In another study exposing Manila clams to the harmful alga *Karenia selliformis*, *P. olseni* infection intensity regressed in toxic algal-exposed clams, suggesting a negative effect of this alga upon *P. olseni* (da Silva et al., 2008). *In vitro* experiments confirmed that *K. selliformis* had negative effects upon *P. olseni* viability, modifying the host–pathogen interaction (da Silva et al., 2008). *In vitro* experiments also demonstrated detrimental effects of another dinoflagellate, *P. minimum* and its exudates, upon *P. olseni* cells (Hégaret et al., 2009). It would thus be interesting to assess the direct effect of *A. ostenfeldii* on *P. olseni* cells to understand the observed effects of *A. ostenfeldii* exposure upon the parasite occurrence within tissues.

## 5. Conclusions

Overall, hemocyte immune functions of Manila clams remained relatively unchanged according to perkinsosis level and *A. ostenfeldii* exposure, highlighting the capacity of clams to maintain homeostasis. Indeed, despite histological damage and partial dysfunction of the digestive gland caused by *A. ostenfeldii* exposure, digestive enzyme activities were not affected, showing that clams were able to maintain these main nutritional functions. Modifications in mitochondrial metabolism and detoxication processes probably participated in maintaining homeostasis. From this study, it appears that the interaction of *A. ostenfeldii* blooms and perkinsosis does not likely affect survival of Manila clam stocks from Arcachon Bay. Considering the roles of hemocytes in immunity, however, the question of a potential consequence of changes in hemocyte morphology and MMP induced by exposure to *A. ostenfeldii* on clam capacity to defend against other more virulent pathogens or other environmental stressors cannot be excluded.

## Conflict of interest

Authors have nothing to declare.

## Author contribution

Conceived and designed the experiment: PS, NLG, CL, XM, CF, HH. Performed the analyses: ML, PS, GR, NH, WM, PMS, AD, MB, NLG, CL, CF, HH. Analyzed the data: ML, PS, GR, NH, HH. Wrote the paper: ML, PS, PMS, CF, HH.

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## Article 3

# Physiological and pathological changes in the eastern oyster *Crassostrea virginica* infested with the trematode *Bucephalus* sp. and exposed to the toxic dinoflagellate *Alexandrium fundyense*

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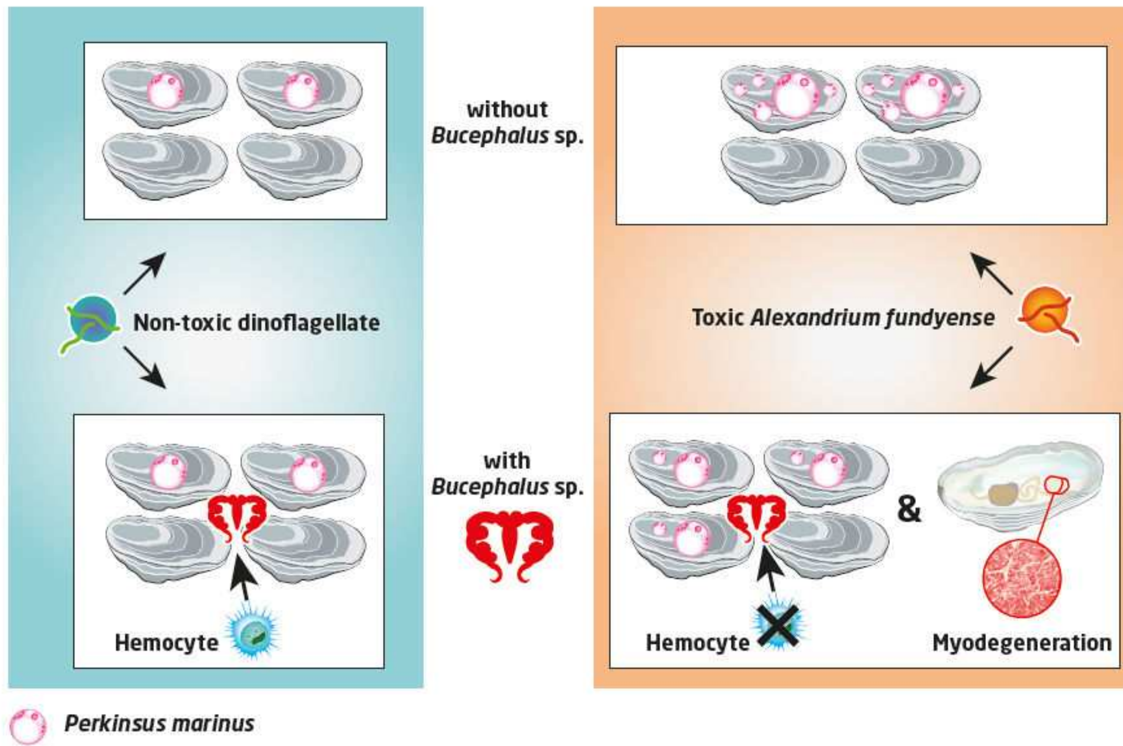
## Highlights

- *A. fundyense* and *Bucephalus* sp increased Dermo susceptibility in eastern oysters.
- *A. fundyense* induced immuno-modulation likely responsible for Dermo susceptibility.
- *A. fundyense* and *Bucephalus* sp. combined altered adductor muscle morphology.

## Graphical abstract



**Abstract**

Effects of experimental exposure to *Alexandrium fundyense*, a Paralytic Shellfish Toxin (PST) producer known to affect bivalve physiological condition, upon eastern oysters, *Crassostrea virginica* with a variable natural infestation of the digenetic trematode *Bucephalus* sp. were determined.

After a three week exposure to cultured *A. fundyense* or to a control algal treatment with a non toxic dinoflagellate, adult oysters were assessed for a suite of variables: histopathological condition, hematological variables (total and differential hemocyte counts, morphology), hemocyte functions (Reactive Oxygen Species – ROS – production and mitochondrial membrane potential), and expression in gills of genes potentially involved in immune responses and cellular protection (MnSOD, CAT, GPX, MT-IV, galectin CvGal, Dominin, Segon).

By comparing individual oysters infested heavily with *Bucephalus* sp. and uninfested individuals, we found altered gonad and digestive gland tissue and an inflammatory response (increased hemocyte concentration in circulating hemolymph and hemocyte infiltrations in tissues) associated with trematode infestation. Exposure to *A. fundyense* led to a higher weighted prevalence of infection by the protozoan parasite *Perkinsus marinus*, responsible for Dermo disease. Additionally, exposure to *A. fundyense* in trematode-infested oysters was associated with the highest prevalence of *P. marinus* infection. These observations suggest that the development of *P. marinus* infection was advanced by *A. fundyense* exposure, and that, in trematode-infested oysters, *P. marinus* risk of infection was higher when exposed to *A. fundyense*. These effects probably have a basis in oyster immune response modulation, as suggested by suppression of the inflammatory response to trematode infestation by *A. fundyense* exposure. Additionally, the combination of trematode infestation and *A. fundyense* exposure caused degeneration of adductor muscle fibers, suggesting alteration of valve movements and catch state, which could increase susceptibility to predation.

**Keywords**: *Crassostrea virginica*; *Alexandrium fundyense*; *Bucephalus* sp.; *Perkinsus marinus*; host-pathogen interaction.

## 1. Introduction

The eastern oyster *Crassostrea virginica*, native to the east coast of North and Central America, is a major commercial species with 144,012 tons produced in 2011 in the USA, which accounted for 75% of worldwide production (FAO, 2014). Infectious diseases of eastern oysters caused by protozoan parasites *Perkinsus marinus* (Dermo), *Haplosporidium nelsoni* (MSX) and *Haplosporidium costale* (SSO) are responsible for economic loss from mortality during epizootics (Ford and Tripp, 1996). The protozoan parasite *P. marinus* has been, in fact, the primary cause of mortality in cultured *C. virginica* on the east coast of the US (Ford and Smolowitz, 2007). Other parasites also may present a serious risk to the oyster industry. Among these are the digenetic trematodes, referred to as bucephalids, that in advanced infestations affect gonadal follicles causing sterility (Lauckner, 1983). Marine bivalves are the first intermediary hosts for bucephalids, fish being the final hosts (Olsen, 1974).

Diseases caused by parasites imply an imbalance in the host-parasite relationship; for example, the host immune response is inadequate to control proliferation. In bivalves, internal defense is based upon an innate immune system composed primarily of hemocyte-mediated cellular mechanisms and humoral factors such as lectins, lysins, and antimicrobial peptides (Cheng, 1996; Hine, 1999; Song et al., 2010; Soudant et al., 2013). The best known immune responses to microbial pathogens involve phagocytosis by individual hemocytes followed by intracellular degradation (Cheng, 1996; Janeway and Medzhitov, 2002; Song et al., 2010; Soudant et al., 2013). Encapsulation by masses of hemocytes is another mechanism of isolation and destruction of pathogens too large for phagocytosis (Cheng, 1981; Feng, 1988; Fisher, 1988, 1986). Cellular immune response to bucephalid trematodes in bivalves usually is low or not detected (Cheng and Burton, 1965; da Silva et al., 2002) and consists mainly, when existent, of hemocyte infiltration (Cheng and Burton, 1965); however, humoral factors seem also to be involved (Feng and Canzonier, 1970).

A hypothesis is that toxic dinoflagellate exposure could increase bivalve susceptibility to disease by altering both defense and physiological condition. HAB intensity and frequency are reported to be increasing (Van Dolah, 2000), and HAB co-occurrence with pathogen infections in marine bivalves is recurrent. Only few studies, however, have reported the effects of these interactions which could lead to modified host-pathogen relationships (Bricelj et al., 2011; da Silva et al., 2008; Hégaret et al., 2009). One such interaction was reported by

Bricelj et al. (2011), who showed that exposure to *Alexandrium tamarense* increased mortality of juvenile clams challenged with *Vibrio tapetis*, the causative agent of Brown Ring Disease.

The present study was undertaken to investigate possible interactions between parasitic diseases and toxic *Alexandrium* in compromising the health of the eastern oyster *Crassostrea virginica*. This study was conducted in spring, when *A. fundyense* blooms may occur along the northeastern coast of the USA (Anderson et al., 2005; Hattenrath et al., 2010; Lopez et al., 2014) and when *P. marinus* intensity and *Haplosporidium* spp. prevalence and intensity in oysters are at the lowest (Ford and Smolowitz, 2007; Ford, 1985). Experimental oysters were found to contain high infestations of the trematode *Bucephalus* sp. We hypothesized that: (1) oyster exposure to *A. fundyense* could modify susceptibility to parasitic infestations, and (2) the interaction of *A. fundyense* and parasites could lead to combined effects upon oyster physiological condition. To test these hypotheses, we examined parasitic infestations and histopathological changes in oysters exposed to cultured *A. fundyense*. In addition, we assessed immune status by determining hematology (total and differential hemocyte counts) and hemocyte functions (ROS production and mitochondrial membrane potential), as well as gene expression of proteins associated with immune responses and cellular protection: a galectin, CvGal, involved in recognition of micro-organisms prior to phagocytosis and also implicated in proliferation of *P. marinus* (Tasumi and Vasta, 2007); the two newly-described *C. virginica* major plasma proteins Dominin (Itoh et al., 2011) and Segon (Xue et al., 2012), for which specific roles are still under investigation; antioxidant enzymes (MnSOD, CAT, GPX); and a metallothionein family (MT-IV) that responded to immune challenge (Jenny et al., 2006; Wang et al., 2010) and was isolated from hemocytes (Jenny et al., 2006). Gene expression analysis was conducted in the gill, an organ rich in hemocytes and in direct contact with the external milieu.

## 2. Materials and methods

### 2.1 Experimental oysters

Eastern oysters *C. virginica* of 9.3 ± 0.1 cm length (mean ± SE) were collected from Milford Harbor (CT, USA) on April 20$^{th}$, 2012, and placed the same day in tanks with running, unfiltered seawater.

### 2.2 Characteristics of the seawater supplied

Throughout the experiment, flow of 30-40 L $h^{-1}$ of unfiltered seawater (containing natural phytoplankton) from Milford Harbor was distributed to the tanks. Temperature of the seawater was not controlled to provide the natural temperature of the local oyster habitat and varied from 10.5 to 15.8°C during the 23 days of the experiment (12.9 ± 0.4°C, mean ± SE). Salinity was 24.2 ± 0.5 ppt (mean ± SE) over the entire experiment. Light cycle in experimental facilities was controlled, with L:D = 14:10, corresponding roughly to the natural cycle when the experiment was conducted.

Biodiversity of phytoplankton in the seawater supplied to the tanks was surveyed during the experiment. 150 mL was sampled every 3 days and fixed with 3% Lugol's solution. Sedimentation columns were used to concentrate the samples, and slides were observed under an inverted light microscope. Two diatoms, *Skeletonema* sp. and *Thalassionema* sp., dominated the phytoplankton community during the experiment, and no harmful algal species were detected.

Total particulate matter (TPM) and particulate organic matter (POM) in the water supplied to the tanks was quantified every 4 days by filtering 300 mL in triplicate through Whatman GF/C filters following the procedure described by Galimany et al. (2011). Briefly, TPM was determined by weighing dried filters (48 h at 60°C). Particulate inorganic matter (PIM) then

was obtained by weighing ashed filters (4 h at 450°C), and POM was calculated as the difference between POM and TPM. TPM in the seawater supplied to the tanks was 13 ± 1 mg $L^{-1}$ (mean ± SE) during the experiment, with POM accounting for 20 ± 1% of the TPM (mean ± SE).

### *2.3 Algal culture*

The non-toxic dinoflagellate *Scrippsiella lachrymosa* (strain CCMP2666) was used as the control alga for the experiment, and the toxic dinoflagellate *Alexandrium fundyense* (BF2 strain, isolated from Gulf of Maine, USA) was used for the toxic-algal exposure. Both dinoflagellate species were cultured at 20°C with 24 h light using f/2-enriched filtered seawater from Milford Harbor (Guillard and Ryther, 1962) in 20-L glass carboy assemblies using aseptic technique (Ukeles, 1973). To maintain consistency in culture quality during the study, cultures were harvested semi-continuously in late-log or early-stationary phase. The Paralytic Shellfish Toxins (PSTs) produced by the BF2 strain of *A. fundyense*, cultured and harvested under these conditions, was previously reported to occur in the following mass proportions: GTX1,4 17.4%; NEO 29.0%; GTX2,3 5.4%; B1 6.2%; STX 6.6%; and C1,2 35.3% (Galimany et al., 2008). Algal cell densities were determined by flow cytometry (FACScan, BD BioSciences, San Jose, CA, USA).

### *2.4 Experimental design and sampling*

170 oysters were distributed haphazardly into ten 18-L tanks (17 oysters per tank). Oysters were acclimated for 3 days to laboratory conditions with no addition of algal culture before being exposed for 23 days (April 23rd to May 17th, 2012) to the two algal treatments, *S. lachrymosa* or *A. fundyense* (5 replicates each). Four pulses of algal culture per day were provided to each tank to simulate tidal delivery. Each pulse of algal culture yielded a final cell count in the tanks of $10^3$ cells $mL^{-1}$ for the two first weeks and $2\times10^3$ cells $mL^{-1}$ the last week ($9\text{-}18\times10^6$ cells per oyster and per day after the $T_0$ sampling). Water flow was stopped for 1 h following algal distribution to allow feeding and prevent immediate washout of the algae. This procedure added the dinoflagellates to the existing, background plankton community described above.

80 oysters (8 oysters per tank) were sampled individually at the end of the acclimation period, prior to dinoflagellate addition ($T_0$) and after 23 days exposure ($T_{23}$). Hemolymph was withdrawn from the adductor muscle of each oyster with a syringe before oysters were shucked, and soft tissues were weighed. Next, the anal-rectal region was excised for *P. marinus* quantification, and a tissue cross-section and a transversal section were fixed for histological analysis. Remaining digestive gland and remaining gills were dissected, individually frozen immediately in liquid $N_2$, and stored at -80°C for toxin quantification and gene expression analysis, respectively. Oysters at $T_0$ were analyzed for *P. marinus* only.

*2.5 Condition index*

Condition index (CI) was determined for 77 oysters at each sampling time, $T_0$ and $T_{23}$, as follows (adapted from Bodoy et al., 1986):

$$CI = \frac{\text{wet flesh weight}}{\text{height} \times \text{width}} \times 100$$

*2.6 Analysis of hemocyte variables*

77 oysters sampled at $T_{23}$ were analyzed for hemocyte variables. Hemolymph withdrawn from adductor muscle was stored temporarily in Eppendorf microcentrifuge tubes held on ice before flow-cytometric analysis. Characteristics determined in live, circulating hemocytes – total (THC) and differential hemocyte counts (granulocytes and hyalinocytes) (in cell $mL^{-1}$), size, and internal complexity (in arbitrary units a.u.), as well as mortality (percentage of dead hemocytes) – were assessed following Hégaret et al. (2003). Functional responses, i.e. production of reactive oxygen species (ROS) (specifically $H_2O_2$ and $O_2^{\bullet-}$) by unstimulated hemocytes and mitochondrial membrane potential (MMP) were determined as described in Delaporte et al. (2003) and Donaghy et al. (2012), respectively. Hemocyte analyses were performed with a FACScan flow-cytometer, and data were processed using WinMDI 2.8.

*2.7 Histopathological observations*

54 oysters sampled at $T_{23}$ were analyzed for histopathology. A 5-mm cross section of soft tissues including gills, mantle, gonad and digestive gland, and a transversal section including adductor muscle, heart and kidney, were excised and fixed in Davidson's fixative for 48 h at 4°C (Shaw and Battle, 1957). Tissue sections then were rinsed in 50% ethanol in filtered seawater and transferred to 70% ethanol, dehydrated in ascending ethanol solutions, cleared with xylene, and embedded in paraffin wax. Six-µm sections were stained with Harris' hematoxylin and eosin (Howard et al., 2004) and read blind under a light microscope.

Histopathological condition was examined per tissue. Intensity of each pathological observation was rated using a six-level semi-quantitative scale ranging from 0 to 2.5 as described in Table 1, according to Lassudrie et al. (2014). Based upon this scale, mean, weighted prevalence of each pathological condition was calculated as described in (1) and used for statistical analysis.

(1) $\bar{x}_p = \frac{\sum_{i=1}^{n} x_i}{n}$ where $\bar{x}_p$ is the mean weighted prevalence for a given pathological condition $p$, $x$ is the intensity of this pathological condition, and $n$ is the total number of individuals examined.

In addition, the sum of mean, weighted prevalence of each histopathological condition per organ was calculated as described in (2).

(2) $\sum_{i=1}^{n} \bar{x}_{p_i}$ where $\bar{x}_p$ is the mean weighted prevalence for a given pathological condition $p$, and $n$ is the total number of pathological conditions counted.

Gonadal maturation stage was determined following a 8-level, semi-quantitative scale (1: sexually undifferentiated, to 8: spawned) adapted from Ford and Figueras (1988) by Powell et al. (1993). Mean maturation stage was calculated as in (1) and used for statistical analysis.

Table 1. Semi-quantitative scale categorizing intensity of histopathological conditions observed (from Lassudrie et al., 2014).

| Level intensity | Occurrence of the pathological condition in the examined tissue area |
|---|---|
| 0.0 | Absence |
| 0.5 | Very low (1-5 total occurrence) |
| 1.0 | Low (> 5 occurrence / presence in all fields at magnification 10x) |
| 1.5 | Moderate (presence in all fields at magnification 20x / covering about 10% of the tissue) |
| 2.0 | High (presence in all fields at magnification 40x / covering about 20% of the tissue) |
| 2.5 | Very high (presence in all fields at magnification 60x / covering about 30% or above of the tissue) |

*2.8 Gene expression analysis in gills*

77 oysters sampled at $T_{23}$ were analyzed for gene expression. Fifty mg of gills, ground with a "Dangoumeau"-like ball grinder in liquid nitrogen, were homogenized in 1 mL of TRI reagent (Sigma-Aldrich), and total RNA was extracted according to manufacturer instructions. To remove possible genomic-DNA contamination, RNA extracts were treated with RTS DNase™ Kit (MO BIO). RNA concentrations were measured with a NanoDrop® ND-8000 UV-Vis Spectrophotometer (Thermo Scientific) at 260 nm, with a conversion factor of 1 OD = 40 µg $mL^{-1}$ RNA). RNA nanochips and Agilent RNA 6000 Nano Reagents (Agilent Technologies) were used to check RNA quality (RNA Integrity Number, RIN, above 8.00). Reverse transcription was carried out on 1 µg of RNA with RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas) with oligo$(dT)_{18}$ primers. The transcript levels of CvGal, Dominin, Segon, MnSOD, CAT, GPX and MT-IV (all the known MT-IV subtypes, A, B and C, were targeted) were determined by qPCR, relative to a constitutively-expressed reference gene, 18S ribosomal RNA, using specific primers either published in the literature or designed using Primer 3 web v. 4.0.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) from nucleotide sequences published in GenBank, to obtain 81 to 240 bp amplicons (Table 2). When non-annotated sequences from *C. virginica* were used, gene identification was performed with BLASTX (Altschul et al., 1997), accepting the result showing the lowest *E*-value (Table 2). To ensure specificity, only primer pairs that amplified a single sequence were used, and each amplicon was sequenced (performed by GATC Biotech) and compared to the expected nucleotide sequence. In addition, each amplified sequence was compared to Perkinsea and Bucephalidea nucleotide sequences from GenBank database using BLASTN (Altschul et al., 1997) to check that no sequence would match with a significant *E*-value.

Amplification efficiency (E) of each primer pair was calculated based upon standard curves from serial dilutions (from 1/10 to 1/10 000) of control cDNA (mix of 10 cDNA samples from uninfested oysters exposed to the control alga) and using the formula: $E=10^{-1/a}$, with $a$ = slope of the linear regression line calculated from Ct = f($\log_{10}$ of dilution). Percentage of efficiency, calculated as (E-1) × 100, was between 95% and 105% for all primers used. Each reaction was performed in triplicate, with 3.5 µL cDNA (1/20 dilution) in a total volume of 10 µL, with 0.75 µL of each primer at 1 µM and 5 µL ABsolute™ QPCR SYBR® Green ROX Mix (Thermo Fisher Scientific) in the Applied Biosystems® 7500 Real-Time System. Reactions were carried out as follows: Taq polymerase activation for 15 min at 95°C followed by 40 cycles consisting of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 min. In addition, a melting curve program was applied from 60°C to 95°C by increasing temperature at a ramp rate of 1% to verify that a single product was observed. The control cDNA and negative controls of water were included in each run. To check for the absence of DNA carryover, negative reverse-transcription controls (each total RNA sample after DNase treatment) also were analyzed. The relative expression ratio (R) of each target gene was calculated following the Pfaffl (2001) formula:

$$R = \frac{(E_{target\ gene})^{\Delta Ct_{target\ gene}}}{(E_{18S})^{\Delta Ct_{18S}}} \quad \text{with } \Delta Ct = Ct_{control\ cDNA} - Ct_{sample}$$

Table 2. Genes analyzed using RT-qPCR, GenBank accession number and associated species. BLASTX result (lowest *E*-value result) performed for gene identification of non-annotated sequences. Previously published primer pairs are referenced.

| Target gene | GenBank accession; species | BLASTX (lowest *E*-value result): GenBank accession; species; *E*-value | Forward primer sequence 5'-3' | Reverse primer sequence 5'-3' | Reference |
|---|---|---|---|---|---|
| 18S rRNA (reference gene) | AB199895.1; *Crassostrea gigas* | | TACAGCCAGGTCATGTCCAA | ACCCCAGTAGTGACGCAGAC | Xue et al., 2012 |
| MT-IV: | | | AACGGGAAAATGTGTGTGCT | CTTGACCTTGCAGCCCTTAC | Brulle et al., 2012 |
| MT-IV A<br>MT-IV B<br>MT-IV C | DQ117912.1,<br>DQ117913.1,<br>DQ117914.1; *Crassostrea virginica* | | | | |
| Catalase | CV087224.1 *C. virginica* | EKC34489.1; *C. gigas*; 2 $10^{-90}$ | ATGGCGTAATCTGGGTCATC | CAAACCCGTCTTCTGCAAAT | Brulle et al., 2012 |
| GPX | CD650160.1 *C. virginica* | EKC25678.1; *C. gigas*; 7 $10^{-87}$ | GATACCACCAAGCGGGACTA | CATTTTCCTGGTGACCGAAT | Brulle et al., 2012 |
| MnSOD | EH646608.1 *C. virginica* | ADR70997.1; *Crassostrea hongkongensis*; 7 $10^{-85}$ | TGGCTGAGGCATTACAGAAA | GGCTGAGGGTATCCCAGAAT | Brulle et al., 2012 |
| Dominin | AB269930.1 *C. virginica* | | AACACCAGCGAGGACTTTGC | GGTGTCACATCCGTGTTCCA | Itoh et al., 2011 |
| Segon | JQ235755.1 *C. virginica* | | GGGGATACCCTCTGGAACAT | GTCAGGTTGGCCTTCAGAAA | Xue et al., 2012 |
| CvGal | DQ779197.1 *C. virginica* | | GAACCGTGTAATGGCGAAGT | TACATTCCCGAGGTCCAGTC | designed |

MT: Metallothionein; CAT: Catalase; GPX: Glutathion Peroxidase; MnSOD: Manganese-dependent Superoxide Dismutase; CvGal: Galectin.

*2.9 Parasitic infestations*

77 oysters for each sampling time, $T_0$ and $T_{23}$, were analyzed for *P. marinus*. A sample of the anal-rectal region from each oyster was incubated for 8 days in the dark at room temperature in Ray's Fluid Thioglycollate Medium (RFTM) as modified by Bushek et al. (1994). Hypnospores of *P. marinus* in tissue squashes then were stained with Lugol's solution and examined under the light microscope. Intensity of infection was determined using the Mackin scale (Mackin, 1962) (0: no parasite detected, to 5: heavy infection). Prevalence of infection, mean weighted prevalence (equation (1)), and mean intensity (considering only infected oysters) were calculated.

Prevalence (n=77) and intensity (n=10) of trematode *Bucephalus* sp. infestation were assessed by histological observations at $T_{23}$. Intensity of infestation was determined as the percentage of the area of *Bucephalus* sp. sporocyst occupation calculated from 10 random fields per oyster (including gills, digestive gland, gonad and mantle) at total magnification 150x, using Image J software, as:

$$\frac{\sum_{i=1}^{10} \%_{field_i}}{10}$$

Other parasites were detected by examination of histological slides on 54 oysters at $T_{23}$. Prevalence of *Haplosporidium* spp. was determined, and weighted prevalence and intensity of the infection were assessed using a semi-quantitative scale followed by a composite rating matrix as published by Kim et al. (2006) for a final rating on a 4-level scale. Intensity of infestation with other parasites was rated on the six-level semi-quantitative scale ranging from 0 to 2.5 described in Table 1. Mean weighted prevalence of each infestation was calculated as described in equation (1) (section 2.8) and used for statistical analysis.

*2.11 Toxin detection*

77 oysters sampled at $T_{23}$ were analyzed for toxins. Digestive gland tissue was ground using a "Dangoumeau"-like ball grinder and frozen in liquid nitrogen. Paralytic Shellfish toxin (PST)

accumulation in the digestive gland was estimated using a PSP ELISA kit (Abraxis), after being extracted from ground digestive gland in HCl 0.1 M (1:1, w:v) and boiled for 5 min, according to manufacturer instructions, leading to acid hydrolysis that can induce chemical conversion of some PST analogues to STX (Vale et al., 2008). In fact, this ELISA assay recognizes mostly STX, and other PSTs only to varying degrees (cross-reactivities of 100% for STX and from 29% to 0% for other PSTs). Consequently, toxicity was expressed as µg STX $kg^{-1}$ of wet digestive gland weight. The principle of this assay is different from bioassay or HPLC methods; therefore, results obtained with the Abraxis PSP ELISA kit should not be compared directly to toxicity obtained with other methods.

*2.12 Statistical analysis*

Considering the low number of oysters infected with *Haplosporidium* spp., no statistical analyses was performed to assess effect of this infection upon pathological and physiological variables.

Effects of algal treatment and trematode infestation were tested upon prevalence of *P. marinus* with non–parametric, Chi-square tests performed independently in each *Bucephalus* sp.-infested or *Bucephalus* sp.-uninfested group, to assess effect of *A. fundyense* exposure. Differences in *P. marinus* infection weighted prevalence were assessed using non-parametric tests: the Mann-Whitney test (effect of algal treatment or trematode infestation, independently) and the Kruskal-Wallis test (non-nested – comparison of four groups categorized by both algal treatment and trematode infestation) followed by the Nemenyi-Damico-Wolfe-Dunn (NDWD) post-hoc test, to detect differences between the four groups defined according to algal exposure and trematode status.

Effects of *Bucephalus* sp. and *P. marinus* infestation status upon PST accumulation were tested with a two-way ANOVA (the factors were infestation with *Bucephalus* sp and with *P. marinus*), and effect of the intensity level of each infection upon PST accumulation was tested using the Kruskal-Wallis test.

Two-way ANOVA was used to analyze effects of trematode infestation, algal treatment, and the interaction, and effects of *P. marinus* infection, algal treatment, and the interaction, upon

hemocyte variables and gene expression, followed by the Least Significant Difference (LSD) post-hoc test, to detect differences between the four groups defined according to algal exposure and trematode status, or algal exposure and *P. marinus* status.

For histopathological changes and other parasitic infestations, the Mann-Whitney test was used to assess the effect of algal treatment, *Bucephalus* sp., and *P. marinus* infestation individually, and the Kruskal-Wallis test followed by the NDWD post-hoc test, to detect differences between the four groups defined according to algal exposure and trematode status, or algal exposure and *P. marinus* status.

Data were log or 1/X transformed, when needed, to meet normality and homoscedasticity assumptions, and percentage data were transformed as Arc-sin of square root. Differences were considered significant when $p<0.05$. Statistics were performed with Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA) and R version 2.15.1 (R Core Team, 2012).

## 3 Results

### *3.1 Parasitic infestations*

Infestation with the trematode *Bucephalus* sp. was detected in histological sections. The microscopic presentation of *Bucephalus* sp. in the tissues consisted of sporocysts containing germ balls and different developmental stages of cercariae (Fig. 2A, B, E). Although trematode sporocyst branches were present mainly in the gonad, they could be observed in connective tissues in all organs and in hemolymph vessels and were sometimes observed breaking the hemolymph-vessel walls. After 23 days of algal exposure, 34% of experimental oysters were infested with the trematode *Bucephalus* sp. (n=77). Prevalence was not significantly different at $T_{23}$ between the *A. fundyense*-treated group (29%; n=38) and the *S. lachrymosa*-treated group (38%; n=39). Trematode sporocyst occupation accounted for 22

± 2% of the tissues in oysters (mean ± SE, n=10 oysters), and no significant difference between algal treatments was detected.

Prevalence of infection with *P. marinus* in oysters at the beginning of the experiment was 65% (n=77) and decreased significantly to 38% after 23 days exposure to the control dinoflagellate ($p<0.01$) (n=39), whereas, it remained at 61% in *A. fundyense*-exposed oysters (n=38) (Fig. 1A), but the difference between algal treatments at $T_{23}$ was not significant.

At the beginning of the experiment, *P. marinus* weighted prevalence was 0.7 ± 0.1 (mean ± SE; n=77) (Fig. 1A), and decreased significantly after 23 days of exposure in *S. lachrymosa*-exposed oysters ($p<0.05$) to 0.4 ± 0.1 (mean ± SE; n=39). In *A. fundyense*-exposed oysters, weighted prevalence of *P. marinus* after 23 days exposure, however, did not vary significantly compared to day 0, remaining at 0.7 ± 0.1 (mean ± SE; n=38), and was thus significantly higher compared to *S. lachrymosa*-exposed oysters ($p<0.05$).

Trematode infestation (not determined at day 0) had no significant effect upon prevalence and weighted prevalence of *P. marinus* at $T_{23}$.

A significant effect of algal exposure was found upon prevalence of *P. marinus* among *Bucephalus* sp.-infested oysters at $T_{23}$. Indeed, 82 % of *Bucephalus* sp.-infested oysters exposed to *A. fundyense* were infected with *P. marinus* (n=11), which was significantly higher compared to prevalence of *Bucephalus* sp.-infested oysters exposed to the control dinoflagellate (40%; n=15) ($p<0.05$) (Fig. 1B). Such a difference was not detected among oysters uninfested with *Bucephalus* sp. (45% of *P. marinus* prevalence).

Intensity of *P. marinus* infection (calculated for infected oysters) was 1.02 ± 0.08 (mean ± SE; n=88) and did not vary significantly during the experiment or with algal treatment or trematode status.

*Haplosporidium* spp. was observed in 9% of the examined oysters (5 of 54 oysters), at intensities ranging from 1 (4 oysters) to 3 (1 oyster) and weighted prevalence of 0.13 / 4. Other microorganisms and parasites were observed on histological slides: *Nematopsis ostrearum* oocysts in all examined organs, RLO (Rickettsia-like organisms) in digestive tract epithelium, platyhelminthe *Turbellaria* sp. in intestinal lumen, and ciliates attached to the epithelia of pericardium, gills, mantle or in digestive tract lumina. All of these organisms were

observed at very low intensities and prevalence: mean weighted prevalence per condition was less than 0.6 / 2.5, and no statistical difference was detected attributable to algal exposure, *Bucephalus* sp. or *P. marinus* infection (n=54 oysters).

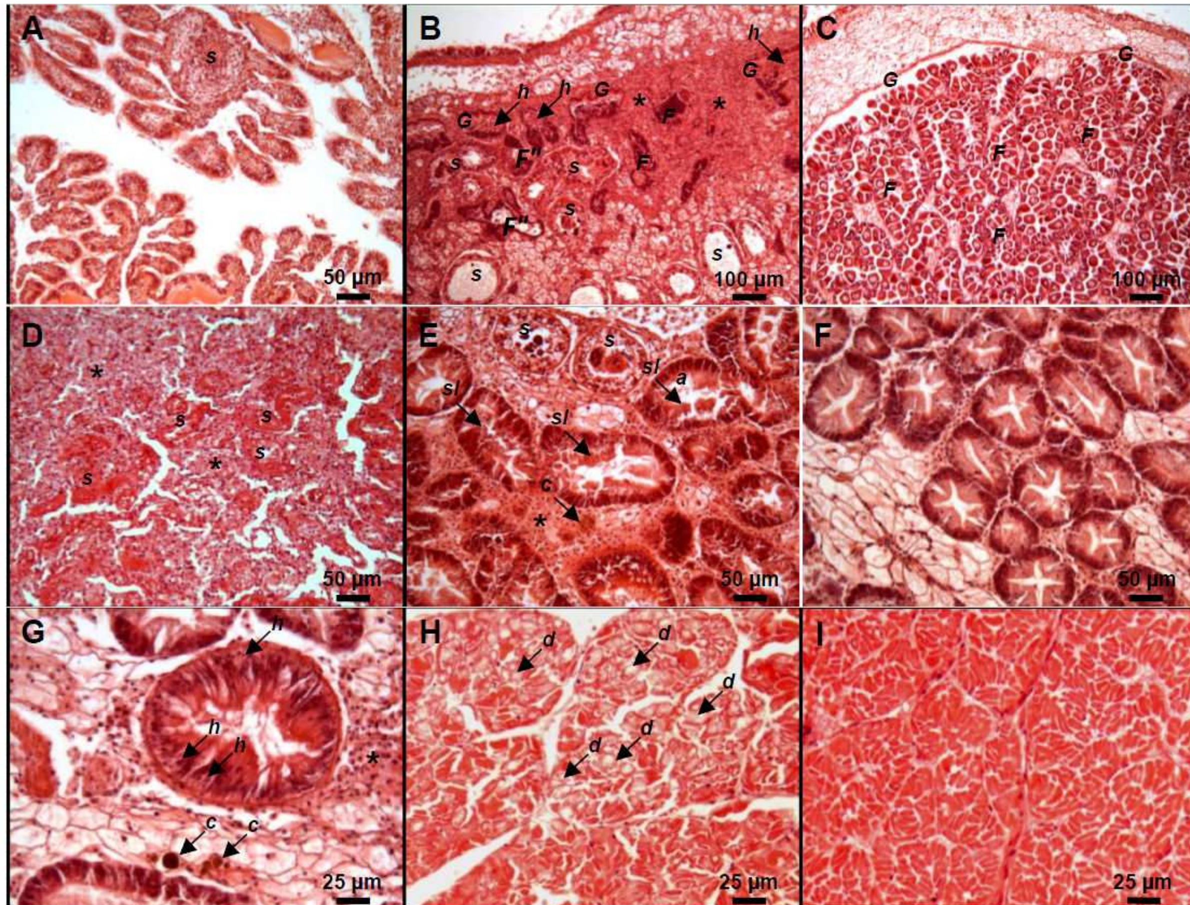



**Figure 2**. Histopathological conditions in experimental oysters *Crassostrea virginica* induced by the trematode *Bucephalus* sp. or its interaction with *Alexandrium fundyense* exposure for 23 days. (A) *Bucephalus* sp. sporocysts (*s*) in gill intraplical sinus inducing disruption of normal gill structure; (B) gonad infested with *Bucephalus* sp. sporocysts (*s*) associated with hemocyte infiltration (*) and hemocytes (*h*) in gonaducts (*G*) and follicles (*F*) lumina; (C) normal gonad of an uninfested oyster, with gametes maturing in follicles (*F*) and visible in gonoducts (*G*); (D) heavy hemocyte infiltration (*) associated with destruction of *Bucephalus* sp. sporocysts (*s*) in the digestive gland; (E) digestive duct and tubule degeneration in a digestive gland infested with *Bucephalus* sp. sporocysts (*s*): epithelium atrophy (*a*) associated with epithelium sloughing into the lumen (*sl*) and hemocyte infiltration (*) and presence of ceroid bodies (*c*); (F) normal digestive ducts and tubules in an uninfested oyster; (G) hemocyte infiltrated (*) around digestive ducts and undergoing diapedesis (*h*) associated with ceroid bodies (*c*) in the digestive gland of *Bucephalus* sp.-infested oysters; (H) degenerated adductor muscle fibers (*d*) in a *Bucephalus* sp.-infested oyster exposed to *A. fundyense*; (I) normal adductor muscle.

*3.2 PST accumulation in the digestive gland*

PST accumulation, as measured with the Abraxis ELISA assay, was 1,030 ± 124 µg STX $kg^{-1}$ of wet digestive gland (mean ± SE, n=38) in *A. fundyense*-exposed oysters after 23 days of exposure; no PST was detected in the control group. No difference in PST measurement was significantly detected according to *Bucephalus* sp. or *P. marinus* infection status or level.

*3.3 Condition index*

No significant effect upon condition index after 23 days of experimental exposure was detected according to algal treatment or *P. marinus* infection; whereas, infestation with the trematode *Bucephalus* sp. was associated with a significantly higher ($p<0.01$) condition index (0.28 ± 0.01, mean ± SE; n= 26) compared to uninfested oysters (0.24 ± 0.01, mean ± SE; n= 51).

*3.4 Histopathology*

Statistical differences related to *A. fundyense* exposure, trematode *Bucephalus* sp., and the algal treatment – *Bucephalus* sp. interaction, *P. marinus*, and the algal treatment – *P. marinus* interaction, upon each histopathological observation in experimental oysters after 23 days of algal exposure, are presented in Table 3.

Intact and partially-lysed cells of *A. fundyense* or *S. lachrymosa* and of other algae from the seawater supplied were observed in intestine lumina of the oysters, regardless of trematode- or *P. marinus*-status.

Growth of *Bucephalus* sp. sporocysts in intraplical sinuses and connective tissue of gills caused disruption of the normal configuration of gill filaments in all infested oysters (Fig. 2A). In addition, in the digestive gland hemolymph sinus of one oyster (of 54 oysters analyzed), heavy focal infiltration with hemocytes was observed around the trematode sporocysts, associated with tissue destruction (Fig. 2D).

Table 3. Statistical differences in weighted prevalence of histopathological conditions in experimental oysters *Crassostrea virginica* according to exposure to either toxic *Alexandrium fundyense* or the control dinoflagellate *Scrippsiella lachrymosa* for 23 days (Mann-Whitney test), infestation with trematodes *Bucephalus* sp. (Mann-Whitney test), between the four conditions defined by algal treatment and *Bucephalus* sp. infestation (Kruskal-Wallis test), according to *Perkinsus marinus* infection (Mann-Whitney test), and between the four conditions defined by algal treatment and *P. marinus* infection (Kruskal-Wallis test). NS: no significant difference. Significant differences were indicated by *: p<0.05; **: p<0.01; ***: p<0.001.

| **Histopathological observations** | **Algal treatment** (n=26-28) | ***Bucephalus* sp. infestation** (n=22-32) | **Algal – *Bucephalus* sp. conditions** (n=9-18) | ***Perkinsus marinus* infection** (n=27) | **Algal – *P. marinus* conditions** (n=9-18) |
|---|---|---|---|---|---|
| **Pericardium** | | | | | |
| - Ceroid bodies in auricle | NS | NS | NS | NS | NS |
| **Kidney** | | | | | |
| - Hemocyte infiltration | ** | NS | ** | NS | NS |
| - Ceroid bodies | NS | NS | NS | NS | NS |
| **Adductor muscle** | | | | | |
| - Hemocyte infiltration | NS | NS | NS | NS | NS |
| - Muscle fiber degeneration | * | * | ** | NS | NS |
| - Ceroid bodies | NS | NS | NS | NS | NS |
| **Gills** | | | | | |
| - Hemocyte infiltration | NS | *** | *** | NS | NS |
| - Ceroid bodies | NS | NS | NS | NS | NS |
| **Mantle** | | | | | |
| - Hemocyte infiltration | NS | *** | ** | NS | NS |
| - Ceroid bodies | NS | NS | NS | NS | NS |
| **Gonad** | | | | | |
| - Maturation | NS | *** | *** | NS | NS |
| - Hemocyte infiltration | NS | *** | *** | NS | NS |
| - Ceroid bodies | NS | ** | * | NS | NS |
| - Gamete degeneration in gonoducts | NS | NS | NS | NS | NS |
| - Gamete degeneration in follicles | NS | ** | ** | NS | NS |
| - Hemocytes in gonoduct and follicle | NS | * | * | NS | NS |
| **Digestive gland connective tissue** | | | | | |
| - Hemocyte infiltration | NS | *** | *** | NS | NS |
| - Ceroid bodies | NS | ** | * | NS | NS |
| **Stomach** | | | | | |
| - Epithelium sloughing into the lumen | NS | NS | NS | NS | NS |
| - Hemocytes in diapedesis | NS | NS | NS | NS | NS |
| - Hemocytes in lumen | NS | NS | NS | NS | NS |
| - Dinoflagellate cells in lumen | NS | NS | NS | NS | NS |
| - Other microalgal cells in lumen | NS | NS | NS | NS | NS |
| - Hemorhage | NS | NS | NS | NS | NS |
| **Intestine** | | | | | |
| - Hemocytes in diapedesis | NS | NS | NS | NS | NS |
| - Hemocytes in lumen | NS | NS | NS | NS | NS |
| - Dinoflagellate cells in lumen | NS | NS | NS | NS | NS |
| - Other microalgal cells in lumen | NS | NS | NS | NS | NS |
| **Digestive ducts and tubules** | | | | | |
| - Hemocyte infiltration surrounding | NS | ** | * | NS | NS |
| **Digestive ducts** | | | | | |
| - Epithelium sloughing into the lumen | NS | * | NS | NS | NS |
| - Hemocyte diapedesis | * | ** | ** | * | * |
| - Hemocytes in lumen | NS | NS | NS | NS | NS |
| - Mucus in lumen | NS | * | ** | NS | NS |
| **Digestive tubules** | | | | | |
| - Epithelium atrophy | NS | ** | * | * | NS |
| - Epithelium sloughing into the lumen | NS | *** | *** | NS | NS |
| - Hemocytes in lumen | NS | * | NS | NS | NS |
| - Mucus in lumen | NS | NS | NS | NS | NS |

Oysters infested with the trematode *Bucephalus* sp. had more intense hemocyte infiltration in the mantle ($p<0.001$), gills ($p<0.001$), gonads ($p<0.001$) (Fig. 2B, C) and digestive gland ($p<0.001$), compared to oysters with no visible trematodes. *Bucephalus* sp. infestation also caused higher weighted prevalence of ceroid bodies in gonadal tissues ($p<0.01$) and in digestive-gland connective tissue ($p<0.01$) (Fig. 2E, G). In the digestive gland, trematode infestation was associated also with increased epithelium sloughing into digestive ducts ($p<0.05$) and tubules ($p<0.001$) (Fig. 2E, F), mucus in digestive duct lumina ($p<0.001$), and hemocytes in digestive-tubule lumina ($p<0.05$). Atrophy of the digestive-tubule epithelium (Fig. 2E, F) was associated with both trematode infestation ($p<0.01$) and *P. marinus* infection ($p<0.05$).

A reduction in the average gonadal maturation stage ($p<0.001$) was detected in *Bucephalus* sp.-infested oysters (Fig. 2B, C, and Fig. 3), with a mean maturation stage of $3.0 \pm 0.2$ in *Bucephalus* sp.-uninfested oysters, corresponding to a mid-gonadal development stage, *vs.* 1.8 $\pm$ 0.1 in infested oysters, corresponding to an early developmental stage.

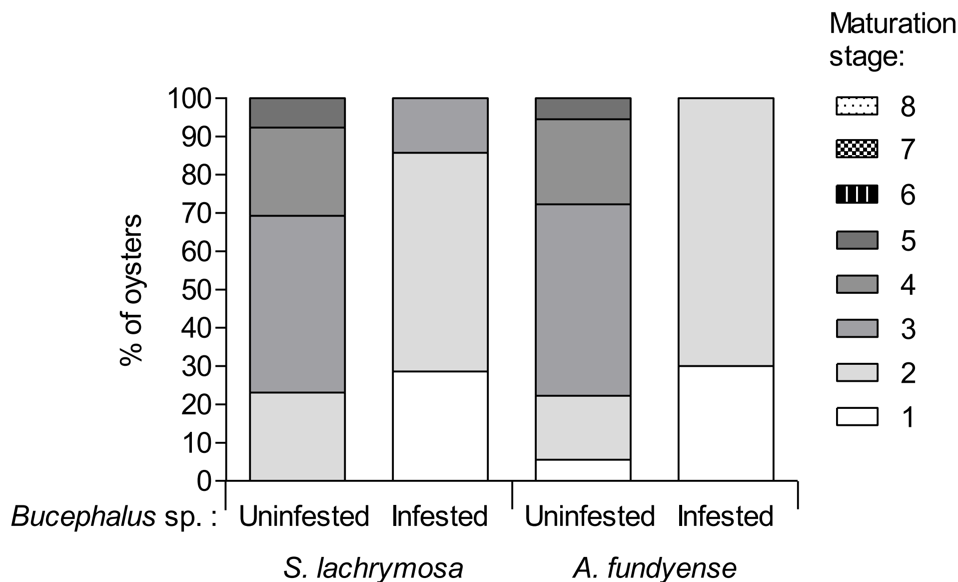



**Figure 3**. Percentages of oysters *Crassostrea virginica* at each gonadal maturation stage following a semi-quantitative scale from 1 (sexually undifferentiated) to 8 (spawned) (adapted from Ford and Figueras, 1988, by Powell et al., 1993) according to algal treatment (toxic *Alexandrium fundyense* or control *Scrippsiella lachrymosa* for 23 days) and trematode *Bucephalus* sp. infestation. N in each group: 13 in *S. lachrymosa* – Uninfested; 13 in *S. lachrymosa* – Infested; 18 in *A. fundyense* – Uninfested; 10 in *A. fundyense* – Infested.

Some histopathological conditions were observed more intensely in response to trematode infestation, but only in oysters exposed to the control alga *S. lachrymosa*: hemocyte infiltration around digestive ducts and tubules ($p<0.05$) (Fig. 2E, F, G, and Fig. 4B) or within gonoduct and follicle lumina ($p<0.05$) (Fig. 2B, C, and Fig. 4D), and degeneration of gametes within follicles ($p<0.01$) (Fig. 2B, C, and Fig. 4E).

In the kidney, hemocyte infiltration was lower in control oysters (exposed to *S. lachrymosa* and without trematodes) than in oysters exposed to *A. fundyense* or with *Bucephalus* sp. ($p<0.01$) (Fig. 4C).

Both trematode infestation ($p<0.01$), *P. marinus* infection ($p<0.05$) and *A. fundyense*-exposure ($p<0.05$) led to higher weighted prevalence of hemocyte diapedesis in digestive ducts (Fig. 2G). As a consequence, trematode-infested oysters exposed to *A. fundyense* had the highest weighted prevalence of hemocyte diapedesis in digestive ducts ($p<0.05$) (Fig. 4A) compared to the other algal treatment – *Bucephalus* sp. groups. The same result was observed in *P. marinus*-infected oysters exposed to *A. fundyense* compared to the other algal treatment – *P. marinus* status groups ($p<0.05$).

Degeneration of muscle fibers in the adductor muscle was significantly more intense in trematode-infested oysters also exposed to *A. fundyense* ($p<0.01$) than in oysters in other groups (Fig. 2H, I and Fig. 4F).

Other histopathological conditions observed did not vary significantly with algal treatment, trematode or *P. marinus* status (Table 3).

The sum of the mean weighted prevalence for each type of hemocyte response (infiltration, diapedesis, presence in lumina), per organ, is presented in Fig. 5A. The following general pattern is apparent: *A. fundyense* exposure, and even more so *Bucephalus* sp. infestation, increased hemocyte responses. Hemocyte responses to the interaction, however, were lower compared to the response to *Bucephalus* sp. alone.

The global pattern for ceroidosis in each organ is presented in Fig. 5B as the sum of the mean, weighted prevalence per organ, illustrating that trematodes *Bucephalus* sp.infestation caused higher intensity of ceroidosis.

The sum of the mean weighted prevalence for each type of degenerative condition (sloughing epithelium, cellular degenerations, mucus in digestive lumina) per organ shows that degeneration was associated principally with *Bucephalus* sp. (Fig. 5C).

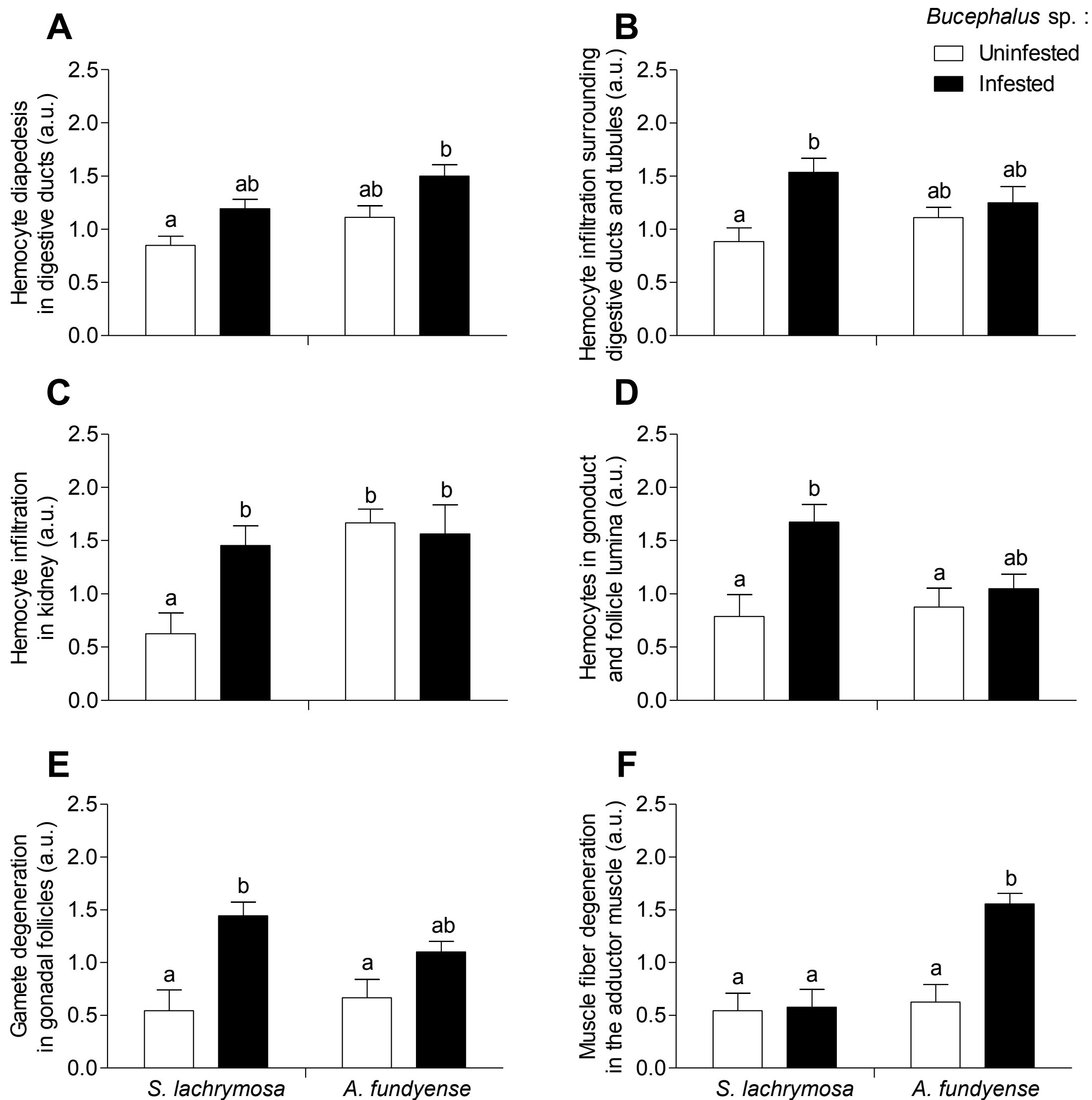



**Figure 4**. Mean weighted prevalences of histopathological conditions modified by the interaction of algal treatment (toxic *Alexandrium fundyense* or control *Scrippsiella lachrymosa*) and infestation with trematodes *Bucephalus* sp. in oysters *Crassostrea virginica* after 23 days of experimental algal exposure. (A) hemocyte diapedesis in digestive ducts; (B) hemocyte infiltration surrounding digestive ducts and tubules; (C) hemocyte infiltration in kidney; (D) hemocytes in gonoduct and follicle lumina; (E) degeneration of gametes in gonadal follicles; (F) muscle fiber degeneration in the adductor muscle. Letters indicate significant differences between conditions (Kruskal-Wallis test followed by post-hoc NDWD test, $p<0.05$). Error bars represent standard errors. N per group: 13 in *S. lachrymosa* – Uninfested; 13 in *S. lachrymosa* – Infested; 18 in *A. fundyense* – Uninfested; 10 in *A. fundyense* – Infested.

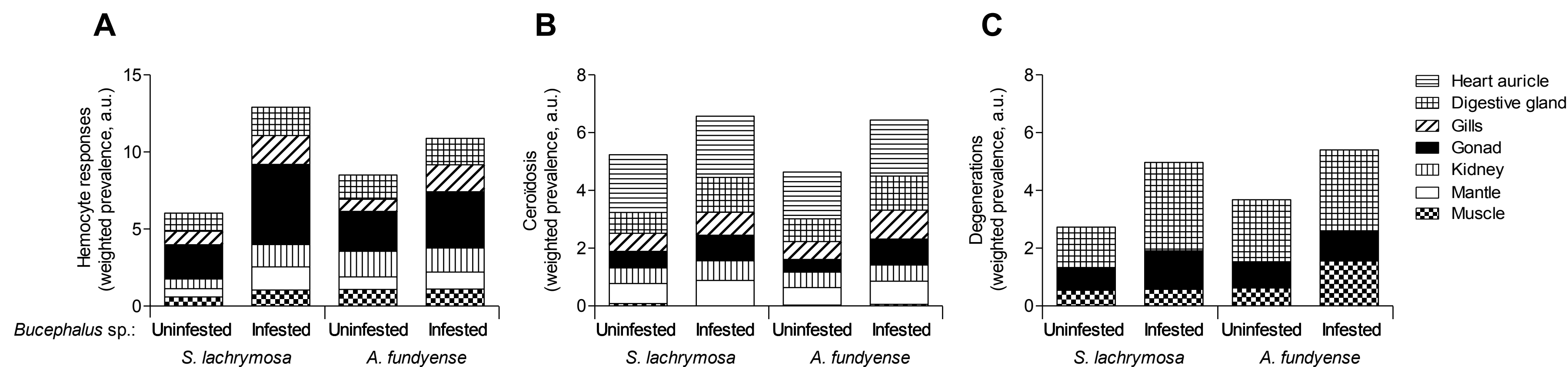



**Figure 5**. Sum of the mean weighted prevalences of each different histopathological conditions, per organ, corresponding to the following categories: (A) hemocyte responses; (B) ceroidosis; (C) degenerations in experimental oysters *Crassostrea virginica* after 23 days of experiment according to algal treatment (toxic *Alexandrium fundyense* or control *Scrippsiella lachrymosa*) and infestation with trematodes *Bucephalus* sp. N per group: 13 in *S. lachrymosa* – Uninfested; 13 in *S. lachrymosa* – Infested; 18 in *A. fundyense* – Uninfested; 10 in *A. fundyense* – Infested.

*3.5 Hemocyte variables*

Percentage of dead hemocytes was lower in trematode-infested oysters than in uninfested oysters (p<0.05). Total hemocyte count was significantly higher in oysters with trematodes and also exposed to the control alga *S. lachrymosa* (p<0.05) (Fig. 6). Other hemocyte variables were not affected significantly by algal treatment, trematodes, or the interaction. When considering the interaction of algal treatment with *P. marinus* infection, MMP of both granulocytes and hyalinocytes was significantly higher in circulating hemolymph of uninfected oysters exposed to the control alga (p<0.05), but other hemocyte variables were not affected.

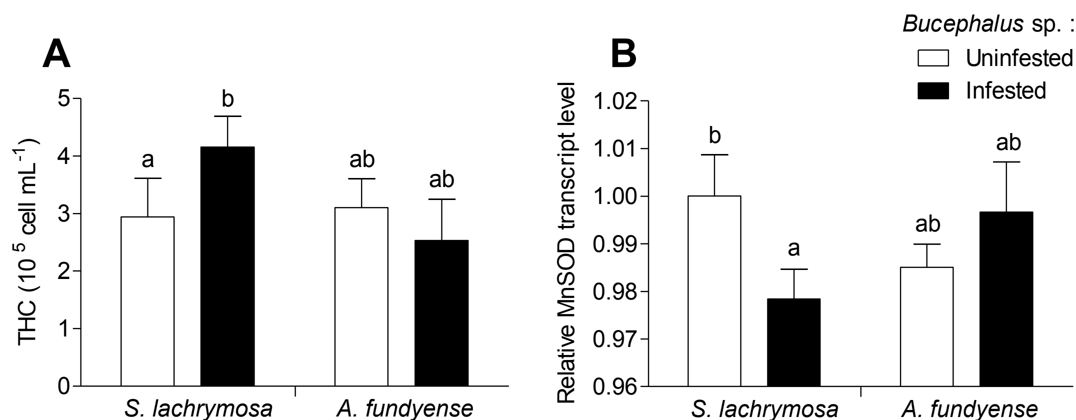



**Figure 6.** (A) Total hemocyte count (THC) from circulating hemolymph and (B) MnSOD gene expression in oyster *Crassostrea virginica* gills according to exposure to toxic *Alexandrium fundyense* or control *Scrippsiella lachrymosa* for 23 days, and trematode *Bucephalus* sp. infestation. Letters indicate significant differences (2-way ANOVA followed by LSD post-hoc test, p<0.05). N per group: 24 in *S. lachrymosa* – Uninfested; 15 in *S. lachrymosa* – Infested; 27 in *A. fundyense* – Uninfested; 11 in *A. fundyense* – Infested.

*3.6 Gene expression*

A significantly lower MnSOD gene expression level (p<0.05) was found in oysters with trematodes and also exposed to the control alga *S. lachrymosa* (Fig. 6). Other gene transcript levels did not vary with algal treatment, trematodes, or the interaction. No significant effect of *P. marinus* infection and its interaction with algal treatment upon gene expression was detected.

## 4 Discussion

Finding a population of eastern oysters, *Crassostrea virginica*, with a high prevalence and severity of infestation with the trematode parasite *Bucephalus* sp. provided an unanticipated opportunity to describe physiological responses of oysters to multiple infectious agents. Additional parasitic infections with *Perkinsus marinus* and *Haplosporidium* spp. were detected, but at lower intensity (and low prevalence for *Haplosporidium* spp.) typical of the season. Both parasites can cause extensive tissue disruption that may persist after infections have declined; however, intensities of *P. marinus* and *Haplosporidium* spp. at the study site have been low for years prior this experiment (Sunila, 2013), consistent with the few pathological effects related to *P. marinus* in this study. Thus, the availability of this oyster population provided a model for experimental investigation of interactions between parasitism with *Bucephalus* sp. and harmful-algal-bloom exposure in affecting oyster health and susceptibility to the other parasitic infections.

Results highlight the principle that harmful algal exposure interacts significantly with both host and parasite, and such interactions underscore the difficulty in diagnosing oyster health in the natural environment where simultaneous, multiple stimuli may invoke oyster responses at tissue, cellular, and molecular levels.

### *4.1 Effects of parasitic infestations*

Infestation with the trematode *Bucephalus* sp. affected experimental oysters from the tissue to the molecular level. Connective tissue in the gonad was the main site of infestation, suggesting that this species was *Bucephalus cuculus*, as observed previously in *C. virginica* by Hopkins (1954), McCrady (1874) and Tennent (1906) (NB: Tennent misidentified it as *Bucephalus haimeanus* at first, see Lauckner, 1983). The tissue occupation by the trematodes was high enough to increase condition index. The main consequence of infestation in the gonad, the most severely affected organ, was reduced gonadal maturation compared to uninfested oysters. In fact, bucephalid sporocysts are known to perturb gonadal development in bivalves, including *C. virginica* (Calvo-Ugarteburu and McQuaid, 1998; da Silva et al., 2002; Hopkins, 1954; McCrady, 1874; Tennent, 1906), because the parasite partly substitutes for the gonadal tissue and uses the host's reproductive energy (Calvo-Ugarteburu and

McQuaid, 1998; Lafferty and Kuris, 2009; Minguez et al., 2012). Energy uptake from the host by the trematodes probably also explains the increased gamete degeneration in gonadal follicles compared to uninfested oysters (in the non-toxic dinoflagellate-exposed oysters). Observation of gill deformation resulting from trematode sporocyst growth suggested that infestation could affect respiratory and feeding performance.

Digestive glands of oysters infested with *Bucephalus* sp. showed degradation of digestive ducts and tubules, as indicated by epithelium sloughing into the lumen, atrophy of digestive tubule epithelium, and presence of mucus in digestive duct lumen. Disarrangement and degeneration of digestive diverticula and gut were observed also by Cheng and Burton (1965) in *C. virginica* infested with *Bucephalus* sp.; both mechanical and physiological causes, such as impaired nutrient uptake, were mentioned as possible mechanisms (Cheng and Burton, 1966; Cheng, 1965). In addition, such degeneration can be typical with *P. marinus* and *H. nelsoni* infections (Farley, 1968; Ford, 1985; Smolowitz, 2013), as shown by digestive tubule epithelium atrophy significantly associated also with *P. marinus* infection. These degenerations could thus also partly result from other, earlier, parasitic infections.

Hemocyte infiltration into several tissues was observed in oysters infested with *Bucephalus* sp., indicating an inflammatory response, as also observed in mussels *P. perna* (da Silva et al., 2002). In our study, however, only one oyster showed focal, heavy hemocyte infiltration associated with partial destruction of trematode sporocyst branches. Effective destruction of *Bucephalus* sp. by hemocytic defense responses is usually not observed in bivalves and has only been rarely reported (Cheng and Burton, 1965; da Silva et al., 2002; Douglass, 1975); whereas, other digenetic trematodes, such as *Proctoeces maculatus* infesting mussels *Mytilus galloprovincialis* can be encapsulated (Villalba et al., 1997). In addition, hemocytes also migrated in response to lesions caused by the parasitic infestations. Indeed, degeneration of gametes in follicles was associated with infiltration and presence of hemocytes in follicle and gonaduct lumina, probably to resorb degenerated material. In digestive glands of oysters exposed to the non-toxic dinoflagellate, degeneration caused by parasitic infections was associated with more intense hemocyte infiltration. This also was associated with more intense hemocyte diapedesis in the digestive-duct epithelia of *P. marinus*- and in *Bucephalus* sp.-infested oysters, and presence of hemocytes in digestive tubule lumina of *Bucephalus* sp.-infested oysters. In hemolymph, the increase in hemocyte concentration in trematode-infested oysters exposed to the non-toxic alga was accompanied by a decrease in dead hemocytes

within trematode-infested oysters. This may result from enhancement of *de novo* hemocyte production, as proposed in previous studies (Hégaret and Wikfors, 2005; Hégaret et al., 2007, 2004; Soudant et al., 2004) and may help to sustain the inflammatory response.

In the two most infested tissues, gonad and digestive gland, presence of *Bucephalus* sp. also was associated with more numerous ceroid bodies (also referred to as brown cells, serous cells, pore cells, or rhogocytes in the literature), as previously observed by Cheng and Burton (1965). Ceroid, which is believed to play a role in detoxication and in the excretory system in bivalves (Haszprunar, 1996; Zaroogian and Voyer, 1995), is constituted of non-degradable material resulting mainly from the accumulation of oxidized proteins and lipids (Yin, 1996). The increased intensity of ceroidosis in affected oysters may indicate oxidative stress induced by the inflammatory response upon trematode infestation. The lower expression level of the mitochondrial antioxidant MnSOD gene detected in gills of trematode-infested oysters exposed to the non-toxic dinoflagellate suggests cellular metabolic modifications that could result from energy uptake from the host. In hemocytes of *P. marinus*-infected oysters exposed to the control dinoflagellate, MMP was lower compared to uninfected oysters, suggesting that *P. marinus* affects cellular metabolic functions of its host. This effect may be a consequence of antioxidant compounds produced by *P. marinus* that protect the parasite from the oxidative burst associated to phagocytosis and enables it to develop within *C. virginica* hemocytes (Anderson, 1999; Schott et al., 2003; Smolowitz, 2013; Volety and Chu, 1995; and see review of Soudant et al., 2013) or be associated with hemocyte apoptosis repression by *P. marinus* (Goedken et al., 2005; Hughes et al., 2010; Ly et al., 2003).

*4.2 Effect of A. fundyense exposure*

In all oysters exposed for three weeks to *A. fundyense*, histological observations of intact or lysed *A. fundyense* cells in intestine lumena, as well as PST accumulation in the digestive gland, demonstrated that oysters ingested and digested this alga. In oysters uninfested by trematodes, increased hemocyte infiltration in response to *A. fundyense* exposure also was detected in connective tissues of kidneys, which form an excretory complex together with the pericardial gland (Morse, 1987). This inflammatory response, which was specific to this organ, may indicate that algal toxins are carried, presumably by hemocytes, toward kidneys to be excreted. Galimany et al. (2008) also suggested that hemocytes could transport PST from

tissues to feces by diapedesis through the digestive epithelium. In fact, hemocyte diapedesis is a typical response of bivalves exposed to toxic *Alexandrium* spp. (Haberkorn et al., 2010b; Lassudrie et al., 2014; Medhioub et al., 2012). Similarly, we observed that *A. fundyense* exposure induced diapedesis of hemocytes through the digestive duct epithelia in oysters uninfested by trematodes.

In addition, *A. fundyense* exposure affected oyster susceptibility to Dermo disease, caused by *P. marinus.* Indeed, although weighted prevalence decreased during the time course of the experiment in oysters exposed to the control alga, no decrease was observed in *A. fundyense*-exposed oysters, resulting in a higher weighted prevalence than in control oysters. This difference may indicate that *A. fundyense* diminished immune functions in exposed oysters, consequently decreasing their ability to repress parasitic infections.

*4.3 Effects of the interaction between parasitic infestations and A. fundyense exposure*

In oysters infested with the trematode *Bucephalus* sp., the exposure to *A. fundyense* was associated with a higher prevalence of *P. marinus*, suggesting that the combined effects of *A. fundyense* and trematodes may promote *P. marinus* infection or at least prevent regression.

The responses of hemocytes to the trematodes that were suppressed by *A. fundyense* exposure (THC, infiltrations in kidney and in digestive gland, hemocytes in gonoduct and follicle lumina) suggest that *A. fundyense* exposure altered immune functions. In fact, deficiencies in immune functions could explain why infection and proliferation of *P. marinus* was enhanced as oyster immune function was weakened. A similar hypothesis was formulated by Galimany et al. (2008) after observation of trematodes (*Gymnophallidae) in A. fundyense*-exposed *mussels*.

Another response to the interaction of *Bucephalus* sp. infestation and *A. fundyense* exposure was degeneration of adductor muscle fibers. Paralytic Shellfish Toxins (PSTs) and bucephalids individually are known to affect adductor muscles of bivalves (Calvo-Ugarteburu and McQuaid, 1998; Haberkorn et al., 2010b; Hégaret et al., 2012; Howell, 1967); however, in the present experiment, only the interaction of both led to a significant increase in myodegeneration. Again, suppression of hemocyte responses could have a role in this

pathology. A first hypothesis considers the potential role of hemocytes in carrying and detoxifying PSTs, which would be less efficient in parasitized oysters. Although the PST content in the digestive gland remained unchanged between oysters with or without trematodes, PST concentration in other tissues may have varied. Another hypothesis considers the role of hemocytes in nutrient digestion and transport, combined with trematode energy uptake, resulting in depressed nutrient supply to the muscle and energy depletion. The hypothesis that *A. fundyense* exposure combined with parasitic infection would modulate energetic metabolism is also supported by the decreased MnSOD gene expression in gills by trematodes, thus possibly altering the anti-oxidant response. The degeneration of adductor muscle fibers could have deleterious consequences upon valve movements and the catch state. Adductor muscle dysfunction also could affect filtration, interfering with respiration and nutrition functions, and could increase susceptibility of oysters to predation and exposure to environmental variations that otherwise could be moderated by shell closure.

**5 Conclusions**

Infestation with trematodes, *Bucephalus* sp., affected oyster, *C. virginica,* physiological functions including reproduction, immunity, and oxidative metabolism. In addition, considering histopathological lesions in gills and digestive tubules, respiration and digestion could be altered. Neither *A. fundyense* blooms nor *Bucephalus* sp. infestation have been reported to be lethal for *C. virginica*. The results of the present study suggest, however, that an *A. fundyense* bloom could intensify the severity of a potentially-lethal disease, Dermo, by impairing hemocyte immune functions, especially in trematode-infested oysters that would be more susceptible to Dermo infections. In addition, oysters with trematode infestations exposed to blooms of PST-producing microalgae may be more susceptible to predation as a consequence of muscle fiber degeneration that could compromise valve-closure movements and the catch state of the adductor muscle.

There is increasing evidence of HAB relationships with climate change (temperature, eutrophication, etc.) (Anderson et al., 2008; Hallegraeff et al., 2003), and HABs can modify host-pathogen interactions, for example by aggravating disease status as demonstrated in the

present study. In this context, a better understanding of complex biotic interactions is needed to predict and possibly prevent new disease outbreaks in bivalve populations.

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# Chapitre 3

## Les effets d'*Alexandrium* sp. sur la physiologie du bivalve peuvent-ils favoriser des infections opportunistes lors de l'exposition du bivalve à un nouvel environnement microbien ?

**Article 4:** *En préparation*

Lassudrie, M., Soudant, P., Nicolas, J.L., Miner, P., Le Grand, J., Lambert, C., Le Goic, N., Fabioux, C., Hégaret, H. Exposure to the toxic dinoflagellate *Alexandrium catenella* modulates juvenile oysters *Crassostrea gigas* hemocyte variables: possible involvement in susceptibility to opportunistic infections. In preparation.

# Article 4

## Exposure to the toxic dinoflagellate *Alexandrium catenella* modulates juvenile oysters *Crassostrea gigas* hemocyte variables: possible involvement in susceptibility to opportunistic infections

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**Abstract**

Toxic dinoflagellate *Alexandrium* sp. exposures have been reported to modulate bivalve interaction with specific pathogens, as well as physiological and immunological variables in bivalves. The Pacific oyster *Crassostrea gigas* is an important economical species widely cultured, which imply transfers to new environments that can be stressful, especially at young age. This study was undertaken to determine if exposure of juvenile oysters to a new microbial environment during an *Alexandrium* c*atenella* bloom, regularly occurring in French oyster bed cultures, could compromise oyster immune status and disrupt the microbiome stability, and thus modify its susceptibility to newly encountered pathogenic or opportunistic environmental bacteria. Juvenile specific pathogen-free (SPF) oysters naïve from the environment were subjected to an artificial bloom of *A. catenella*, simultaneously with a new microbial environment, simulated by a cohabitation challenge with previously field-exposed oysters.

Exposure to *A. catenella* induced hemocyte responses, possibly related to both PSTs and other extracellular compounds. Challenge with a new microbial environment provoked an inflammatory response, possibly as a response to bacterial virulence factors. Although hemocyte responses to a putative bacterial challenge were modulated by *A. catenella* exposure, thus demonstrating possible detrimental effects of the interaction of both stressors, the oyster microbiome remained stable. Overall, results of this study suggest that a short-term *A. catenella* exposure, combined with exposure to a new microbial environment have likely compromised physiological condition of the juvenile oysters but did not lead to a septicemic bacterial infection.

**Keywords** : *Crassostrea gigas*; *Alexandrium*; harmful algal blooms; microbiome; opportunistic pathogen; hemocyte

## 1 Introduction

The pacific oyster *Crassostrea gigas* is the most exploited bivalve species, with over 6 $10^5$ tons produced worldwide in 2012 (FAO, 2014). Recurrent losses caused by infectious diseases have been impacting aquaculture industry over the past decades (Bower et al., 1994; Lafferty et al., 2015; Lauckner, 1983). Oyster farming practices usually include numerous transfers of oysters at all life stages, especially of spat and juveniles grown in hatchery, that transit in ponds before being transferred to the oyster farming area in open seawater (Helm and Bourne, 2004). Along with these farming processes, oysters have to regularly face new abiotic and biotic environments. Biotic changes include interactions with new micro-organism communities, which can enter the oyster organism via filtration and feeding processes. Parts of these micro-organisms are transient, while another parts may become resident and thus modify the oyster microbiome (King et al., 2012). Whereas microbiota provide protection from pathogens to their hosts (Kamada et al., 2013), host-associated microbial communities themselves can act as a source of opportunistic pathogens under environmental perturbations or in immuno-compromised hosts (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Garnier et al., 2007; Olson et al., 2014). In addition, specific pathogenic agents have been involved in major diseases in bivalves including the herpesvirus OsHV-1µVar that is associated to recent massive mortality phenomenon of *Crassostrea gigas* spat and juveniles (Cochennec-Laureau et al., 2011; EFSA Panel on Animal Health and Welfare, 2010; Jenkins et al., 2013; Martenot et al., 2011; Renault et al., 2012; Schikorski et al., 2011a, 2011b; Segarra et al., 2010), and several bacterial species and strains from the *Vibrio* genus (Garnier et al., 2007; Gay et al., 2004; Paillard et al., 2004). A recent study showed that *Vibrio* communities in the oyster hemolymph are associated with the surrounding environment (Wendling et al., 2014).

Phytoplankton is another major actor in biotic interactions. Constituting oyster diet, it may also have deleterious effects when harmful algae are involved. Dinoflagellates are the most represented group causing harmful algal blooms (HABs) with species of the genus *Alexandrium*, producing Paralytic Shellfish Toxins (PST) and / or spirolides, both neurotoxic. *Alexandrium* sp. exposures can alter bivalve physiological processes and tissue integrity of bivalves (Cucci et al., 1985; Gainey and Shumway, 1988a, 1988b; Galimany et al., 2008a; Haberkorn et al., 2010b; Landsberg, 2002; Lassudrie et al., 2014; Medhioub et al., 2012; Shumway, 1990), and were reported to modulate host-

pathogen interactions in a few studies (Bricelj et al., 2011; Lassudrie et al., in rev., subm.). Immuno-suppression induced by *Alexandrium* sp. exposure was suggested to be responsible for these modifications of host-pathogen interactions (Bricelj et al., 2011), possibly through modulation of hemocyte variables (Lassudrie et al., in rev.). In fact, many studies reported effects of *Alexandrium* sp. exposure upon bivalve hemocytes (Haberkorn et al., 2010a; Hégaret et al., 2011, 2007).

Considering the effects of *Alexandrium* sp. upon host-pathogen interactions and physiological and immunological variables, we hypothesized that exposure of juvenile oysters to a new microbial environment during an *Alexandrium catenella* bloom could compromise oyster immune status and modify susceptibility to newly pathogenic or opportunistic bacteria, possibly by misbalancing oyster microbiome.

This study investigated the possible biotic interactions between juvenile oysters *C. gigas*, grown in hatchery, with a new biotic environment defined by (i) a punctual, artificial bloom of *A. catenella*, and (ii) the simulation of a new microbial environment induced by cohabitation with oysters previously exposed to the field, known to release and transmit pathogens (Petton et al., 2013). Responses of circulating hemocytes associated with bacterial community composition and toxin accumulation were assessed in order to investigate the possible effect of a toxic dinoflagellate upon oyster susceptibility to new bacterial environmental, through the study of the microbiome and associated immune responses. This study is the first, to our knowledge, to assess the effect of a HAB upon bivalve microbiome.

## 2 Material and methods

### *2.1 Algal cultures*

*Tisochrysis lutea* (Bendif & Probert) (T-*Iso*) was used as diet during acclimation and maintenance stages at 5 × $10^5$ cells $mL^{-1}$. T-*Iso* was cultured in 300-L cylinders containing seawater enriched with Conway medium (Walne, 1966) at 20°C with continuous light (200 µmol photons $m^{-2}$ $s^{-1}$). T-*Iso* was harvested after 3 to 5 days of growth, at a cell density approaching 1 × $10^7$ cells $mL^{-1}$.

The dinoflagellate *Alexandrium catenella* (Whedon & Kofoid) strain VGO676, PST producer (Lassus et al., 2007), isolated in 2003 from Thau lagoon (France) was used for toxic algal exposure, and *Heterocapsa triquetra* (Ehrenberg) Stein, strain HT99PZ (isolated from Penzé Bay, France in 1999) was used as a control non-toxic dinoflagellate. Both strains were provided by the Phycotoxin laboratory, Ifremer, Nantes (France). Both dinoflagellate cultures were grown in L1 medium (Guillard and Hargraves, 1993) at 17°C with a light:dark cycle of 12:12h and were harvested during exponential growth phase, at a cell density approaching 5 × $10^4$ cells $mL^{-1}$.

Algal cell densities were determined by counts using Malassez and Nageotte cells under a light microscope.

### *2.2 Specific Pathogen-Free (SPF) oysters*

The Pacific oysters, *Crassostrea gigas* (Thunberg), used in this study came all from a single cohort, produced in April 2011 in the Argenton Ifremer facilities (France) following a standardized procedure to obtain OsHV-1-free diploid-oysters described by Petton et al. (2013). Screening for OsHV-1 DNA was conducted by qPCR (following the standard procedure described in Pépin et al., 2008) a first time during D-larval stage and at 3 month old following thermal challenge, and returned all negative (analyses by IDHESA, Quimper, France). At the beginning of the experiment (September 2011), oysters were 5 month-old, measured 30 to 40 mm length and weighed 3.2 ± 0.2 g (total wet weight; mean ± SE).

*2.3 Field-exposure*

On September 1th, 2011, a subsample of the SPF oysters was transferred in an oyster farming area of the Bay of Brest, at Pointe du Chateau (48° 20' 06.19" N, 4° 19' 06.37" W), during a period of low mortality events in this location (5 % cumulative mortality in one month, RESCO, http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-nationaux/Resultats-nationaux-2011/Mortalite-par-site-et-par-classe-d-age). No harmful algal blooms were detected during this period (VELYGER and RESCO networks, Ifremer). After 2 weeks in the field (i.e. September 19th 2011), oysters were transferred to the experimental facilities on the first day of experiment and were used to challenge SPF oysters by cohabitation, as described below.

*2.4 Experimental design and sampling*

Experimental design is summarized in Figure 1.

On September 15th, 2011, 432 SPF oysters were distributed in 15-L tanks (36 SPF oysters per tank, 12 tanks) and acclimated for 4 days fed continuously with T-*Iso* at 3-5 × $10^5$ cell $mL^{-1}$. At the end of this acclimation period, on September 19th, 10 field-exposed oysters per tank, held in a net, were added to six of these tanks. The SPF oysters that were thus maintained in cohabitation with these field-exposed oysters were designated as "challenged". In the six other tanks, 10 other SPF oysters per tank, held in a net, were added to obtain the same number of oysters in all tanks. The oysters in these tanks were then designated as "unchallenged". In addition, 3 "challenged" tanks and 3 "unchallenged" tanks were exposed continuously to 1 × $10^2$ cell $mL^{-1}$ of the toxic dinoflagellate *A. catenella*, whereas the other 6 tanks were exposed to the same concentration of a control, non-toxic dinoflagellate, *H. triquetra.*

During the whole experiment, 1-µm-filtered and UV-sterilized seawater was supplied to the tanks (10 mL $min^{-1}$, i.e. one tank renewal every 24h) with aeration at 20°C, close to temperature in the field (VELYGER and RESCO networks, Ifremer, http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-nationaux/Resultats-nationaux-2011/Mortalite-par-site-et-par-classe-d-age).

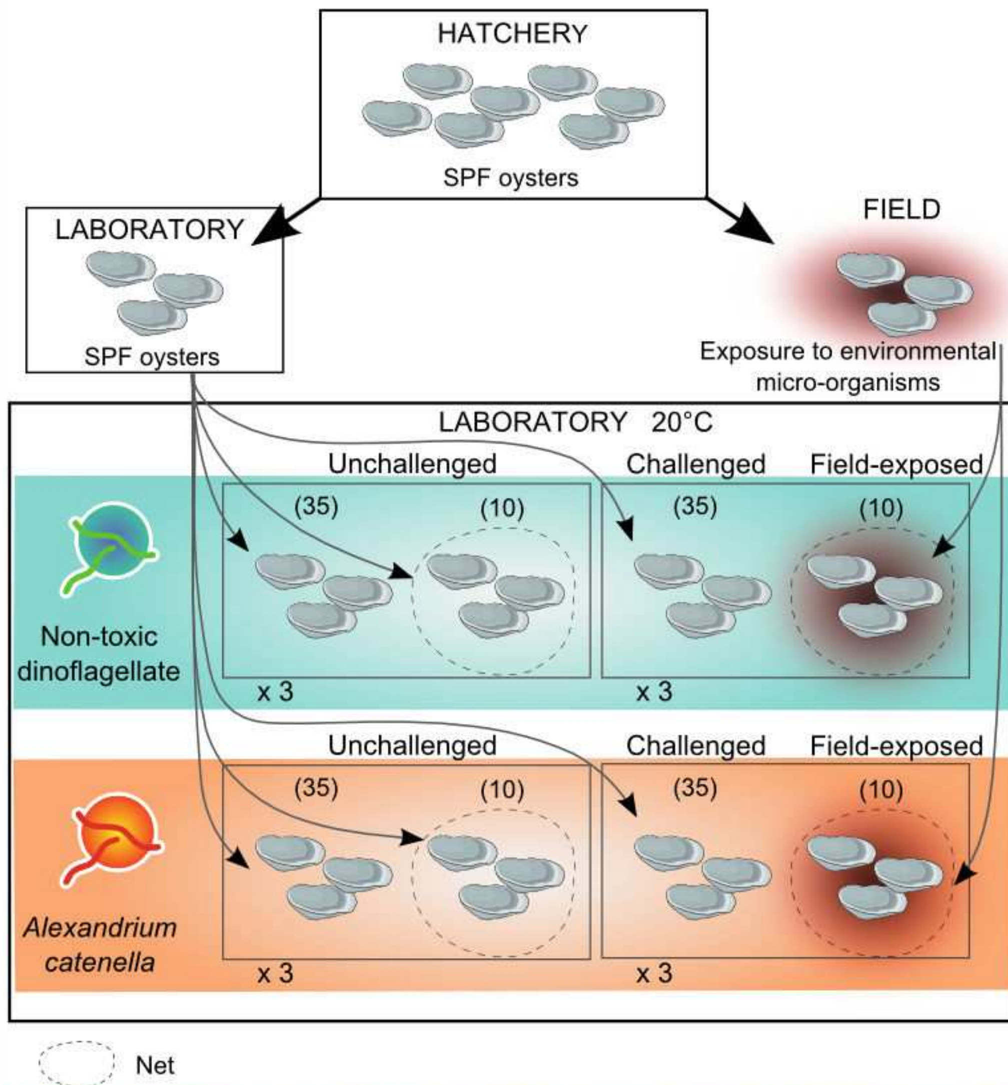


**Figure 1.** Scheme of the experimental design. All oysters used (5 months-old) were produced in the hatchery following a standardized process to obtain Specific Pathogen-Free (SPF) oysters. SPF and field-exposed oysters were sampled prior to exposure and cohabitation ($T_0$); challenged and unchallenged oysters were sampled after 4 days and 9 days of algal exposure and cohabitation with field-exposed oysters ($T_4$ and $T_9$).

## *2.5 Sampling*

Oysters were sampled after 4 and 9 days of exposure (12 oysters per tank per sampling time). For each oyster, hemolymph was withdrawn and immediately analyzed for hemocyte variables. In eight oysters per tank, mantle was dissected for OsHV-1 and *Vibrio* sp. quantification as well as bacterial community sequencing, and digestive gland was dissected for toxin quantification, and frozen in liquid nitrogen before being stored

at -80°C prior to analyses. In four oysters per tank, transversal sections were cut for parasite detection using histological analysis.

No mortality occurred during the experiment.

*2.6 OsHV-1 and* Vibrio *sp. quantifications by qPCR*

Quantification of OsHV-1 and bacteria related to the *Vibrio* genus was performed in the mantles of two SPF oysters per tank and ten field-exposed oysters at $T_0$, three SPF oysters per tank at $T_4$, and three SPF oysters and three field-exposed oysters per tank at $T_9$.

DNA extraction was performed with the QIAamp DNA Mini Kit (QIAgen), from 20 mg of wet mantle, following the manufacturer instructions. 100 µL of molecular biology grade-water (DNase-free) were used for elution. Nucleic acid concentration was measured with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (conversion factor: 1 OD = 50 µg $mL^{-1}$ DNA) at 260 nm and purity was checked using the 260 / 230 nm and 260 / 280 nm ratios.

A standard protocol was followed to quantify OsHV-1 DNA (OsHV-1 reference as well as OsHV-1µVar) using qPCR (Pépin, 2013; Pépin et al., 2008) with the HVDP-F – HVDP-R primer pair (forward, HVDP-F 5'-ATT-GAT-GAT-GTG-GAT-AAT-CTG-TG-3', reverse, HVDP-R 5'-GGT-AAA-TAC-CAT-TGG-TCT-TGT-TCC-3'), targeting OsHV-1 polymerase sequence. Each reaction was performed in triplicate and carried out in a final volume of 15 µL containing each HVDP-F and HVDP-R primers at a final concentration of 5 µM, 7.5 µL of IQ SYBR Green Supermix and 5µL of DNA samples diluted at 3 ng $\mu L^{-1}$. Each run included a no template control (water), a positive control (DNA from OsHV-1-infected oyster), and six standards (from $10^5$ to $10^0$ OsHV-1 copies $\mu L^{-1}$), prepared by successive ten-fold dilutions of a stock solution of OsHV-1 genomic DNA at $5 \times 10^6$ copies $\mu L^{-1}$ extracted from purified virus particles (Le Deuff and Renault, 1999). The standard curve obtained was used to calculate the percentage of amplification efficiency (% E) described in equation (1), which was comprised between 90% and 110%, and quantification of the samples was determined by comparing Ct values.

(1) % E = $(10^{-1/a} - 1) \times 100$; with *a* = slope of the linear regression calculated from Ct = f($\log_{10}$ of dilution).

The MyIQ2 Thermocycler (Biorad) was used with the following thermal profile: 1 cycle of enzyme activation (95°C, 3min.), 40 cycles of amplification/detection (95°C, 30s.; 60°C, 1min.; 72°C, 1min.), and a final step for melting temperature curve analysis (80 cycles, 95°C to 55°C, decreasing the temperature by 0.5°C after each cycle, 10s). The specificity of the PCR products was systematically checked with the melting temperature (Tm) value calculated from the dissociation curve.

For *Vibrio* quantification, qPCR was used following a standard protocol adapted from Thompson et al. (2004), with the 567F – 680 R primer pair, targeting the 16S rRNA gene (forward: 567F, 5'-GGC-GTA-AAG-CGC-ATG-CAG-GT-3'; reverse: 680R, 5'-GAA-ATT-CTA-CCC-CCC-TCT-ACA-G-3'). Reactions were performed in triplicate and carried out in a final volume of 25 µL containing each 567F and 680 R primers at a final concentration of 70 nM, 12.5 µL of ABsolute QPCR SYBR Green ROX Mix and 5µL of DNA samples diluted at 50 ng $\mu L^{-1}$. Each run included a no template control (water) and six standards ranging from $2.1 \times 10^{0}$ to $2.1 \times 10^{5}$ CFU $\mu L^{-1}$, prepared by successive ten-fold dilutions of purified DNA from cultured *V. aestuarianus* 02/041 strain, counted on Marine Agar (Difco) plates. The standard curve obtained was used to calculate the percentage of amplification efficiency (% E) as described above in equation (1) which was comprised between 85% and 100%, then quantification of the samples was determined by comparing Ct values. The Applied Biosystems 7300 Real Time PCR System was used with the following thermal profile: 1 cycle of polymerase activation (95°C for 15 min.), 40 cycles of amplification/detection (95°C for 15 s.; 60°C for 1 min), and a dissociation stage for melting temperature curve analysis (95°C for 15 s, 60°C for 30 s, 95°C for 15 s).

Results were expressed as number of *Vibrio* sp. CFU $mg^{-1}$ of wet mantle, upon which statistical analyses were performed. Additionally, quantification expressed as *Vibrio* sp. CFU $ng^{-1}$ total DNA, which followed a linear, significant relationship with *Vibrio* sp. CFU $mg^{-1}$ of wet mantle (Pearson product moment correlation, correlation coefficient=0.96, $p<0.001$, n=114), was mentioned. *Vibrio* sp. detection was considered positive when Ct<38, associated to a Tm corresponding to *Vibrio* sp DNA amplicon (81.5-83.5°C). Moreover, when the Ct was higher than the Ct of the most diluted

standard, although sample was considered positive for *Vibrio* sp. detection, quantification could not be determined and was thus estimated as 0.

*2.7 Bacterial community analysis*

*Library preparation and 454 pyrosequencing.* DNA previously extracted from mantle was used for sequencing. Thirty three samples were processed, each of them consisting of a pool of equal concentrated DNA, extracted from 3-6 individuals from the same tank and sampling time. Replicated samples per condition (n=3, except for unchallenged oysters exposed to *H. triquetra* n=2, and SPF oysters at $T_0$: n=1) were analyzed at $T_0$, $T_4$ and at $T_9$.

A nested PCR method was used to avoid co-amplification of oyster DNA (Prosdocimi et al., 2013; Wegner et al., 2013). The 1$^{st}$ PCR consisted in the amplification of a ~1500 bp sequence with the forward SAdir (5'-AGA-GTT-TGA-TCA-TGG-CTC-AG-3') and reverse S17rev (5'-CGG-YTA-CCT-TGT-TAC-GAC-3') primer pairs, specific for Eubacteria (Kalmbach et al., 1997), comprising the region targeted in the 2$^{nd}$ PCR. Final PCR reactions of 50 µL contained 100 ng of DNA which was mixed with 10 µL of GoTaq reaction Buffer (Promega; 1X final concentration) which contained $MgCl_2$ (1.5 µM final concentration), 0.2 mM of dNTP, 0.2 µM of each primer, 0.25 µL of GoTaq G2 polymerase (Promega; 0.025U/µL final concentration) and 22.75 µL of molecular grade water. Duplicate were run for each sample, with a NTC (no template control) and a positive control (*V. aestuarianus* 02/041 DNA) for each primer pair. A touchdown thermal cycling program was used: initial denaturation and polymerase activation of 2 min at 95°C followed by a low number of 15 cycles of denaturation, annealing and extension, with 45 s at 95°C, 45 s at 58°C then decrementing of 0.4°C per cycle (last cycle: 52°C) and 1 min 30 s at 72°C, and a final extension of 5 min at 72°C. The 2$^{nd}$ PCR amplified a ~520 bp sequences length, corresponding to the variable regions V2-V3 of the 16S rRNA gene, using universal bacterial primers 27F (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG -3') and 533R (5'-TTA-CCG-CGG-CTG-CTG-GCA-C-3') modified at the 5' position by the addition of Multiplex Identifiers (MIDs), consisting in unique 10 bases-barcode sequences, to discriminate each sample (Table S1). The DNA product from the first PCR was diluted at 1/100, and 10 µL was used in the 2$^{nd}$ PCR, performed in triplicate for each products of the 1$^{st}$ PCR (i.e. six final replicates for each

sample and for the blank extraction), following the same protocol as the 1st PCR. The touchdown thermal cycling program was: 2 min at 95°C, 35 cycles with 45 s at 95°C, 45 s at 63°C then decrementing of 0.3°C per cycle (last cycle: 52.5°C) and 30 s at 72°C, and a final extension of 5 min at 72°C. These PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The sole presence of the desired amplicon was then verified on gel electrophoresis. After DNA concentration was determined with Quant-iT PicoGreen dsDNA assay (Invitrogen), PCR products were pooled at equal concentration. DNA sequences of 400-800 bp were selected with Pippin Prep and purified with Agencourt AMPure XP (Beckman, USA). Purity and quality were verified with a NanoDrop (Thermo Scientific) and an Agilent 2100 bioanalyzer (Agilent Technologies). Sequencing was performed by the pyrosequencing method, using 454 adaptators added at each sequence tail, on the 454 Roche Genome Sequencer FLX using Titanium chemistry, by Beckman Coulter Genomics (Danvers, MA, USA), generating reads from both ends with a read length of ~500 bp.

*Data processing.* The Quantitative Insights Into Microbial Ecology 1.8.0 (QIIME) pipeline (Caporaso et al., 2010b) was used for processing of raw sequences with quality scores. Sequences were split into multiple libraries corresponding to each sample based on their respective barcode. Valid reads should comply with the following conditions: sequence length comprised between 350 and 600 bp, no mismatch with the primers and one of the used barcodes, quality score ≥ 25 and no singleton. Operational taxonomic units (OTUs) were picked using uclust_ref (Edgar, 2010) at a 97% similarity threshold, searching against reference sequences from Greengenes v13.8 database (chimera-checked, DeSantis et al., 2006). A unique representative sequence for each OTU was set based on the most abundant sequences. Sequences were assigned with UCLUST algorithm to the genus level, with similarity threshold values of 0.97 and a minimum of 75% of closest blast hit with the Greengenes taxonomy as reference database (McDonald et al., 2012). Pairwise alignment was built with uclust in PyNAST (Caporaso et al., 2010a) against the Greengenes core set alignment and filtered to remove highly variable regions. OTUs corresponding to chloroplasts were removed (considered as artifacts from microalgae attached to the mantle surface).

Potentially pathogenic taxa included genera described as pathogenic in animals and were determined according to Paillard et al. (2004), Wegner et al. (2013) and PATRIC web resources (Wattam et al., 2014).

### *2.8 Parasite detection by histology*

Two diagonally-slanted, 5-mm sections of soft tissue, including gills, mantle, digestive gland, intestine and gonad, were excised. Additionally, a section of adductor muscle was sampled. Tissues were fixed immediately in Davidson's solution (Shaw and Battle, 1957) for 24 h. Tissues then were transferred into 70% ethanol, dehydrated in ascending ethanol solutions, cleared with Claral®, and embedded in paraffin wax. Five-µm sections were stained with Harris' hematoxylin and eosin (Howard et al., 2004), and observed under a light microscope for any parasitic infestation visible in histological slides.

### *2.9 Toxin accumulation*

PST accumulation was measured individually in the digestive gland (8 oysters per tank exposed to *A. catenella*; 1 oyster per tank exposed to the control alga *H. triquetra*) with the PSP ELISA kit (Abraxis). PST extraction was performed 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. Dilutions of this homogenate was used in the ELISA assay. Toxicity was expressed as µg STX $kg^{-1}$ of wet digestive gland weight.

### *2.10 Analysis of hemocyte variables*

Hemolymph withdrawn from adductor muscle was stored temporarily in Eppendorf microcentrifuge tubes held on ice before flow-cytometric analysis. Characteristics determined in live circulating hemocytes: total (THC) and differential hemocyte counts (granulocytes and hyalinocytes) (in cell $mL^{-1}$), size, and internal complexity (in arbitrary units, a.u.), as well as mortality (percentage of dead hemocytes) – were

assessed following Hégaret et al. (2003). Functional responses, i.e. production of reactive oxygen species (ROS) (specifically $H_2O_2$ and $O_2^{\bullet -}$) by unstimulated hemocytes was determined as described in Delaporte et al. (2003) and Lambert et al. (2003). Hemocyte analyses were performed with a FACScalibur flow-cytometer (BD), and data were processed using WinMDI 2.8 software. Impossibility to withdraw hemolymph from individual oysters was recorded.

*2.11 Statistical analyses*

Differences in *Vibrio* sp. DNA quantification according to "Algal exposure", "Challenge condition" "Field-exposure" were tested at each sampling time with Mann-Whitney test, to assess the effect of each factor individually, and with Kruskal-Wallis test followed by Nemenyi-Damico-Wolfe-Dunn (NDWD) post-hoc test to compare each group and identify possible combined effects between factors.

In *A. catenella*-exposed oysters, differences in measurements of PST accumulation regarding challenge condition, field-exposure and sampling time were estimated with Kruskal-Wallis test followed by NDWD post-hoc test.

Hemocytes variables were first analyzed with 3-way ANOVA to test effects of "Time", "Algal exposure" and "Challenge condition" and their interactions. Then, when "Time" was not significant, this factor was removed from the analysis, thus data were analyzed over the entire experiment with 2-way ANOVA followed by LSD post-hoc test. When the factor "Time" was significant, 2-way ANOVA testing the effects of "Algal exposure", "Challenge condition" and the interaction was performed at each sampling time, followed by LSD post-hoc test. When needed, data were transformed as log(X+1) or 1/X to meet normality of residuals and homoscedasticity. Percentage data were transformed as Asin(sqrt(X/100)). Multiple correlations were tested among hemocyte variables, *Vibrio* sp. quantification and PST accumulation using Spearman rank correlations. In addition, differences in the difficulty to withdraw hemolymph from the adductor muscle between the four conditions defined by algal exposures and challenge conditions (i.e. four conditions: "Unchallenged – *A. catenella*"; "Unchallenged – *H. triquetra*"; "Challenged – *A. catenella*"; "Challenged – *H. triquetra*") were tested at $T_4$

and at $T_9$ with a Chi-square test with Holm-Bonferroni correction for multiple comparison.

Microbiome data were analyzed with rarefaction curves for Shannon's H to visualize alpha-diversity in each condition. Differences in Shannon's H diversity index between conditions were tested with Kruskal-Wallis test. Beta-diversity was assessed in the whole dataset, and in the potentially pathogenic OTUs only, using Bray-Curtis distance matrices built after Asin(sqrt(X/100)) transformation of relative abundance of OTUs in each sample. Nonmetric multidimensional scaling (NMDS) and Analysis of Similarity (ANOSIM) were performed to visualize and statistically test the effects of "Algal exposure" and "Challenge condition" at $T_4$, and "Algal exposure", "Challenge condition" and "Field-exposure" at $T_9$. $T_0$ data were represented in the figures but were excluded from statistical analyses because of the lack of replicate for SPF condition. Differences between the sums of potentially pathogenic relative abundance according to experimental conditions were tested with Kruskal-Wallis test.

Differences were considered significant when $p<0.05$ for all statistical tests. Statistical analyses were performed using Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA), R version 2.15.1 (R Core Team, 2012), and PRIMER6 software (PRIMER-E Ltd). All values were expressed as mean ± standard error.

## 3 Results

### *3.1 OsHV-1 DNA quantification*

OsHV-1 DNA was not detected in any analyzed mantle samples, regardless of the sampling time ($T_0$, $T_4$, $T_9$) or the experimental condition (field-exposed, challenged or unchallenged oysters; exposed to *Alexandrium catenella* or *Heterocapsa triquetra*) (n=114).

### *3.2* Vibrio *sp quantification*

Over the entire experiment, bacteria related to the *Vibrio* genus were detected in the mantle of 93 ± 3% of the oysters, and their prevalence in oysters was not significantly different between conditions.

At $T_0$, a significantly higher amount of *Vibrio* sp. and related bacteria DNA was detected in mantle of field-exposed oysters ($2.5 \pm 0.8 \times 10^3$ CFU $mg^{-1}$ wet mantle or $1.1 \pm 0.3 \times 10^0$ CFU $ng^{-1}$ total DNA, n=10) compared to SPF oysters ($2.6 \pm 1.0 \times 10^2$ CFU $mg^{-1}$ wet mantle or $1.1 \pm 0.4 \times 10^{-1}$ CFU $ng^{-1}$ total DNA, n=24) (Figure 2).

After 4 days of experiment, no significant difference in *Vibrio* sp. DNA quantification was detected among unchallenged and challenged oysters, exposed to *H. triquetra* or *A. catenella* ($4.3 \pm 0.7 \times 10^2$ CFU $mg^{-1}$ wet mantle or $2.0 \pm 0.3 \times 10^{-1}$ CFU $ng^{-1}$ total DNA, n=36).

After 9 days of experiment, no significant difference in *Vibrio* sp. quantification was detected among algal exposure, challenge condition or field-exposure, although *Vibrio* sp. load tended to remain higher in field-exposed oysters ($1.3 \pm 0.7 \times 10^3$ CFU $mg^{-1}$ wet mantle or $6.9 \pm 3.1 \times 10^{-1}$ CFU $ng^{-1}$ total DNA, n=18) compared to SPF oysters (both unchallenged and challenged) ($4.8 \pm 1.2 \times 10^1$ CFU $mg^{-1}$ wet mantle or $3.0 \pm 0.7 \times 10^{-2}$ CFU $ng^{-1}$ total DNA, n=36).

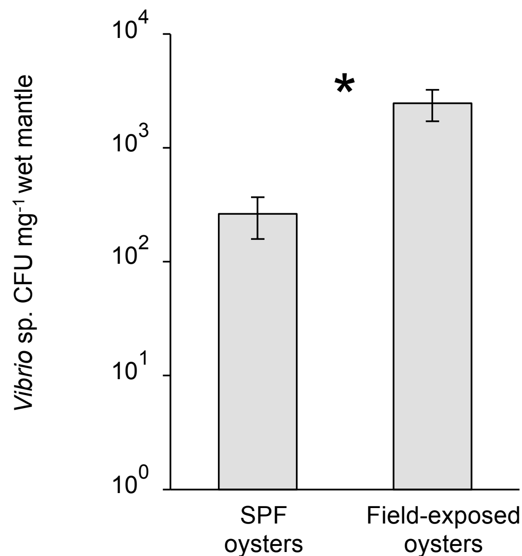


**Figure 2.** Quantification of *Vibrio* sp. in mantle of Specific Pathogen-Free ("SPF") oysters or oysters held two weeks in the field ("field-exposed") before the beginning of the experiment ($T_0$). *: significant difference between conditions, $p<0.05$ (Mann-Whitney test). Mean ± SE. N=24 SPF oysters and 10 field-exposed oysters.

*3.3 Bacterial community in the mantle*

Pyrosequencing of the 33 samples generated a total of 654,003 raw reads with a median size of 476 bp. After trimming, assignment and alignment, 189 genera were represented. A few OTUs belonging to Proteobacteria phylum largely dominated the bacterial assemblage (>90% in each condition) (Figure 3). The dominant OTUs were assigned to the Betaproteobacteria class, mainly represented by the genera *Ralstonia* (>30%), *Pelomonas* (>30%), and by an unidentified genus from Comamonadaceae family (>10%), and to the Alphaproteobacteria class, with mainly the genus *Sphingomonas* (>4%). These dominant taxa corresponded to bacteria that are not reported to be pathogenic. Shannon's H rarefaction curves were very similar between experimental conditions and time of sampling (Figure S1) and no significant difference of Shannon's H index was detected. Significant differences in beta-diversity (Bray-Curtis distances) were detected between $T_4$ and $T_9$ (ANOSIM; R=0.172, $p<0.05$), thus effects of experimental conditions were analyzed separately at each sampling time. At $T_9$, a significant effect of field-exposure upon beta-diversity was detected (ANOSIM, R=0.440, $p<0.001$), as visualized in the NMDS (Figure 4). Algal exposure and challenge condition did not induce any significant changes at $T_4$ and at $T_9$, with similar patterns of OTU composition found between these conditions.

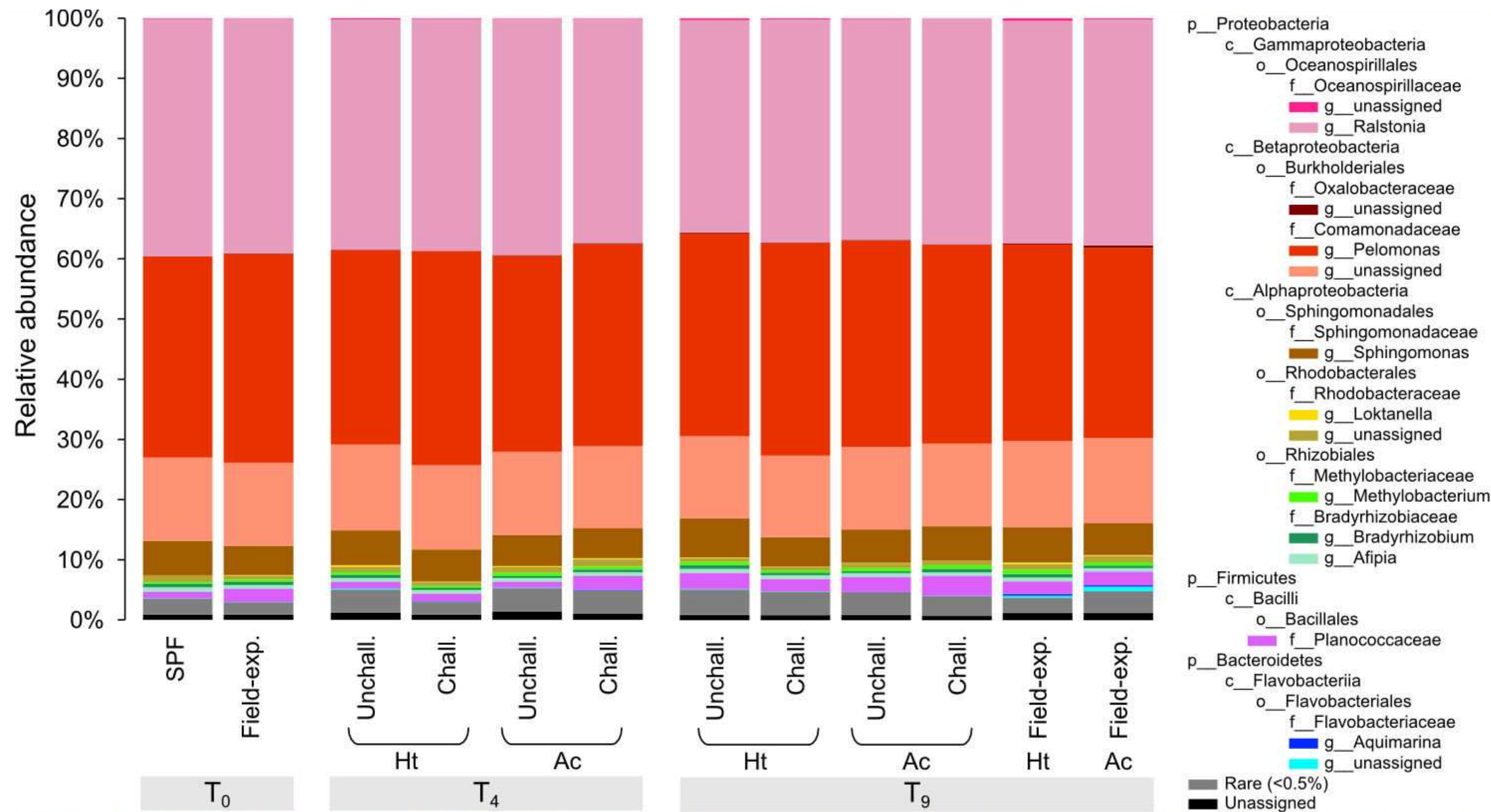


**Figure 3.** Mean relative abundance of juvenile oyster microbiome in mantle according to field-exposure or to challenge ("SPF": Specific Pathogen-Free; "Field-exp.": field-exposed ;"Unchall.": unchallenged; "Chall.": challenged by cohabitation with field-exposed oysters) and algal treatment ("Ht": *Heterocapsa triquetra*; "Ac": *Alexandrium catenella*), at the beginning of the experiment ("$T_0$"), after 4 ("$T_4$") and 9 days ("$T_9$") of experiement. p_: phylum; c_: class; o_: order; f_: family; g_: genus. Rare OTUs represented less than 0.5% of the whole dataset. N=2-3 samples (pool of 3 to 6 oysters).

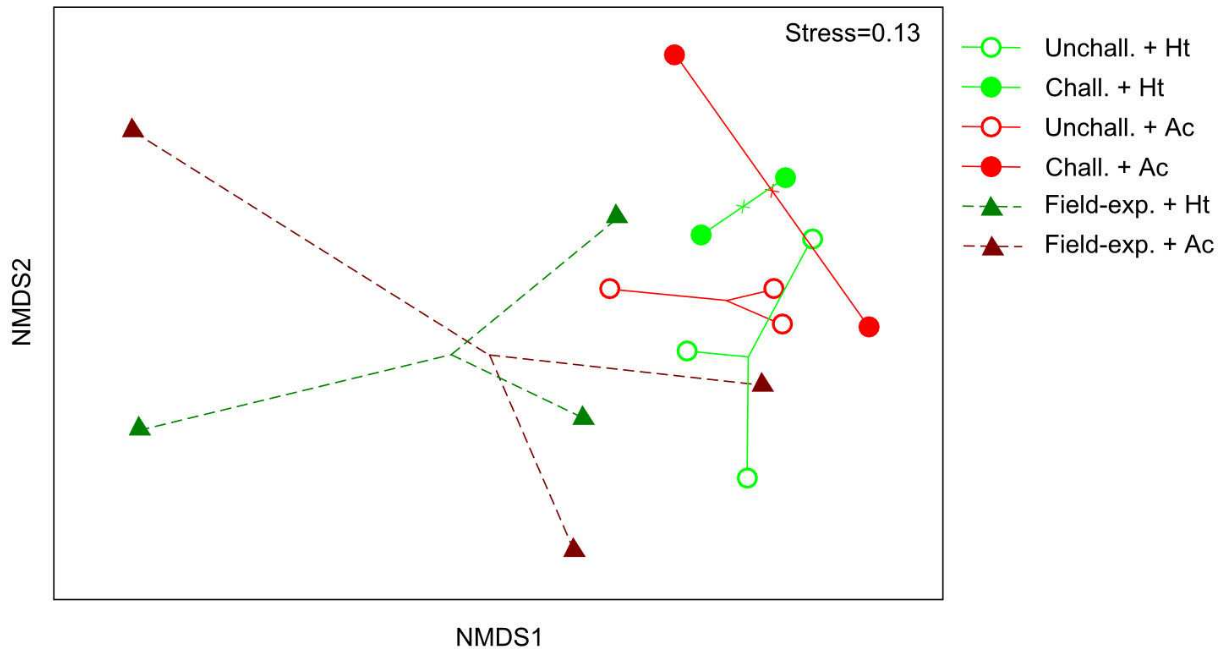


**Figure 4.** Non-metric multidimensional scaling (Bray-Curtis distances) of the whole bacterial communities in juvenile oyster mantle after 9 days of experiment, according to challenge condition or field exposure ("Unchall.": unchallenged; "Chall.": challenged by cohabitation with field-exposed oysters; "Field-exp.": field-exposed) and algal treatment ("Ht": *Heterocapsa triquetra*; "Ac": *Alexandrium catenella*). Symbols represent bacterial community of each sample (pool of 3 to 6 oysters) connected with group centroids.

Potentially pathogenic bacteria identification included 17 OTUs (Figure 5). Significant effect of time upon beta-diversity (Bray-Curtis distances) was detected (ANOSIM; R=0.345, $p<0.01$), thus effects of experimental conditions were analyzed separately at each sampling time. Field-exposure (at $T_9$) also induced a significant difference in beta-diversity of potentially pathogenic OTUs (Bray-Curtis distances; ANOSIM, R=0.232, $p<0.05$), and no significant difference was detected at $T_4$ and at $T_9$ according to algal exposure and challenge condition (NMDS). No significant difference between sums of relative abundances (relatively to the whole bacterial community) of potentially pathogenic bacteria was detected between conditions; however trends could be observed (Figure 5): at $T_0$, field-exposed oysters tended to contain a higher relative abundance of potentially pathogenic bacteria compared to SPF oysters. This trend could not be observed anymore at $T_9$ when comparing field-exposed oysters to unchallenged and challenged oysters. At $T_4$ and $T_9$, challenged-oysters exposed to *A. catenella* contained a non significant higher relative abundance of potentially pathogenic bacteria compared to the other conditions.

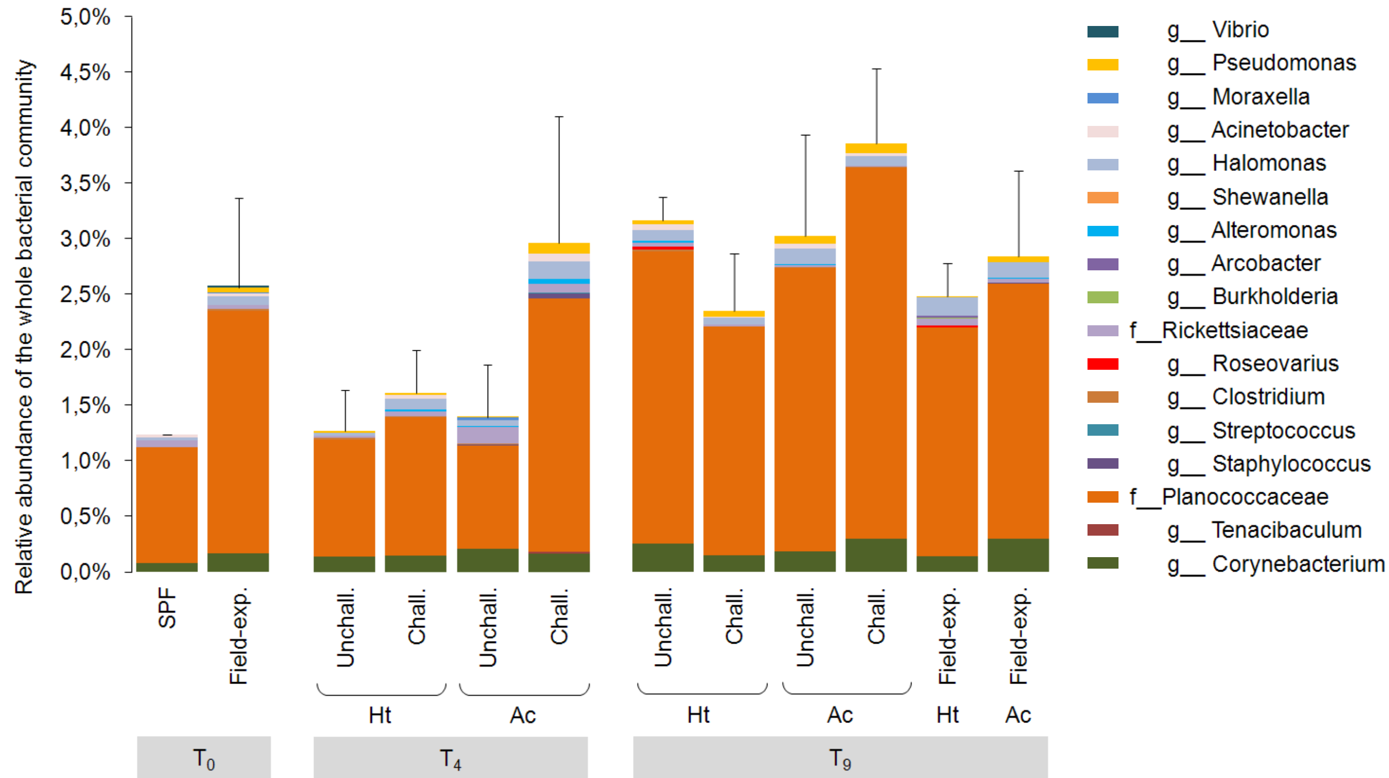


**Figure 5**. Mean relative abundance of potentially pathogenic bacteria of juvenile oysters in mantle according to challenge or field-exposure ("SPF": Specific Pathogen-Free; "Field-exp.": field-exposed ;"Unchall.": unchallenged; "Chall.": challenged by cohabitation with field-exposed oysters) and algal treatment ("Ht": *Heterocapsa triquetra*; "Ac": *Alexandrium catenella*), at the beginning of the experiment ("$T_0$"), after 4 ("$T_4$") and 9 days ("$T_9$") of experiement. f_: family; g_: genus. N=2-3 samples (pool of 3 to 6 oysters). Error bars represent standard error of the mean of the sum of relative abundances. No significant difference was detected among groups (Kruskal-Wallis test).

### *3.4 Parasite detection by histology*

No parasite infestation was detected in histological slides.

### *3.5 PST accumulation*

No PST was detected in the digestive glands of oysters exposed to the non-toxic dinoflagellate *H. triquetra*, challenged or unchallenged, at both $T_4$ and $T_9$, and in field-exposed at $T_9$ (n=24 per condition and per sampling time).

After 4 days of experiment, PST were detected in the digestive gland of *A. catenella*-exposed oysters, and no significant difference was detected between challenged and unchallenged oysters (n=24 per condition). After 9 days of experiment, however, challenged oysters accumulated significantly 2.6-fold less PSTs than unchallenged oysters (n=24 per condition) and field-exposed oysters (n=24 per condition) (Figure 6).

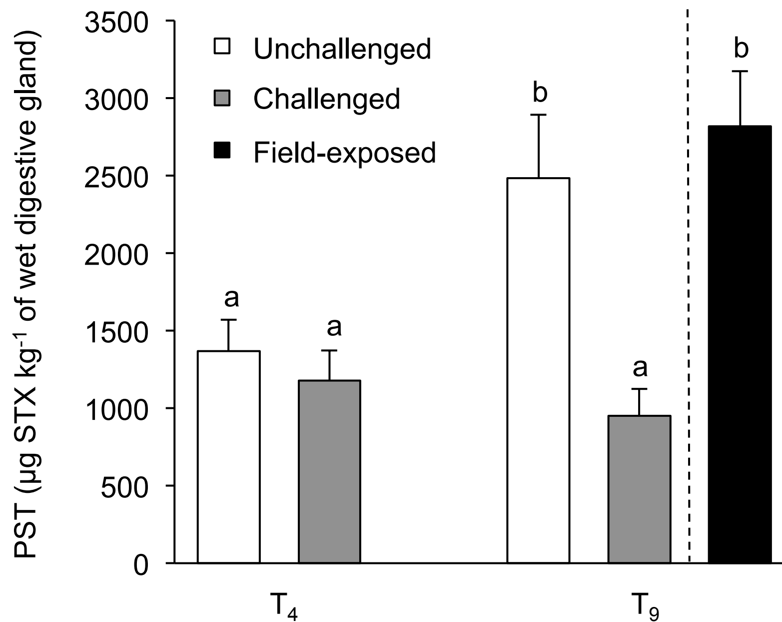


**Figure 6.** Paralytic Shellfish Toxin (PST) accumulation after 4 ($T_4$) and 9 ($T_9$) days of exposure to *Alexandrium catenella* in the digestive glands of unchallenged, challenged and field-exposed juvenile oysters (analyzed at $T_9$ only). Field-exposed oysters were analyzed only at $T_9$. Letters indicate significant differences between conditions (Kruskal-Wallis test followed by NDWD post-hoc test; $p<0.05$). Mean ± SE. N=24 per condition.

### *3.6 Hemocyte variables*

Statistical effects of algal exposure (*A. catenella* or to control *H. triquetra*), "challenge" (challenged by cohabitation with field-exposed oysters or control unchallenged) upon hemocyte variables of juvenile *Crassostrea gigas* are reported in Table 1 and represented in Figure 7. When significant effect of time of sampling ($T_4$ and $T_9$, i.e. 4 and 9 days of experiment) was detected, results were examined at $T_4$ and $T_9$, otherwise, results were considered over the entire course of the experiment.

Over the entire experiment, the percentage of granulocytes (Figure 7B) was significantly higher in *A. catenella*-exposed oysters compared to oysters exposed to the control dinoflagellate, and varied only according to algal exposure without being affected by cohabitation with field-exposed oysters.

Both *A. catenella* exposure and challenge condition led to significantly higher ROS production in hemocytes (Figure 7C) and granulocyte internal complexity (Figure 7D) during the entire experiment; and to higher granulocyte size (Figure 7E) after 4 days of experiment. After 9 days of experiment, however, only the effect of challenge condition persisted upon granulocyte size (Figure 7E), which was significantly higher in challenged oysters compared to unchallenged oysters.

Interaction of both algal exposure and challenge condition significantly impacted total hemocyte count (THC; Figure 7A), hyalinocyte internal complexity (Figure 7G) and size (Figure 7H) over the entire experiment. In oysters exposed to the control dinoflagellate *H. triquetra*, significant higher THC (Figure 7A) and hyalinocyte complexity (Figure 7G) were measured in challenged oysters compared to unchallenged oysters. Additionally, in unchallenged oysters, *A. catenella* exposure led to higher THC (Figure 7A) and hyalinocyte complexity (Figure 7G). In *A. catenella*-exposed oysters, however, measurements of these variables were not higher in response to challenge condition.

Finally, after 4 days of experiment, a significant synergistic effect was detected with the interaction of both *A. catenella* exposure and challenge by cohabitation with field-exposed oysters, causing an increase in mortality of hemocytes (both granulocytes and

hyalinocytes, Figure 7F, 7I). After 9 days of experiment, however, no significant change was detected between different conditions.

Additionally, a significant difference ($p<0.05$) in the possibility to withdraw hemolymph was noted between conditions, at $T_9$ only. Indeed, at $T_9$, whereas hemolymph was successfully withdrawn from 83% of the control oysters, i.e. unchallenged oysters exposed to the non-toxic *H. triquetra*, only 43% of the other oysters (unchallenged exposed to *A. catenella*, or challenged, regardless of the algal exposure) were successfully bled.

Multiple correlations between hemocyte variables, PST accumulation and *Vibrio* sp. quantification over the course of the experiment ($T_4$ and $T_9$) are presented in Table 2. Most of hemocyte variables were significantly correlated with each other. In addition, positive significant correlations were detected between PST accumulation and THC, percentage of granulocytes, hyalinocyte size and complexity, and ROS production. *Vibrio* sp. quantification showed significant positive correlation with ROS production only.

**Table 1**. Statistical effects of “time” (4 or 9 days of experiment), “algal exposure” (*Alexandrium catenella* or control *Heterocapsa triquetra*), “challenge” (challenged by cohabitation with field-exposed oysters or unchallenged) upon hemocyte variables of juvenile oysters *Crassostrea gigas*, either over the entire experiment (“$T_4 + T_9$”) when no significant effect of “time” was detected, or, when significant effect of “time” was detected, after 4 days of experiment (“$T_4$”), after 9 days of experiment (“$T_9$”). NS: No significant difference; * Significant difference indicated by $p<0.05$; ** Significant difference indicated by $p<0.01$ (including factor “time”: 3-way ANOVA or without factor “time”: 2-way ANOVA); “-”: no analysis. N in each group is indicated.

| Hemocyte variables | Over the entire experiment ($T_4 + T_9$) | | | | $T_4$ | | | $T_9$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Time | Algal exposure | Challenge | Interaction Algal exposure × Challenge | Algal exposure | Challenge | Interaction Algal exposure × Challenge | Algal exposure | Challenge | Interaction Algal exposure × Challenge |
| | N=51-63 | N=50-64 | N=51-63 | N=23-36 | N=29-34 | N=30-33 | N=12-18 | N=21-30 | N=21-30 | N=10-20 |
| **THC** | NS | *** | NS | * | - | - | - | - | - | - |
| **% granulocytes** | NS | *** | NS | NS | - | - | - | - | - | - |
| **Granulocyte size** | * | - | - | - | ** | * | NS | NS | * | NS |
| **Hyalinocyte size** | NS | *** | NS | * | - | - | - | - | - | - |
| **Granulocyte complexity** | NS | ** | ** | NS | - | - | - | - | - | - |
| **Hyalinocyte complexity** | NS | *** | * | * | - | - | - | - | - | - |
| **ROS production** | NS | *** | *** | NS | - | - | - | - | - | - |
| **Granulocyte mortality** | ** | - | - | - | * | * | * | NS | NS | NS |
| **Hyalinocyte mortality** | ** | - | - | - | *** | *** | *** | NS | NS | NS |

THC: total hemocyte count; ROS: reactive oxygen species.

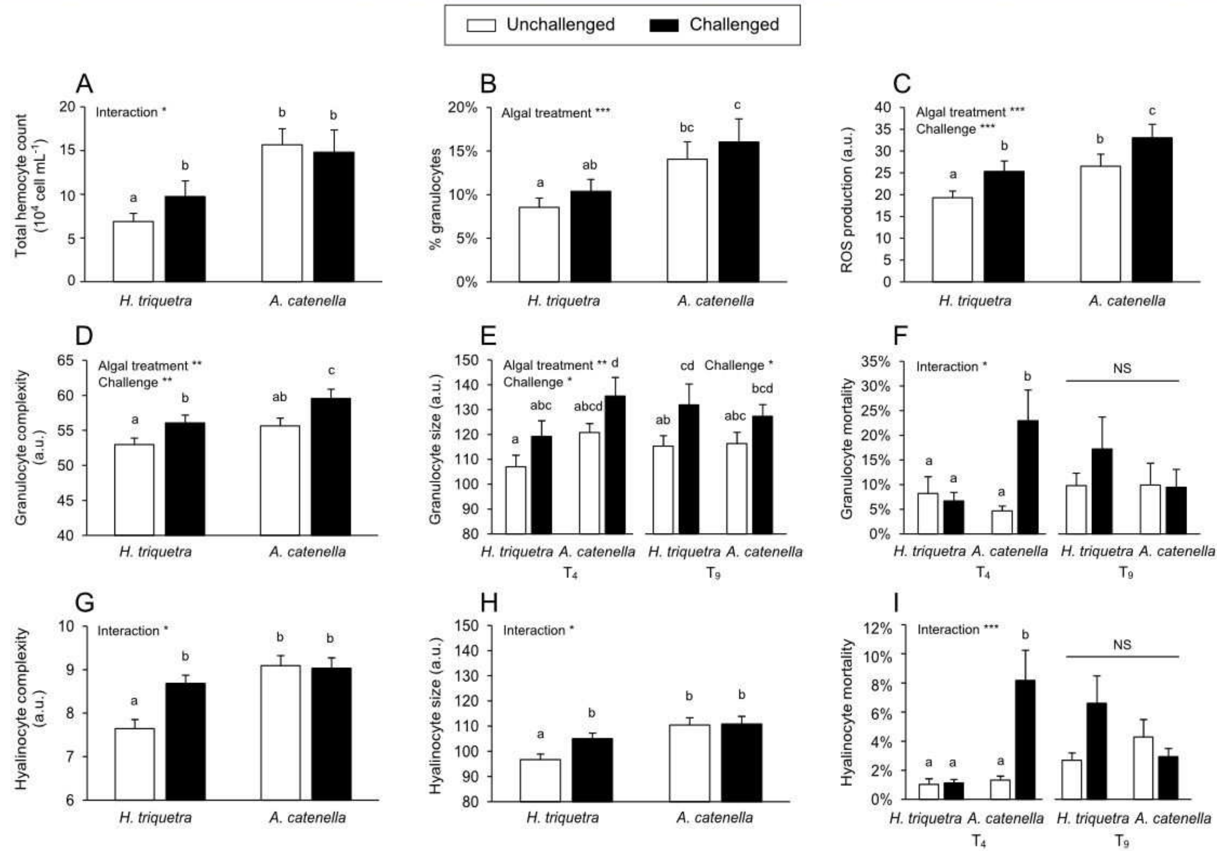


**Figure 7**. Hemocyte variables in the circulating hemolymph of oysters *Crassostrea gigas* according to "challenge condition" (unchallenged or challenged by cohabitation with field-exposed oysters) and "algal treatment" (*Heterocapsa triquetra* or *Alexandrium catenella*), either over the entire experiment ($T_4 + T_9$) when no statistical effect of the time of sampling was detected, or at $T_4$ and $T_9$ separately, as specified, when effect of the time of sampling was significant (3-way ANOVA, with "Time", "Challenge condition" and "Algal treatment" as main factors and their interactions). (A) Total hemocyte count; (B) percentage of granulocytes; (C) ROS production; (D) Granulocyte complexity; (E) Granulocyte size: (F) Granulocyte mortality (percentage of dead granulocytes); (G) Hyalinocyte complexity: (H) Hyalinocyte size; (I) Hyalinocyte mortality (percentage of dead hyalinocyte). NS: No significant difference; Significant differences are indicated by *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ (2-way ANOVA). Letters indicate significant differences between groups (LSD post-hoc test). Mean ± SE. $N_{T4}$= 12-18 oysters per group, $N_{T9}$= 10-20 oysters per group, $N_{T4+T9}$ =23-36 oysters per group.

**Table 2.** Spearman rank correlations between hemocyte variables, PST accumulation in the digestive gland, and *Vibrio* sp. quantification in the mantle of experimental juvenile oysters *Crassostrea gigas* (all conditions: unchallenged or challenged, and exposed to *Alexandrium catenella* or *Heterocapsa triquetra*). Data from both sampling times $T_4$ and $T_9$ were used. Spearman rank correlation coefficient, (N) and significance ($p$<0.05 are in bold, *NS* when not significant) are indicated.

| Hemocyte variables | % granulocytes | Granulocyte size | Hyalinocyte size | Granulocyte complexity | Hyalinocyte complexity | ROS production | Granulocyte mortality | Hyalinocyte mortality | PST accumulation | *Vibrio* sp. quantification |
|---|---|---|---|---|---|---|---|---|---|---|
| **THC** | 0,306<br>(114)<br>**0,0015** | 0,05<br>(114)<br>NS | 0,35<br>(114)<br>**0,0002** | 0,16<br>(114)<br>NS | 0,37<br>(114)<br>**0,0001** | 0,38<br>(114)<br>**0,0001** | -0,03<br>(112)<br>NS | 0,27<br>(112)<br>**0,0040** | 0,51<br>(82)<br>**0,0000** | 0,22<br>(51)<br>NS |
| **% granulocytes** | | 0,21<br>(114)<br>**0,0278** | 0,17<br>(114)<br>NS | 0,57<br>(114)<br>**0,0000** | 0,44<br>(114)<br>**0,0000** | 0,13<br>(114)<br>NS | 0,03<br>(112)<br>NS | 0,27<br>(112)<br>**0,0039** | 0,23<br>(82)<br>**0,0372** | -0,19<br>(51)<br>NS |
| **Granulocyte size** | | | -0,10<br>(114)<br>NS | 0,56<br>(114)<br>**0,0000** | 0,21<br>(114)<br>**0,0237** | 0,06<br>(114)<br>NS | 0,60<br>(112)<br>**0,0000** | 0,47<br>(112)<br>**0,0000** | 0,16<br>(82)<br>NS | -0,12<br>(51)<br>NS |
| **Hyalinocyte size** | | | | 0,01<br>(114)<br>NS | 0,83<br>(114)<br>**0,0000** | 0,40<br>(114)<br>**0,0000** | -0,36<br>(112)<br>**0,0001** | -0,21<br>(112)<br>**0,0246** | 0,28<br>(82)<br>**0,0115** | 0,14<br>(51)<br>NS |
| **Granulocyte complexity** | | | | | 0,24<br>(114)<br>**0,0100** | 0,08<br>(114)<br>NS | 0,38<br>(112)<br>**0,0001** | 0,44<br>(112)<br>**0,0000** | 0,21<br>(82)<br>NS | -0,22<br>(51)<br>NS |
| **Hyalinocyte complexity** | | | | | | 0,40<br>(114)<br>**0,0000** | -0,10<br>(112)<br>NS | -0,07<br>(112)<br>NS | 0,34<br>(82)<br>**0,0022** | 0,01<br>(51)<br>NS |
| **ROS production** | | | | | | | -0,12<br>(112)<br>NS | -0,24<br>(112)<br>**0,0126** | 0,42<br>(82)<br>**0,0002** | 0,31<br>(51)<br>**0,0274** |
| **Granulocyte mortality** | | | | | | | | 0,63<br>(123)<br>**0,0000** | 0,06<br>(88)<br>NS | -0,06<br>(56)<br>NS |
| **Hyalinocyte mortality** | | | | | | | | | 0,10<br>(88)<br>NS | -0,03<br>(56)<br>NS |
| **PST accumulation** | | | | | | | | | | 0,21<br>(57)<br>NS |

THC: total hemocyte count; ROS: reactive oxygen species; PST: paralytic shellfish toxin; NS: not significant.

## 4 Discussion

This study aimed to evaluate the ability of *Alexandrium catenella*, a toxic dinoflagellate previously reported to decrease herpesvirus OsHV-1 infection in oyster *Crassostrea gigas* (Lassudrie et al., subm.), to modulate hemocyte functions, possibly associated with oyster susceptibility to opportunistic infections from a new environment.

### *4.1 Hemocyte activation by* A. catenella *exposure*

Exposure to *A. catenella* induced a clear stimulation of oyster hemocyte responses, possibly upon PST accumulation. Indeed, PST accumulation was positively correlated with density of circulating hemocytes, morphological changes (increase of size and complexity) and ROS production. Similarly, Haberkorn et al. (2010) reported hemocyte morphological changes upon *Alexandrium minutum* exposure, as well as positive correlation between PST accumulation and hemocyte ROS production in *C. gigas*. Based on functional and morphological studies, other authors suggested that the different hemocyte sub-populations (granulocytes and hyalinocytes) would represent different stages of the same cell, reflecting differentiation in response to environmental challenges (Hine, 1999; Rebelo et al., 2013; Wikfors and Alix, 2014). Hemocyte could thus evolve in different functional types, by shifting from one morphologically-based sub-population to another, but also inside the same sub-population. In the present study, the morphological changes correlated with PST accumulation in the hyalinocyte sub-population suggest a specific functional differentiation of hyalinocytes in response to increasing PSTs, for wound repair or for detoxication. In fact higher number of circulating hemocytes and lower proportion of hyalinocytes in circulating hemolymph may reflect migration of hyalinocytes towards the tissues that accumulated PSTs. Indeed, infiltration in tissues constitute a typical response to *Alexandrium* sp. exposure in bivalves (Galimany et al., 2008a; Haberkorn et al., 2010a, 2010b; Lassudrie et al., 2014). These authors suggested that hemocyte infiltration could be related to detoxication, considering their occurrence in the digestive gland, the main organ accumulating PSTs in bivalves (Bricelj and Shumway, 1998; Guéguen et al., 2008; Lassus et al., 2007), and the associated observation of hemocyte diapedesis across the digestive epithelium. Hemocytes undergoing diapedesis were believed to either carry toxins out of the tissues towards the lumen of the stomach and intestine to be expelled

within the faeces, or to isolate toxic algal cells by encapsulation (Galimany et al., 2008a, 2008b). Deleterious effects of the contact with algal cells and their extracellular compounds in gills and mantle have been suggested after histological observations of inflammatory reactions associated with tissue lesions (Estrada et al., 2007; Lassudrie et al., 2014). Indeed, *Alexandrium* sp. extracellular compounds have been reported to have allelopathic, hemolytic, ichtyotoxic, and oxidative properties (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011). In the present study, a response to these compounds in *A. catenella*-exposed oysters could have increased the percentage of granulocytes in circulating hemolymph, which were larger and more complex in exposed-oysters than in controls, independently from PST content.

In contrast with our results, Hégaret et al. (2007) did not report any strong modifications of hemocyte variables after exposure to *A. catenella* in adult oysters. These differences may be explained by the young age and life history (i.e. grown exclusively in hatchery) of *C. gigas* used in the present study, making them more sensitive. Indeed, Bricelj et al. (2011), described higher vulnerability of juvenile Manila clams to *A. tamarens*e exposure compared to adults, with observation of high mortality and burrowing incapacity.

*4.2 Hemocyte activation induced by challenge*

Challenge of specific pathogen-free (SPF) oysters by cohabitation with oysters previously held in the field also led to marked hemocyte responses. Enhancement of hematopoïesis was suggested by higher total hemocyte count (THC) in hemolymph of challenged oysters. Furthermore, size and complexity increased in both hyalinocytes and granulocytes, and were correlated with ROS production. This may reflect an increase of metabolic activity (Donaghy et al., 2012) and / or an immune related response associated with phagocytosis against pathogenic microorganisms (Hine, 1999). Indeed, ROS production was correlated with *Vibrio* sp. load. Altogether, the hemocyte responses suggested an early immune response to exposure to non-self particles, putatively micro-organisms carried by field-exposed oysters, which actually contained a higher load of *Vibrio* sp. than SPF oysters at the beginning of the cohabitation, suggesting bacterial colonization of field-exposed oysters from the environment.

### *4.3* Vibrio *load and bacterial community stability*

Few Operational Taxonomic Units (OTUs) belonging to Proteobacteria phylum largely dominated the bacterial assemblage in oyster mantle, consistent with other studies of oyster microbiome (King et al., 2012; Lokmer and Wegner, 2014; Trabal Fernández et al., 2014; Wegner et al., 2013). Non-pathogenic, commensal bacteria dominated the bacterial communities, with OTUs assigned to the Betaproteobacteria class, mainly represented by the genera *Ralstonia*, *Pelomonas*, and by an unidentified genus from Comamonadaceae family, and to the Alphaproteobacteria class, with the genus *Sphingomonas*. After 9 days of experiment, significant differences in beta-diversity observed in previous field-exposed oysters compared to non-field-exposed ones indicated that the whole bacterial assemblage was probably driven by field exposure oysters, as was the potentially pathogenic (to bivalve or to other animal models) bacterial community. Although challenge by cohabitation were previously reported to successfully transmit pathogenic agents (De Decker and Saulnier, 2011; Petton et al., 2013), no significant modification of total *Vibrio* load and of bacterial assemblage was detected in the mantle of challenged oysters compared to unchallenged ones.

Despite the absence of clear modification of bacterial community associated with oyster mantle after cohabitation with field-exposed oysters, the strong hemocyte responses suggest either the involvement of pathogenic bacterial strains (not identified for this study) or of opportunistic pathogens, that would provoke an early immune response, without having induced detectable infection. Virulence factors contained in extracellular products of *Vibrio aesturianus* strain 01/32, for example, were demonstrated to enhance ROS production in hemocytes, inducing a possible toxic effect upon the cell effectors of the immune defense (Labreuche et al., 2006a, 2006b; Lambert et al., 2003). This effect was produced despite very low infection intensity in hemolymph, suggesting colonization of other compartments (Labreuche et al., 2006a). Similar effects of virulence factors may have triggered the hemocyte responses observed in challenged oysters in the present study.

Finally, the global microbiome homeostasis may have been maintained thanks to these hemocyte responses, which would participate in the elimination of invading pathogens. Consistently with the absence of global bacterial community shift, Lokmer and Wegner (2014) reported relative robustness of bacterial assemblage in *C. gigas* hemolymph against injection of pathogenic *Vibrio* sp. Indeed, microbiome homeostasis is often conserved despite stressful conditions (Erwin et al., 2012; Pita et al., 2013; Wegner et al., 2013), likely participating in host acclimation and health status (Rosenberg et al., 2007).

### *4.4 Interaction between challenge and* A. catenella *exposure*

The increase in hemocyte variables (THC, size, complexity and ROS production) induced by challenge condition was not exacerbate when combined to *A. catenella*-exposure, despite the strong stimulating effects of *A. catenella* exposure in unchallenged condition. These could be associated with (i) toxic effect of *A. catenella* exposure to bacteria or to their virulence factor expression; (ii) cumulative effects of both *A. catenella* exposure and new microbial environment challenge leading to a plateau in terms of hemocyte responses.

For the first hypothesis, both PSTs and extracellular compounds produced by *A. catenella* may have impaired potentially pathogenic agents themselves or the production of their virulence factors, thus decreasing the need for bivalve immune response. As mentioned above, *Alexandrium* sp. can produce other extracellular compounds than PSTs that can have deleterious effects upon different target cells (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011). In addition, *Alexandrium* cells can release PSTs extracellularly, especially during encystment that widely occurs when grazed by bivalves (Hégaret et al., 2008; Laabir et al., 2007; Lefebvre et al., 2008; Persson et al., 2012). Previous studies already demonstrated direct negative effects of HABs on bivalve pathogens (Hégaret et al., 2010, 2009). Moreover, possible toxic effects of PSTs upon prokaryotes, including Gram-negative bacteria such as *Vibrio* sp., have been reported, with alteration of sodium and potassium fluxes (Pomati et al., 2003).

The other hypothesis is that both challenge and *A. catenella* exposure would be partially antagonistic upon hemocyte responses, or that responses would have reached a plateau that limits any further increase in hemocyte variable levels. In fact, PSTs or extracellular compounds produced by *A. catenella* could have interfered with pathogen recognition receptors (PRRs) or immune signaling pathways that trigger the cellular immune responses (Song et al., 2010). However, the synergistic effect of both challenge and *A. catenella* exposure upon hemocyte mortality observed at $T_4$ suggested cytotoxic effects, possibly resulting in necrosis of hemocytes. Alternately, when hemocytes reached their plateau in terms of response, apoptosis may have been activated. Apoptosis is indeed an important mechanism of immune defense against intra-cellular pathogens (Sokolova, 2009) that was activated by *A. catenella* in oyster hemocytes (Medhioub et al., 2013). In another study, exposure to the PST-producer *A. fundyense* inhibited oysters *C. virginica* hemocyte responses (i.e. infiltrations in tissues) to trematode infestation. This inhibition of hemocyte responses was suspected to be responsible for immuno-suppressive effects, since exposed oysters contained a higher parasitic load compared to oysters exposed to a non-toxic dinoflagellate (Lassudrie et al. in rev.). Although no obvious infection could be clearly evidenced from *Vibrio* sp. load or from the NMDS analysis of bacterial community in this study, one must consider that shift from healthy to diseased status may be due to subtle differences in pathogen or opportunist load, undetectable by ordination analyses. Such phenomenon was previously described in human diseases (Duran-Pinedo et al., 2014; Galimanas et al., 2014). Infection with a virus (other than OsHV-1) that would also not have been detected with the methods used here may also have occurred. Thus, in the present study, the possibility that health of oysters may be compromised by undetectable micro-organisms is not excluded. Moreover, the relative abundance of potentially pathogenic bacteria tended to be higher in oysters subjected to both challenge and *A. catenella* exposure and hemocyte ROS production was positively correlated with *Vibrio* load. Overall, health status seemed to be affected in both challenged and *A. catenella*-exposed oysters, as also suggested by the difficulty to withdraw hemolymph from 57 % of this oyster groups after 9 days of experiment (as opposed to 17 % in the control group). Such difficulties to withdraw hemolymph from bivalves infected with pathogenic agents or exposed to toxic dinoflagellates were recurrently noticed in different laboratories (G. H. Wikfors, C. Lambert, P. Soudant, H. Hégaret, pers. comm.), and were assumed to be associated with compromised health status. The mechanisms underlying this

reaction, however, remain unclear (e.g. compromised muscle drainage by hemolymph). Finally, the lower PST accumulation in challenged oysters may indicate alterations of feeding processes such as filtration, ingestion or digestion by putative pathogenic agents. Casas (2002) and Flye Sainte-Marie et al. (2007) observed negative effects of perkinsosis (caused by *Perkinsus olseni* syn*. atlanticus*) and Brown Ring Disease (BRD, caused by *Vibrio tapetis*), respectively, upon clearance rate of Manila clams, and was associated with alterations of digestive diverticula similar to starvation symptoms in clams with BRD (Plana and Le Pennec, 1991). Measure of feeding processes in oysters subjected to the same conditions as in the present experiment would be useful to further explore this hypothesis.

Surprisingly, PST accumulation in field-exposed oysters was similar to those in unchallenged oysters after 9 days of exposure to *A. catenella*. The lower PST accumulation in challenged compared to unchallenged oysters suggests an early response to a new disturbance, possibly opportunistic or pathogenic micro-organisms which would not have been able to induce a septicemic infection. In fact, bacteria also considered as non-pathogenic for their host have been reported to induce hemocyte responses, as shown for example in *M. galloprovincialis* challenged with *V. splendidus* (a bacterium species not considered pathogenic for *Mytilus* sp., Balbi et al., 2013), or in Manila clam *Venerupis philippinarum* challenged with a non-pathogenic *Vibrio tapetis* strain (Le Bris et al., 2014).

## 5 Conclusions

Exposure to *A. catenella* induced a strong inflammatory response related to PSTs and probably also to other toxic compounds produced by *A. catenella.* Additionally, putative bacterial virulence factors would have led to an inflammatory response in challenged oysters. Although hemocytes responses to the putative bacterial challenge were modulated by *A. catenella* exposure, demonstrating possible cytotoxic effects of the interaction of both stressors, oyster microbiome remained stable, suggesting no strong effect upon susceptibility to infection by opportunistic bacteria. The maintenance of homeostasis, as demonstrated by the high stability of the microbiome to different stressors, seemed to be achieved thanks to the initiation of hemocyte responses.

However, feeding-related physiological processes may have been impaired, as suggested by the lower PST accumulation.

Although previous studies showed modulations of host-pathogen interactions in bivalves by exposure to *Alexandrium* spp. (Lassudrie et al., in rev., subm.), results of this study suggest that short-term *A. catenella* exposure, combined with exposure to environmental micro-organisms, may have compromised physiological conditions, without inducing clear pathogenic or opportunistic infection. Further studies involving ecophysiological measures could help validate and better understand the physiological mechanisms occurring, and determine the implication of these effects upon energy allocation and growth (Kooijman, 2010).

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Supplementary material

**Table S1**. Sequences of the MID-tagged primer pair used for 454 pyrosequencing, targeting the V2-V3 region of the 16S rRNA gene. MID sequence are in italics.

| Primer forward name | Primer forward_MIDsequence 5'-3' | Primer reverse name | Primer reverse_MIDsequence 5'-3' |
|---|---|---|---|
| **MID001_16S-0027F** | *ACGAGTGCGTAGAGTTTGATCCTGGCTCAG* | **MID001_16S-0533R** | *ACGAGTGCGT*TTACCGCGGCTGCTGGCAC |
| **MID002_16S-0027F** | *ACGCTCGACA*AGAGTTTGATCCTGGCTCAG | **MID002_16S-0533R** | *ACGCTCGACA*TTACCGCGGCTGCTGGCAC |
| **MID003_16S-0027F** | *AGACGCACTC*AGAGTTTGATCCTGGCTCAG | **MID003_16S-0533R** | *AGACGCACTC*TTACCGCGGCTGCTGGCAC |
| **MID004_16S-0027F** | *AGCACTGTAG*AGAGTTTGATCCTGGCTCAG | **MID004_16S-0533R** | *AGCACTGTAG*TTACCGCGGCTGCTGGCAC |
| **MID005_16S-0027F** | *ATCAGACACG*AGAGTTTGATCCTGGCTCAG | **MID005_16S-0533R** | *ATCAGACACG*TTACCGCGGCTGCTGGCAC |
| **MID006_16S-0027F** | *ATATCGCGAG*AGAGTTTGATCCTGGCTCAG | **MID006_16S-0533R** | *ATATCGCGAG*TTACCGCGGCTGCTGGCAC |
| **MID007_16S-0027F** | *CGTGTCTCTA*AGAGTTTGATCCTGGCTCAG | **MID007_16S-0533R** | *CGTGTCTCTA*TTACCGCGGCTGCTGGCAC |
| **MID008_16S-0027F** | *CTCGCGTGTC*AGAGTTTGATCCTGGCTCAG | **MID008_16S-0533R** | *CTCGCGTGTC*TTACCGCGGCTGCTGGCAC |
| **MID010_16S-0027F** | *TCTCTATGCG*AGAGTTTGATCCTGGCTCAG | **MID010_16S-0533R** | *TCTCTATGCG*TTACCGCGGCTGCTGGCAC |
| **MID011_16S-0027F** | *TGATACGTCT*AGAGTTTGATCCTGGCTCAG | **MID011_16S-0533R** | *TGATACGTCT*TTACCGCGGCTGCTGGCAC |
| **MID013_16S-0027F** | *CATAGTAGTG*AGAGTTTGATCCTGGCTCAG | **MID013_16S-0533R** | *CATAGTAGTG*TTACCGCGGCTGCTGGCAC |
| **MID015_16S-0027F** | *ATACGACGTA*AGAGTTTGATCCTGGCTCAG | **MID015_16S-0533R** | *ATACGACGTA*TTACCGCGGCTGCTGGCAC |
| **MID016_16S-0027F** | *TCACGTACTA*AGAGTTTGATCCTGGCTCAG | **MID016_16S-0533R** | *TCACGTACTA*TTACCGCGGCTGCTGGCAC |
| **MID017_16S-0027F** | *CGTCTAGTAC*AGAGTTTGATCCTGGCTCAG | **MID017_16S-0533R** | *CGTCTAGTAC*TTACCGCGGCTGCTGGCAC |
| **MID018_16S-0027F** | *TCTACGTAGC*AGAGTTTGATCCTGGCTCAG | **MID018_16S-0533R** | *TCTACGTAGC*TTACCGCGGCTGCTGGCAC |
| **MID019_16S-0027F** | *TGTACTACTC*AGAGTTTGATCCTGGCTCAG | **MID019_16S-0533R** | *TGTACTACTC*TTACCGCGGCTGCTGGCAC |
| **MID020_16S-0027F** | *ACGACTACAG*AGAGTTTGATCCTGGCTCAG | **MID020_16S-0533R** | *ACGACTACAG*TTACCGCGGCTGCTGGCAC |
| **MID021_16S-0027F** | *CGTAGACTAG*AGAGTTTGATCCTGGCTCAG | **MID021_16S-0533R** | *CGTAGACTAG*TTACCGCGGCTGCTGGCAC |
| **MID022_16S-0027F** | *TACGAGTATG*AGAGTTTGATCCTGGCTCAG | **MID022_16S-0533R** | *TACGAGTATG*TTACCGCGGCTGCTGGCAC |
| **MID025_16S-0027F** | *TCGTCGCTCG*AGAGTTTGATCCTGGCTCAG | **MID025_16S-0533R** | *TCGTCGCTCG*TTACCGCGGCTGCTGGCAC |
| **MID026_16S-0027F** | *ACATACGCGT*AGAGTTTGATCCTGGCTCAG | **MID026_16S-0533R** | *ACATACGCGT*TTACCGCGGCTGCTGGCAC |
| **MID027_16S-0027F** | *ACGCGAGTAT*AGAGTTTGATCCTGGCTCAG | **MID027_16S-0533R** | *ACGCGAGTAT*TTACCGCGGCTGCTGGCAC |
| **MID028_16S-0027F** | *ACTACTATGT*AGAGTTTGATCCTGGCTCAG | **MID028_16S-0533R** | *ACTACTATGT*TTACCGCGGCTGCTGGCAC |
| **MID029_16S-0027F** | *ACTGTACAGT*AGAGTTTGATCCTGGCTCAG | **MID029_16S-0533R** | *ACTGTACAGT*TTACCGCGGCTGCTGGCAC |
| **MID030_16S-0027F** | *AGACTATACT*AGAGTTTGATCCTGGCTCAG | **MID030_16S-0533R** | *AGACTATACT*TTACCGCGGCTGCTGGCAC |
| **MID031_16S-0027F** | *AGCGTCGTCT*AGAGTTTGATCCTGGCTCAG | **MID031_16S-0533R** | *AGCGTCGTCT*TTACCGCGGCTGCTGGCAC |
| **MID032_16S-0027F** | *AGTACGCTAT*AGAGTTTGATCCTGGCTCAG | **MID032_16S-0533R** | *AGTACGCTAT*TTACCGCGGCTGCTGGCAC |
| **MID033_16S-0027F** | *ATAGAGTACT*AGAGTTTGATCCTGGCTCAG | **MID033_16S-0533R** | *ATAGAGTACT*TTACCGCGGCTGCTGGCAC |
| **MID035_16S-0027F** | *CAGTAGACGT*AGAGTTTGATCCTGGCTCAG | **MID035_16S-0533R** | *CAGTAGACGT*TTACCGCGGCTGCTGGCAC |
| **MID036_16S-0027F** | *CGACGTGACT*AGAGTTTGATCCTGGCTCAG | **MID036_16S-0533R** | *CGACGTGACT*TTACCGCGGCTGCTGGCAC |
| **MID037_16S-0027F** | *TACACACACT*AGAGTTTGATCCTGGCTCAG | **MID037_16S-0533R** | *TACACACACT*TTACCGCGGCTGCTGGCAC |

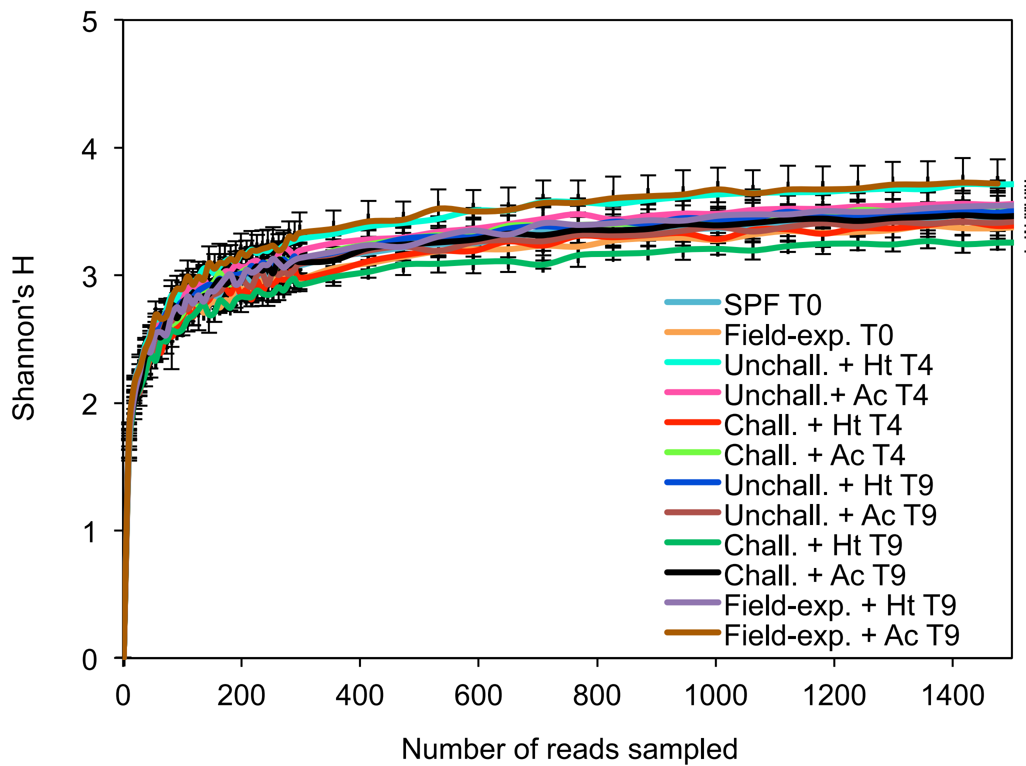


**Figure S1**. Alpha-diversity of bacterial communities from the mantle of *Crassostrea gigas* oysters as represented by rarefaction curves of Shannon's H, obtained from 10 resamples, according to challenge condition or field exposure ("Unchall.": unchallenged; "Chall.": challenged by cohabitation with field-exposed oysters; "Field-exp.": field-exposed) and algal treatment ("Ht": *Heterocapsa triquetra*; "Ac": *Alexandrium catenella*), after 4 ("T4") and 9 days ("T9") of experiment. Mean ± SE.

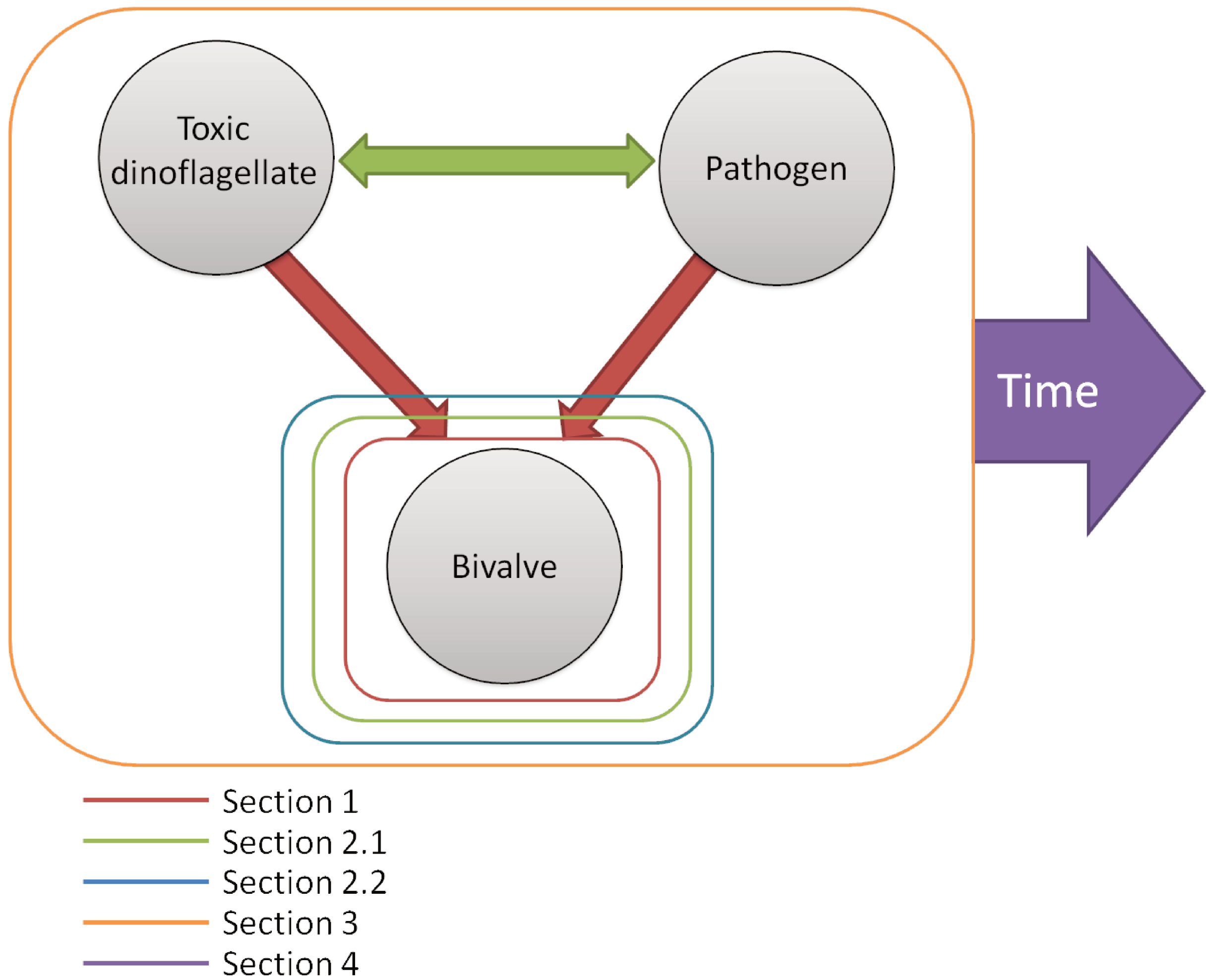


Figure 7. Compartiments de l'interaction tripartite bivalve – dinoflagellé toxique – agent pathogène discutés dans chaque section de la partie *Discussion générale et perspectives*.

# Discussion générale et perspectives

## 1 Modulation espèces-spécifique des interactions biotiques bivalve – pathogène – *Alexandrium* sp.

Harvell et al. (1999) avaient émis l'hypothèse que les efflorescences de microalgues toxiques augmentent la susceptibilité aux maladies chez les organismes marins. C'est effectivement le cas dans certaines interactions (Figure 7); les résultats de l'article 3 (chapitre 2, Lassudrie et al., in rev.) présentés dans cette thèse suggèrent qu'une efflorescence d'*Alexandrium fundyense* augmente la susceptibilité de l'huître américaine *Crassostrea virginica* à la prolifération de *Perkinsus marinus*. De même, Galimany et al. (2008) avaient observé que l'exposition à *A. fundyense* favorisait la colonisation par des parasites trématodes chez la moule bleue *Mytilus edulis*. Cet effet **« négatif »** du dinoflagellé toxique sur le bivalve serait d'autant plus exacerbé que l'animal est infecté par d'autres parasites (article 3, chapitre 2, Lassudrie et al., in rev.). Un détournement de l'énergie de l'hôte vers le parasite et une augmentation des coûts énergétiques associés à la défense immunitaire et la réparation tissulaire peuvent résulter en un affaiblissement physiologique global du bivalve (Casas, 2002; Flye-Sainte-Marie et al., 2009, 2007). En plus des effets sur les relations hôte – pathogène, l'effet combiné parasite – dinoflagellé toxique pourrait impacter d'autres relations, de type proie – prédateur notamment, comme le suggèrent les résultats de l'article 3 (chapitre 2, Lassudrie et al., in rev.) d'après lesquels l'effet combiné des trématodes *Bucephalus* sp. et d'*A. fundyense* provoque des pathologies du muscle adducteur, organe indispensable au maintien de la fermeture des valves qui constituent la première ligne de défense contre les agressions extérieures. Une conclusion similaire avait été émise par Hégaret et al. (2012) dont les résultats démontrent que non seulement la prévalence de bactéries Rickettsiales augmente chez des pétoncles chiliens *Argopecten purpuratus* après une exposition à *Alexandrium catenella*, mais aussi que la durée de leur réponse de fuite à un de leur prédateur, l'étoile de mer, est raccourcie, ce qui est associé à des altérations des fibres musculaires. **Les résultats soulignent l'inter-connectivité des différentes interactions biotiques, et ainsi l'importance de les considérer dans leur ensemble.**

Cependant, l'exposition à *Alexandrium* sp. n'aboutit pas systématiquement à des modulations « négatives » de l'interaction hôte – pathogène pour le bivalve. Ainsi, l'effet combiné d'*Alexandrium ostenfeldii* et du parasite protozoaire *Perkinsus olseni* sur des palourdes *Ruditapes philippinarum* du bassin d'Arcachon (article 2, chapitre 2, Lassudrie et al., 2014) n'entraîne ni modification de la relation hôte – pathogène initiale, ni d'effet synergique, c'est-à-dire plus important que ceux qui résulteraient de la somme des effets de chaque facteur (agent pathogène et dinoflagellé toxique) sur les critères physiologiques examinés. Ces effets peuvent donc être définis comme « **neutres** ». De même, Hégaret et al. (2007a) n'ont observé aucun effet du dinoflagellé *Karenia mikimotoi* sur la même interaction *R. philippinarum* - *P. olseni*. En France, cette relation hôte – parasite est considérée comme stable car, malgré des prévalences atteignant régulièrement 100%, l'intensité de l'infection reste modérée (Binias et al., 2014, 2013; da Silva et al., 2008; Dang et al., 2013; de Montaudouin et al., 2010; Hégaret et al., 2007a), et le parasite n'a encore jamais été associé à des phénomènes de mortalités massives comme en Corée du Sud (Choi et al., 2005; Hamaguchi et al., 1998; Liang et al., 2001; Park et al., 1999). Ainsi, une efflorescence d'*A. ostenfeldii,* en dépit des dommages tissulaires et cellulaires qu'elle provoque chez les palourdes de la population étudiée, ne semble pas suffire à déstabiliser l'équilibre actuel de l'interaction palourde – *P. olseni*. En revanche, dans d'autres populations où l'impact de *P. olseni* est plus sévère, la question de l'effet d'*Alexandrium* sp. comme facteur aggravant des mortalités se pose.

L'exposition de naissain d'huîtres creuses *Crassostrea gigas* à *Alexandrium catenella* semble avoir un effet « **positif** » pour le bivalve en altérant le processus d'infection (i.e. de transmission ou de prolifération) de l'herpesvirus OsHV-1 (article 1, chapitre 1, Lassudrie et al., subm.), impliqué dans les phénomènes de mortalités qui déciment les stocks de naissain depuis 2008 en France et qui depuis ont touché d'autres pays notamment en Europe et en Océanie (EFSA Panel on Animal Health and Welfare, 2010; Jenkins et al., 2013; Paul-Pont et al., 2014). De plus, l'exposition à l'herpesvirus (article 1, chapitre 1, Lassudrie et al., subm.) et à des micro-organismes de l'environnement (article 4, chapitre 3, Lassudrie et al., in prep.) altèrent le processus d'accumulation des toxines (PSTs), et diminue donc potentiellement les effets de ces toxines. De tels effets pourraient être liés à **des interactions directes entre *A. catenella* et ces pathogènes,** ou

a des **altérations des processus physiologiques liés à la filtration et la nutrition de l'huître**, diminuant l'entrée des pathogènes dans l'organisme ou l'ingestion et la digestion des cellules algales contenant les PSTs. Dans ce cas, les effets considérés comme « positifs » en fonction des critères « infection » et « accumulation de toxines » cités précédemment, seraient la conséquence d'altérations physiologiques qui pourraient à plus long terme avoir un impact négatif sur le bivalve.

**Les résultats obtenus au cours de cette thèse démontrent qu'il n'existe pas de profil de réponse unique à ces interactions tripartites bivalves – *Alexandrium* sp. – pathogènes. Ils mettent au contraire en évidence l'aspect « espèces – spécifiques » de ces interactions,** qui sont probablement également propres à chaque population. De plus, l'effet de l'interaction tripartite dépend aussi de l'état initial de l'interaction hôte – pathogène (bivalves infectés ou naïfs) et de sa stabilité. Les résultats de ce travail de thèse démontrent l'importance de mieux appréhender **les interactions entre les pathogènes et les microalgues toxiques,** mais aussi les interactions de ces deux facteurs biotiques s**ur la physiologie de l'hôte**, à différentes échelles d'organisation.

# 2 Les facteurs qui modulent l'interaction tripartite

## 2.1 Interactions directes entre *Alexandrium* sp. et agents pathogènes ?

Bien qu'au cours de cette thèse l'intérêt ait été porté sur le compartiment « bivalve » de l'interaction tripartite, les résultats obtenus soulignent la nécessité de **considérer les interactions directes entre *Alexandrium* sp. et les pathogènes**, qui sont susceptibles d'avoir des conséquences pour le bivalve (Figure 7).

Ainsi, le dinoflagellé *K. selliformis* produit des **composés extracellulaires toxiques** pour le parasite *P. olseni,* qui peuvent conduire à une diminution du taux d'infection chez les palourdes *R. philippinarum* (da Silva et al., 2008). D'autres études ont mis en évidence que les composés extracellulaires d'*Alexandrium* spp., ont des effets cytotoxiques: allélopathiques, hémolytiques, ichtyotoxiques et oxydatifs sur divers organismes unicellulaires (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011a). De plus, les PSTs, en partie extracellulaires (Lefebvre et al., 2008; Persson et al., 2012), peuvent modifier les flux de sodium et de potassium de différents prokaryotes tels que

les bactéries à Gram négatif (Pomati et al., 2003), dont font partie les vibrions, et qui sont les bactéries les plus impliquées dans les maladies de bivalves (Paillard et al., 2004). Dans l'article 1 (chapitre 1, Lassudrie et al., subm.), nous proposons que ces composés extracellulaires puissent également avoir des effets toxiques sur l'herpesvirus. L'enveloppe lipidique du virus pourrait par exemple avoir été altérée par des espèces réactives de l'oxygène (Reactive Oxygen Species ; ROS) provenant de ces composés extracellulaires (Flores et al., 2012).

Des travaux visant à caractériser les composés extracellulaires des dinoflagellés toxiques et leurs effets *in vitro* sur d'autres micro-organismes ont été initiés au LEMAR et dans d'autres laboratoires (da Silva et al., 2008; Flores et al., 2012; Lelong et al., 2011a). Ces tests *in vitro* pourraient être appliqués à d'autres micro-organismes pathogènes affectant les bivalves afin d'améliorer la compréhension des mécanismes impliqués dans les interactions tripartites bivalve – pathogène – dinoflagellé toxique.

Inversement, des micro-organismes pathogènes tels que **les bactéries peuvent impacter les micro-algues toxiques**, dont *Alexandrium* sp., notamment lorsque leur proximité physique est favorisée par chimiotactisme positif ou par adhérence à la paroi des cellules algales (voir revue de Doucette, 1995). Les bactéries peuvent produire des composés bioactifs qui peuvent interférer avec la physiologie de l'algue. Ainsi, un métabolite capable d'inhiber la croissance, voire d'être létal pour plusieurs espèces de micro-algues toxiques a été isolé par Ishio et al. (1989). Plus récemment, une souche bactérienne d'actinomycète possédant des effet algicides envers *A. tamarense* a été isolée (Bai et al., 2011). Des exopolymères peuvent aussi être produits par les bactéries, et modifier la dynamique d'une efflorescence, notamment en favorisant la formation d'agrégats qui conduisent à la sédimentation des cellules algales (Kirchman, 1993). Par ailleurs, la communauté bactérienne associée aux micro-algues toxiques peut avoir un effet sur la production de toxines (Doucette et al., 1998; Hold et al., 2001; Lelong et al., 2014), et donc potentiellement sur leur accumulation par les bivalves.

La nécessité de comprendre ces effets est d'autant plus pertinente que, comme d'autres micro-algues, les **dinoflagellés toxiques peuvent être vecteurs de micro-organismes pathogènes** lorsque ces derniers se fixent sur les cellules algales (Doucette, 1995; Rivera et al., 2012).

La poursuite des recherches sur ces interactions serait pertinente pour mieux les comprendre, bien qu'en pratique des limitations techniques existent, notamment l'incapacité de cultiver l'herpesvirus ou certaines bactéries. Cependant, le suivi de la dynamique et de la physiologie des algues dans de l'eau contenant ces virus, ou en présence de bivalves infectés pourrait être envisagé. L'utilisation de marqueurs fluorescents permettant d'estimer l'état physiologique de l'algue, à travers la viabilité, le métabolisme associé à la production de chlorophylle, ou encore les métabolismes primaire ou secondaire (activité des estérases, quantité de lipides intracellulaires, etc.) associé aux techniques de microscopie de fluorescence et de cytométrie en flux (Lelong et al., 2011b) permettrait d'explorer les effets de ces micro-organismes pathogènes sur les dinoflagellés toxiques.

## 2.2 Implication des réponses physiologiques des bivalves

L'état physiologique initial du bivalve est l'un des paramètres qui module le résultat de l'interaction tripartite (Samain and McCombie, 2007). Ainsi, les variables physiologiques du bivalve mesurées à l'issue d'une interaction bivalve – pathogène – *Alexandrium* sp. sont la résultante (i) de l'état physiologique initial du bivalve, et de (ii) l'effet d'*Alexandrium* sp. et du pathogène. Finalement, ces modifications physiologiques peuvent traduire leurs rôles dans les modulations ou la stabilité de l'interaction tripartite, ou au contraire en être la conséquence (altérations par exemple) (Figure 7).

**Les hémocytes** sont des cellules circulantes **impliquées dans de multiples fonctions physiologiques** chez les bivalves, notamment l'immunité, la nutrition, la minéralisation coquillère et la réparation tissulaire. Cependant, les mécanismes opérant lors de leur implication dans ces différentes fonctions sont très peu connus chez les bivalves, notamment en raison du **manque de connaissances sur les différenciations fonctionnelles et surtout sur l'hématopoïèse** (i.e. la production *de novo* d'hémocytes) (cf Donaghy et al 2009 ; Hine 1999). Les hypothèses les plus récentes concernant leur **différenciation fonctionnelle** proposent que les sous-populations d'hémocytes circulants distinguées par critères morphologiques (cellules de type souche ou « blast-like cells », hyalinocytes, granulocytes) correspondent à différentes phases d'un seul

type cellulaire. Ces différentes phases seraient liées à différents stades de maturité ou à différentes fonctions. La différentiations pourrait intervenir entre sous-populations mais aussi au sein d'une même sous-population (Rebelo et al., 2013; Wikfors and Alix, 2014)

Ainsi, les variables hémocytaires mesurées chez les bivalves au cours de ce travail de thèse suggèrent que ces cellules assurent plusieurs fonctions en réponse à l'interaction tripartite bivalve – pathogène – *Alexandrium* sp. Ces observations permettent d'émettre des hypothèses sur le rôle des hémocytes dans ces interactions, et plus généralement d'améliorer l'état actuel des connaissances sur le lien entre hématologie, morphologie et fonctions, encore mal compris.

### 2.2.1 Immunité, réparation tissulaire, détoxication : rôle des hémocytes ?

L'exposition à des micro-organismes potentiellement pathogènes, ou leur présence dans l'organisme des bivalves infectés, a entraîné en général, des infiltrations dans les tissus, une augmentation de la concentration en hémocytes dans l'hémolymphe, et une augmentation de leur taille et de leur complexité interne (articles 2 et 3, chapitre 2, Lassudrie et al., 2014, in rev. ; article 4, chapitre 3, Lassudrie et al., in prep.). **Ces réponses hématologiques et morphologiques suggèrent qu'une augmentation de la production d'hémocytes et une différenciation sont probablement associées à la réponse immunitaire (phagocytose, encapsulation), à leur migration sur le lieu d'infection, mais aussi éventuellement à la réparation des lésions provoquées par l'infection** (Cheng, 1996; Hine, 1999; Soudant et al., 2013).

D'une façon générale, au cours de ces travaux de thèse, l'exposition à *Alexandrium* sp. a activé la production d'hémocytes circulants chez les bivalves, augmenté leur taille et leur complexité et provoqué leur infiltration dans les tissus (article 2 et 3, chapitre 2, Lassudrie et al., 2014 ; Lassudrie et al., in rev.; article 4, chapitre 3, Lassudrie et al., in prep.). **Les hyalinocytes semblent jouer un rôle particulier dans la réponse à *Alexandrium* sp**. En effet, leurs modifications morphologiques corrélées aux quantités de PSTs accumulées dans la glande digestive et à la production de ROS, peuvent traduire une activité métabolique accrue (Donaghy et al., 2012). **Cela suggère une différenciation fonctionnelle, soit pour la réparation des lésions provoquées par les**

**PSTs, soit pour l'isolement des cellules algales présentes dans la lumière de l'intestin, ou encore pour la détoxication des tissus.**

Plusieurs auteurs ont suggéré que des hémocytes observés en diapédèse dans l'épithélium digestif et autour des cellules algales dans la lumière de l'intestin ou encore dans les faeces utiliseraient un mécanisme comparable à l'encapsulation afin de **protéger les tissus internes des composés toxiques excrétés par ces algues** (Galimany et al., 2008b; Hégaret et al., 2009). Cependant, l'observation d'hémocytes autour des cellules algales n'a pas été fréquente au cours de ces travaux de thèse (articles 2 et 3, chapitre 2, Lassudrie et al., 2014 ; Lassudrie et al., in rev).

Les observations d'infiltrations et de diapédèses et les modifications morphologiques ont également été rapportées chez des bivalves exposés à des xénobiotiques (Farley, 1988; Sunila, 1984), ce qui, dans le cas d'une exposition à un dinoflagellé toxique, suggère un rôle des hémocytes dans la **détoxication en transportant les toxines ou leur produits à l'extérieur des tissus** (Galimany et al., 2008a, 2008b; Hégaret et al., 2009).Franchini et al. (2003) ont détecté des yessotoxines (toxines lipophiles) dans les hémocytes de moules, soutenant cette hypothèse.

Par ailleurs, Wikfors et Alix (2014) suggèrent l'existence d'un lien entre les modifications morphologiques et **l'apoptose des hémocytes**. Ce phénomène de mort cellulaire programmée, impliqué particulièrement dans la **défense immunitaire** contre les pathogènes intracellulaires (Sokolova, 2009), peut être stimulé par l'exposition aux dinoflagellés toxiques (Hégaret et al., 2009; Medhioub et al., 2013).

Enfin, la présence des toxines dans les hémocytes pourrait également impacter leur **osmolarité**, et être à l'origine des changements morphologiques détectés (Franchini et al., 2003; Malagoli and Ottaviani, 2004).

Cependant, la nature des différenciations morphologiques des hémocytes de bivalves en réponse à *Alexandrium* sp. diffère en fonction des études (Galimany et al., 2008a; Haberkorn et al., 2010a; Hégaret et al., 2007b). Ces différences intrinsèques sont liées aux modèles étudiés, comme précédemment évoqué, mais aussi probablement **à l'état physiologique du bivalve, entraînant l'implication simultanée des hémocytes dans plusieurs fonctions** qui pourraient interférer entre elles, indépendamment de la réponse à *Alexandrium* sp.

**La répression des réponses hémocytaires (THC, morphologie, ROS…) généralement mesurée lors de l'interaction des bivalves avec *Alexandrium* sp. et un agent pathogène** pourrait aussi s'expliquer par des interférences entre les réponses liées à différentes fonctions (Figure 8). En effet, la combinaison des deux facteurs biotiques entraine une réponse hémocytaire ne correspondant pas à la simple addition des effets provoqués par chacun de ces facteurs (articles 2 et 3, chapitre 2, Lassudrie et al., in rev., 2014; article 4, chapitre 3, Lassudrie et al., in prep.), mais suggère plutôt une **interaction antagoniste** entre les effets de ces deux facteurs biotiques sur les hémocytes. Un état **immunodéprimé** peut en résulter, comme le suggère l'augmentation de la charge parasitaire (*P. marinus*) chez l'huître américaine exposée à *A. fundyense* dans l'article 3, (chapitre 2, Lassudrie et al., in rev.) Cependant, ces réponses ne mènent pas systématiquement à des modulations de l'interaction hôte – pathogène (article 2, chapitre 2, Lassudrie et al., in rev.), et pourraient alors être expliquées par les faibles intensités de réponses associées à chacun des facteurs, insuffisantes pour entraîner des interférences.

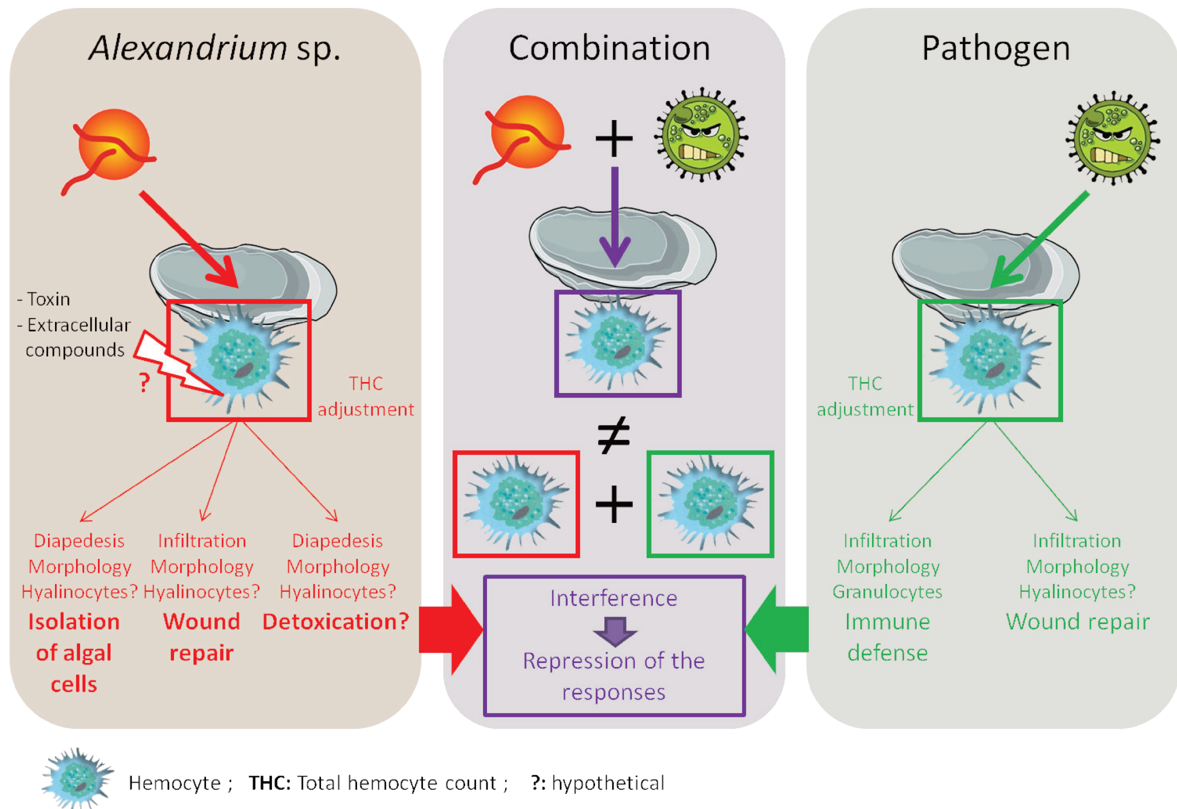


Figure 8. Réponses des hémocytes circulants des bivalves (i) exposés à *Alexandrium* sp., (ii) infectés par des agents pathogènes, et (ii) à la combinaison des deux, d'après les résultats obtenus dans cette thèse (articles 2 et 3, chapitre 2, Lassudrie et al., 2014, in rev. ; article 4, chapitre 3, Lassudrie et al., in prep.).

**D'autre part, les résultats de ce travail de thèse soulignent la capacité adaptative des hémocytes circulants via une grande plasticité qui leur permet de répondre rapidement et efficacement à l'invasion de particules étrangères**. Les résultats de l'article 4 (chapitre 3, Lassudrie et al., in prep.) associent pour la première fois (à notre connaissance) chez un bivalve la mesure des variables hématologiques à l'analyse de la communauté bactérienne, déterminée par des techniques de séquençage nouvelle génération. L'activation des réponses hémocytaires face à un nouvel environnement microbien, associée à une forte stabilité du microbiome chez l'huître, y compris lors d'une exposition à *A. catenella*, illustre le rôle clef des hémocytes dans le **maintien de l'homéostasie**, comme l'avaient suggéré Donaghy et al. (2009).

**En dépit de rapides modulations, le renouvellement du système hémocytaire et son maintien font preuve d'une globale robustesse,** malgré la combinaison de stress biotiques expérimentée au cours de ces travaux de thèse. Les modifications des hémocytes, que ce soit d'un point de vue hématologique, morphologique ou fonctionnel, n'indiquent pas d'altération importante ou irréversible de leurs fonctions. En effet, l'article 2 (chapitre 1, Lassudrie et al., 2014) illustre la **réversibilité des modulations** hémocytaires chez la palourde *R. philippinarum* après une exposition à *Alexandrium* sp., suivie d'une semaine de dépuration. Cette réversibilité avait également été notée par Galimany et al. (2008) chez la moule bleue *M. edulis.* Cette homéostasie des hémocytes, en accord avec de précédentes observations (Donaghy et al., 2009; Galimany et al., 2008a; Haberkorn et al., 2010a; Hégaret et al., 2007b), est un enjeu vital pour les bivalves afin d'éviter des répercussions sur les autres fonctions essentielles qui lui sont associées (nutrition, minéralisation coquillière,…).

**Finalement, les modifications des variables hémocytaires induites par les interactions biotiques expérimentées ici peuvent être considérées comme des réponses adaptatives efficaces si elles ont permis de maintenir l'homéostasie de l'état physiologique du bivalve. Elles sont au contraire considérées comme des réponses insuffisantes et / ou comme la conséquence d'altérations induites par ces stress biotiques en cas d'observations de lésions à plus grande échelle.** Dans le cas présent, l'approche intégrative utilisée a notamment permis d'associer ces mesures cellulaires à des observations histologiques (articles 2 et 3, chapitre 2, Lassudrie et al., 2014, in rev.).

### 2.2.2 Perturbation de l'homéostasie cellulaire et de l'intégrité tissulaire

La description de **réponses inflammatoires** dans ces travaux de thèse ou dans la littérature, traduites principalement par **l'infiltration massive d'hémocytes dans les tissus,** ont souvent été associée à des **lésions de ces tissus** provoquées par les composés toxiques d'*Alexandrium* sp. ou par la colonisation par des agents pathogènes, comme l'ont également observé d'autres auteurs (articles 2 et 3, chapitre 2, Lassudrie et al., 2014, in rev. ; Choi et al., 2005; Haberkorn et al., 2010b; Hégaret et al., 2010; Medhioub et al., 2012) L'exposition à *Alexandrium* sp. provoque typiquement des lésions dans les tissus digestifs, qui sont non seulement en contact avec les composés extracellulaires produits par les algues, mais interagissent aussi avec les PSTs ou les spirolides, puisqu'ils accumulent la majorité de ces toxines (article 2, chapitre 2, Lassudrie et al., 2014; Medhioub et al., 2012). Les PSTs peuvent être métabolisées par les bivalves (Bricelj and Shumway, 1998; Guéguen et al., 2011, 2008), ce qui pourrait engendrer la production d'espèces réactives de l'oxygène (ROS) de la même manière que certains xénobiotiques lors de leur métabolisation (Regoli and Giuliani, 2014). Les ROS, bien qu'essentiels car impliqués dans de nombreux processus cellulaires, sont hautement toxiques pour les constituants cellulaires en concentration élevée, et peuvent notamment altérer les membranes cellulaires en réagissant avec les lipides qui les composent (péroxydation lipidique) et endommager les protéines et l'ADN (Bartosz, 2009; Stowe and Camara, 2009). Le manteau et les branchies peuvent également être altérés par l'exposition à *Alexandrium* sp.. (article 2, chapitre 2, Lassudrie et al., 2014) Ces effets sont probablement imputables principalement aux composés extracellulaires, car ces tissus accumulent les PSTs et spirolides en concentrations moindres comparativement à la glande digestive. Une implication du stress oxydant dans ces dommages a été suggérée relativement à la diminution d'activité d'une enzyme antioxydante, la Superoxyde Dismutase (SOD), et à l'observation de céroïdes (granules mélanisés composés de molécules oxydées) (article 2, chapitre 2, Lassudrie et al 2014 ;Yin, 1996). Cette hypothèse est confortée par plusieurs études reportant la modulation de l'activité d'enzymes antioxydantes ou de leurs transcrits dans cet organe (Fabioux et al., subm.; Romero-Geraldo and Hernández-Saavedra, 2012) ainsi que la mesure de péroxydation lipidique (Estrada et al., 2007) lors d'expositions à des dinoflagellés toxiques.

De plus, des effets synergiques de l'exposition à *Alexandrium* sp. et aux pathogènes, ont été observés sur le muscle (article 3, chapitre 2, Lassudrie et al., in rev.). Hégaret et al. (2009) ont également observé des effets synergiques de l'exposition au dinoflagellé *Prorocentrum minimum* et du parasite *P. olseni* sur l'intégrité du muscle et des ovocytes des palourdes *R. philippinarum*. L'énergie détournée par le parasite, ainsi que le coût énergétique des fonctions physiologiques impliquées dans la réponse (défense immunitaire, réparation tissulaire, détoxication), pourraient induire des compromis pour l'allocation d'énergie, au détriment d'une ou plusieurs fonctions, et ainsi induire ce type de lésions.

Finalement, ces lésions sont susceptibles d'affecter les fonctions physiologiques associées aux organes touchés (respiration, nutrition, reproduction…).

### 2.2.3 Filtration, ingestion et digestion : étapes clés dans la modulation de ces interactions ?

D'après les résultats des articles 3 et 4 (chapitre 1 et 4, Lassudrie et al., subm., in prep.), la réduction des activités de filtration, d'ingestion et de digestion pourraient jouer un rôle important dans les modulations des interactions tripartites. En effet, **l'infection par des agents pathogènes**, ainsi que **l'accumulation de toxines** est intimement liée à la quantité de pathogènes et de cellules algales qui pénètrent dans l'organisme.

Plusieurs facteurs peuvent influencer ces activités physiologiques, comme des lésions tissulaires, qui peuvent altérer le fonctionnement des organes (cf. paragraphe précédent). La capacité des bivalves à adapter leur activité alimentaire en fonction de la composition en plancton peut aussi modifier les taux de filtration et d'ingestion. La diminution, notamment du taux de filtration en présence de micro-algues toxiques (*Alexandrium* sp. inclus), a été largement rapportée dans la littérature avec de fortes différences inter-spécifiques (Contreras et al., 2011; Lassus et al., 2007, 1999; Shumway, 1990). D'autre part, une diminution de la filtration peut être induite lors d'une infection par un pathogène en déséquilibrant la balance énergétique du bivalve (Casas, 2002; Flye-Sainte-Marie et al., 2009, 2007). Il est important de considérer qu'un lien rétroactif existe entre le taux de filtration et le taux d'infection. **Ainsi, la filtration engendre l'accumulation de micro-organismes pathogènes qui a leur tour peuvent**

**inhiber en partie la filtration lorsque l'infection ou la virulence du pathogène devient suffisamment sévère pour induire un déséquilibre énergétique.**

Des mesures écophysiologiques (taux de filtration, d'ingestion et d'assimilation) lors de l'exposition simultanée à *Alexandrium* sp. et à des pathogènes pourraient répondre aux interrogations soulevées par l'observation des modulations des interactions bivalve – pathogènes et bivalve – *Alexandrium* sp. au cours de ce travail de thèse.

**L'utilisation d'un modèle bioénergétique basé sur la théorie des budgets d'énergie dynamiques (DEB,** Dynamic Energetic Budget ; Kooijman, 2010) serait un moyen d'améliorer la compréhension des processus métaboliques impliqués dans les interactions tripartites. Ce type d'approche permet en effet de prendre en compte l'état physiologique (reproduction notamment) de l'animal. De plus, ces modèles, développés à l'échelle de l'organisme, sont extrapolables à l'échelle d'une population (Martin et al., 2013). Un modèle DEB a été utilisé pour l'étude d'interactions hôte-pathogène chez la palourde *R. philippinarum* (Flye-Sainte-Marie et al., 2009). En outre, l'intégration des effets de composés toxiques, dont leur métabolisation, sur les grandes fonctions physiologiques est une des problématiques à l'origine de cette théorie (Kooijman 2010). Le cheminement théorique et conceptuel de cette approche a donné naissance à une version simplifiée de la théorie DEB, la variante DEB Tox (Jager and Selck, 2011). L'application d'un modèle DEB Tox à l'étude de l'influence de l'état physiologique initial de l'huître *C. gigas* sur l'accumulation de toxines et la détoxication, dans le but d'estimer les coûts énergétiques liés à l'exposition à *Alexandrium* sp. en fonction de l'état physiologique de l'animal fait actuellement l'objet d'une thèse au LEMAR (E. Pousse). A terme, il serait imaginable de coupler ces modèles (interaction bivalve – pathogène et interaction bivalve – toxines) pour l'étude des interactions tripartites afin d'estimer deux paramètres étroitement liés : (i) l'influence de l'état physiologique initial du bivalve sur sa réponse dans l'interaction tripartite, et (ii) l'effet de l'interaction tripartite sur la physiologie de l'hôte.

# 3 Des interactions à intégrer dans un environnement global

L'étude des interactions entre efflorescences toxiques et pathogènes sur la physiologie des bivalves constitue une première étape dans la considération de l'animal dans son environnement global, et fait l'objet des chapitres 1 et 2 de cette thèse (articles 1, 2 et 3 : Lassudrie et al., subm., 2014, in rev.). **Le troisième chapitre propose une approche plus globale** de l'étude des interactions entre le bivalve et son environnement biotique, en considérant *Alexandrium* sp, le bivalve, et l'ensemble de la communauté bactérienne qui est associée à l'animal lors d'un changement d'environnement microbien (article 4, Lassudrie et al., in prep.). **L'accumulation différentielle de toxines en fonction de l'environnement microbien auquel les huîtres ont été confrontées souligne la nécessité de considérer les interactions biotiques dans leur ensemble.** De plus, *Alexandrium* sp. produit des composés allélopathiques capables de modifier la structure de la communauté phytoplanctonique (Weissbach et al., 2010), et ainsi potentiellement la physiologie des bivalves via une modification de leur régime alimentaire (Delaporte et al., 2003; Hégaret et al., 2004).

D'autres études ont mis en avant l'influence de facteurs abiotiques sur l'interaction bivalve – *Alexandrium* sp.**,** notamment de certains métaux lourds (cadmium et cuivre), qui diminuent l'accumulation de PSTs dans les tissus de *C. gigas* (Haberkorn et al., 2014). L'influence des contaminants chimiques sur les interactions bivalve – pathogènes (bactéries ou parasites) a été largement rapporté dans la littérature, et dans une moindre mesure, les effets des agents pathogènes sur l'interaction bivalve – contaminants chimiques (revues de Morley, 2010; Morley et al., 2006; Sures, 2008a, 2008b). Bien que les effets sur les virus aquatiques des contaminants en général soient très peu connus (Morley, 2010), des métaux lourds (cuivre et mercure) ont démontré des signes de toxicité envers un virus (birnavirus) détecté dans des palourdes (Chou et al., 1998). Enfin, d'autres facteurs abiotiques, comme la température, la salinité, le pH, peuvent modifier les relations hôtes – pathogènes chez les bivalves (Asplund et al., 2014; Harvell et al., 1999; Mackenzie et al., 2014).

**L'environnement définit aussi la tolérance de l'agent pathogène, et peut modifier sa virulence.** La survie du parasite de la palourde *P. olseni* peut notamment être compromise par des contaminants d'origine anthropiques, des xénobiotiques ou des composés pharmacologiques (Bushek et al., 2007; Elandalloussi et al., 2005; Lund et al., 2005). Des paramètres abiotiques naturels, tels que la température ou la salinité, sont également d'importants facteurs régulant la tolérance de l'agent pathogène, ou l'expression de ses facteurs de virulence. Par exemple, le parasite *P. marinus*, pathogène de l'huître *C. virginica*, ne tolère pas de salinités inférieures à 9 (Ragone and Burreson, 1993). A l'inverse, **l'environnement bactérien associé à *Alexandrium* sp. peut impacter sa production de toxines** (Doucette et al., 1998; Hold et al., 2001).

Ces paramètres environnementaux peuvent constituer des variables forçantes à intégrer aux modèles DEB dans le but de considérer l'ensemble des facteurs pouvant modifier la physiologie du bivalve.

# 4 Evolution de l'interaction : quelques considérations temporelles

Les efflorescences de dinoflagellés toxiques, en zones tempérées, sont en général des phénomènes de courte durée (quelques semaines), saisonniers et récurrents. Ainsi, il est important de distinguer leurs effets à deux échelles de temps : à court terme (effets d'une efflorescence ponctuelle) et à long terme (effets d'efflorescences récurrentes). D'autre part, l'échelle de temps liée à la dynamique de l'interaction hôte – pathogène et le développement subséquent des maladies sont variables en fonction des modèles considérés (Figure 7).

**Les conséquences d'une exposition ponctuelle à *Alexandrium* sp. sur l'évolution de l'interaction hôte – pathogène pourraient ainsi varier en fonction de la dynamique temporelle associée à cette dernière** (Figure 9). Les systèmes hôte – pathogène étudiés au cours de cette thèse peuvent être classés en deux catégories, en fonction de l'échelle de temps liée à leur dynamique :

- **Un premier système hôte – pathogène, qui évolue sur une échelle de temps comparable à celle de l'efflorescence toxique, avec des conséquences rapides et**

**intenses de l'infection, associé à une maladie considérée comme aigüe**. Ainsi, l'interaction naissain d'huîtres *C. gigas* – herpesvirus est associée chaque année en France à un pic de mortalités sur quelques semaines (par exemple, augmentation de 3 à 70% de mortalités cumulées d'un lot de naissain sur 12 jours dans l'étang de Thau en 2013 ; Fleury, 2014), et ne touche que les stades naissain et juvénile. Les résultats de l'article 1 (chapitre 1, Lassudrie et al., subm.) démontrent que dans le cas d'une primo-infection, une efflorescence d'*A. catenella* altère la transmission et / ou la prolifération du virus. Ainsi, à l'échelle de la population, l'impact sur le phénomène de mortalité pourrait être modulé par l'efflorescence si elle se produit avant le pic de mortalités (Figure 9A).

- **Un second système hôte – pathogène, évoluant sur une échelle de temps plus grande, avec une dynamique d'interaction durable**, associé aux parasites *P. olseni*, *P. marinus* et *Bucephalus* sp., qui n'induisent pas d'épisodes de mortalités sévères sur les populations de bivalves étudiées. Bien qu'à court terme, une efflorescence toxique puisse favoriser le développement de l'infection parasitaire (article 3, chapitre 2, Lassudrie et al., in rev.), il ne semble pas réaliste de considérer qu'une unique exposition puisse avoir un impact à plus long terme (Figure 9B). L'équilibre de l'interaction hôte – pathogène se rétablirait probablement après l'exposition, comme le suggère l'étude de da Silva et al. (2008). Leurs travaux indiquent que l'intensité de l'infection par *P. olseni* de palourdes *R. philippinarum* est diminuée après 2 et 3 semaines d'exposition à une concentration élevée en dinoflagellé toxique *Karenia selliformis*. Puis, lorsque cette concentration algale diminue pendant 3 semaines, l'intensité d'infection se rétablit à son niveau d'origine (Figure 9C).

En revanche, des effets **à long terme de la récurrence d'efflorescences toxiques** sur la dynamique d'interaction hôte – pathogène à l'échelle de la population sont envisageables. Le recours à des suivis *in situ* et à des modèles épidémiologiques contribuerait à l'étude de ces effets. Un modèle SIIPS (« Susceptibles, Infected, and Infective Particles in Space ») a notamment été utilisé récemment pour caractériser la dynamique d'infection d'une population d'huitres américaines *C. virginica* par le parasite *P. marinus*, et validé par des données issues de suivis *in situ* (Bidegain et al., 2014). Ce modèle prend en compte notamment la filtration et la densité en bivalves, qui jouent un rôle sur la dynamique de certaines maladies infectieuses en réduisant la quantité de particules infectieuses dans l'eau et ainsi la probabilité de primo-infection.

En parallèle, des particules infectieuses peuvent être relâchées dans le milieu par les animaux infectés (alors qualifiés « d'infectieux »), via les faeces ou par les animaux morts. L'intégration à un tel modèle de l'impact d'efflorescences toxiques sur la susceptibilité de l'hôte, dans le but d'évaluer les conséquences sur la dynamique de la maladie à l'échelle de la population et sur la structuration de cette population, serait particulièrement utile dans le domaine de la conchyliculture et de la restauration écologique. La construction d'un tel modèle intégrant l'effet des dinoflagellés toxiques nécessiterait également d'estimer leur impact sur la susceptibilité du bivalve à l'agent pathogène, et donc en amont, d'améliorer la compréhension des processus d'interactions à l'échelle de l'organisme.

Finalement, la question d'un impact sur la co-évolution du système hôte – pathogène face aux efflorescences toxiques se pose (Koella et al., 1998). En effet, des adaptations des populations de bivalves à des efflorescences toxiques récurrentes, reflétées par des réponses physiologiques différentielles entre populations (Navarro et al., 2014), peuvent aboutir à l'apparition de génotypes résistants (Bricelj et al., 2010, 2005). De plus, des effets directs de ces efflorescences sur les micro-organismes pathogènes sont possibles (revus dans cette discussion), et pourraient également conduire à des adaptations chez ces micro-organismes.

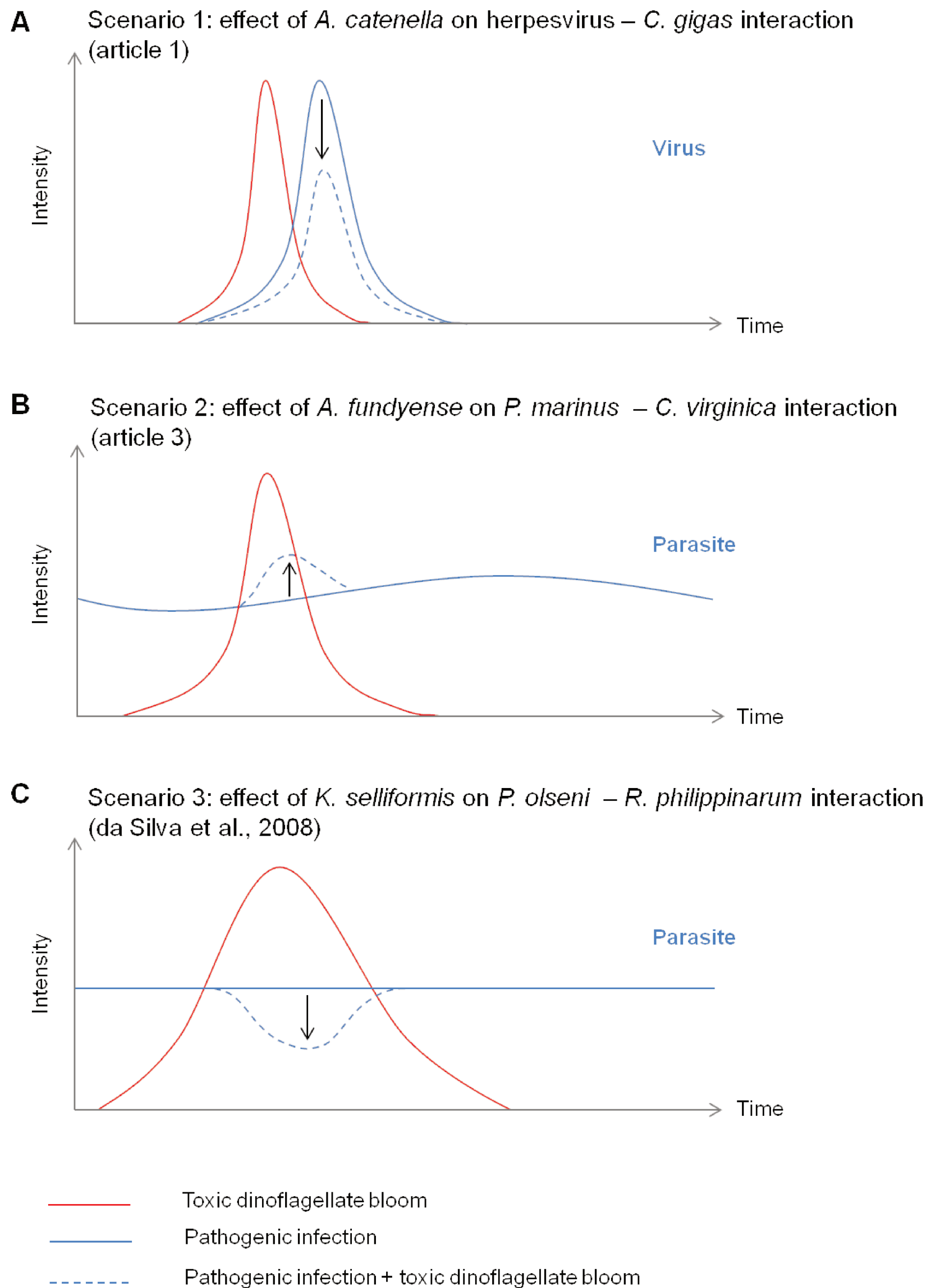


Figure 9. Scénarios hypothétiques de l'effet d'une efflorescence de dinoflagellés toxiques sur la dynamique temporelle du taux d'infection de différents systèmes hôte-pathogène à l'échelle de la population, basés sur des études expérimentales ; (A) scénario 1 : système naissain d'huître *Crassostrea gigas* – herpesvirus OsHV-1 lors d'une efflorescence d'*Alexandrium catenella* (d'après l'article 1, chapitre 1, Lassudrie et al., subm.) ; (B) scénario 2 : système huître *C. virginica* – parasite protozoaire *Perkinsus marinus* lors d'une efflorescence d'*A. fundyense* (d'après l'article 3, chapitre 2: Lassudrie et al., in rev.) ; (C) scénario 3 : système palourde *R. philippinarum* – parasite protozoaire *P. olseni* (d'après da Silva et al., 2008).

# Conclusion

L'objectif de cette thèse était d'évaluer les effets combinés d'une exposition ponctuelle au dinoflagellé toxique *Alexandrium* sp., et d'agents pathogènes sur la physiologie des bivalves au travers de l'étude de différentes interactions tripartites bivalve – pathogène – *Alexandrium* sp. Ces travaux ont permis de répondre aux questions posées au début de ce manuscrit.

**Ces travaux mettent en évidence qu'une exposition à *Alexandrium* sp. peut impacter le processus de primo-infection d'un mollusque bivalve par un agent pathogène, et moduler des interactions hôte – pathogène établies.**

L'exposition à *Alexandrium catenella* inhibe en effet partiellement le processus d'infection ou de prolifération de l'herpesvirus chez du naissain d'huîtres du Pacifique *Crassostrea gigas*.

Le phénomène inverse est observé lors de l'exposition d'huîtres américaines *C. vriginica* à *A. fundyense*, qui favorise la prolifération du parasite protozoaire *Perkinsus marinus*. L'effet combiné d'*A. fundyense* et d'un autre parasite, le trématode *Bucephalus* sp., accroît également la susceptibilité de l'huître à *P. marinus*.

**De plus, cette thèse révèle que l'environnement microbien peut moduler l'interaction bivalve – *Alexandrium* sp.**

En effet, les huîtres juvéniles *C. gigas* exposées à *A. catenella* accumulent moins de toxines lorsqu'elles sont également exposées à l'herpesvirus ou à un nouvel environnement microbien. Ces résultats pourraient s'expliquer par des altérations des processus de filtration, d'ingestion et de digestion.

**Enfin, ce travail montre que les modulations de la relation hôte - pathogène induites par l'exposition à *Alexandrium* sp. peuvent être associées à des impacts sur les paramètres physiologiques…**

La répression des réponses hémocytaires engendrée par l'effet combiné d'*A. fundyense* et du trématode *Bucephalus* sp chez les huîtres *C. virginica* pourrait traduire une immunodépression à l'origine de leur susceptibilité accrue à *P. marinus*. De plus, un effet synergique d'*A. fundyense* et du trématode *Bucephalus* sp. provoque des lésions du muscle adducteur de *C. virginica*, reflétant un affaiblissement physiologique global.

**…mais l'effet d'*Alexandrium* sp. sur les paramètres physiologiques n'induit pas nécessairement de modulation hôte – pathogène, ou d'infection opportuniste!**

L'exposition à *A. ostenfeldii* entraîne des lésions des branchies, du manteau et des tissus digestifs chez la palourde *Venerupis philippinarum*. Pourtant, ces lésions n'impactent pas l'équilibre de son interaction avec le parasite *Perkinsus olseni*, et la stabilité des variables hémocytaires qui y est associée reflète la capacité du bivalve à maintenir l'homéostasie du système circulatoire baignant ces hémocytes.

D'autre part, l'exposition à *A. catenella* réprime les réponses hémocytaires d'huitres juvéniles *C. gigas* à un nouvel environnement microbien et suggère une altération de leurs conditions physiologiques. En dépit de ces effets, le microbiome de l'huître reste stable, et n'indique aucune infection opportuniste.

Il ressort de ce travail que les modulations de ces interactions sont fortement espèces – spécifiques. Ainsi un profil global de réponse ne peut pas être dressé mais nécessite des études au cas par cas. Cependant, ces travaux permettent une meilleure évaluation des risques pour la santé du bivalve résultant des interactions biotiques qui se produisent régulièrement *in situ*, et ce à travers différents scénarios incluant les facteurs 'statut infectieux' et 'accumulation toxinique'.

Enfin, ces interactions biotiques sont en constante évolution, notamment du fait des actions anthropiques : transferts de bivalves entre zones d'exploitation, rejets d'eaux de ballast, contaminations organiques et chimiques, etc. et accroissent la nécessité d'études *in situ*, intégrées sur une large échelle spatio-temporelle.

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