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Anne-Sophie Pavaux

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Sorbonne Université

Ecole Doctorale des Sciences de l'Environnement d'Ile de France (ED 129)

Laboratoire d'Océanographie de Villefranche

UMR 7093 – SU – CNRS

Ecologie chimique du dinoflagellé toxique
***Ostreopsis cf. ovata* en Méditerranée Nord Occidentale**

Thèse présentée par Anne-Sophie PAVAUX

En vue de l'obtention du grade de Docteur de Sorbonne Université

Spécialité : Ecologie Marine

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Pavaux A-S., Berdalet E., Lemée R. Chemical ecology of the benthic dinoflagellates genus *Ostreopsis* : à review. En préparation.

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Pavaux A-S., Rostan J., Guidi-Guilvard L., Marro S., Gasparini S., Lemée R. Chemical ecology of the benthic toxic dinoflagellate *Ostreopsis cf. ovata* in N.W. Mediterranean Sea. Visite volontaire au Natural History Museum of Denmark, Janvier 2019, Copenhague, Danemark – Présentation orale.

Pavaux A-S., Ternon E., Dufour L., Marro S., Thomas O., Lemée R. *Artemia franciscana* as a simple and pertinent model to check *Ostreopsis* spp. toxicity. Projet Assemblée + APOTOX, National University of Galway, Mars 2019, Galway, Irlande – Présentation orale.

Pavaux A-S., Ternon E., Dufour L., Rostan J., Guidi-Guilvard L., Marro S., Gasparini S., Thomas O., Lemée R. Chemical ecology of the benthic toxic dinoflagellate *Ostreopsis cf. ovata* in N.W. Mediterranean Sea. GdR PHYCOTOX, Mai 2019, Brest, France – Présentation orale : Prix de la meilleure présentation.

Pavaux A-S., Ternon E., Dufour L., Marro S., Thomas O., Lemée R. The use of *Artemia franciscana* to assess the toxicity of benthic dinoflagellates genus *Ostreopsis*. Gordon Research Conference – Phycotoxins and Mycotoxins, June 2019, Boston, USA – Poster.

Boisnoir A., Pascal P.Y., **Pavaux A-S.**, Shizas N. V., Marro S., Blasco T., Lemee R. Ingestion of potentially toxic benthic dinoflagellates by harpacticoid copepods. SeventIMCO, July 2019, Evora, Portugal – Présentation orale.

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LISTES DES ABBREVIATIONS

ACN : Acétonitrile
ANR : Agence Nationale de la Recherche
ANSES : Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du travail
ASP : Amnesic Shellfish Poisoning
AZA : Azaspiracide
BPC : Base Peak Chromatogram
CAT : Catalase
CE : Collision Energies
Cellules/gPF : nombre de cellules par gramme de poids frais de macroalgues
CFP : Ciguatera Fish Poisoning
DMSO : Dimethylsulfoxyde
DMS : Diméthylsulfide
dNTP : Désoxyribonucléotides
DSP : Diarrheic Shellfish Poisoning
DTX : Dinophysistoxines
EC₅₀ : Median effect concentration
EFSA : Autorité européenne de sécurité des aliments
EN : Endométabolome
Eq. : Equivalent
ESI-MS/MS : ElectroSpray Ionization-tandem Mass Spectrometry
EX : Exométabolome
FW : Fresh Weight
GdR MediatEC : Groupement de Recherche Médiation chimique dans l'environnement – Ecologie Chimique
GEOHAB : Global Ecology and Oceanography of Harmful Algal Blooms
GYM : Gymnodimine
HA : Hemolytic Assay
HAB : Harmful Algal Bloom
HNA : Hemolytic Neutralization Assay
HPLC-ESI-MS/MS : High Performance Liquid Chromatography – ElectroSpray Ionization tandem Mass Spectrometry
HTD : Heterotrophic dinoflagellate
IFREMER : Institut français de Recherche pour l'exploitation de la mer
ITS : Internal Transcribed Spacer
LC₅₀ : Median lethal concentration
LC-HRMS : Liquid Chromatography – High Resolution Mass Spectrometry
LC-FLD : Liquid Chromatography with Florescence Detection
LD₅₀ : Median lethal dose
LSU : Large ribosomal subunit
LT₅₀ : Median lethal time
MBA : Mouse BioAssay
MCCV : Mediterranean Culture Collection of Villefranche
MCE : Marine Chemical Ecology
MC-PAM : Multi-Color Pulse-Amplitude-Modulated
MeOH : Méthanol
MT6 : Métallothioneine 6
MU : Mouse Unit
N2a : Neuroblastoma cell line cytotoxicity assay
nd : not detectable

NSP : Neurotoxic Shellfish Poisoning
NOAA : National Oceanic and Atmospheric Administration
OCEAN-15 : *Ostreopsis* Chemical Ecology and Allelopathy Network (Projet ANR)
OVTX : Ovatoxine
PCA : Principal Components Analysis
PCR : Polymerase Chain Reaction
PLS-DA : Partial Least Squares-Discriminant Analysis
PLTX : Palytoxine
PnTX : Pinnatoxine
pPLTX : putative Palytoxine
PS II : Photosystème II
PSP : Paralytic Shellfish Poisoning
PTX : Pectenotoxine
PUAs : PolyUnsaturated Aldehydes
PUFAs : PolyUnsaturated Fatty Acids
PVDF : PolyVinylidene Fluoride
QC : Quality Control
qPCR : PCR quantitative
QTOF : Quadripole-temps de vol
RT : Remaining Tissue
Rt : Retention time
SPX : Spirolide
UHPLC : Chromatographie Liquide à Ultra Haute Performance
VIP : Variable Importance in Projection
v/v : volume/volume
WF : Whole Flesh
WHOI : Woods Hole Oceanographic Institute
YTX : Yessotoxine

Préambule

Cette thèse a été financée par l'Ecole Doctorale 129 (ED 129 - Sciences de l'Environnement) de Sorbonne Université.

Ce travail s'inscrit principalement dans le cadre du projet multipartenaires (LOV - Sorbonne Université et CNRS, ANSES, Université de Chimie de Galway et IFREMER Nantes) ANR OCEAN-15 (Ostreopsis Chemical Ecology and Allelopathy Network) dont l'objectif principal est de comprendre les effets des changements climatiques sur le métabolisme secondaire d'Ostreopsis cf. ovata afin d'anticiper les modifications potentielles de la toxicité et les effets écologiques qui en découlent. Plus précisément, cette thèse s'insère dans la tâche 3 de ce projet dont l'objectif est d'étudier les impacts écologiques de ces toxines.

Une partie du travail effectué lors de mon doctorat est également intégrée dans le projet européen CoCliMe (<https://www.coclimate.eu/>) dont l'objectif est de fournir des services climatiques en relation avec les qualités des eaux marines littorales (en particulier en relation avec les microalgues nuisibles). J'ai également pu bénéficier d'un soutien financier dans le cadre du projet européen Assemble + (Association of European Marine Biological Laboratories Expanded - <http://www.assembleplus.eu/>) afin de faire des analyses chimiques au sein du Laboratoire Marine Biodiscovery à Galway, Irlande, pendant 3 semaines au printemps 2019.

L'objectif principal de mon travail de thèse est d'améliorer nos connaissances sur les rôles écologiques des toxines produites par le dinoflagellé Ostreopsis cf. ovata en mer Méditerranée. Dans un premier temps, j'ai réalisé une étude exhaustive de la bibliographie sur ce sujet, qui sera publiée sous forme de revue. J'ai ensuite étudié les interactions chimiques avec les compétiteurs, puis j'ai évalué l'impact des phycotoxines sur des prédateurs (copépodes benthiques et planctoniques). Enfin, puisque la nécessité s'est rapidement fait sentir, j'ai développé un modèle biologique simple permettant d'évaluer rapidement la toxicité des cellules d'O. cf. ovata.

Afin de répondre à mon objectif principal, j'ai combiné des études expérimentales in situ et en laboratoire. J'ai également utilisé une approche métabolomique, technique indispensable dans l'étude de la communication chimique, qui a permis l'étude de l'endo- et de l'exométabolome d'O. cf. ovata.

Ce manuscrit s'articule ainsi autour de 7 chapitres :

- *Le premier chapitre introductif traite de l'écophysiologie et de l'écologie chimique des espèces du genre *Ostreopsis*.*
- *Le second chapitre détaille les relations allélopathiques qu'*Ostreopsis cf. ovata* entretient avec les producteurs primaires et notamment une diatomée benthique compétitrice ;*
- *Les chapitres 3 et 4 traitent respectivement de l'impact d'*Ostreopsis cf. ovata* sur les copépodes benthiques et planctoniques ;*
- *Le chapitre 5 s'intéresse aux effets de ce dinoflagellé sur les consommateurs secondaires en prenant l'exemple de l'oursin comestible *Paracentrotus lividus**
- *Le chapitre 6 aborde la mise en place d'un test écologique pour tester facilement, rapidement et manière pertinente, la toxicité des espèces du genre *Ostreopsis*.*
- *Le chapitre 7 constitue une conclusion des travaux et présente les perspectives envisagées.*

Le titre et les résumés des articles réalisés en collaboration au cours de ma thèse, sont ajoutés en annexe.

Chapitre 1. Introduction

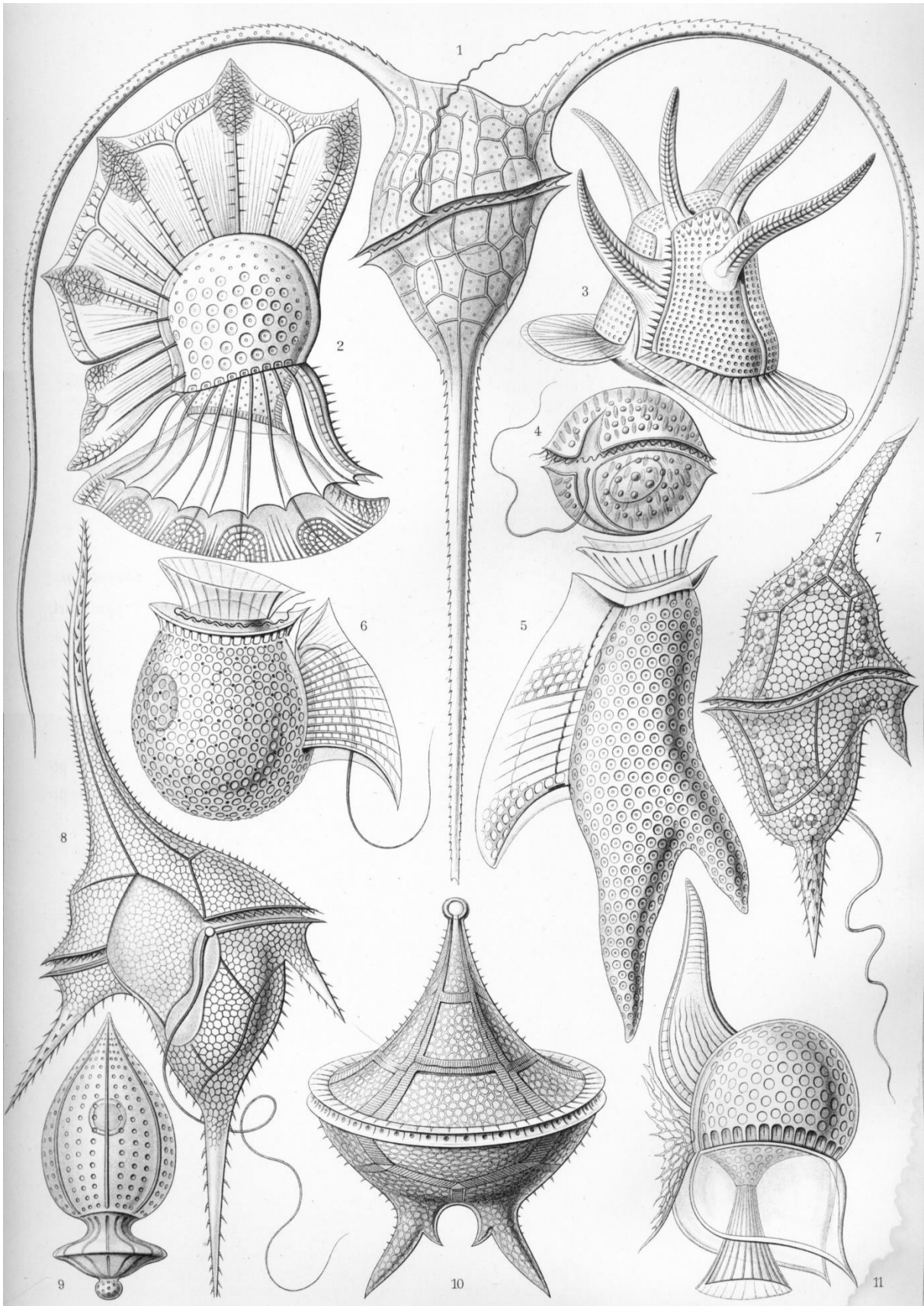


Illustration of armoured dinoflagellates from Ernst Haeckel's "Kunstformen der Natur" (1904)

1. Ecologie chimique marine

L'écologie chimique marine est une science pluridisciplinaire relativement jeune dont l'objectif est de mieux comprendre l'implication de la signalétique chimique produite par les organismes marins (Paul et al., 2006; Paul & Ritson-Williams, 2008) dans l'organisation et le fonctionnement des écosystèmes marins (Hay, 1996; McClintock & Baker, 2001; Pohnert et al., 2007). L'impact de ces signaux chimiques sont encore mal reconnus (Hay, 2009). Ils affectent pourtant les comportements individuels et les processus à l'échelle des populations, mais également l'organisation des communautés par des effets en cascade (Hay and Kubanek, 2002). Ils affectent ainsi les transferts d'énergie, la dynamique des réseaux trophiques et la structure des écosystèmes (Schwartz et al., 2016) en intervenant dans la quête de nourriture, les communications intraspécifiques, les interactions de compétition, de prédation et d'allélopathie, mais également dans la sélection des habitats et des partenaires sexuels (Hay, 2009; Poulson et al., 2009; Sieg et al., 2011). L'un des exemples les plus étonnants illustrant l'importance de ces signaux est celui des oiseaux procellariiformes. Le diméthylsulfure (DMS), composé excrété par le phytoplancton lorsqu'il est brouté par le zooplancton (Dacey and Wakeham, 1986), agit comme un guide olfactif pour ces oiseaux et leur permet de repérer les zones océaniques productives où leur prise de nourriture sera un succès (Nevitt, 2000; Nevitt et al., 1995). Ils ont pour cela développé une neuroanatomie particulière leur permettant de détecter ce métabolite à plusieurs dizaines de kilomètres (Nevitt, 2008; Nevitt and Bonadonna, 2005). Un autre exemple montrant l'importance de ces signaux chimiques dans les écosystèmes marins est l'utilisation, par les crabes juvéniles *Libinia dubia*, des métabolites secondaires produits par la macroalgue *Dictyota menstrualis* pour décorer leurs carapaces afin de diminuer leur prédation (Stachowicz and Hay, 1999). Les signaux intervenant dans ces processus sont encore assez mal étudiés, mais cette discipline connaît un véritable essor ces dernières années (McClintock and Baker, 2001), essor illustré par la création de groupements de recherche au niveau national (GdR Mediatec : Médiation Chimique dans l'Environnement - Ecologie Chimique <https://www.gdr-mediatec.cnrs.fr/>) et international (International Society of Chemical Ecology <https://chemecol.org/>) dont l'objectif est de fédérer des scientifiques d'horizons variés (chimistes, biologistes et écologues) pour explorer le rôle fonctionnel de ces signaux et leur coévolution.

La communication chimique est régie par les métabolites secondaires, c'est-à-dire des produits métaboliques non essentiels à la croissance végétative des organismes producteurs (Amsler, 2008; Hay, 1996). Les métabolites secondaires sont d'une incroyable diversité en termes de structure et de fonction. Ils sont regroupés sous le terme de phéromones lorsque liés aux interactions intraspécifiques et plutôt qualifiés d'allélochimiques pour les relations interspécifiques (Bagnères and Hossaert-Mckey, 2017 - Figure 1). Parmi ces substances allélochimiques, on distingue les kairomones et les allomones,

respectivement bénéfiques pour le récepteur et l'émetteur, et les synomones qui sont bénéfiques à la fois pour l'émetteur et le récepteur (Brown et al., 1970). Les voies métaboliques associées à la production de ces métabolites sont souvent complexes et requièrent une importante quantité d'énergie (Chakraborty et al., 2018; Steppuhn and Baldwin, 2008). Cette production de métabolites secondaires, sans intérêt du point de vue du métabolisme basal, soulève de nombreuses questions et quelques hypothèses sont avancées pour expliquer cette production malgré le coût énergétique associé. Ces métabolites pourraient tout d'abord constituer des déchets chimiques sans fonction au regard du métabolisme primaire (Ianora et al., 2006). Ces médiateurs chimiques pourraient également avoir de multiples fonctions, ce qui permettrait de limiter les coûts énergétiques liés à la biosynthèse de plusieurs composés (Wink and Schimmer, 2010). Enfin, chez les organismes unicellulaires, ces métabolites pourraient être conservés intracellulairement afin de diminuer leur prédation par d'éventuels brouteurs. Dans ce dernier cas, les cellules productrices n'ont pas besoin de libérer puis de reconstituer le stock de ces composés (Ianora et al., 2011).

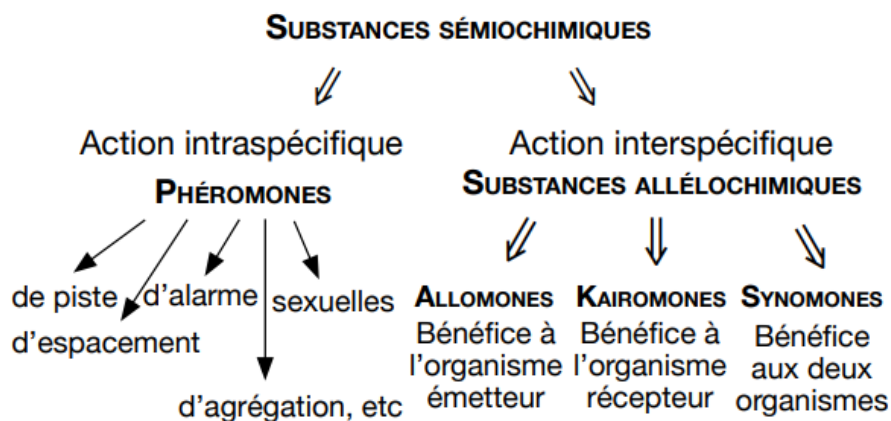


Figure 1 : Les différents types de médiateurs chimiques (Brossut, 1997).

Les signaux chimiques ont un rôle important à toutes les échelles spatiales et temporelles de la formation des efflorescences phytoplanctoniques (Cembella, 2003 - Figure 2). Aux petites échelles spatiales (entre 1 μm et 1 mm) correspondant à des échelles temporelles de l'ordre de quelques minutes, les processus intracellulaires relatifs au cycle de vie vont engendrer la production de substances qui vont agir sur les interactions trophiques et allélopathiques. Ces signaux sont ensuite intégrés aux échelles de temps et d'espace supérieures, c'est-à-dire au niveau des traits d'histoire de vie des organismes. Ils influencent également des processus à plus grandes échelles tels que le broutage et les migrations en déterminant la nage, la sédimentation et la flottaison. L'influence de ces signaux est également importante au niveau de la formation, voire même de la dispersion des efflorescences. La communication chimique est ainsi produite à l'échelle subcellulaire mais les effets

biologiques de ces signaux chimiques peuvent couvrir de larges échelles spatiales et temporelles. Seule la stabilité de la molécule devient alors limitante.

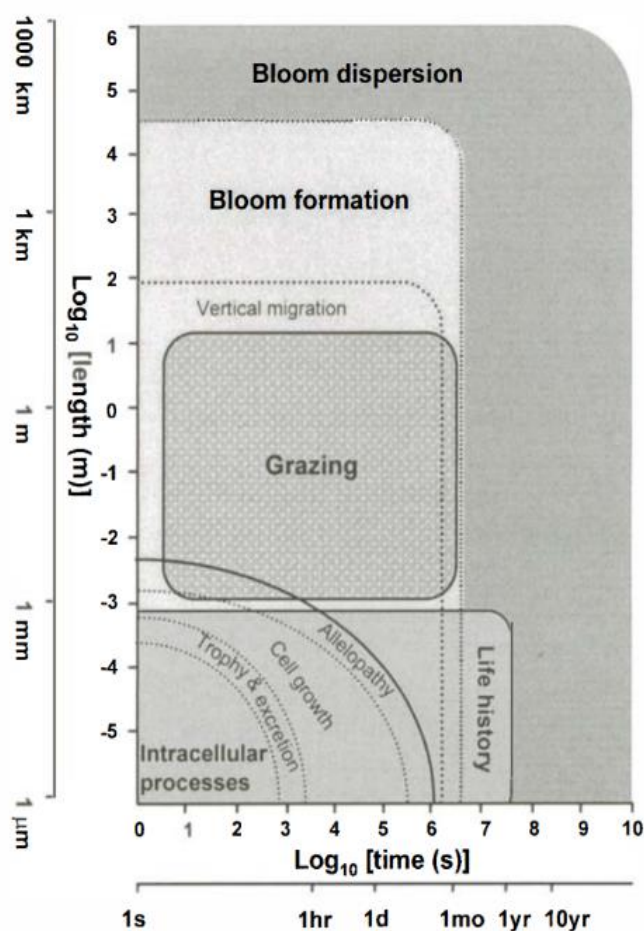


Figure 2 : Schéma représentant les niveaux d'organisation fonctionnelles régis par la communication chimique dans les efflorescences d'algues toxiques planctoniques. L'axe des abscisses représente l'échelle temporelle (en échelle logarithmique) et celui des ordonnées l'échelle spatiale (également en échelle logarithmique). Les signaux chimiques sont issus de processus cellulaires mais agissent à différents niveaux d'organisation : à l'échelle subcellulaire (ex : excrétion, croissance), à méso-échelle (ex : broutage, parasitisme, allélopathie) ainsi qu'à l'échelle de la formation et la dispersion des efflorescences. L'importance de cette communication dans l'évolution des traits d'histoire de vie est également représentée (Cembella, 2003).

Les difficultés majeures de cette discipline sont liées aux faibles quantités de métabolites produits. En effet, l'identification structurale et fonctionnelle de ces composés nécessite une quantité importante de métabolites. Ces composés peuvent également agir en synergie avec d'autres métabolites plutôt que sous la forme de composés purs, voire être dégradés rapidement pour éviter les erreurs de communication (Hay, 2009), rendant la séparation et l'identification structurale de ces composés complexes. A titre d'exemple, les toxines issues du métabolisme secondaire de certains unicellulaires sont produits à hauteur de quelques picogrammes par cellule : 0,5 à 7 pg.cellules⁻¹ d'acide domoïque, une toxine amnésiante, sont ainsi produits par les diatomées du genre *Pseudo-nitzschia* (Bates et al., 1991; Lundholm et al., 2018; Tammilehto et al., 2015).

Les efflorescences d'algues toxiques illustrent parfaitement comment la production de métabolites secondaires affecte la structure et le fonctionnement des écosystèmes. Les phycotoxines produites par certaines espèces de microalgues peuvent affecter l'approvisionnement en eau, les

activités de pêcheries, voire les activités récréatives humaines (Paerl and Otten, 2013), générant des pertes économiques pouvant se chiffrer en milliards de dollars (Hoagland et al., 2002; Hoagland and Scatasta, 2006; Shumway et al., 2018). L'impact économique annuel des efflorescences d'algues néfastes est ainsi estimé à plus de 83 millions de dollars aux États-Unis (Ritzman et al., 2018). Les microalgues toxiques constituent donc un modèle pertinent pour les études en écologie chimique. Le rôle écologique des toxines (et des métabolites secondaires en général), dans les interactions biotiques (allélopathie, prédation, lutte contre les infections et le parasitisme, coopération intra- et interspécifique) que ces microalgues entretiennent avec leur environnement est en effet encore peu connu (Berry et al., 2008; Holland and Kinnear, 2013; Leflaive and Ten-Hage, 2007; Legrand et al., 2003; Schatz et al., 2007; Sugg and VanDolah, 1999).

2. Application de l'écologie chimique à l'étude des efflorescences d'algues nuisibles.

Les efflorescences d'algues nuisibles (Harmful Algal Bloom en anglais – HAB) sont des proliférations exceptionnelles d'espèces qui vont affecter les écosystèmes par des effets non chimiques (=algues nuisibles) conduisant à l'anoxie ou causant des dommages mécaniques et physiques préjudiciables, ou des effets chimiques (=algues toxiques) liés à la production de phycotoxines (Granéli 2007; Smayda, 1997a). Ces efflorescences sont décrites dans la littérature depuis plus de 150 ans (Francis, 1878), mais une augmentation de l'incidence et de l'intensité de ces événements aussi bien dans les eaux douces que marines a été observée ces dernières années (Hoagland et al., 2002). Le nombre de cas d'efflorescences responsables d'intoxications de type paralysantes causées par des fruits de mer (ou Paralytic Shellfish Poisoning – PSP en anglais) a ainsi été multiplié par 10 entre 1970 et 2015 (Figure 3).

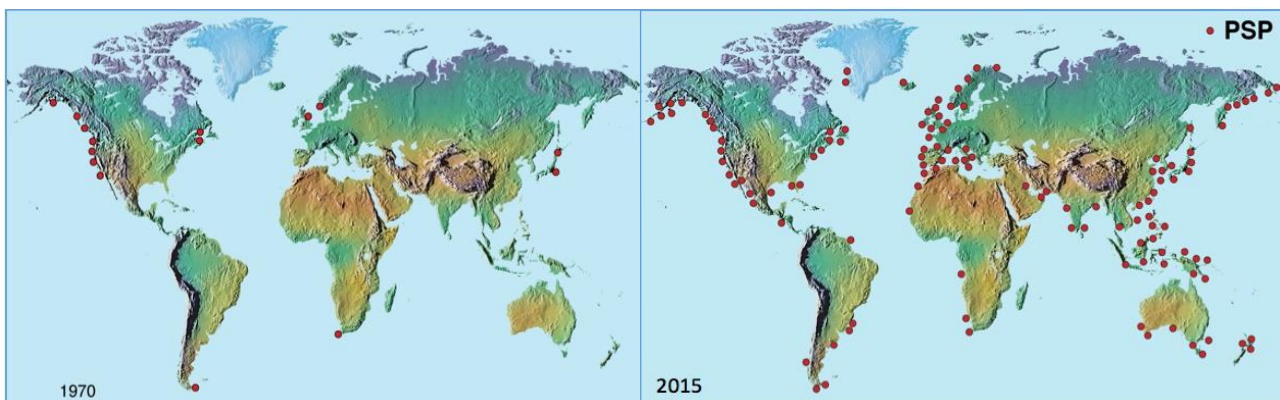


Figure 3 : Evolution du nombre d'efflorescences d'algues toxiques provoquant des intoxications par fruits de mer de type paralysante entre 1970 et 2015 (Sources : National Oceanic and Atmospheric Administration – NOAA and Woods Hole Oceanographic Institution - WHOI

<https://www.whoi.edu/website/redtide/regions/world-distribution/>).

A ce jour, il reste difficile de savoir si l'intensification de ces efflorescences est plutôt due à une fréquence d'échantillonnage plus élevée ces dernières années ou à d'autres facteurs, qu'ils soient d'origines naturelles et/ou anthropiques. Les changements climatiques globaux et l'eutrophisation sont souvent considérés comme des facteurs potentiellement déterminants dans l'augmentation de ces événements (Figure 4 : Anderson et al., 2002, 2008 ; Fu et al., 2012; GEOHAB, 2001; Glibert et al., 2005; Glibert et al., 2010; Glibert and Burkholder, 2011; Griffith and Gobler, 2019; Heisler et al., 2008; Moore et al., 2008; O'Neil et al., 2012; Paerl et al., 2011; Paerl and Huisman, 2008; Paerl and Paul, 2012; Visser et al., 2016). Le rôle du transport des cellules et des cystes par les eaux de ballast, ainsi que l'augmentation de l'aquaculture et/ou de la surpêche sont également considérés car ils vont permettre aux algues nuisibles de dominer les communautés phytoplanctoniques en modifiant les équilibres trophiques (GEOHAB, 2001; Griffith and Gobler, 2019; Hallegraeff, 1993).

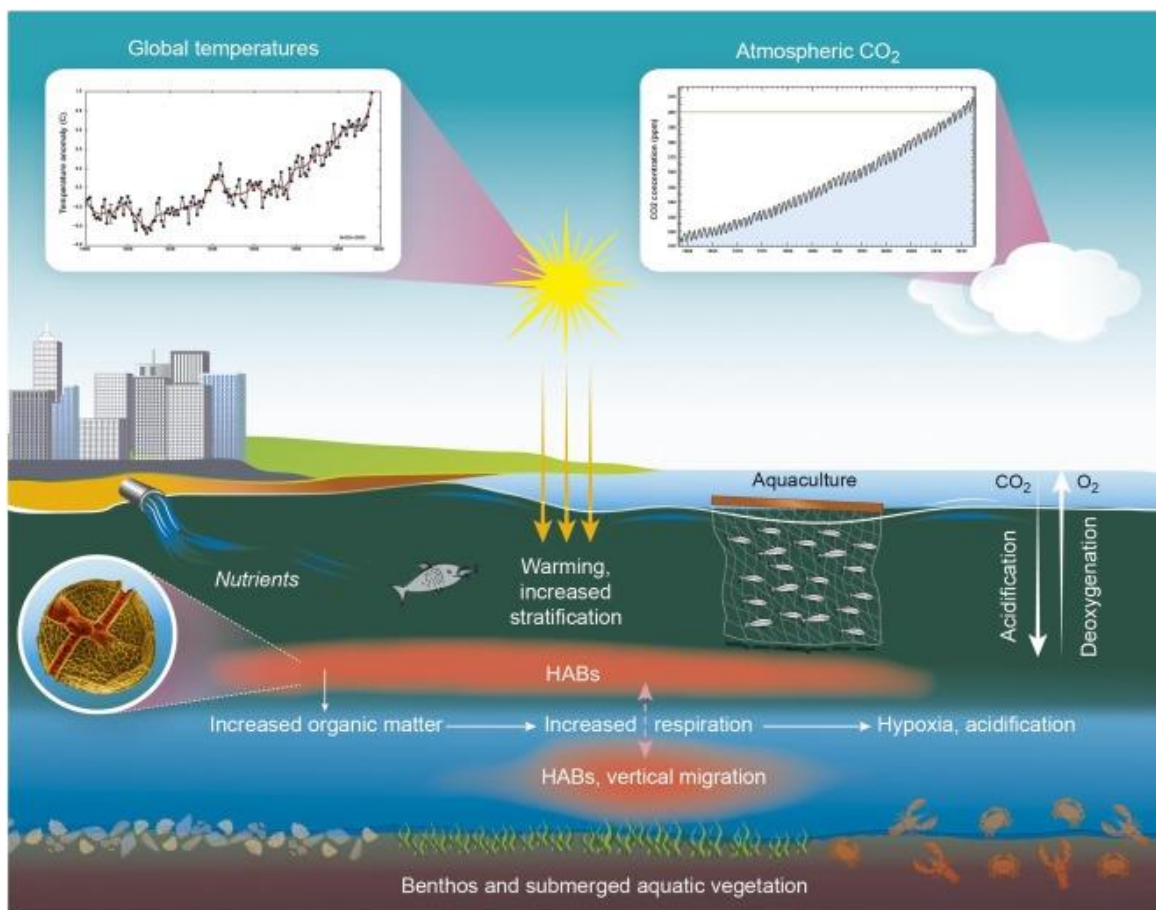


Figure 4 : Bilan des facteurs de stress climatiques qui peuvent potentiellement déterminer l'intensité et la fréquence des efflorescences d'algues nuisibles. L'élévation globale des températures, l'augmentation des concentrations de CO₂ atmosphériques ainsi que l'eutrophisation décrites depuis le début du 20^{ème} siècle peuvent potentiellement favoriser de manière indépendante et interactive les HABs (Griffith and Gobler, 2019).

De nombreuses études se sont focalisées sur les conséquences de ces phycotoxines au niveau de la santé humaine (Backer et al., 2015; James et al., 2010; Munday et al., 2012; Picot et al., 2011). Ces toxines sont d'ailleurs souvent classées selon les symptômes humains qu'elles provoquent (Tableau 1) : on retrouve les toxines paralysantes (PSP), neurotoxiques (NSP), diarrhéiques (DSP), amnésiques (ASP) et la ciguatera (CFP).

Tableau 1 : Classification des phycotoxines selon les symptômes qu'elles provoquent chez l'Homme. Les espèces ainsi que les toxines associées sont également énumérées (Traduit depuis Sidharta, 2005).

Intoxication	Organismes	Toxines produites	Symptômes
ASP Amnesic Shellfish Poisoning	<i>Pseudo-nitschia</i> spp.	Acide domoïque	En 24h : nausées, vomissements, maux de ventre, diarrhées. En 48h : symptômes neurologiques comme étourdissements, maux de tête, désorientation, pertes de mémoires à court terme, difficultés respiratoires et coma.
DSP Diarrheic Shellfish Poisoning	<i>Dinophysis</i> spp. <i>Prorocentrum lima</i>	Acide okadaïque	En 30 minutes : diarrhées sévères (92%), nausées (80%), vomissements (79%), maux de ventres et frissons.
NSP Neurotoxic Shellfish Poisoning	<i>Gymnodinium</i> <i>breve</i>	Brévéttoxines	Diarrhées, vomissements, maux de ventre, suivis par des troubles neurologiques
PSP Paralytic Shellfish Poisoning	<i>Alexandrium</i> spp., <i>Gymnodinium</i> <i>catenatum</i> , <i>Pyrodinium</i> <i>bahamense</i> var. <i>compressum</i>	Saxitoxines	Potentiellement fatal (14%), mort dans les 24 heures. Dans les cas non létaux, sensations de fourmillements, engourdissements, ataxie, vertiges, somnolence, fièvre, éruptions cutanées.
CFP Ciguatera Fish Poisoning	<i>Gambierdiscus</i> spp., <i>Prorocentrum</i> spp., <i>Ostreopsis</i> spp., <i>Coolia monotis</i>	Ciguatoxines, maitotoxines	Initialement maux de ventre, diarrhées, vomissements, suivis de douleurs musculaires, d'étourdissements, anxiété, engourdissements et picotements au niveau de la bouche et de l'extrémité des membres

L'émergence de nouveaux outils de chimie analytique tels que la métabolomique (Fiehn et al., 2007; Kuhlisch and Pohnert, 2015; Poulin and Pohnert, 2019; Prince and Pohnert, 2010) ont toutefois permis la détection de nouveaux analogues voire de nouvelles toxines dites émergentes, souvent encore non soumises aux législations européennes et mondiales (Silva et al., 2015 - Tableau 2). Le rôle écologique des phycotoxines dans la structuration des écosystèmes est encore mal appréhendé (Cembella, 2003).

De manière générale, l'importance même des interactions biotiques est également rarement prise en compte dans la modélisation des dynamiques phytoplanctoniques conduisant à la formation des efflorescences (Ivanora et al., 2006). Ces toxines peuvent pourtant conférer un avantage écologique

en diminuant la prédation ou en influençant la composition et la dynamique des communautés phytoplanctoniques (Zabaglo et al., 2016).

Tableau 2 : Les toxines émergentes

Phycotoxines	Organismes producteurs	Symptomatologies	Références
Azaspiracides (AZAs)	<i>Protoberidinium crassipes</i>	Nausées, vomissements, diarrhées	Furey et al., 2010
Dinophysistoxines (DTXs) = analogues de l'acide okadaïque	<i>Dinophysis</i> spp.	DSP	Vale and Botana, 2008
Gymnodimines (GYMs)	<i>Gymnodinium</i> spp.	NSP	Seki et al., 1995
Ovatoxines/Palytoxine (OVTXs/PLTX)	<i>Ostreopsis</i> spp. <i>Palythoa</i> spp.	Nausées, vomissements, goût métallique, maux de ventre, parasthésie des extrémités, détresse respiratoire, convulsion, vertiges...	Alcala et al., 1988; Ciminiello et al., 2012a; Yasumoto et al., 1986
Pectenotoxines (PTXs)	<i>Dinophysis</i> spp.	DSP	Draisci et al., 1996
Pinnatoxines (PnTXs)	Peridinoid dinoflagellate	Symptômes proches des gymnodimines	Hellyer et al., 2011; Rhodes et al., 2010
Spirolides (SPXs)	<i>Alexandrium ostenfeldii</i> <i>A. peruvianum</i>	Symptômes proches des gymnodimines	Munday et al., 2012
Yessotoxines (YTXs)	<i>Protoceratium reticulatum</i> , <i>Lingulodinium polyedrum</i> and <i>Gonyaulax spinifera</i>	Symptômes apparentés aux brevéttoxines et ciguatoxines	Paz et al., 2008

Une intensification des efflorescences du dinoflagellé benthique toxique *Ostreopsis* cf. *ovata* a été signalée en mer Méditerranée depuis une quinzaine d'années (Cohu et al., 2013; Mangialajo et al., 2011; Parsons et al., 2012; Rhodes, 2011). Des épisodes de contamination humaine, liés à ces efflorescences, ont même été signalés notamment en Espagne, Italie et Algérie, où plus de 200 personnes ont été hospitalisées à plusieurs reprises entre 2001 et 2006 (Brescianini et al., 2006; Illoul et al., 2012; Vila et al., 2001). Des phénomènes de mortalité de masse d'invertébrés benthiques ont également été décrits pendant ces efflorescences toxiques (Ciminiello et al., 2006; Shears and Ross, 2009; Totti et al., 2010; Vale and Ares, 2007). Même si de nombreuses études se sont intéressées à l'impact de ces toxines sur l'homme (Beau et al., 2017; de Haro, 2011; Kermarec et al., 2008; Tichadou et al., 2010), le rôle écologique des toxines et leurs potentielles implications dans ces mortalités de masse ne sont encore que très peu documentés et nécessitent plus d'études afin de comprendre les mécanismes impliqués.

3. Cas du dinoflagellé toxique *Ostreopsis cf. ovata*

3.1. Ecologie d'*Ostreopsis cf. ovata*

Ostreopsis cf. ovata est un dinoflagellé benthique (Figure 5a) qui produit une substance mucilagineuse composée d'un réseau de trichocystes et de filaments de polysaccharides (Escalera et al., 2014; Honsell et al., 2013). Ce mucus a un rôle d'adhérence permettant aux cellules de se développer sur des substrats biotiques (macroalgues) ou abiotiques (Figure 5b et c - Mangialajo et al., 2011) dans des zones peu profondes et généralement abritées (Mangialajo et al., 2008). Le rôle de défense de cette matrice mucilagineuse, notamment contre les brouteurs, est également de plus en plus décrit (Giussani et al., 2015).

Lorsque les conditions sont favorables (généralement l'été sur les côtes méditerranéennes françaises), *O. cf. ovata* prolifère et peut atteindre des concentrations benthiques de plus de 2 millions de cellules par gramme de poids frais de macroalgue (Mangialajo et al., 2008). Les cellules vont également être remises en suspension et atteindre des concentrations planctoniques allant jusque 200 000 cellules.mL⁻¹ (Berdalet, comm. pers.) et former des fleurs d'eau en surface (Figure 5c). Les mécanismes expliquant cette migration sont encore mal connus mais les phénomènes de remise en suspension des cellules benthiques dans la colonne d'eau, sous l'effet de l'hydrodynamisme, sont souvent avancés.



Figure 5 : Cellules d'*Ostreopsis cf. ovata* (MCCV 054) en culture (a), recouvrant les macroalgues (b) et les rochers *in situ* (c). Une remise en suspension des cellules est visible sur la photo b.

L'intensification de la fréquence des efflorescences d'*Ostreopsis cf. ovata* en mer Méditerranée a conduit à une augmentation des études portant sur l'écologie et l'écophysiologie de ce dinoflagellé. Une meilleure connaissance des paramètres abiotiques qui influencent la croissance de ce dinoflagellé, permet de mieux déterminer la niche écologique d'*O. cf. ovata* mais également de faciliter son maintien en culture, au laboratoire.

Température

De nombreuses études affirment que le paramètre clef pour la prolifération des dinoflagellés benthiques toxiques est la température de l'eau (Ballatine et al., 1988; Morton et al., 1982). Pourtant, pour *O. cf. ovata*, aucune corrélation ne peut être observée entre l'abondance des microalgues et la température de l'eau (Pistocchi et al., 2011). En effet, les préférences thermiques obtenus via les études *in situ* et en laboratoire sont divers, souvent compris entre 19°C et 29°C, et varient entre les années (Accoroni et al., 2011; Ciminiello et al., 2006; Cohu et al., 2013; Granéli et al., 2008; Mangialajo et al., 2008; Pezolesi et al., 2012; Simoni et al., 2004; Totti et al., 2010). Ces différences sont souvent expliquées par les variations intrapécifiques en lien avec les localisations géographiques des souches.

Salinité

Une corrélation négative entre la salinité et la croissance a été mise en évidence, *in situ*, pour *O. cf. ovata* proliférant le long des côtes hawaïennes (Parsons and Preskitt, 2007). L'étude de Morton et al. (1992) a plutôt montré un effet négatif de la diminution de la salinité sur la croissance. Les conclusions inverses obtenues par les différentes études (Pezolesi et al., 2012; Rhodes et al., 2000) laissent d'avantage présager un rôle secondaire de la salinité dans la croissance cellulaire d'*O. cf. ovata* (Pistocchi et al., 2011).

Hydrodynamisme

Les études qui se sont focalisées sur le rôle de l'hydrodynamisme dans le développement des efflorescences d'*O. cf. ovata* sont unanimes et décrivent qu'*O. cf. ovata* se développe préférentiellement dans des sites abrités et peu profonds (Accoroni et al., 2011, 2012; Barone, 2007; Shears and Ross, 2009; Totti et al., 2010). Ces études ont également montré l'importance de l'hydrodynamisme dans la régulation des efflorescences d'*O. cf. ovata* puisque des abondances significativement plus importantes d'*O. cf. ovata* sont retrouvées dans les sites abrités. Néanmoins, les observations réalisées à Villefranche-sur-Mer et à Nice (Lemée, comm. pers) ne sont pas si tranchées : les sites agités peuvent supporter de très fortes abondances d'*Ostreopsis cf. ovata*, mais celles-ci sont fugaces à cause de l'hydrodynamisme important qui emporte plus fréquemment les microalgues.

Nutriments

La disponibilité en nutriments est souvent considérée comme un facteur important dans l'apparition et le contrôle des efflorescences de dinoflagellés toxiques. Le consensus existant autour de l'enrichissement des zones côtières sous l'effet des activités anthropiques, amène à une eutrophisation des écosystèmes côtiers considérés comme un facteur explicatif de l'intensification des efflorescences d'algues toxiques au niveau mondial. Toutefois, même si cette relation semble claire

pour les dinoflagellés toxiques planctoniques (Parsons and Dortch, 2002), elle l'est beaucoup moins pour les dinoflagellés benthiques. En effet, de manière globale, *O. cf. ovata* se développe aussi bien dans des zones eutrophes (Accoroni et al., 2011) qu'oligotrophes (Shears and Ross, 2009). Aucune relation claire ne peut être déterminée entre les abondances cellulaires en *O. cf. ovata* et les concentrations en nutriments inorganiques (Parsons and Preskitt, 2007; Shears and Ross, 2009; Vila et al., 2001). La capacité de mixotrophie (par osmotrophie) d'*O. cf. ovata* ne semble pas non plus pouvoir expliquer le succès de son développement (Jauzein et al., 2017). Il reste à savoir si sa capacité de phagotrophie de plastes de macroalgues rouges décrite par Lee and Park (2018), peut faciliter sa prolifération.

Substrats

En terme de substrats, les études ont montré qu'*O. cf. ovata* était aussi bien capable de se développer sur des substrats durs abiotiques que sur des substrats biotiques, avec une préférence pour les macrophytes affichant une architecture tridimensionnelle complexe (Totti et al., 2010; Vila et al., 2001). Cette affinité pour les thalles complexes est expliquée par une réponse différente de ce morphotype de thalle à l'action des vagues limitant ainsi le décrochement des cellules d'*O. cf. ovata* (Accoroni et al., 2016).

Lumière

Les études réalisées en laboratoire suggèrent une préférence pour les fortes intensités lumineuses mais les irradiances ne sont pas toujours précisées (Monti and Cecchin, 2012; Morton et al., 1992). En milieu naturel, les concentrations cellulaires d'*O. cf. ovata* diminuent rapidement avec la profondeur, ce qui soutient une éventuelle préférence pour les fortes intensités lumineuses (Brissard et al., 2014; Cohu et al., 2013).

En bilan, les variations des facteurs écologiques classiques (température, salinité, hydrodynamisme, concentrations en sels nutritifs, mixotrophie, substrats) ne permettent pas, pour l'instant, d'expliquer les proliférations d'*Ostreopsis cf. ovata* en mer Méditerranée. Il est par conséquent important d'étudier plus en détails les interactions biotiques, régies par le biais des métabolites secondaires produits par ce dinoflagellé.

3.2. Ecologie chimique d'*Ostreopsis cf. ovata*

La description des différentes espèces d'*Ostreopsis*, de leur distribution ainsi que des toxines associées sont détaillées dans la revue de la littérature qui suit. L'objectif de cette revue est de faire un point sur les connaissances en écologie chimique pour toutes les espèces d'*Ostreopsis*, même si la plupart des études concernent *Ostreopsis cf. ovata*. Les relations qu'*Ostreopsis* sp. entretient avec son environnement biotique sont ainsi détaillées afin de comprendre les impacts d'*Ostreopsis cf. ovata* à la fois sur les producteurs primaires, mais également sur les consommateurs de différents niveaux trophiques, aussi bien benthiques que planctoniques. Les effets sur les bactéries, virus et autres protistes hétérotrophes sont également détaillés, ainsi que la rétroaction de ces différents organismes en réponse à l'exposition à *O. cf. ovata*.

La présentation de cette partie sous forme d'une revue a pour principal objectif de valoriser le travail de synthèse effectué, sous forme d'une publication qui sera soumise à *Frontiers in Marine Science* dès que possible.

Chemical ecology of the benthic dinoflagellate genus *Ostreopsis*: a review

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3.2.1. Introduction

3.2.1.1. Marine Chemical Ecology (MCE)

Marine Chemical Ecology is a cross-disciplinary and emergent science which gives insights into chemical compounds that shape interactions among organisms and their environment and thus influences the structure, ecology, organization and functioning of marine communities (Hay, 1996; McClintock and Baker, 2001; Pohnert et al., 2007). Chemical signals and cues (released intentionally or not, respectively) are omnipresent in marine systems and play critical roles at different scales, by e.g. affecting food web dynamics, communication, mating choices and behavior, dominance hierarchies determination and stabilization, feeding and habitats selection, community organization, and potentially in ecosystem-level processes as well (e.g. nutrient and carbon cycling and storage : Bakus et al., 1986; Hay, 2009, 1996; Pawlik, 1992; Schwartz et al., 2016; Sieg et al., 2011). In general, these chemical cues and signals are poorly studied due to their low concentrations in the water and in the air and their rapid degradation (likely to prevent miscommunication), making their sampling, isolation and structural elucidation very difficult. Fortunately, recent improvements in chemical technologies with the rise of high-throughput approaches have led to major advances in the understanding of the role of these compounds, notably in benthic systems (Kuhlisch and Pohnert, 2015; Prince and Pohnert, 2010).

3.2.1.2. MCE of Harmful Algal Blooms (HABs)

Chemical communication is particularly important in the life history and evolutionary strategies of eukaryotic microalgae, which live at the low Reynolds numbers scale, than for larger marine macro-organisms functionally dependent on optical, acoustical and tactile sensing (Ianora et al., 2011; Sieg et al., 2011). Chemical cues and signals synthesized at the small cellular level are dispersed by molecular diffusion assisted by turbulence and thus can operate at relatively larger scales influencing trophic interactions and life history (Cembella, 2003). By fostering intra- and interspecific

cooperation, or as defense mechanisms against predators, parasites or competitors (Poulson et al., 2009; Prince et al., 2008; Schwartz et al., 2016) chemical cues and signals can play a role in the formation of blooms of different species. However, the information of chemical signals in the domain of HABs is very limited mainly due to the inherent technical difficulties of their study. To date, some studies have focused on allelopathy as an effective mechanism that could reinforce high cell density blooms but could not be a main or direct cause of HABs (Jonsson et al., 2009). Benthic marine ecosystems are supposed to be a source of great diversity of chemical signals and cues since the competition for space and substrate is higher than in pelagic systems (Hay, 2009). Moreover, compared to the three-dimensional space experienced by planktonic cells, benthic systems are essentially bi-dimensional, which imposes unique constraints with distinct advantages for the effectiveness of chemical ecological interactions. The importance of chemical cues and signals in benthic HAB dynamics has not been yet well studied.

3.2.1.3. Objectives of the review

The objectives of this work were to review the present state of knowledge on the MCE concerning the biology and dynamics of the benthic dinoflagellate genus *Ostreopsis*. This genus has attracted scientific and social interest due to their negative impacts on certain benthic marine fauna, the production of toxins chemically close to the potent palytoxin (PLTX) associated to dramatic seafood borne poisonings in tropical areas, and the link of its blooms to mild respiratory and cutaneous irritations in beach users in temperate latitudes. The diversity, distribution and toxins produced by the *Ostreopsis* species are presented first, followed by a synthesis of the knowledge on the health impacts associated to aerosolized toxins. Next, a review of the literature on the potential chemical interactions between *Ostreopsis* spp. and their environment, from virus and bacteria to large herbivorous and carnivorous macroorganisms is presented. Finally, some key scientific aspects to improve knowledge on MCE of *Ostreopsis* spp. are suggested.

3.2.2. The genus *Ostreopsis*

3.2.2.1. Species and distribution

The genus *Ostreopsis* and the type species *Ostreopsis siamensis* were first described a century ago from the Gulf of Siam (Thailand) by Schmidt (1901). Since then, 9 other species of *Ostreopsis* have been identified: *O. ovata* (Fukuyo, 1981), *O. lenticularis* (Fukuyo, 1981), *O. heptagona* (Norris et al., 1985), *O. labens* (Faust and Morton, 1995), *O. mascarenensis* (Quod, 1994), *O. belizeanus*, *O. caribbeanus* and *O. marinus* (Faust, 1999) and more recently, *O. fattorussoi* (Accoroni et al., 2016a). A first characterization of *Ostreopsis* species is based on morphological approaches combining size, shape and thecal plates pattern (Hoppenrath et al., 2014). Molecular approaches based on the nuclear

rDNA internal transcribed spacer region (ITS1 and ITS 2), partial nuclear LSU (D1/D2 domains) and 5.8S rDNA gene (Penna et al., 2010, 2005) are also key in the identification and determination of phylogeny link. These methods allowed, for example, the description of *Ostreopsis* sp. by Tartaglione et al. (2016), as a new species, *O. fattorussoi*. With the most recent progresses in chemical analysis, toxin profile can also become a taxonomic characteristic. Nevertheless, a big part of the taxonomy of the genus *Ostreopsis* remains unclear and controversial due to the high morphological and cell size variability within the different species and the fact that original descriptions lacked genetic analysis. In particular, there is still a debate regarding the taxonomy of the most widespread species, *O. cf. ovata* and *O. cf. siamensis* since their first description was based on morphological features only.

Initially considered a tropical genus, *Ostreopsis* is increasing its biogeographic distribution to temperate latitudes (Parsons et al., 2012 – Table 3). Indeed, *Ostreopsis cf. ovata* has been isolated in Australia, Brazil, China (Malacca, South China Sea), Cook Islands, Caribbean, French Polynesia, Hawaii, Indian Ocean, Japan, Malaysia, New Caledonia, New Zealand, Mediterranean, Venezuela, Vietnam (Table 3). Other *Ostreopsis* species that appeared to be circumscribed to narrower regions, are also found in new localities. For instance, *Ostreopsis fattorussoi* isolated from the Eastern Mediterranean could be also growing in the Atlantic coast of the Iberia Peninsula although it has not been demonstrated yet (Accoroni et al., 2016a). The wide expansion of the genus *Ostreopsis* is considered a potential problem on human health and wellbeing (economy, tourism, beach quality) issues due to the production of PLTX analogues whose mode of action is still not clear (see section 2.2.2.3.4. of this review).

3.2.2.2. Toxins: Secondary metabolites synthesized by *Ostreopsis* species

Several species in the *Ostreopsis* genus are considered potentially harmful to humans and other marine organisms due to the production of palytoxin (PLTX) like compounds (Table 1). PLTX is the most potent non-bacterial toxin of biological origin, initially isolated in 1971 from the tropical soft coral genus *Palythoa*, and now recognized as 42-OH-PLTX (see revision by Ciminiello et al., 2011 and references there in Poli et al., 2018).

Progresses in chemical analysis technology, in particular on HR LC-MS/MS and NMR have revealed the high diversity of PLTX analogues (Table 1) and eight *Ostreopsis* species have been described as toxic. Ostreocin D was the first analogue isolated from *O. siamensis* by Usami et al. (1995) and other PLTX analogues, namely, ovatoxins, ostreocines, mascarenotoxin or ostreotoxins *sensu lato*, have been characterized for *O. fattorussoi*, *O. lenticularis*, *O. mascarensis*, *O. cf. ovata* and *O. siamensis* (Deeds and Schwartz, 2010; Mercado et al., 1994; Parsons et al., 2012). However, toxicity of *O. heptagona*, *O. labens* and *O. rhodesiae* has only been described using biological assays and toxins involved in these effects have not already been identified (Table 1). In the case of *O. cf. ovata*, up to

12 ovatoxins, from a to k and the isobaric PLTX (isoPLTX, Garcia-Altarets et al., 2015) have been identified among the different isolated strains, and the toxinologic profile constitutes a taxonomic characteristic (e.g. Accoroni et al., 2016). A main limitation to ascertain the health impacts risks associated to *Ostreopsis* blooms is the lack of standards for the variety of toxins, all them complex and with high molecular weight (molecular formulas varying between $C_{129}H_{223}N_3O_{55}$ and $C_{131}H_{227}N_3O_{52}$).

Phycotoxins in general and the palytoxin group as well, are considered secondary metabolites and their physiological role in the cells is unknown. Environmental factors such as temperature, salinity, light or nutrients, but also the characteristics of the strains (isolation site, age and growth phase of the strain in culture) may influence toxin production (Ciminiello et al., 2012a; Granéli et al., 2011; Pezzolesi et al., 2012; Pezzolesi et al., 2014; Scalco et al., 2012; Vanucci et al., 2012a). However, it has been suggested that other microorganisms co-occurring along the *Ostreopsis* blooms could be involved in the toxin production as well. Tosteson et al. (1989) and Ashton et al. (2003) found, in cultures, that bacteria associated with the surfaces or the extracellular matrices of *O. lenticularis* were correlated with the high dinoflagellate toxicity during the stationary phase of the cultures. Biological assays and analytical chemistry analysis are both used for toxin presence detection and/or quantification. Mouse Bioassay (prohibited in Europe since 2005 for ethical reasons), Haemolysis Neutralization Assay (HNA) or *Artemia* bioassay have been long recognized for toxicity quantification (Bignami, 1993; Botana, 2014; Delaney, 1984). Advantages given by analytical chemistry analysis (i.e. LC-MS/MS) are the absence of restrictions in terms of reproducibility, specificity, precision and consistency. Unfortunately, this methodology relies on very expensive technical equipment, it requires high qualified personnel skills and it produces significant chemical waste (it is not environmentally friendly).

3.2.2.3. Health problems in humans: an indication of the *Ostreopsis* Chemical Ecology

As mentioned in the introduction, chemical ecology is focused the communication among organisms mediated by chemical compounds. Somehow, health impacts in humans due to exposure to PLTX analogues constitute an indication of *Ostreopsis* chemical ecology. Health impacts can occur: i) after consumption of potentially PLTX contaminated seafood, ii) from direct contact with zoanthids in the natural environment or in home aquaria, or iii) after direct exposure to seawater and/or aerosol during *Ostreopsis* blooms. The characterization of human poisonings potentially due to exposure to PLTX analogues was reviewed by Tubaro et al. (2011) and it is briefly presented next. The effects of *Ostreopsis* spp. in different marine organisms have also been reported and are presented in sections 2.2.3.

3.2.2.3.1 Seafood borne PLTX poisonings

Severe illness and even lethal fatalities after consumption of potentially PLTX contaminated seafood have been scarcely reported in tropical and subtropical latitudes since the 1970s so far (see Tables 1, 2 and 3 in Tubaro et al. (2011)). Among them, only a few cases were confirmed through direct PLTX analogues detection in seafood leftovers. In many other cases, screening tests on seafood samples collected or purchased after or before the poisoning episode combined or not with clinical observations and symptoms, concluded that consumption of PLTX contaminated seafood was the causative reason of the health disorders. Symptoms include nausea, tiredness, diarrhea, vomiting, dizziness, numbness of the extremities, muscle cramps, paresthesia, restless-ness, respiratory distress and bradichardia. In dramatic cases, patients died after 15h to 4 days following seafood poisonings, even after hospitalization. Unusual metallic taste of the seafood is one of the common signs noted by potential PLTX intoxication.

In the Mediterranean coasts, the proliferation of *O. cf. ovata* and *O. cf. siamensis* since the end of the 21th century raised concern about the potential risk of seafood intoxication. Biré et al. (2013) and Brissard et al. (2014) reported the presence of toxin content in macrofauna (mainly sea urchin, rock shell, crab and flathead mullet) sampled during *O. cf. ovata* blooms. OVTX-a was the most abundant toxin detected, and in some cases PLTX equivalents accounted on average for 223 to 392.2 $\mu\text{g}\cdot\text{kg}^{-1}$ of whole flesh, exceeding the threshold of 30 $\mu\text{g}\cdot\text{kg}^{-1}$ in shellfish recommended by the European Food Safety Authority (EFSA). The key and unsolved questions are whether *Ostreopsis* was the ultimate organism synthesizing the PLTX-like compounds that contaminated the seafood and, how toxins can be transferred through the food webs. Fortunately, until now, seafood intoxications due to PLTX analogues have not been occurred in the Mediterranean zone yet.

3.2.2.3.2 Health impacts by direct contact with zoanthids in the natural environment or in home aquaria

Zoanthids have become common in hobby aquarists worldwide. Deeds and Schwartz (2010) described the health impacts on a person that inhaled a potent irritative compound released after pouring boiling water or bleach in aquaria with the aim to eliminate a colony of zoanthids. Within 20 min after exposure, the man experienced rhinorrhea and coughing, and difficulty breathing and light headedness which progressed to severe fits of coughing and chest pain 4h later. At the local hospital he was administered an anti-inflammatory corticosteroid and pain medication, an inhaled steroid treatment and cough suppressant for one months. A follow-up examination by a pulmonary specialist 2 weeks post exposure diagnosed the patient with asthma-like symptoms of bronchial inflammation and bronchoconstriction. Similar cases have been reported in the last years (see Tables 4 and 5 in Tubaro et al. (2011)), one last case affecting a whole family, with 3 little kids in the UK in August 2019.

Table 3 : Representative information about the range of toxin content in described *Ostreopsis* species from different locations. The literature review is not exhaustive. * = Identity of the isolate(s) confirmed genetically confirming to which described or cryptic (ribotype) species they belong. Abbreviations: Equivalent (eq.); Hemolytic Assay - ability of cellular extract to induce erythrocyte cell lysis versus a saponin control (HA); HPLC-ESI-MS/MS High-performance liquid chromatography-electrospray ionisation tandem mass spectrometry; Ovatoxin (OVTX); Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS); Mouse Bioassay - toxicity determined by intraperitoneal injection of *Ostreopsis* cellular extracts (MBA); Mouse Units, the 24 h LD₅₀ dose estimated using ~20 g mice (MU); Not Detectable = below detection limits (nd); neuroblastoma cell line cytotoxicity assay (N2a); putative palytoxin (pPLTX) like toxin = isobaric PLTX (García-Altres et al., 2015). More information on toxicity tests as well as on other *Ostreopsis* strains (that can become new species) is found in Tester et al., 2019.

Species	Toxins / Formula	Quantification	Distribution	References
<i>O. belizeanus</i> (Faust, 1999)	Unknown		Belize, Caribbean Sea	Faust, 1999
<i>O. caribbeanus</i> (Faust, 1999)	Unknown		Puerto Rico, Belize, Caribbean Sea	Faust, 1999
<i>O. fattorussoi</i> (Accoroni et al., 2016a)	isobPLTX C ₁₂₉ H ₂₂₃ O ₅₄ N ₃ Ovatoxin-a C ₁₂₉ H ₂₂₃ N ₃ O ₅₂ Ovatoxin-d C ₁₂₉ H ₂₂₃ N ₃ O ₅₃ Ovatoxin-e C ₁₂₉ H ₂₂₃ N ₃ O ₅₃ Ovatoxin-i C ₁₃₁ H ₂₂₅ O ₅₃ N ₃ Ovatoxin-j1 C ₁₃₁ H ₂₂₅ O ₅₄ N ₃ Ovatoxin-j2 C ₁₃₁ H ₂₂₅ O ₅₄ N ₃ Ovatoxin-k C ₁₃₁ H ₂₂₅ O ₅₅ N ₃	LC-HRMS 0.06 to 2.8 pg eq. PLTX/cell	Canari island, Crete, Cyprus island, Lebanon, Puerto Rico, Atlantic coast of Iberia	Accoroni et al., 2016a; David et al., 2013; Penna et al., 2014, 2010; Tartaglione et al., 2016
<i>O. heptagona</i> (Norris et al., 1985)	Unknown but extracts described as toxic to mice (in Accoroni et al., 2016)		Knight Key, Florida, Gulf of Mexico	Norris et al., 1985; Rhodes, 2011
<i>O. labens</i> (Faust and Morton, 1995)	Unknown but described as toxic in Rhodes et al. (2002)	Artemia assay Death of 24h old larvae of <i>Artemia</i> in standard assay	Belize, Caribbean Sea; Malaysia, Japan,	Faust and Morton, 1995; Rhodes, 2011

<i>O. lenticularis</i> (Fukuyo, 1981) Includes the cryptic species <i>Ostreopsis</i> sp. 5 in Chomérat et al., 2019	Ostreotoxin 1 Ostreotoxin 3	N2a analysis EC ₅₀ (PLTX) = 1191 ± 175 pg.mL ⁻¹ (O ⁻ condition) LC-UV-MS/MS analysis Under detection levels	Australia, Caribbean, French Polynesia, Indian Ocean, Malaysia, Mexican Pacific, New Caledonia, New Zealand, Philippines, Venezuela, Vietnam	Ashton et al., 2003; Carnicer et al., 2015b*; Chomérat et al., 2019*; Mercado et al., 1995; Sato et al., 2011; Tosteson et al., 1989
<i>O. marinus</i> (Faust, 1999)	Unknown	No pPLTX activity by HA using sheep erythrocytes	Caribbean, Indian Ocean, Vietnam	Carnicer et al., 2015a*; Faust, 1999
<i>O. mascarenensis</i> (Quod, 1994)	Mascarenotoxin-A C ₁₂₇ H ₂₂₁ N ₃ O ₅₀ Mascarenotoxin-B nd	HPLC-ESI-MS/MS: 0.04 pg eq.PLTX/cell Mouse bioassay: LD ₅₀ = 900 µg.kg ⁻¹ (intraperitoneal injection)	Rodrigues and Reunion Islands, Indian Ocean	Lenoir et al., 2004; Ramos and Vasconcelos, 2010
<i>O. ovata</i> (Fukuyo, 1981)	p-PLTX C ₁₂₉ H ₂₂₃ N ₃ O ₅₄ Ovatoxin-a C ₁₂₉ H ₂₂₃ N ₃ O ₅₂ Ovatoxin-b C ₁₃₁ H ₂₂₇ N ₃ O ₅₃ Ovatoxin-c C ₁₃₁ H ₂₂₇ N ₃ O ₅₄ Ovatoxin-d C ₁₂₉ H ₂₂₃ N ₃ O ₅₃ Ovatoxin-e C ₁₂₉ H ₂₂₃ N ₃ O ₅₃ Ovatoxin-f C ₁₃₁ H ₂₂₇ N ₃ O ₅₂ Ovatoxin-g C ₁₂₉ H ₂₂₃ N ₃ O ₅₁ Ovatoxin-h C ₁₂₉ H ₂₂₅ N ₃ O ₅₁ Ostreol A C ₆₇ H ₁₁₂ N ₂ O ₂₃ Mascarenotoxin-a C ₁₂₇ H ₂₂₁ N ₃ O ₅₀ Mascarenotoxin-c C ₁₂₉ H ₂₂₁ N ₃ O ₅₁	LC-HRMS pPLTX: 0.1 to 24.8 pg eq. PLTX/cell OVTX a: 0.065 to 171 pg eq. PLTX/cell OVTX b: 2 to 205 pg eq. PLTX/cell OVTX c: 0.7 to 37 pg eq. PLTX/cell OVTX d+e: 2 to 80 pg eq. PLTX/cell OVTX f: ND - 17 pg eq. PLTX/cell OVTX g: 0.35 to 24.8 pg eq. PLTX/cell	Australia, Brazil, China, Cook Islands, Caribbean, French Polynesia, Hawaii, Indian Ocean, Japan, Korea, Malaysia, New Caledonia, New Zealand, Mediterranean, Eastern Russia, Tasmania, Venezuela, Vietnam	Ben Gharbia et al., 2017; Brissard et al., 2015, 2014; Ciminiello et al., 2012, 2011, 2008, 2006; Garcia-Altarets et al., 2015; Giussani et al., 2015; Granéli et al., 2011*; Guerrini et al., 2010*; Honsell et al., 2013; Hwang et al., 2013; Nascimento et al., 2012b*; Penna et al., 2005; Pezzolesi et al., 2012*; Rossi et al., 2010; Sechet et al., 2012*; Selina and Levchenko, 2011; Tawong et al., 2014*; Vanucci et al., 2012b
<i>O. rhodesiae</i> (Verma et al., 2016)	Unknown but described as toxic in Verma et al. (2016)	LC-MS/MS: No PLTX analogs Fish gill cell line assay for toxicity: ≥98.5% decrease of gill cell line RTgill-W1 viability	Heron Reef Lagoon, southern Great Barrier Reef, Coral Sea, Australia	Verma et al., 2016

<p><i>O. siamensis</i> (Schmidt, 1901)</p>	<p>Ostreocin A C₁₂₇H₂₁₉N₃O₅₄ Ostreocin-D C₁₂₇H₂₁₉N₃O₅₃ Ostreocin-E1 C₁₂₇H₂₁₇N₃O₅₂ Ostreocin-B C₁₂₇H₂₁₉N₃O₅₃ pPLTX</p>	<p>Haemolysis Neutralization Assay 0.3 pg PLTX eq/cells Mouse bioassay: LD₅₀ = 0.75 µg.kg⁻¹ (intraperitoneal injection) Mediterranean strains = sub-fg levels of PLTX/cells and no toxicity by MBA Japanese Strains contained Ostreocin- b + Ostreocin-d + pPLTX (nd-0.8 pg eq. PLTX/cell). Total: nd-16 pg PLTX eq/cells</p>	<p>Eastern Atlantic coast, Australia, Hawaii, Japan, Madagascar, Mediterranean Sea, Mexico, New Zeland, Reunion Island, Thailand, Venezuela, Vietnam</p>	<p>Cagide et al., 2009; Ciminiello et al., 2013; Meunier et al., 1997; Ramos and Vasconcelos, 2010; Rhodes et al., 2002, 2000; Terajima et al., 2019, 2018; Tichadou et al., 2010; Tindall and Miller, 1988; Ukena et al., 2001; Verma et al., 2016*</p>
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3.2.2.3.3 Mild respiratory and cutaneous symptoms after direct exposure to seawater and/or aerosol during *Ostreopsis* blooms

In the last 15 years, in several Mediterranean and Brazilian beaches, *Ostreopsis* blooms have been associated to mild cutaneous and respiratory symptoms and general malaise in beach users, workers and inhabitants of the first coastline, after direct contact with water and/or after inhalation of marine aerosols (Table 2). Symptoms include rhinorrhea, pharyngeal pain, dry or mildly productive cough, nose irritation, general malaise, headache, fever ($\leq 38^{\circ}\text{C}$), eye irritation and/or dermatitis (Durando et al., 2007; Tichadou et al., 2010). Most of the symptoms disappeared within a few hours without specific medication when people moved away from the affected area in the vicinity of the *Ostreopsis* bloom; in only a few severe cases hospitalization was required (Durando et al., 2007). It has been hypothesized that PLTX analogues could be the most plausible agent causing the observed symptoms, which have some similarities, although much softer, with those experienced by aquarists (section 2.3.2), while a metallic taste has also been reported by people on snorkeling, windsurfing, or by researchers when sampling *Ostreopsis* blooms. However, OVTX-a and isoPLTX have been only rarely found in the aerosol (Ciminiello et al., 2014). It has been also suggested that *Ostreopsis* fragments, mucus, and/or associated biota (including bacteria, cyanobacteria or other pico- or nanoeukaryotes) could be present in the aerosol and cause the health disorders. It should be noted here that symptoms do not occur concurrently with high cell *Ostreopsis* densities neither along the bloom, but only during certain periods. Vila et al. (2016) conducting a parallel epidemiology and ecology survey found that symptoms occurred during the end of the exponential phase and beginning of the stationary phase of the bloom, at least in 2013 in a Mediterranean hot spot. This trend has been approximately repeated in the following years in that hot spot (Berdalet, Vila, Abós-Herràndiz, unpublished). Still an open question is how toxins and/or fragment of *Ostreopsis* can be aerosolized and whether these toxins are the causative agent of the disorders.

3.2.2.3.4 Toxicity on mammalian cells and mode of action

The main biological target of PLTX seems to be the Na^+/K^+ -ATPase, a plasma membrane pump involved in the maintenance of trans-membrane ionic gradients of animal cells, essential for cellular functions and life (Habermann, 1989; Rossini and Bigiani, 2011; Wu, 2009). Impairment of the membrane pump by PLTX results in its conversion into a non-selective pore for monovalent cations, thereby destroying the transmembrane ionic gradient and triggering several adverse biological effects, some life threatening (Artigas and Gadsby, 2003; Habermann, 1989).

However, these studies have been conducted *in vitro* on mammalian cell tissues and the actual *in vivo* toxicity of the molecule is still poorly understood. In the 1970's, semi-purified PLTX was tested in a broad range of animal species through several routes of administration. Thus, a reduced or even

increased toxicity of the pure molecule are both possible. More recently, high purity PLTX administration in mice showed that toxicity is dependent upon the route of administration (Munday, 2011). Also Poli et al. (2018) characterized the toxicity and basic histopathological effects of PLTX (derived from Japanese *Palythoa tuberculosa*), the analogue 42-OH-PLTX (from Hawaiian *P. toxica*) and ovatoxin-a (isolated from a Japanese strain of *Ostreopsis ovata*) after intraperitoneal (IP) and aerosol administration to rats. All toxin preparations showed similar potency towards mouse erythrocytes in the erythrocyte hemolysis assay and interactions with the anti-PLTX mAb (antibody). Intraperitoneal LD₅₀ values between 0.92 and 3.26 µg·kg⁻¹ consistent with published values, and aerosol LD₅₀ values of 0.063 to 0.045 µg·kg⁻¹ confirmed the exquisite potency of PLTX suggested by the literature. Most commonly affected tissues were the lungs, liver, heart, salivary glands, and adrenal glands.

Table 4 : Reports of mild respiratory, cutaneous and/or general malaise symptoms in humans after exposure to aerosols in *Ostreopsis cf. ovata* blooms affected beaches.

Year	Location	Affected people (n)	Reference
1998	NW Med (Spain)	-	Vila et al., 2008
1998, 2000, 2001	Tirrenian (Italy)	~100	Sansoni et al., 2003
2001, 2003, 2004	S Adriatic (Italy)	28	Gallitelli et al., 2005
2004	NW Med (Spain)	74 (estimated~200)	Vila et al., 2008
2005, 2006	Ligurian (Italy)	228, 19	Brescianini et al., 2006
2005	Genoa (Italy)	209	Durando et al., 2007
2006	NW Med (Spain)	37	Àlvarez, 2006
2006-2009	France	47	Tichadou et al., 2010
2006	SW Med (Spain)	57	Barroso García et al., 2008
2009	SW Med (Algeria)	150-200	Illoul et al., 2012
2010	Adriatic (Croatia)	7	Pfannkuchen et al., 2012
2013	NW Med (Spain)	13	Vila et al., 2016

3.2.2.3.5 Release of toxins in sea water/aerosolization

In section 2.2.2.3.3., it was explained that some blooms of *Ostreopsis* spp. have been related to mild respiratory impacts on beach users, workers and inhabitants exposed to marine aerosols. Here we will describe the main factors affecting aerosolization of toxins and cells. It is well recognized that microorganisms including some microalgae can be dispersed by air and survive in the aerosols (e.g., as reviewed by Tesson et al. (2016) and references there in; Genitsaris et al. (2011). Microorganisms become airborne mainly due to drop formation at the water surface microlayer, which is enriched with biological material in all aquatic habitats (Schlichting, 1974; Wilson et al., 2015). Foam drops of a diameter larger than 40 µm are formed by wind friction and breaking wave crests at wind speeds

exceeding 7 to 11 m·s⁻¹. Bubble bursting due to waves, rainfall, boat traffic, or supersaturation of gases in the water produces film drops (1 µm to 10 µm diameter), projected in various directions, and vertically emitted jet drops (6 µ to 100 µm diameter). Recreational activities (i.e., waterskiing, using personal watercraft, swimming, or wading) can also generate aerosols. Once emitted, airborne turbulent kinetic energy drags the microorganisms further up into the atmosphere.

The few available studies indicate that microalgae are found at very low concentrations (10⁻⁴ to 10⁴ cells per m⁻³) (Mayol et al., 2014) and with heterogeneous distributions, which requires high-volume sampling technology. High-volume sampling on filters or agar plates can be achieved by different instruments, but high-volume sampling into liquids is more complicated, as high airflows may lead to evaporation and reduce collection efficiency. Once collected combination of microscopy and molecular tools are contributing to the identification of the airborne microalga. The main microalgal groups found in bioaerosols are chlorophytes ("green algae"), bacillariophytes (diatoms, mainly small *Nitzschia*) and cyanobacteria. Airborne microalgae are recognized as allergens and antigens. They are the cause of severe medical issues, including respiratory allergies (e.g., hay fevers), asthmatic attacks, dermatitis and skin lesions, rhinitis, and disturbances in lymphatic systems or vital organs (e.g., protothecosis, see review in Ashok and Gupta, 1998). In addition, certain aerosolized algal toxins are the causes of further human illnesses, namely, microcystins produced by cyanobacteria, brevetoxins synthesized by *Karenia brevis* and ovatoxins and palytoxin analogues generated by *Ostreopsis* species. So far, the detection of *Ostreopsis* cf. *ovata* and its related toxins in the aerosol have been conducted by Casabianca et al. (2013) and (Ciminiello et al., 2014). Casabianca et al. (2013) used air pump samplers (CAV-A/mb, Barcelona, Spain) equipped with 15 cm diameter QM-A quartz filters (Whatman, Maidstone, UK). Two air samplers located at 0.5 m and 3 m above sea level, respectively, pumped air continuously for 3 days during an *Ostreopsis* cf. *ovata* bloom at a flow rate of 30 m³·h⁻¹. Filters were replaced every 6 or 7 h and were divided in two portions to analyze, respectively, the concentration of *Ostreopsis* cell concentrations by qPCR and toxins by the hemolytic assay and by liquid chromatography with fluorescence detection (LC-FLD). *Ostreopsis* cells were detected in the aerosol by qPCR, exhibiting a marked variability along the bloom although not always directly related to the concentrations of the epiphytic *Ostreopsis* in the water; the authors noted that wind and other environmental factors could have affected these findings. Epiphytic *Ostreopsis* contained ca. 1.2 pg total PLTX analogues per cell but toxins were not detected in the aerosol. Different technical aspects (e.g. toxin binding to glass tubes, not efficient retention by the filters) any need to be overcome. Ciminiello et al. (2014) also employed AirCube COM2 (Analitica Strumenti srl., Italy) equipped with 47 mm diameter glass/quartz microfiber filters (Whatman, Maidstone, UK) and SAS PCR (VWR International (PBI, Italy). In this case, air and a collecting fluid (50 mL distilled water in 2009) flow together through a nozzle were poured into a vessel with a coil; the collecting fluid was circulated continuously to prolong the contact time

with aerosols and to enrich itself in toxins and/or cells. Both air samplers were located 10 m from the seashore and worked continuously for 1.5–6 h. Air flow speed was 10 L.min⁻¹ in 2009 and 30 L.min⁻¹ in 2010. The presence of ovatoxins in the aerosol at levels of 2.4 pg of ovatoxins per liter of air was confirmed for the first time but palytoxins by inhalation exposure toxicological data were not available. Overall, the presence of both cells and toxins in the aerosols provided by these studies opened new possibilities to increase the understanding of the *Ostreopsis*-related respiratory syndrome.

3.2.3. MCE of *Ostreopsis* spp

3.2.3.1. Introduction

As described in the former section, toxins, the secondary metabolites synthesized by *Ostreopsis*, may be involved in some human health impacts associated to the blooms of this benthic dinoflagellate. Here, the described and potential impacts on different marine organisms and environments as part of the chemical ecology of *Ostreopsis* are reviewed.

3.2.3.2. Interactions with bacteria, viruses and parasites

Interactions between HAB species and bacteria remain still poorly studied although these connections may play important roles in the biology and the ecology of these organisms. Bacteria may interact with algae within the phycosphere (Bell and Mitchell, 1972) as free-living bacteria, attached to the algal cell surface or occurring as intracellular symbionts. Algae-bacteria interactions can be synergistic, mutualistic, as commensalism or symbiotic (e.g., by producing cytokinins - phytohormones, Maruyama et al., 2008). Some vitamins and ion chelators produced by bacteria are involved in nutrient recycling facilitating nutrient incorporation by algae (Jasti et al., 2005), while, in return, bacteria use microalgae exudates (Sarmiento and Gasol, 2012). Furthermore, competition, parasitism or antagonist interactions also occur. Bacteria could interfere microalgal life cycle (Sawayama et al., 1993) and growth of some HAB species (Doucette, 1995; Fukami et al., 1992; Hare et al., 2005; Kim et al., 2009). Bacterial exopolymers can increase the tendency of cells to flocculate and enhance sinking rates and degradation of decaying (harmful and innocuous) blooms (Decho, 1990). In turn, algae could also control bacteria growth by the production of antibiotic (Trick et al., 2011), or hydroxyl radicals (Oda et al., 1992) and other selected anti-bacterial growth substance (Bell et al., 1974).

These interactions exhibit spatio-temporal scales variability (Doucette, 1995; Mayali et al., 2011). Phylogenetically diverse bacterial groups with different functional roles could modulate phytoplankton and HABs bloom dynamics. While some bacteria could microalgal cells during the initiation and exponential phases of the blooms, antagonist or parasitic bacteria would proliferate before the declining period and saprophytic or scavenger bacteria will occur after bloom termination when the availability of phytodetritus is high.

Bacterial community associated to *Ostreopsis* species appear to be species and even clone specific (Tosteson et al., 1989) and vary with the bloom phase, and likely, with the habitat. *Alphaproteobacteria* (83 % relative abundance - genera *Ruegeria*, *Jannaschia* and *Erythrobacter-Gammaproteobacteria* (13%) and *Flavobacteria* (7%) were identified during the early *Ostreopsis* cf. *ovata* bloom period, while *Alphaproteobacteria* (66%) and *Flavobacteria* (19%) were predominant in peak bloom phases (Guidi et al., 2018; pratoci et al., 2016). Bacterial communities associated to *Ostreopsis lenticularis* included single species belonging to the *Cytophaga-Flavobacter-Bacteroides* complex (Pérez-Guzmán et al., 2008). Bacteria-algae interactions can be very strong given that some microalgae are unable to grow in axenic conditions in culture confirming the predominant role of bacteria in algal cycle. This has also been observed in some *Ostreopsis* cultures.

Some studies indicate that bacteria could directly or indirectly modulate production and degradation of microalgals toxins (Albinsson et al., 2014) although the results appear study- and species- dependent and contradictory. While associated bacteria were linked to the production of paralytic shellfish toxin by *Alexandrium* spp. (Gallacher et al., 1997), higher toxin levels were found in bacteria-free than in non-axenic cultures of *Alexandrium tamarense* (Hold et al., 2001) and *A. lusitanicum* (Wang et al., 2004). Ashton et al. (2003) reported a toxicity loss of *Ostreopsis lenticularis* when exposed to elevated temperature and explained it by the reduction of *Ostreopsis* associated *Pseudomonas* and *Alteromonas*. Axenic cultures of (Vanucci et al., 2012a) were also characterized by lower toxin content in late stationary phase compared to non-axenic cultures. *Ostreopsis* cf. *ovata* cell concentration along a bloom was correlated with the abundance of planktonic, free-living *Vibrio* (Bellés-Garulera et al., 2016) and a high proportion of *Vibrio* were found attached to particles, suggesting that *Ostreopsis* bloom could provide biological colonizable surfaces (fragments of thecae or mucilage) for bacteria. The study suggested that bacteria could be involved in wound infections reported by beach users during *Ostreopsis* blooms. In fact, *Ostreopsis* blooms were reported in heavily antropogenically impacted environments (e.g. Cencini, 1998).

The role of the mucus produced by *Ostreopsis* (a mucilaginous matrix, described as a network of trichocysts embedded with acid polysaccharides filaments by Honsell et al., 2013) and associated bacteria in the respiratory irritations associated to the microalgal bloom is not clarified yet.

Parasites infection may play critical roles in the regulation of HABs. It has been already described for the planktonic toxic dinoflagellate *Alexandrium fundyense* which present an up-regulation of genes associated to defense against the parasite *Amoebophyra* and thus controlling host mortality (Lu et al., 2016, 2014). The algal metabolite dimethylsulphide (DMS) was identified as the density-dependent cue of the presence of potential dinoflagellate host (*Alexandrium minutum*, *Amphidinium carterae* and *Karlodinium veneficum*) that triggers zoospore activation and release from

the dormant sporangium of *Parvilucifera sinerae* (Garcés et al., 2012). *Ostreopsis* spp. was also infected by this generalistic parasitoid (Garcés et al., 2013).

3.2.3.3. Interactions with heterotrophic protists and ciliates

Heterotrophic dinoflagellates (HTDs) and ciliates have been described as effective grazers on HAB species (Jeong et al., 2015; Tillmann, 2004). For example, Matsuyama et al. (1999) demonstrated that predation by *Polykrikos kofoidii* can contribute to the cessation of a *Gymnodinium catenatum* bloom.

In the case of *Ostreopsis*, only one study has investigated the feeding of various HTDs and one ciliate on *O. cf. ovata* (Yoo et al., 2015). Even if all HTDs tested in this work were potentially able to ingest *O. cf. ovata* based on cell size criteria, only *Gyrodinium dominans*, *Gyrodinium moestrupii*, *Oxyrrhis marina* and *Polykrikos kofoidii* were able to feed on this toxic dinoflagellate by engulfment whereas *Pfiesteria piscicida* fed by using a peduncle. The study suggested that these HTDs can feed on *O. cf. ovata* by enzymes involved in the detection, capture, ingestion and digestion of toxic dinoflagellates. Lack of these enzymes in the naked ciliate *Strombilidium* sp. and the HTDs *Gyrodinium spirale*, *Protoperidinium bipes*, and *Stoeckeria algicida* would hamper feeding on *O. cf. ovata*.

3.2.3.4. Interactions with flora

A relatively small but recent number of studies focus on interactions between *Ostreopsis* sp. and flora, microphytobenthos and macrophytes, suggesting their impact on this dinoflagellate bloom dynamic. In these studies, the degree of interaction has been measured using different physiology and morphology parameters of the involved organisms, namely, growth rate (Ben Gharbia et al., 2017; García-Portela et al., 2016), fluorescence (Ben Gharbia et al., 2017; Ternon et al., 2018), swimming speed (Yoo et al., 2015), adherence capability (García-Portela et al., 2016), cysts formation (Accoroni et al., 2016b), cellular integrity (variation of shape, size, nucleus position using lipid and nuclear staining; Pichierri et al., 2017) and toxin production (Ben Gharbia et al., 2017).

3.2.3.4.1 Microalgae

Some studies have shown that particular secondary metabolites (i.e. low molecular weight compounds not directly involved in basic life processes) could considerably affect HABs dynamic and growth (Fistarol et al., 2003; Uchida et al., 1999; Yamasaki et al., 2010). As an example, the dinoflagellate *Karenia brevis* release extracellular compounds during blooms to strongly inhibit the growth of competitive species, and thus induce planktonic community changes (Prince et al., 2008). These sorts of interactions have also been addressed concerning the impact of *O. cf. ovata* on diatoms and other dinoflagellates, as well as the retroactions of these microalgae on *O. cf. ovata*.

A strong inhibitory effect of *O. cf. ovata* on microalgae was observed in the *in situ* study of Accoroni et al. (2016b). In particular, the diversity index decreased significantly and motile diatoms increased during *O. cf. ovata* bloom. Motile life diatom phase would be favored by the *Ostreopsis* mucous matrix, illustrating the importance of the mucus in the interactions between *O. cf. ovata* and its environment. Giussani et al. (2015) had already documented the toxicity of the mucus in biological assays. Recently, Ternon et al. (2018) using co-cultures assays (with diatoms) suggested a contact-toxicity of *Ostreopsis* cells, probably mediated by the mucus, which could retain the toxins and other compounds and avoid their dilution in the surrounding environment.

Monti and Cecchin (2012) observed weak effects on the growth of the dinoflagellates *Coolia monotis* and *Prorocentrum minimum* when exposed to filtrates of *O. cf. ovata* cultures obtained during its exponential phase. This highlights the importance of growth stages considered in the experiments. Indeed, variation of the toxin content among the growth stage has already been described by different studies (Brissard et al., 2014; Pezzolesi et al., 2016; Vanucci et al., 2012b; Pavaux et al., 2019 - In prep.). García-Portela et al. (2016), using mixed cultures of different *Ostreopsis* sp. strains and toxic and non-toxic dinoflagellates, demonstrated differences of toxicity among the *Ostreopsis* strains. Indeed, *O. cf. ovata* was more toxic than *O. sp.* «Lanzarote type», highlighting the necessity to study the intraspecific variability of the interactions of *Ostreopsis* sp. with other organisms. The chemical compounds involved in the negative interaction of *Ostreopsis* and other microalgae (specially diatoms) are poorly unknown and secondary metabolites other than the OVTXs group could be at play, as suggested by Ternon et al. (2018).

In turn, planktonic and benthic diatoms can affect *O. cf. ovata* physiology, morphology and growth. An inhibition of *O. cf. ovata* growth rate by 57 % and 78 % was reported in cultures exposed to, respectively, filtrates of the planktonic diatoms *Skeletonema marinoi* and *Thalassiosira* sp. (Pichierri et al., 2017). Similar inhibition of growth, deleterious effects and genotoxic damages were also observed on *Ostreopsis* cultures exposed to filtrates of other benthic diatoms (e.g., *Tabularia affinis*, *Proschkinia complanatoides* and *Navicula* sp.). These studies suggested the production of allelopathic compounds by all tested diatoms, especially released after cell destruction (Pohnert, 2000). These diatoms are well known to produce a large family of polyunsaturated aldehydes (PUAs - Wichard et al., 2005). Pichierri et al. (2017) found that exposure to a range of PUAs (2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal) concentrations (from 3 to 36 $\mu\text{mol.L}^{-1}$) caused *O. cf. ovata* growth inhibition, decrease in photosynthesis efficiency, increase of abnormal cells (motionless, decrease in dimensions, contraction of cytoplasm and formation of abnormal vesicle-like structure) and a decrease in cell integrity (chromatin dispersion, lack of autofluorescence of the chlorophyll and larger lipid bodies).

To conclude, there are still too few studies to understand interactions between *Ostreopsis* spp. and other microalgae and they are mainly focused on *O. cf. ovata*. However, a strong strain/species dependent effect of *Ostreopsis* on co-occurring community was demonstrated. Moreover, the predominant role of the mucilaginous matrix, as “concentrator of secondary metabolites” was suggested.

3.2.3.4.2 Macroalgae

Biotic substrate preferences of *Ostreopsis* species has already been demonstrated in several studies (e.g. Totti et al., 2010). This preference of substrate by *Ostreopsis* sp. could be explained in part, by the macroalgal thallus surface and morphology: complex, three dimensional flexible and branched thalli favored cell attachment (Accoroni et al., 2016a; Cochu et al., 2013; Meroni et al., 2018). These thalli would also be more suitable for *O. cf. ovata* (Accoroni et al., 2012; Vila et al., 2001) resistance to wave action. *Ostreopsis* cells might be removed more easily by wave action on low ramified thalli compared to complex thalli. Higher abundances of *Ostreopsis cf. ovata* were also found on the turf dominated community i.e. in a complex intricate matrix of small Corallinales, Ceramiales and other filamentous algae (Meroni et al., 2018) as previously reported in Vila et al. (2001) for *Ellisolandia elongata* and in Blanfuné et al. (2015) for *Jania rubens*.

The production of allelopathic compounds by macroalgae could also explain substrate preferences by *Ostreopsis* sp. Macroalgae are known to produce a large amount of secondary metabolites sometimes used in biotechnology as antibiofouling coatings (Hellio et al., 2002) or as a way to control HABs (Hu and Hong, 2008; Tang et al., 2015). Thus, it is reasonable to hypothesize that these metabolites could selectively determine *Ostreopsis* spp. substrate preference. *Rhodymenia pseudopalmata* (Florideophyceae) have little effects on *Ostreopsis* growth and only in powder (Accoroni et al., 2015b). The seagrasses *Zostera noltei* and *Cymodocea nodosa* have also a low impact on *Ostreopsis cf. ovata* growth although *Zostera noltei* have been described as a producer of phenolic acids inhibiting the growth of *Alexandrium catenella* (Laabir et al., 2013). *Ulva rigida* (Ulvophyceae) and *Dyctyota dichotoma* (Phaeophyceae) had a strong effect on *O. cf. ovata* by decreasing its growth, physiology, morphology, toxin production and behaviour with a decrease of cell adherence (Accoroni et al., 2015b; Ben Gharbia et al., 2017). The potential allelochemical compounds produced by this macroalgae are still poorly known but could mainly include phenols and polyunsaturated fatty acids (PUFAs - Ben Gharbia et al., 2017).

In summary, macroalgae morphology is decisive for *Ostreopsis* spp. colonization but allelochemicals produced by macroalgae could also play a key role. More studies are needed to understand which compounds are involved in interactions between *O. cf. ovata* and macroalgae, to identify and purify them in order to test them directly in biological assays.

3.2.3.5. Interactions with fauna

3.2.3.5.1 Impacts of *Ostreopsis* spp. blooms on fauna in tropical and temperate areas

As indicated above, human intoxications have been reported in tropical areas mainly due to the consumption of fishes or crustaceans contaminated with PLTX or its analogs without any indication of the effects of these toxins on the vector organisms. This suggests a direct ingestion, at least for herbivorous, of *Ostreopsis* sp. cells by these contaminated organisms. Even if no human intoxication due to the consumption of fishes or crustaceans was reported in temperate areas, massive mortality of invertebrates occurred during *Ostreopsis* sp. bloom. Mass mortalities of sea urchins were reported in Italy (Vale and Ares, 2007), Algeria (Illoul et al., 2012), Brazil (Granéli et al., 2002), New Zealand (Shears and Ross, 2009), Spain (Vila et al., 2008) and France (Blanfuné et al., 2012). Impacts on other sessile and mobile benthic organisms such as cirripeds, mussels, limpets and cephalopods were also observed (Ciminiello et al., 2006; Totti et al., 2010). Even if the role of oxygen depletion and high sea water temperature could be involved in those mass mortalities (Shears and Ross, 2009), more and more authors rather suggest that these mortalities were a consequence of some ecotoxic effects associated to *Ostreopsis* sp. blooms (Sansoni et al., 2003; Simoni et al., 2003; Vila et al., 2008).

3.2.3.5.2 Direct interactions between marine fauna and *Ostreopsis* spp

Ostreopsis sp. is an epibenthic genus, growing attached to macroalgae or rocks (e.g. Carnicer et al., 2015; Cochu et al., 2013, 2011; Penna et al., 2010) thanks to the production of a mucilaginous matrix (Escalera et al., 2014; Honsell et al., 2013). Cells are also released in the water column, even if the link between planktonic and benthic abundances is still unclear (Mangialajo et al., 2011), and can also aggregate on the water surface, forming what was called “sea-flowers”. *Ostreopsis* spp. could thus have different impacts on marine ecosystems depending on whether cells are benthic, planktonic or aggregated at surface.

3.2.3.5.2.1. Interactions of fauna with benthic *Ostreopsis* spp. cells

When *Ostreopsis* spp. is attached on benthic substrates, it can interact with its surrounding community and notably with meiobenthic organisms. Indeed, these benthic organisms live in close contact with *Ostreopsis* spp. and could potentially feed on this toxic dinoflagellate. To study the impact of *O. cf. ovata* on benthic organisms, bioassays are commonly used in order to compare the sensitivity of various organisms to this toxic dinoflagellate (Table 5). For instance, the benthic copepod *Tigriopus fulvus* and the amphipod *Corophium insidiosum* are both sensitive organisms since their median lethal concentration (LC₅₀) values after 96 hours of exposition are respectively 10.11 et 11.81 cell.mL⁻¹ (Prato

et al., 2011). Other organisms, such as the harpacticoid copepod *Sarsamphiascus cf. propinquus*, co-occurring with *Ostreopsis cf. ovata* in summer, has been demonstrated as very resistant to toxins produced by *Ostreopsis cf. ovata* (Pavaux et al., 2019) with LC₅₀(48h) values higher than 20 000 cells.mL⁻¹ so at least 250 times higher than another benthic copepod, *Tigriopus fulvus* (Faimali et al., 2012), suggesting potential acclimation phenomenon to the presence of *Ostreopsis*. However, it remains complex to compare results of these studies since life stages of the studied organism varies from one study to another. Parameters used to determine the sensitivity also varies between studies, with sometimes the determination of LC₅₀ values at 24, 48 or 96 hours. Moreover, the strong influence on the determination of this parameter (LC₅₀) has been demonstrated by Faimali et al. (2012) since toxicity appears to increase with the temperature of culture. Indeed, LC₅₀ values of the barnacle *Amphibalanus amphitrite* are respectively equal to 1416.95 and 192.26 at 20 and 25°C. This suggests the need of standardizing the methods to test the toxicity of *Ostreopsis* spp. on benthic organisms.

Moreover, a reprotoxic effect of *O. cf. ovata* has been suggested by the significant decrease of number of nauplii of copepods described in the *in situ* study of Guidi-Guilvard et al. (2012) and confirmed by laboratory experiments by Pavaux et al. (2019), with a decrease of fecundity and fertility ratios of the benthic copepod *S. cf. propinquus*. These reprotoxic effects lead to changes in benthic communities.

By forming aggregates that cover macroalgae, *Ostreopsis* spp. cells are also ingested by macroherbivores such as sea urchins or herbivorous fish. Numerous studies have already been quantified PLTX and analogs in these two groups (Amzil et al., 2012; Biré et al., 2013, 2015; Brissard et al., 2014; Milandri et al., 2010) using biological assays (HNA) or toxin analysis by HPLC-MS. *Paracentrotus lividus* (an edible and herbivorous sea urchin) and *Sarpa salpa* (an herbivorous fish) accumulated high concentrations of PLTX-group toxins (respectively 423 and 361 µg.kg⁻¹), making these organisms good sentinel species for the presence of PLTXs in marine organisms (Biré et al., 2015).

Quantification of toxins seems to be organ-dependent: no toxins were found in sea urchins gonads although it reached to 423 µg eq PLTX.kg⁻¹ in the digestive tube (Brissard et al., 2014). The study of Bauder et al. (2001) demonstrated that scallops exposed to Diarrheic Shellfish Toxins (DSP) produced by *Prorocentrum lima* mostly accumulated toxins in viscera (76 %) but also in the gonads (12 %) with a quick detoxification. After the depuration period, toxins were only found in the gut suggesting that the toxins were poorly bound to the tissues with the exception of the viscera. This low affinity of toxins for tissues excepted for viscera, could explain the absence of toxins in sea urchins gonads documented in the work of Biré et al. (2015, 2013). Neves et al. (2018) also reported that the ingestion of *O. cf. ovata* cells by adult sea urchins have an impact on their offspring (dead and abnormal larvae, arrest of embryonic development – Table 5). These development anomalies persist at least 8 months after the exposition to *Ostreopsis cf. ovata* and seems to be transmitted from the female

gonads with the involvement of the nitric oxide pathway since the transcription of several genes directly or indirectly modulated by nitric oxide showed transcription variations (Neves et al., 2018).

3.2.3.5.2.2. Interactions of fauna with planktonic *Ostreopsis* spp. cells

During its planktonic phase, *Ostreopsis* cf. *ovata* could reach to 385 601 cell·L⁻¹ (e.g. Mangialajo et al., 2011) and thus can affect planktonic organisms but also bivalves and other benthic filter-feeders. Numerous studies have quantified toxins in bivalves due to sanitary impacts and economic losses caused on local economies due to prohibitions of shellfish harvesting and commercialization during these toxic events (Accoroni et al., 2011; Amzil et al., 2012; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009; Lemée et al., 2012). Concentrations of toxins quantified in mussels (*Mytilus galloprovincialis*) varies from 28 to 228 µg.kg⁻¹ (Table 5). Quantities of toxins found in other filter-feeders such as the mussel *Perna canaliculus* or the bivalve *Arca noae* were well below and frequently found only in trace amounts (Rhodes et al., 2002, 2000), probably due to the use of biological assays to quantify the toxins in these organisms leading to an underestimation of toxins concentration.

Mechanisms of toxicity have been addressed by exploring histological impairments on *Mytilus galloprovincialis* adults (Gorbi et al., 2012, 2013). These studies showed a limited role of oxidative stress and suggested that toxic effects of the compounds produced by *Ostreopsis* sp. could be rather mediated by immune response than oxidative stress. At last, toxins produced by *Ostreopsis* cf. *ovata* had a strong impact on the planktonic ephyrae larvae of the jellyfish *Aurelia* sp. (Giussani et al., 2016) and embryos and eggs of the sea urchin *Paracentrotus lividus* (Caroppo and Pagliara, 2011; Migliaccio et al., 2016; Pagliara and Caroppo, 2012). The sea urchin *Lytechinus variegatus* also showed reduced fertilization success and arrest of embryonic development at the gastrula stage when exposed to *Ostreopsis* cf. *ovata* cells (Neves et al., 2018).

Table 5 : Organisms in direct interaction with *Ostreopsis* spp. Half maximal effective concentration (EC₅₀) ; Digestive Tube (DT); Hemolysis Neutralization Assay (HNA); Median Lethal concentration (LC₅₀) ; Liquid Chromatography–Mass Spectrometry (LC-MS); Media, lethal time (LT₅₀); Mouse Bioassay (MBA); Remaining tissue (RT); Whole flesh (WF).

Species/Strain	Organisms	Stage	Effects	Reference	
• Interactions with epibenthic cells of <i>Ostreopsis</i> spp.					
<i>Ostreopsis ovata</i> isolated by A. Penna	<i>Tigriopus fulvus</i> (Fisher, 1860)	I-II stage Nauplii	Mortality	LC _{50-48h-20°C} = 1486.74 cells.mL ⁻¹ LC _{50-48h-25°C} = 250.12 cells.mL ⁻¹	Faimali et al., 2012
<i>Ostreopsis</i> cf. <i>ovata</i>	<i>Tigriopus fulvus</i> (Fisher, 1860)		Mortality	LC _{50-24h-20°C} > 4000 cells.mL ⁻¹	Found in Giussani et al., 2016. Unpublished data
<i>Ostreopsis ovata</i> isolated by A. Penna	<i>Amphibalanus amphitrite</i> (Darwin, 1854)	II-III stage Nauplii	Mortality	LC _{50-48h-20°C} = 1416 cells.mL ⁻¹ LC _{50-48h-25°C} = 192 cells.mL ⁻¹	Faimali et al., 2012
<i>Ostreopsis</i> cf. <i>ovata</i>	<i>Amphibalanus amphitrite</i> (Darwin, 1854)		Mortality	LC _{50-24h-20°C} > 4000 cells.mL ⁻¹	Found in Giussani et al., 2016. Unpublished data
<i>Ostreopsis</i> cf. <i>ovata</i> Natural samples Ligurian Sea (44°3'27''N; 9°55'43''E)	<i>Dinophylus gyrocoliatius</i> (Schmidt, 1857)	Juveniles	Mortality	LT _{50-24°C} (3500 cells.mL ⁻¹) = 2h LT _{50-24°C} (1500 cells.mL ⁻¹) = 3.4h LT _{50-24°C} (200 cells.mL ⁻¹) = 12.25h ± 3.18	Simonini et al., 2011
<i>Ostreopsis ovata</i> isolated by A. Penna	<i>Paracentrotus lividus</i> (Lamarck, 1816)	Juveniles	Mortality	LC _{50-48h-20°C} = 168 cells.mL ⁻¹	Privitera et al., 2012
<i>Ostreopsis</i> cf. <i>ovata</i> Natural samples Gulf of Taranto	<i>Tigriopus fulvus</i> (Fisher, 1860)	Nauplii	Mortality	LC _{50-96h-25°C} = 10.11 cell.mL ⁻¹	Prato et al., 2011
	<i>Corophium insidiosum</i> (Crawford, 1937)	2-4 mm body length	Mortality	LC _{50-96h-25°C} = 11.81 cell.mL ⁻¹	
	<i>Sphaeroma serratum</i> (Fabricius, 1787)	2-4 mm body length	Mortality	LC _{50-96h-25°C} = 214.81 cell.mL ⁻¹	
<i>Ostreopsis siamensis</i> CAWD73/74/75/96	<i>Haliotis virginea</i> (Gmelin, 1791)	Larvae	Mortality	Not lethal within 24h	Rhodes et al., 2000
<i>Ostreopsis</i> cf. <i>ovata</i> MCCV 054	<i>Sarsamphiascus</i> cf. <i>propinquus</i> (Sars G.O., 1906)	Adults	Mortality	LC _{50-24h-24°C} > 20 000 cell.mL ⁻¹	Pavaux et al., 2019

<i>Ostreopsis cf. ovata</i> Natural samples from French and Italian NW Med. coasts	Phytoplankton meiofauna		Community composition	72 % decrease of nauplii	Guidi-Guilvard et al., 2012
<i>Ostreopsis cf. ovata</i> MCCV 054	<i>Sarsamphiascus cf. propinquus</i> (Sars G.O., 1906)	Females	Reproduction	Decrease of fertility and fecundity ratios	Pavaux et al., 2019
<i>Ostreopsis cf. ovata</i> Natural samples (French Mediterranean coasts)	<i>Paracentrotus lividus</i> (Lamarck, 1816)		Toxin quantification	LC-MS/MS: 77 to 361 $\mu\text{g eq PLTX.kg}^{-1}$	Amzil et al., 2012
<i>Ostreopsis cf. ovata</i> Field sample (Villefranche-sur-Mer)	<i>Paracentrotus lividus</i> (Lamarck, 1816)		Toxin quantification	LC-MS/MS: 103 to 423 $\mu\text{g.kg}^{-1}$ Hemolytic test: 179 to 270 $\mu\text{g.kg}^{-1}$	Brissard et al., 2014
	<i>Sarpa salpa</i> (Linnaeus, 1758)		Toxin quantification	LC-MS/MS: 33 to 152 $\mu\text{g.kg}^{-1}$	
<i>Ostreopsis ovata</i> Field samples (Tyrrhenian Sea)	Sea urchins		Toxin quantification	HNA: 5 to 99 pPLTX $\mu\text{g.kg}^{-1}$ LC-MS/MS: < LOQ pPLTX $\mu\text{g.kg}^{-1}$ 87 to 164 OVTXa $\mu\text{g.kg}^{-1}$	Milandri et al., 2010
<i>Ostreopsis cf. ovata</i> Field samples Nice harbour and Villefranche-sur-mer	<i>Paracentrotus lividus</i> (Lamarck, 1816)		Toxin quantification	Haemolytic assay : 256.6 $\mu\text{g/kg}$ (WF) LC-MS/MS: 192.5 $\mu\text{g/kg}$ (WF)	Biré et al., 2013
	<i>Sarpa salpa</i> (Linnaeus, 1758)		Toxin quantification	Haemolytic assay : 230.0 $\mu\text{g/kg}$ (DT) LC-MS/MS: 191.2 $\mu\text{g/kg}$ (WF)	
<i>Ostreopsis cf. ovata</i> Field samples - Rochambeau site, Villefranche-sur-mer	<i>Paracentrotus lividus</i> (Lamarck, 1816)		Toxin quantification	Haemolytic assay : 159.2 $\mu\text{g/kg}$ (DT)	Biré et al., 2015
	<i>Sarpa salpa</i> (Linnaeus, 1758)		Toxin quantification	Haemolytic assay : 361.0 $\mu\text{g/kg}$ (DT)	
• Interactions with planktonic cells of <i>Ostreopsis sp.</i>					
<i>Ostreopsis ovata</i> isolated by A. Penna	<i>Paracentrotus lividus</i> (Lamarck, 1816)	Competent larvae	Mortality	LC _{50-48h-20°C} = 168 cells.mL ⁻¹	Privitera et al., 2012
<i>Ostreopsis cf. ovata</i> CBA29-2012 (Genoa)	<i>Aurelia sp.</i>	Polyp	Reproduction/early stages	Unaffected (100 cells.mL ⁻¹)	Giussani et al., 2015
<i>Ostreopsis cf. ovata</i> Marche coast (Italie NW Adriatic sea)	<i>Mytilus galloprovincialis</i> (Lamarck, 1819)	Adults	Histological impairments	<u>Immune system</u> : - Decrease of granulocytes - Phagocytosis activity decrease - Reduced lysosomal membrane stability <u>Histology</u> : - Decrease of the digestive gland wall thickness - Dilatation of tubules	Gorbi et al., 2013

				<ul style="list-style-type: none"> - Haemocytes infiltration into digestive gland - Decrease of neutral lipids quantity <u>Oxidative stress</u> : limited role	
<i>Ostreopsis cf. ovata</i> Field sample from Adriatic Sea (Portonevo & Passetto sites)	<i>Mytilus galloprovincialis</i> (Lamarck, 1819)	Adults	Histological impairments	<ul style="list-style-type: none"> - Inhibition of Na⁺/K⁺ATPase and acetylcholine esterase activity <u>Immune system</u> : Reduced lysosomal membrane stability <u>Oxidative stress</u> : limited role	Gorbi et al., 2012
<i>Ostreopsis spp.</i> Cultures + field samples (North Aegean coasts)	<i>Mytilus galloprovincialis</i> (Lamarck, 1819) <i>Venus verrucosa</i> <i>Odiolus barbatulus</i>		Toxin quantification	MBA: Qualitative analysis HNA: 33.3 to 97 pPLTX µg.kg ⁻¹	Aligizaki et al., 2008
<i>Ostreopsis cf. ovata</i> Natural samples (French Mediterranean coasts)	<i>Mytilus galloprovincialis</i> (Lamarck, 1819)		Toxin quantification	LC-MS/MS: 28 to 217 µg eq PLTX.kg ⁻¹	Amzil et al., 2012
<i>Ostreopsis cf. ovata</i> Filed samples (Adriatic Sea)	<i>Mytilus galloprovincialis</i> (Lamarck, 1819)		Toxin quantification	MBA + HNA: Qualitative analysis	Gorbi et al., 2012
<i>Ostreopsis ovata</i> Field samples (Tyrrhenian Sea)	Mussels		Toxin quantification	HNA: 103 pPLTX µg.kg ⁻¹ LC-MS/MS: < LOQ pPLTX µg.kg ⁻¹ - 131 to 228 OVTXa µg.kg ⁻¹	Milandri et al., 2010
<i>Ostreopsis siamensis</i> CAWD75 (New Zeland)	Greenshell™ mussels Pacific oysters Scallops		Toxin quantification	MBA: qualitative analysis HNA: 0.3 pg eq. PLTX.cell ⁻¹	Rhodes et al., 2000
<i>Ostreopsis siamensis</i> CAWD75 (New Zeland)	<i>Perna canaliculus</i> (Gmelin, 1791) <i>Pecten novaezalandiae</i> (Reeve, 1852) <i>Crassostrea gigas</i> (Thunberg, 1793)		Toxin quantification	MBA (qualitative analysis): none to death HNA: trace to 1 ng.g ⁻¹	Rhodes et al., 2002
<i>Ostreopsis cf. ovata</i> Field samples - Rochambeau site Villefranche-sur-mer	<i>Arca noae</i> (Linnaeus, 1758)		Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	Biré et al., 2015

<i>Ostreopsis cf. ovata</i> CBA29-2012 (Genoa)	<i>Aurelia sp.</i>	Ephyrae	Reproduction/early stages	% Alteration of Pulsation Frequency EC _{50-24h} = 5.32 cells.mL ⁻¹ Immobility EC _{50-24h} = 10.50 cells.mL ⁻¹	Giussani et al., 2015
<i>Ostreopsis cf. ovata</i> ITAC501	<i>Paracentrotus lividus</i> (Lamarck, 1816)	Eggs	Reproduction/early stages	- Gametes: % fertilization decrease; less toxic for eggs than sperms - Embryos: no segmentation	Caroppo and Pagliara, 2011
<i>Ostreopsis cf. ovata</i> Natural samples (Gaiola MPA)	<i>Paracentrotus lividus</i> (Lamarck, 1816)	Embryos	Reproduction/early stages	Fertilization and developmental impairments Alteration of several marker genes	Migliaccio et al., 2016
<i>Ostreopsis cf. ovata</i> Strain UNR-05 Armação dos Búzios (Rio de Janeiro)	<i>Lytechinus variegatus</i> (Lamarck, 1816)	Embryos	Reproduction/early stages	Stronger effect on larvae than on fertilization success: - Reduced fertilization - Arrest of embryonic development (gastrula stages at 400 cells.mL ⁻¹) - Increase of dead and abnormal larvae	Neves et al., 2018
<i>Ostreopsis cf. ovata</i> (whole cells + lysate) Natural samples (Ionian Sea)	<i>Paracentrotus lividus</i> (Lamarck, 1816)	Embryos	Reproduction/early stages	<u>Whole cells</u> : Developmental delay or anomalous cell division <u>Cell lysate</u> : Growth inhibition at 1.87 mg.mL ⁻¹	Pagliara and Caroppo, 2012
• Interactions with surface cells of <i>Ostreopsis spp.</i>					
<i>Ostreopsis cf. ovata</i> Field samples Nice harbour and Villefranche-sur-mer	<i>Patella spp</i>		Toxin quantification	Haemolytic assay : 12.7 µg/kg (WF)	Biré et al., 2013
	<i>Mugil cephalus</i> (Linnaeus, 1758)		Toxin quantification	Haemolytic assay : 392.2 µg/kg (DT) LC-MS/MS: 359.1 µg/kg (WF)	
<i>Ostreopsis cf. ovata</i> Field samples - Rochambeau site Villefranche-sur-mer	<i>Patella spp.</i>		Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	Biré et al., 2015
<i>Ostreopsis cf. ovata</i> Field samples Genoa, Nice, Villefranche-sur-Mer	<i>Paracentrotus lividus</i> (Lamarck, 1816)		Density	Any decrease of <i>P. lividus</i> densities related to high abundances of <i>O. cf. ovata</i>	Blanfuné et al., 2012
	<i>Patella spp.</i>			No significant decrease of densities in Villefranche-sur-mer and Nice ; Significant decrease in Genoa potentially linked to <i>O. cf. ovata</i> blooms	

3.2.3.5.2.3. Interactions of fauna with sea-surface *Ostreopsis* spp. cells

Toxins quantified both in the digestive tube and in the whole flesh of the mullet *Mugil cephalus* were high and reached to $392.5 \mu\text{g}\cdot\text{kg}^{-1}$ which can be explained by the grazing behavior of this animal that feeds directly on *Ostreopsis* cf. *ovata* cells aggregates on the water surface (Biré et al., 2013). Other organism from the mediolittoral area such as the limpets *Patella* spp. are also exposed to surface cells of *Ostreopsis* cf. *ovata* and up to now, studies have been mainly focused on toxin quantification in the whole flesh that reached up to $12.7 \mu\text{g}\cdot\text{kg}^{-1}$, well below the threshold value established by the EFSA (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009). The study of [Blanfuné et al. \(2012\)](#) reported a significant decrease of *Patella* spp. densities during the 2008 *O. cf. ovata* bloom in Genoa, attributed to the poisoning of the shellfish by ingestion of *O. cf. ovata* cells.

To conclude, most studies have focused on the effect of *O. cf. ovata* and revealed reprotoxic effect on both planktonic and meroplanktonic organisms. Toxins are mainly found in the digestive tubes of contaminated organisms and their concentrations appeared to be independent of the way of *O. cf. ovata* exposure (epibenthic, planktonic or surface cells) since toxin concentrations found in the mullet *Mugil cephalus* feeding on surface cells of *O. cf. ovata*, were comparable to values found in sea urchins' digestive tubes, feeding on epibenthic cells. Lastly, toxins are found in herbivorous and omnivorous organisms, regardless of the diet.

3.2.3.5.3 Indirect interactions between *Ostreopsis* spp. and marine fauna

Toxin concentrations found in carnivorous and omnivorous organisms suggested bioaccumulation of toxins within the trophic web (Biré et al., 2013, 2015; Faimali et al., 2012; Giussani et al., 2016; Milandri et al., 2010; Pezzolesi et al., 2012). Toxins quantified in both the crab *Eriphia verrucosa* (carnivorous) and the gastropod *Stramonita haemastoma* (carnivorous) raised up to 58.6 and $26.4 \mu\text{g}\cdot\text{kg}^{-1}$ (Fresh Weight) respectively. Octopuses also accumulated more than $446 \mu\text{g}\cdot\text{kg}^{-1}$ (Milandri et al., 2010) suggesting a bioaccumulation of toxins among the trophic web which might have played a role in the ecosystem suffering with stranding of octopuses.

Other studies reported a strong sensitivity of *Dicentrarchus labrax* fish juveniles to *O. cf. ovata* raising the question of the impact on the structure of marine ecosystems (Faimali et al., 2012; Giussani et al., 2016; Pezzolesi et al., 2012). Indeed, these fish are unable to eat *Ostreopsis* cells, the observed toxicity was exclusively due to contact with *Ostreopsis* cells.

To conclude, high accumulation of *Ostreopsis* toxins has been documented in various marine organisms suggesting the potential bioaccumulation among the trophic web with threshold higher than those recommended by EFSA. More studies are needed to better understand the transfer of toxins among the trophic web as already described for other toxic dinoflagellate such as *Gambierdiscus*

species and ciguatoxins (Ledreux et al., 2014). Moreover, the few existing studies have reported strong differences of sensitivity between organisms hence the need of more studies to investigate potential acclimation and detoxification phenomena on some organisms. At last, the reprotoxicity of *Ostreopsis* sp. have been demonstrated for benthic organisms which can lead to changes in benthic communities.

3.2.4. Overall vision of the impacts of *Ostreopsis* spp. on ecosystem structure and functioning

3.2.4.1. Ecotoxicology studies

The studies on *Ostreopsis* spp. chemical ecology summarized above show negative effects of this dinoflagellate on different trophic levels and suggest subsequent negative effects on the structure of the ecosystems. At the level of primary producers, *Ostreopsis* sp. negatively impact diatoms and other dinoflagellates with which it co-occurs, regardless of the exposure pathway (extracellular released compounds or whole living cells) leading to changes in microphytobenthic communities. Concerning secondary producers, a strong reprotoxic effect has also been reported. Herbivorous organisms feeding on *Ostreopsis* sp. (by e.g., the benthic copepod *Sarsamphiascus* cf. *propinquus*) or organisms feeding on macroalgae supporting its populations (e.g., sea urchins -*Paracentrotus lividus*- or fish -*Sarpa salpa*-) could accumulate very high quantity of toxins without short term lethal effects. However, a clear decrease of fecundation success, fertility and fecundity ratios and abnormal development effects (that lasted for many months after the exposition), was reported for various organisms such as benthic copepods and sea urchins (Table 5). This effect on the progeny could also strongly affect the structure of ecosystems. Despite several studies report invertebrate mass mortalities (Shears and Ross, 2009; Totti et al., 2010), no studies have been conducted to determine if these mortalities could genuinely be attributed to toxins produced by *Ostreopsis* spp. Overall, the transfer to toxin through the food webs poses a risk of seafood poisoning in humans through consumption of the contaminated organisms, not reported in temperate areas yet, but, unfortunately, with some dramatic cases in tropical areas.

Table 6 : Organisms in indirect interaction with *Ostreopsis* spp. Half maximal effective concentration (EC₅₀); Median lethal concentration (LC₅₀); Median lethal time (LT₅₀).

Species/Strain	Organisms	Authority	Effects	Reference	
<i>Ostreopsis ovata</i> Field samples (Tyrrhenian Sea)	<i>Octopuses</i>		Toxin quantification HNA: 446 pPLTX µg.kg ⁻¹ LC-MS/MS: 115 pPLTX µg.kg ⁻¹ 971 OVTXa µg.kg ⁻¹	Milandri et al., 2010	
<i>Ostreopsis</i> cf. <i>ovata</i> Field samples Nice harbour and Villefranche-sur-mer	<i>Diplodus annularis</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : 10.6 µg/kg (DT)	Biré et al., 2013
	<i>Diplodus sargus</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : 5.1 µg/kg (DT)	
	<i>Eriphia verrucosa</i>	Forskål, 1775	Toxin quantification	Haemolytic assay: 38.4 µg/kg (WF) LC-MS/MS: 58.6 µg/kg (WF)	
	<i>Maja squinado</i>	Herbst, 1788	Toxin quantification	Haemolytic assay : 8.3 µg/kg (WF)	
	<i>Mullus surmuletus</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < LOQ	
	<i>Muraena helena</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < LOQ	
	<i>Octopus vulgaris</i>	Cuvier, 1797	Toxin quantification	Haemolytic assay : 18.5 µg/kg (RT)	
	<i>Scorpaena porcus</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : 3.7 µg/kg (DT)	
	<i>Serranus scriba</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < LOQ	
	<i>Stramonita haemastoma</i>	Linnaeus, 1767	Toxin quantification	Haemolytic assay: 34.1 µg/kg (WF) LC-MS/MS: 26,4 µg/kg (WF)	
	<i>Symphodus tinca</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : 1.8 µg/kg (DT)	
<i>Ostreopsis</i> cf. <i>ovata</i> Field samples Rochambeau site, Villefranche-sur-mer	<i>Coris julis</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	Biré et al., 2015
	<i>Diplodus vulgaris</i>	Geoffroy Saint-Hilaire, 1817	Toxin quantification		
	<i>Eriphia verrucosa</i>	Forskål, 1775	Toxin quantification	Haemolytic assay : 13.0 µg/kg (WF)	
	<i>Hexaplex trunculus</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : 40.4 µg/kg (WF)	
	<i>Gobiidae</i>		Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Maja squinado</i>	Herbst, 1788	Toxin quantification	Haemolytic assay : 51.3 µg/kg (WF)	
	<i>Mullus surmuletus</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Muraena helena</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Octopus vulgaris</i>	Cuvier, 1797	Toxin quantification	Haemolytic assay : 19.9 µg/kg (RT)	

	<i>Saurida undosquamis</i>	Richardson, 1848	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Scorpena porcus</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Serranus scriba</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Symphodus melops</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Symphodus ocellatus</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Symphodus roissali</i>	Risso, 1810	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Symphodus rostratus</i>	Bloch, 1791	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Symphodus tinca</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Thalassoma pavo</i>	Linnaeus, 1758	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
	<i>Xantho poressa</i>	Olivi, 1792	Toxin quantification	Haemolytic assay : < 10 µg/kg (WF)	
<i>Ostreopsis ovata</i> isolated by A. Penna	<i>Dicentrarchus labrax</i>	Linnaeus, 1758	Mortality	LC _{50-48h-20°C} = 540 cells.mL ⁻¹	Faimali et al., 2012
<i>Ostreopsis cf. ovata</i>	<i>Dicentrarchus labrax</i>	Linnaeus, 1758	Mortality	LC _{50-48h-20°C} = 679 cells.mL ⁻¹	Found in Giussani et al., 2016. Unpublished data
<i>Ostreopsis cf. ovata</i> Strain OOAN0601	<i>Dicentrarchus labrax</i>	Linnaeus, 1758	Mortality	100% mortality after 45h to 72h exposed to <i>O. cf. ovata</i> (between 425 to 2367 cells.mL ⁻¹)	Pezzolesi et al., 2012

Some organisms are able to protect themselves to the presence of *Ostreopsis* spp. by different mechanisms. Some diatoms such as the competitive diatom *Licmophora paradoxa* produce secondary metabolites which negatively affect *Ostreopsis* cf. *ovata* at different levels of organization. Some copepods commonly exposed to *Ostreopsis* cells have also demonstrated higher resistance to other species, suggesting a possible acclimation to *Ostreopsis* spp. Acclimation processes and detoxification and storage mechanisms of organisms exposed to *Ostreopsis* sp. have been rarely observed although they have already been described for other toxic dinoflagellates. A rapid detoxification of asparacids by *Mytilus edulis* (Jauffrais et al., 2012), of DSP produced by *Prorocentrum lima* by the scallop *Argopecten irradians* (Bauder et al., 2001) or gymnodimine by the carpet shell *Ruditapes decussatus* (Walid et al., 2010) has been already been demonstrated. Other studies have also demonstrated interspecific differences in toxins accumulation linked to toxins sensitivity and determined by neurologic, physiologic and behavior responses (Bricelj and Shumway, 1998). Toxicity also varies according to immersion time, the size of organism, or the off-bottom position (Bricelj and Shumway, 1998). Genes involved in detoxification mechanisms of oysters exposed to *Gymnodinium catenatum* have been already described and are involved in antioxidant defense, cell detoxification, intermediate immune response activation and stress response (García-Lagunas et al., 2013).

Acclimation processes have been already demonstrated for copepods exposed to *Alexandrium* spp. Indeed, populations of *Acartia hudsonica* from Massachussets (USA) to New Brunswick (Canada), commonly exposed to this dinoflagellate are more resistant than naïve populations, which failed to acclimate to the exposition to this toxic dinoflagellate (Colin and Dam, 2002).

Toxins quantified in cephalopods and carnivorous, herbivorous and omnivorous fish (Table 6) raise the question of bioaccumulation of OVTXs along the food web. Toxins circulation through the food webs from benthic unicellular algae to herbivorous fish has already been demonstrated for ciguatoxins (Ledreux et al., 2014). Another hypothesis is the incorporation of toxins into higher organisms through zooplankton (Baustian et al., 2018) and more specially through copepods or fecal pellets (Lehtiniemi et al., 2002). Copepods and other zooplankton organisms have been already described as vectors of the toxins up the food web for microcystins (Smith and Haney, 2006), nodularin (Lehtiniemi et al., 2002), okadaic acid (Maneiro et al., 2000) and PSP (Teegarden and Cembella, 1996).

The presence of *Ostreopsis* sp. could also favor some organisms. Indeed, particles produced by *Ostreopsis* sp. blooms i.e. theca fragments and mucus, respectively rich in cellulose and carbohydrates, provide biological colonizable surfaces and a good source of organic carbon, which can favor the growth of some bacteria such as *Vibrio* (Bellés-Garulera et al., 2016). This positive association for pathogenic bacteria increases the toxicity of *Ostreopsis* for human populations.

3.2.4.2. Importance of the mucus in *Ostreopsis* spp. interactions

The functional role of the mucilaginous matrix produced by *Ostreopsis* sp. is still unknown. Mucus production is considered as an adaptation to benthic life (Escalera et al., 2014; Parsons et al., 2012) and described as a complex structure composed by a network of long fibers, deriving from trichocysts extruded through thecal pores and by an amorphous matrix of acid polysaccharides (Honsell et al., 2013). The production of mucilaginous matrix is common in benthic dinoflagellates (MacKenzie et al., 2002) and plays a role in cellular adhesion to surfaces with the formation of a transparent film embedding many cells, providing a clear example of ecosystem engineering (Escalera et al., 2014). Its role in defense against grazers, in increasing buoyancy and metabolic self-regulation has already been investigated in other microalgae (Reynolds, 2007). The role of the mucilaginous matrix as a method of defense against grazers or to capture larger organisms has indeed been documented for other mixotrophic dinoflagellates (Blossom et al., 2012; Kjørboe and Titelman, 1998). For *Ostreopsis* sp., its role in predation and competition has been previously argued. Toxic effect of *O. cf. ovata* on co-occurring diatom is mediated by contact between cells, suggesting the role of the mucus in the competition as a storage for toxins in order to avoid dilution of toxins in the environment (Ternon et al., 2018). The toxicity of the mucus was also documented by Giussani et al. (2015) with a significant toxic effect of *O. cf. ovata* mucus on *Artemia salina* and supported by images obtained by atomic force microscopy. However, this role is poorly documented mainly due to the difficulty to separate cells from this mucus.

The role of this mucilaginous matrix in mixotrophic behavior was also reported (Barone, 2007). Even if mixotrophy is a common way of nutrition in harmful algal species (Burkholder et al., 2008), this heterotrophic behavior in *Ostreopsis* sp. is also poorly documented. Indeed, mixotrophic behavior of *Ostreopsis* sp. was first described by Faust and Morton (1995) since they observed *Ostreopsis labens* cells with large ingested particles within the cytoplasm. In 2012, Fraga et al. linked the presence of reddish round inclusions inside *Ostreopsis* cells through light microscopy, to a mixotrophic behavior of *Ostreopsis* species and confirmed by the study of Almada et al. (2017) which showed evidence of phagotrophy using LysoSensor staining even if the prey inside the cells were not discernable. Using molecular approaches, Lee and Park (2018) defined these red bodies as digestive vacuoles containing red macroalgae (Class Florideophyceae) ingested as spores.

3.2.4.3. Regulation of *Ostreopsis* bloom by biotic interactions

There is a growing awareness that HABs may have been increasing globally in the last 40 years and may continue to increase under the fast coming scenarios of global warming. HABs have strong impacts on human health and economy (Adams et al., 2018; Hoagland et al., 2002; Larkin and Adams, 2007). Hence the necessity to better understand factors and mechanisms favoring bloom formation

and maintenance. The formation, maintain and declining of such events is mainly attributed to biotic factors combining chemical (salinity, nutrients..) and physical processes (currents, upwelling, river flow..) associated to anthropogenic inputs leading to eutrophication (Sellner et al., 2003). However, this review highlights the number, the importance and the complexity of interactions between *Ostreopsis* sp. and its environment. The question is whether or not these interactions could regulate blooms initiation and decline. Most harmful bloom-forming species produce toxins even if adaptive significance of this synthesis is still unclear. It has been suggested that toxins confer some advantages in reaching very high cell densities (Anderson et al., 1998) by providing a defense against pathogens, grazers and parasites. This hypothesis is supported by the study of Selander et al. (2006) describing that toxin-producing species are less preferred by predators and that predator presence induces toxin production. In the case of *Ostreopsis* cf. *ovata*, it has been noted that benthic copepods are able to actively select non-toxic diatom in a mixture (Pavaux et al., 2019).

The role of these toxins in limiting growth and survival of competitors via allelopathic processes is poorly documented. Ternon et al. (2018) have demonstrated the importance of the toxins in negative effects on competitive diatom (and mediated by the mucus) and the potential involvement in chemical control by *O. cf. ovata* on its competitor. However, there is no consensus on the characterization of toxins produced by HABs as phytochemicals. Indeed, the work of Tillmann and John (2002) has rather shown that toxins produced by *Alexandrium* spp. are not involved in allelochemical defense mechanism. In view of conflicting results obtained, it is difficult to conclude about the role of the toxins as allelochemicals.

One hypothesis is that allelopathy could be an important mechanism to favor HAB by suppressing the growth of competitors (Smayda, 1997b). However, the importance of allelopathy is such mechanisms is difficult to conclusively demonstrate in the field (Prince et al., 2008). Mechanisms for allelopathy has already been proposed and included oxidative damage, loss of competitor mobility, inhibition of photosynthesis, inhibition of enzymes and membrane damage (Legrand et al., 2003). For *Ostreopsis* sp. a strong negative effect has been demonstrated on its competitor by inhibiting growth and fluorescence rates leading to a diminution of competitors' abundances. The production of allelopathic compounds explain in particular the success of *Karenia brevis* blooms (Kubanek et al., 2005; Prince et al., 2008). However, meta-analysis conducted by Jonsson et al. (2009) rather supported a significant effect of allelopathic mechanisms only at high cell densities since at a beginning of a bloom, cell abundances are too low to concentrate enough infochemicals.

Predation as a mechanism to favor Ostreopsis sp. Bloom: The balance between growth- and loss-promoting factors determine the abundance of harmful species. Predation by zooplankton is the largest mortality factor for phytoplankton (Calbet and Landry, 2004). Factors such as size, shape,

nutritional value are traditionally exposed to explain predation pressure. However, the production of chemical deterrents can also affect predation pressure. Predation of *Ostreopsis* sp. by meiofauna organisms has been poorly documented, but studies on this subject have demonstrated low ingestion rate of this dinoflagellate by benthic copepods (Pavaux et al., 2019). Heterotrophic ciliates seems to be active predators of *O. cf. ovata* (Matsuyama et al., 1999). Predation pressure mediated by heterotrophic ciliates seems to be higher than benthic copepods ones, in regard to *Ostreopsis* species and could potentially participate in population decline, already demonstrated by Solé et al. (2006). However, contradictory results have been documented by theoretical study of Mitra and Flynn (2006) describing predatory activation as a factor promoting HAB.

Parasitism to control Ostreopsis sp. blooms: Eukaryotic microparasites infecting bloom-forming dinoflagellates, belonging to *Parvilucifera* (Perkinsozoa) and *Amoebophrya* (Syndiniales) genus, have already been reported in the literature (Park et al., 2004). The infection of *Alexandrium tamarense* by the parasitoid *Amoebophrya* sp. has been notably described as a way to control the blooms of this species (Lu et al., 2016, 2014). In the case of *Ostreopsis* sp., no study has described specific parasite infection of this dinoflagellate although, it could be a way to control proliferation of this dinoflagellate.

3.2.5. Perspectives and new tools to study MCE of *Ostreopsis* spp.

This review highlights that, in a relatively short time, i.e. along the 4-6 weeks of average *Ostreopsis cf. ovata* bloom duration in the Ligurian Sea (Mediterranean Sea), this dinoflagellate affects the ecosystems structure and functioning. It can be envisaged that, in a context of global change leading to an intensification of *Ostreopsis* sp. blooms, these effects could increase, increasing the ecosystem imbalances already posed by anthropogenic pressures in the coastal zone. The question that arises is whether the ecosystems could adapt to increased *Ostreopsis* sp. blooms, in terms of detoxification and acclimation processes. Most research is focused on *Ostreopsis cf. ovata*, both in terms of toxin quantification and impacts on other marine organisms given its wider distribution in the world

Next, we present as a main outcome of this review the scientific priorities to be addressed to estimate the effect of *Ostreopsis* sp. on ecosystems, i.e., its chemical ecology. These include: establishing an ecotoxicology experimental harmonization protocol with (1) ecologically realistic concentrations of microalgal cells, (2) toxin quantification of the tested strains, and (3) the use of a common organisms to facilitate comparisons among studies.

3.2.5.1. Harmonization of bio-assays

Several studies have documented the extreme sensitivity of *Artemia franciscana* and *Artemia salina* when exposed to *Ostreopsis* cells, cell filtrates, and toxin extracts, highlighting the relevance of this *Artemia* as a model organism (Table 7). In order to compare the toxicity of strains, cell filtrates and toxin extracts, it requires a protocol harmonization in terms of temperature used, of measured parameters and units (i.e. median lethal concentration - LC₅₀ - at 24/48/96 hours, lethal time -LT₅₀ or Effect Concentration - EC₅₀ expressed in mg.mL⁻¹ or cells.mL⁻¹) and larval stages. Indeed, Faimali et al. (2012) have demonstrated strong differences of toxicity of *Ostreopsis ovata* strains cultivated at 20 or 25°C with a higher toxicity of strains maintained at 25°C. This bioassay presents a wide range of advantages compare to other bioassays such as the Mouse Bioassay (MBA) in terms of ethics, cost, time and simplicity. However, the responses of *Artemia* sp. exposed to toxins produced by *Ostreopsis* sp. are not specific since we only measured the mortality rates of this organism. The usefulness of this bioassay is based on the assessment of *Ostreopsis* strains toxicity but also on a pre-screening of other toxic metabolites produced by these species. Indeed, the production of other toxic by *Ostreopsis* sp. which do not belong to the OVTX family, has been suggesting by the study of Ternon et al. (2018) and confirmed by the work of Pavaux et al. (2019 – In prep.), showing the toxicity of *Ostreopsis* cf. *ovata*'s fractions free of OVTXs. This highlight the importance of this bioassay for a pre-screening of toxic fractions before using untargeted analysis by HPLC-MS/MS. Most of the time, the quantification of toxins produced by *Ostreopsis* sp. is done by targeted analysis, i.e. by the quantification of ions belonging to the family of OVTXs (Three transitions per analogue are monitored for OVTX-a to -f and isob-PLTX: [M+2H]²⁺, [M+2H-H₂O]²⁺ and [M+3H-H₂O]³⁺, Brissard et al., 2014). Thanks to this bioassay, it would be more interesting to do untargeted analysis on fragments previously shown as toxic with the bioassay.

3.2.5.2. Metabolomics and other « omics » tools

The development of “omics” techniques, which allowed the simultaneous and large-scales study of genes, transcripts, proteins and metabolites without *a priori* on their biological functions, also constitute an important tool for the study of new toxic compounds. In the field of marine chemical ecology, metabolomics approaches are the most commonly used (Kuhlisch and Pohnert, 2015; Poulin and Pohnert, 2019; Prince and Pohnert, 2010).

Table 7 : *Artemia* bio-assay for *Ostreopsis* toxicity. Median lethal concentration (LC₅₀); Median lethal time (LT₅₀).

Species/Strain	Organisms	Stage	Effects	References
<i>Ostreopsis ovata</i> isolated by A. Penna	<i>Artemia salina</i> (Linnaeus, 1758)	II-III stage nauplii	LC _{50-48h-20°C} = 12 cells.mL ⁻¹ LC _{50-48h-25°C} < 4 cells.mL ⁻¹	(Faimali et al., 2012)
<i>Ostreopsis cf. ovata</i> Isolated from Lazio & Marche	<i>Artemia sp.</i>	Nauplii	EC ₅₀ calculated but not expressed	(Guerrini et al., 2008)
<i>Ostreopsis cf. ovata</i>	<i>Artemia salina</i> (Linnaeus, 1758)	Nauplii	LC _{50-24h-20°C} = 15 cells.mL ⁻¹	Found in Giussani et al., 2016. Unpublished data
<i>Ostreopsis cf. ovata</i> CBA29-2012	<i>Artemia salina</i> (Linnaeus, 1758)	II – III stages larvae	LC _{50-48h-20°C} = 24.83 / 16.67/ <4 cells.mL ⁻¹ (exponential, stationary and late stationary phase)	(Giussani et al., 2015)
<i>Ostreopsis cf. ovata</i>	<i>Artemia salina</i> (Linnaeus, 1758)	Nauplii	Immobility EC _{50-48h-20°C} < 4 cells.mL ⁻¹	Found in Giussani et al., 2016. Unpublished data
<i>Ostreopsis cf. ovata</i> UNR-05	<i>Artemia salina</i> (Linnaeus, 1758)	Adults	LT _{50-24°C} (200 cells.mL ⁻¹) = 3h	(Neves et al., 2017)
<i>Ostreopsis cf. ovata</i> (cell lysate) Natural samples (Ionian Sea)	<i>Artemia salina</i> (Linnaeus, 1758)	Nauplii (24h after hatching)	LC _{50-24h-25°C} = 0.03 mg.mL ⁻¹	(Pagliara and Caroppo, 2012)
<i>Ostreopsis cf. ovata</i> Strain OOAN0601 (Marche Region)	<i>Artemia sp.</i>	Nauplii	LC _{50-24h-20°C} (live cells) = 8 cells.mL ⁻¹ ± 5 = 115 pg.mL ⁻¹ ± 72 LC _{50-24h-20°C} (lysed cells) = 96 cells.mL ⁻¹ ± 6 = 1376 pg.mL ⁻¹ ± 86 LC _{50-24h-20°C} (extract) = 80 cells.mL ⁻¹ ± 7 = 1146 pg.mL ⁻¹ ± 272	(Pezzolesi et al., 2012)
<i>Ostreopsis cf. ovata</i> Natural samples Gulf of Taranto	<i>Artemia franciscana</i> (Kellogg, 1906)	II – III stages larvae	LC _{50-48h-25°C} = 1.63 cell.mL ⁻¹	(Prato et al., 2011)
<i>Ostreopsis siamensis</i> CAWD73/74/75/96	<i>Artemia salina</i> (Linnaeus, 1758)		LT ₅₀ (250 cells.mL ⁻¹) = 24h Time until Morbidity ₅₀ (250 cells.mL ⁻¹) = 4h	(Rhodes et al., 2000)
<i>Ostreopsis ovata</i> Mediterranean Sea	<i>Artemia salina</i> (Linnaeus, 1758)		Correlation (p<1%) between <i>O. cf. ovata</i> concentration and death of <i>A. salina</i>	(Simoni et al., 2004)
<i>Ostreopsis ovata</i> Mediterranean Sea	<i>Artemia salina</i> (Linnaeus, 1758)		Correlation between <i>A. salina</i> mortality after 24 h and the number of <i>O. ovata</i> cells	Vale et al., 2007 in Ramos and Vasconcelos, 2010
<i>Ostreopsis cf. ovata</i> <i>Ostreopsis fattorussoi</i> Mediterranean Sea	<i>Artemia franciscana</i> (Kellogg, 1906)	II – III stages larvae	LC _{50 – 24h} (MCCV 054) = 81.96 ± 63.3 cells.mL ⁻¹ LC _{50 – 24h} (MCCV 055) = 0.25 ± 0.90 cells.mL ⁻¹ LC _{50 – 24h} (MCCV 057) = 0.0012 ± 0.00033 cells.mL ⁻¹ LC _{50 – 24h} (MCCV 058) = 0.023 ± 0.039 cells.mL ⁻¹ LC _{50 – 24h} (Dissolved OVTXs) = 5.96 ± 2.12 cells.mL ⁻¹ LC _{50 – 24h} (Dissolved PLTX) = 48.42 ± 14.23 cells.mL ⁻¹	Pavaux et al., 2019. In prep.

This recent discipline allows the analysis of a wide range of low molecular weight metabolites of a cell, an organism or excreted, in a given context (Fiehn et al., 2007). One of the distinctive features of the metabolomics approach is that, due to strong diversity of physico-chemical properties of molecules produced by an organism, it is today impossible to describe exhaustively the metabolome with the actual analytic techniques. A method currently used in metabolomic is an approach combining LC-MS/MS and molecular networking and employed as a rapid analytical method to detect toxins (Allard et al., 2016; Quinn et al., 2017; Watrous et al., 2012; Yang et al., 2013). Mass spectrometry-based molecular networking relies on the principle that structurally similar molecules share similar MS/MS fragmentation patterns, thus giving a visual representation of molecular relatedness of compounds in a sample (Watrous et al., 2012). For instance, this technic allowed the quantitative identification of the lipopeptide malyngamide C by different strains of cyanobacteria (Winnikoff et al., 2014). More and more studies use metabolomic approaches to investigate biotic interactions between HAB and their environment (Giacometti et al., 2013; Poulin et al., 2018; Poulson-Ellestad et al., 2014) but this approach is still barely applied to *Ostreopsis* spp. study. The production of organic compounds not linked to OVTXs family, using notably untargeted metabolomics approach, reported by Ternon et al. (2018) underline the urgent need to characterize *Ostreopsis* spp. metabolome.

The review of McLean (2013) on “omics” tools have demonstrating the relevance of genomics, transcriptomics and proteomics approaches to advance our understanding on the ecology of HAB. These techniques could give insights in metabolic and regulatory pathways involved in toxins and other secondary metabolites production and the mechanisms of action of these metabolites in the target organisms. These techniques are still not very widely applied to *Ostreopsis* spp. although proteomic and metabolomics coupled-analyses lead to a better understanding of allelopathy interactions between *Karenia brevis* and co-occurring species (Poulson-Ellestad et al., 2014).

3.2.5.3. Isolation and toxicity evaluation of each PLTX analogs

To date, only pure palytoxine standard is commercialized, no OVTX standard are available which restricts our knowledges on bioactivity and structure of PLTX analogs. Indeed, these studies need large amount of pure toxins. Poli et al. (2018) have reported differences of toxicity between PLTX and some of its congeners (42-OH-PLTX and OVTXa) by exposing rats to these toxins trough intraperitoneal routes and aerosol exposures and confirmed results already published by others colleagues (Ciminiello et al., 2010; Pelin et al., 2016; Suzuki et al., 2012; Tubaro et al., 2011). Suzuki et al., 2012 have evaluated huge differences of toxicity between OVTX a and PLTX: OVTX-a is 10 to 100 times less potent than the other but data obtained by (Poli et al., 2018) showed only minimal differences. Such differences confirmed difficulties associated with the use of the toxins due to the use of different analogs between Mediterranean and Adriatic strains.

Chapitre 2. Interactions allélopathiques entre *Ostreopsis* cf. *ovata* et une diatomée co-occurente

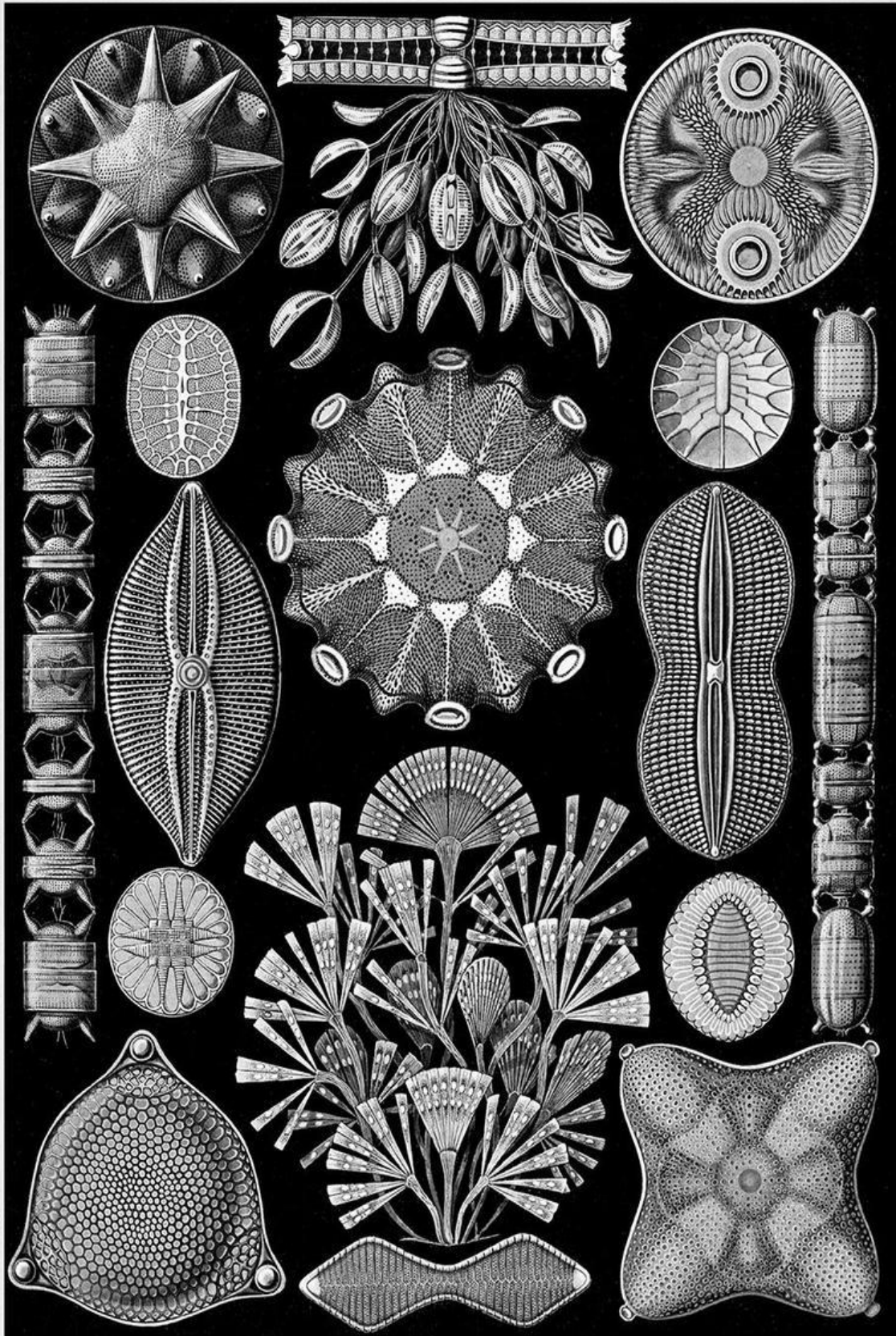


Illustration of diatoms. The 84th plate from Ernst Haeckel's "Kunstformen der Natur" (1904)

1. Présentation de l'étude

Dans le cadre de mes travaux de Master 2, j'ai étudié les interactions chimiques existant entre *Ostreopsis cf. ovata* et la diatomée benthique *Licmophora paradoxa*. Des expériences *in vitro* associées à des observations *in situ* datant de l'été 2015 suggèrent une compétition entre ces deux microalgues (Marro et Lemée, comm. pers.). Elles possèdent des niches écologiques proches mais *L. paradoxa* disparaît avec la prolifération des cellules d'*O. cf. ovata*. L'hypothèse de travail était alors que les toxines produites par *O. cf. ovata* pouvaient affecter le développement de la diatomée.

Afin de confirmer cette hypothèse, des tests biologiques permettant de d'évaluer l'effet des filtrats (exométabolome) sur les cultures algales, ont été couplés avec des co-cultures. Un système de co-culture innovant a été développé afin de permettre la culture sans contact des deux partenaires grâce à une séparation membranaire autorisant uniquement l'échange de métabolites secondaires. Les paramètres étudiés afin d'estimer le potentiel effet néfaste d'*O. cf. ovata* sur la diatomée et la rétroaction de celle-ci, sont la croissance cellulaire, l'activité photosynthétique du photosystème II ainsi que la croissance des communautés bactériennes associées à chacune des macroalgues. Une approche de métabolomique a également été utilisée afin d'étudier l'endo- ainsi que l'exométabolome des deux partenaires.

2. Allelopathic interactions between the benthic toxic dinoflagellate *Ostreopsis cf. ovata* and a co-occurring diatom

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Allelopathic interactions between the benthic toxic dinoflagellate *Ostreopsis cf. ovata* and a co-occurring diatom

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Keywords: *O. cf. ovata*, benthic diatom, exometabolome, allelochemistry, photosynthetic activity

2.1. Abstract

For decades the microphytobenthos assemblage in the coastal Mediterranean Sea has been regularly colonized by the toxic benthic dinoflagellate *Ostreopsis cf. ovata*. This harmful algal species is a toxin producer and occupies the same ecological niche as various diatoms. Surprisingly, there are only few insights reported on the physiological responses of diatoms to blooms of *O. cf. ovata*. The chemical interactions of *O. cf. ovata* with the co-occurring diatom *Licmophora paradoxa* was studied using a bioassay (measuring impact of cell-free culture filtrate) and a co-culture approach (separate by a membrane) to investigate the effects of the exometabolome and its mode of action. Bioassays highlighted a toxic effect of the exometabolome of *O. cf. ovata* on the diatom photosynthetic activity. However, the co-cultures revealed that these toxic effects do not occur through remote allelopathy. Contact or close interactions between cells of the two species is most likely needed to impair the diatom growth. Ovatoxins are suspected to be the toxic metabolites secreted by *O. cf. ovata* although the current set of data did not give confirmation of this assumption. Interestingly, the exometabolome of *L. paradoxa* impaired the growth and the photochemistry of *O. cf. ovata* in both bioassays and co-cultures. Some biomarkers possibly involved for the effect were identified using a metabolomic approach and may correspond to oxylipins, however a bacterial source of the bioactive metabolites is also considered.

2.2. Introduction

The concept of planktonic allelopathy originated from the “paradox of the plankton” by (Hutchinson, 1961) who outlined that the large diversity of planktonic species in an ecosystem with limiting resources could only coexist thanks to a close balance among cell concentrations, nutrients and light availability. Later, as the roles of swimming, mixotrophy and allelopathy were better understood, Hutchinson’s theory was revised (Smayda, 1997b).

Allelopathy is the release of chemical compounds, the so-called allelochemicals, by individual of a population, which can have negative effects on individuals of another species in the community (Ianora et al., 2011; Legrand et al., 2003; Rice, 2012). Allelochemicals are specialized metabolites biosynthesized by the cells likely involved in a chemical competition that contribute to the success of certain species (Wolfe, 2000). Consequently, allelopathy can exhibit a major impact driving the succession of species in the planktonic communities (Keating, 1977), and is now recognized as a key component of the competition between microalgae (Schwartz et al., 2016 for a review). Nevertheless, our understanding of those interactions is still very limited, especially due to the difficulty of identifying the allelochemicals involved, their structure complexity, but also their fates in the water medium (Gillard et al., 2013; Selander et al., 2015).

Among microalgae, dinoflagellates can form blooms (Harmful Algal Bloom, HAB) usually associated with important toxicities. The oceans are currently facing an increase of HABs events, inducing serious socioeconomic impacts in many maritime regions (Anderson et al., 2012; Zingone and Oksfeldt Enevoldsen, 2000). The factors facilitating HABs are still controversial although environmental factors like an increase in temperature, the availability of nutrients or the stratification of the water column are frequently suggested (Pitcher et al., 2010). The toxins produced by these micro-algal species may be a key-factor explaining their ecological success. They are suspected to act as allelochemicals influencing biotic interactions and may, therefore, be involved in structuring the community. Even if poorly understood, the ecological role of toxins may be deterrence against grazers as (Adolf et al., 2006; Hong et al., 2012; Kubanek et al., 2007; Selander et al., 2006), help for prey catching (Blossom et al., 2012; Breckels et al., 2011) or structuration of microbial communities (Sipler et al., 2014; Weissbach et al., 2010). One major question in HAB research has been whether these algal toxins play a role in establishing or maintaining the bloom events (Kubanek et al., 2005; Prince et al., 2008).

Benthic dinoflagellates of the genus *Ostreopsis* Schmidt are known to be the origin of seafood poisoning in tropical areas and some species are toxic by direct contact or inhalation of bioaerosols in temperate regions (Berdalet et al., 2017). In the Mediterranean Sea, blooms of *Ostreopsis* cf. *ovata* have been related to several respiratory and dermatological symptoms together with general weakness, headache, fever and eye irritations Vila et al. (2016) for a review). These toxic episodes led

to a thorough investigation of the chemical content of these species which revealed the presence of palytoxin analogs, named ovatoxins a to h; (Brissard et al., 2015; Ciminiello et al., 2010b, 2012a). Ovatoxins were thought to be responsible for the observed toxicity (Amzil et al., 2012; Ciminiello et al., 2008). The ecological impact of *O. cf. ovata* has not been fully assessed despite preliminary studies on members of the benthic and planktonic communities such as macro-algae (Accoroni et al., 2015b), microalgae (García-Portela et al., 2016; Monti and Cecchin, 2012; Vila et al., 2001), crustaceans (Faimali et al., 2012), sea urchins (Amzil et al., 2012; Brissard et al., 2014; Privitera et al., 2012; Shears and Ross, 2009), bivalves (Amzil et al., 2012), jellyfish (Giussani et al., 2016), *Artemia* (Neves et al., 2017) and cephalopods (Lopes et al., 2013). Whilst various benthic microalgae were shown to co-occur in the same ecological niche as *O. cf. ovata*, like diatoms (*Coscinodiscus* sp. Ehrenberg, 1844 ; Vila et al., 2001) or dinoflagellates (*Coolia monotis* Meunier, *Prorocentrum lima* Ehrenberg, 1844 ; (García-Portela et al., 2016; Vila et al., 2001); the growth of other species seem to be affected by the presence of *O. cf. ovata* (*Licmophora paradoxa* Lyngbye, 1828, *Coscinodiscus* spp.; Lemee, pers. observations). Allelopathic interactions may be involved in the ability of some species to co-occur (or not) with *O. cf. ovata*. The chemical interactions between toxic dinoflagellates and competitors are very specific with either reduced or enhanced growth observed among species (Kubanek et al., 2005; Poulson-Ellestad et al., 2014; Prince et al., 2008). Weak to moderate allelopathic interactions have been observed between *O. cf. ovata* and *Coolia monotis* but also *Prorocentrum minimum* (Pavillard) Schiller in the Atlantic and Mediterranean areas respectively (García-Portela et al., 2016; Monti and Cecchin, 2012). Other competitors should be investigated together along with the nature and the mode of action of their potential allelochemicals.

Since microalgal toxins are not necessarily involved in allelopathic interactions (Kubanek et al., 2005; Poulson-Ellestad et al., 2014; Prince et al., 2008), the analysis of the extracellular toxin content may be insufficient to fully understand the mechanisms of allelopathy. Metabolomic approaches represent a potent analytical and statistical tool that can lead to the identification of biomarkers possibly involved in the chemical communication (see for a review Kuhlisch and Pohnert, 2015) as they enable the untargeted detection of a large panel of specialized metabolites. The recent identification of L-dipropine as a kairomone secreted by diatoms (Gillard et al., 2013) demonstrated the efficiency of metabolomics in the investigation of allelochemicals. In this context, we studied the allelopathic interactions between *O. cf. ovata* and one of its potential competitor, the diatom *Licmophora paradoxa*. The choice of the diatom species was motivated by its co-occurrence in the benthic assemblage of the northwestern Mediterranean Sea, as well as a marked decrease in its abundance observed when the dinoflagellate bloom starts (Lemée, pers. obs.). Bioassays (measuring impact of cell-free culture filtrate) and a co-culture (microalgae separated by a membrane) were both used to investigate a putative chemical interaction, followed by targeted and untargeted metabolomics

analyses to further determine the metabolites responsible for this interaction, focusing first on ovatoxins, and then on other potential allelochemicals.

2.3. Methode

2.3.1. Microalgal cultures

Strains of *Ostreopsis cf. ovata* (MCCV 054) and *Licmophora paradoxa* (MCCV 033) originally isolated from France (bay of Villefranche-sur-mer in 2014, Rochambeau site), were cultured in 100 mL and 300 mL flasks in the laboratory (for the bioassay and co-culture, respectively) in autoclaved and filtered aged-seawater, at a salinity of 38 g.L⁻¹, adding nutrients at L1 concentration. All flasks were incubated at 24 °C under a 14:10 D/L cycle (250 μmol m⁻² s⁻¹) for 7 days (exponential phase) before the transfer of the cells for the experiments.

2.3.2. Bioassays

A first assessment of a chemical mediation between both species was undertaken by observing the responses of the algal cells to the addition of the cell-free filtrate obtained from the other microalgal culture: (i) *L. paradoxa* cells versus *O. cf. ovata* cell-free filtrate, (ii) *O. cf. ovata* cells versus *L. paradoxa* cell-free filtrate. A control was also performed for both species adding sterile L1 medium instead.

An aliquot of 2 mL was collected from the 100 mL culture flasks for cell counting. Then, the homogenized remaining algal culture was split into a 70 mL volume of culture to obtain the cell-free filtrate and 30 mL to perform the bioassay. The 70 mL aliquots were centrifuged (1 800 g for 15 min) to retrieve the cells and the supernatant was filtered through a 0.2 μm membrane (Whatman RC 58) to remove any remaining bacteria.

Using the remaining 30 mL of culture, twelve culture tubes were inoculated with 2 mL of L1 medium and 2.5 mL of *O. cf ovata* culture (3 505 cells.mL⁻¹) and the same was done with *L. paradoxa* (7 886 cells.mL⁻¹). All tubes were incubated for 3 days at the conditions previously described to reach the log phase. Cell-free filtrates were then added to the culture tubes at a 1:10 ratio (500 μL) and the cultures were further incubated for 48 h. The physiological state of the cells was assessed by measuring the *in-vivo* fluorescence (see details of the method below) at +24 h.

2.3.3. Co-cultures

2.3.3.1. Device

The co-culture device was home-designed and based on the system developed by (Paul et al., 2013) and consists of two flat-bottom glass bottles with a 100 mm flat edge opening on their median part and a 30 mm round neck on the top (Figure 6). The flat-bottom bottles were chosen to allow an optimal growth of the benthic microalgae. Both bottles are connected together by holding a clamp and a 0.22 μm hydrophilic PolyVinylidene Fluoride (PVDF) membrane filter (142 mm diameter, Durapore[®] Millipore GVWP14250, Merck Millipore, Cork Ireland) was placed to separate the two growth chambers. The top opening was used for filling and sampling purpose and was closed using porous foam. All items of this device (bottles, clamp, membrane filters and foam) were autoclaved (120 °C, 20 min) before use and were assembled under a laminar hood.

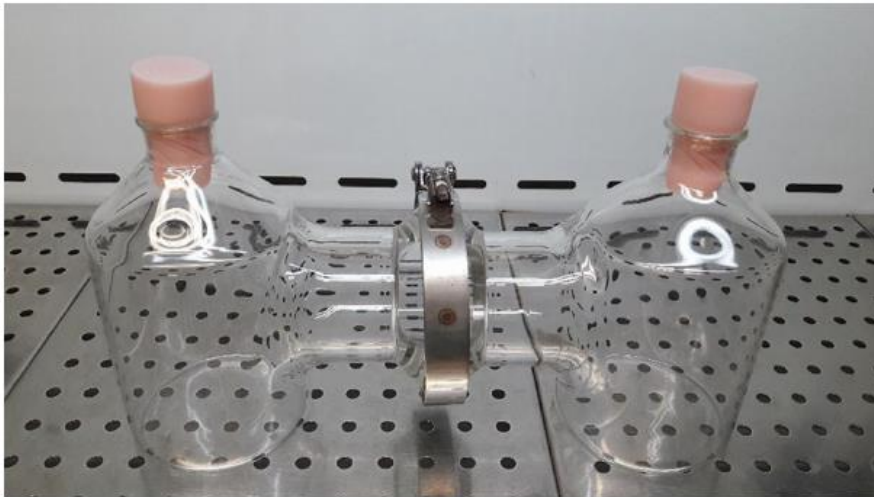


Figure 6 : Co-culture device with the two flat growth chambers connected by the metabolome was performed at day 0 using the initial 300mL clamp.

2.3.3.2. Experiments

Following the assemblage of all parts, the co-culture chambers were filled up with 700 mL of sterile L1 medium before cells inoculation. A total of 9 devices (18 chambers) were used in order to have the following conditions in triplicate: (i) *control 1 (C1)* consisted of one chamber free of cells (Blank chamber) and the other inoculated with *O. cf. ovata*, (ii) *control 2 (C2)* consisted of one chamber free of cells (Blank chamber) and the other inoculated with *L. paradoxa*, and (iii) *treatment 3 (T3)* consisted of one chamber inoculated with *O. cf. ovata* and the other with *L. paradoxa*. Cell concentrations at the time of inoculation were measured as 1 147 cell mL⁻¹ for the dinoflagellate and 7 215 cell mL⁻¹ for the diatom, corresponding to a ratio of 6:1 (*O. cf. ovata*: *L. paradoxa*) that is consistent with a smaller biovolume of *L. paradoxa* regarding *O. ovata*. The cells inoculation was performed at Day 0 and no measurements were performed before day 3 to allow a period of adaptation. The experiments lasted for 13 days and aliquots of seawater were sampled in all chambers

at days 0, 3, 5, 7, 10, 12 and 13 for further analysis. Manual agitation was performed twice a day for homogenization by gently stirring each device to ensure diffusion through the membrane.

2.3.3.3. Cell counting

An aliquot of 11 mL was collected at each sampling day and fixed with acidic lugol (HCl 4 % v/v) before being stored at 4 °C until analysis. Even if a more acidic solution (4 mM HCl) was reported to ensure mucus dissolution (Guerrini et al., 2010), it was not used in our case since preliminary results indicated cell lysis at this pH. Cell counting was performed using a HIAC (Hiac/Royco, Pacific Scientific Instruments) following a size range of 2-80 µm (Stramski et al., 2002).

2.3.3.4. Photosynthetic activity

PSII (PhotoSystem II) function was assessed by fast repetition rate fluorometry (Kolber et al., 1998) using a MC-PAM (Multi-Color Pulse-Amplitude-Modulated, Heinz Walz GmbH, Effeltrich, Germany). For a full description of the method see Cosgrove and Borowitzka (2006). In this study, only the maximum quantum yield of PSII (Fv/Fm ratio) was used (Kolber et al., 1998). The Fv/Fm ratio gives insight into the photosynthetic capacity and the current light adaptation state of a sample. An aliquot of 1.5 mL was collected in all chambers of the co-cultures at each sampling day and all aliquots were quickly transferred to a glass cuvette containing a stir bar to avoid cell sedimentation. Prior to fluorescence measurement, samples were dark-acclimated at 24 °C for 15 min to re-open PSII reaction centers and relax non-photochemical quenching. The fluorescence measurement protocol consisted of saturation pulses of light (intensity 15, width 300 ms, gain 3). Curve-fitting software provided with the instrument (PAMwin V3.20W) was used to obtain Fv/Fm. All curve fits and fluorescence transients were manually inspected in real time.

2.3.3.5. Microbial community

Analysis of the abundance of heterotrophic bacteria was performed at each sampling day. The methods and the results are presented and further discussed in the Supplemental Information.

2.3.3.6. Study of the metabolomes

2.3.3.6.1 Extraction of the endo- and exo-metabolome of both microalgae

Sampling for the study of the endo- and the exo-metabolome was performed at day 0 using the initial 300 mL culture flasks (used for inoculation) and at day 13 using the content of all chambers (700 mL). The volume of culture was gently passed through two filters of decreasing porosity (8 µm Nitrocellulose SCWP, Millipore, and 0.2 µm regenerated cellulose RC 58, Whatman) using a manual

pump to subsequently remove algal and bacterial cells from the medium. The cell-free filtrate and the 8 µm filters were used for the analysis of the exometabolome (EX) and the endometabolome (EN) respectively. The 8 µm filters (EN) were placed in Teflon tubes and further extracted in an ultrasonic bath using 3 x 5 mL of MeOH in an ice bath for 40 min (*O. cf. ovata*) and 3 x 5 mL of MeOH:CH₂Cl₂ (1:1) for 20 min (*L. paradoxa*) respectively. Extracts were passed through a Whatman filter paper and evaporated under a stream of nitrogen after transfer to a 20 mL glass vial. The dry extracts were solubilized in MeOH at a concentration of 10 mg.L⁻¹ and further stored at -20 °C until analysis. The cell-free filtrate (EX) was extracted onto C₁₈ discs (Restek®) under *vacuum* to trap dissolved organic compounds (i.e. potential allelochemicals). The extraction and elution using the C₁₈ disc was performed following the procedure provided by the supplier with MeOH as the eluent (2 mL for activation, 8 mL for conditioning and 2 x 10 mL for elution). The organic extracts were evaporated to dryness under a stream of nitrogen and solubilized in 500 µL of MeOH before being stored at -20 °C until analysis.

2.3.3.6.2 Analysis of the separating PVDF membrane

To test any adsorption of the ovatoxins and other metabolites onto the PVDF membranes, the separating membranes were cut into pieces and introduced in a 20 mL vial for extraction with 5 mL of MeOH in an ultra-sonic bath for 15 min. The resulting extracts were further filtered on 0.2 µm and evaporated using a SPD111 SpeedVac (ThermoSavant, Model RH12-28) before resolubilization into 300 µL of MeOH. All samples were stored at -20 °C until analysis.

2.3.3.7. UHPLC-HRMS analyses

On-line UHPLC-UV-HRMS analysis was performed using a Dionex system Ultimate 3000 equipped with an autosampler and a Dionex Ultimate 3000 diode array dual absorbance wavelength detector, connected to a QToF mass spectrometer fitted with an electrospray ionization source (Bruker Impact II). Mass spectra were recorded in positive mode due to the presence of a primary amine in ovatoxins (Brissard et al., 2015; Ciminiello et al., 2012a). UHPLC separation was achieved on a FluoroPhenyl column (ACQUITY UPLC CSH 1.7 µm, 130 Å, 2.1 x 100 mm, Waters) using a linear elution gradient of H₂O/CH₃CN/formic acid from 90:10:0.1 (v/v/v, isocratic from 0 to 2 min) to 0:100:0.1 (v/v/v, isocratic from 12 to 15 min) at a flow rate of 0.4 mL min⁻¹. The injected volume was 10 µL and detection set at 210, 236, and 263 nm. The spectrometer analyzer parameters were set as follows: nebulizer sheath gas, N₂ (5.8 psi); dry gas, N₂ (4 L.min⁻¹); capillary temperature, 180 °C; capillary voltage, 4,500 V; end plate offset, 500 V; collision gas, He; collision energy, 7 eV. Data were acquired in the positive mode, at the 200 to 3000 *m/z* range.

The evolution of the EX and the EN was followed by UHPLC-HRMS using untargeted metabolomics but also ovatoxins quantification. The ovatoxins quantification was also used to analyze the PVDF membrane content. Metabolomics approach for the analysis of EX involved the preparation of Quality Control (QC) samples by combining 20 μL of each sample in a 2 ml vial. These QC samples were injected every 6 samples to allow chromatogram alignment during data treatment. All EX samples were analyzed following a random order. Ovatoxins quantification was performed by extracting the corresponding ions from the full scan analyses in each EN, PVDF and EX samples using their $[M+2H]^{2+}$ ion, listed in Table 8. Quantitative determination of ovatoxin-a, -b, -c, -d, -e, -f and -g in extracts and medium was carried out using palytoxin as a standard (Wako Chemicals GmbH, Neuss, Germany), assuming that their ionization potential is identical, at concentrations of 1, 2.5, 5, 7.5 and 10 $\mu\text{g}/\text{mL}$. From this calibration curve, we were also able to set the limit of detection of the instrument at 2 $\mu\text{g}/\text{mL}$ for this family of compounds. A collision energy (CE) of 40 eV was applied for bi-charged ions $[M+2H]^{2+}$ of ovatoxins to give the characteristic fragment ions at m/z 327, 343 or 371 (A moiety; Table 8). The biomarkers ions highlighted by the untargeted metabolomic analysis were also fragmented at 40 eV or at 70 eV if not sufficient to detect any fragment.

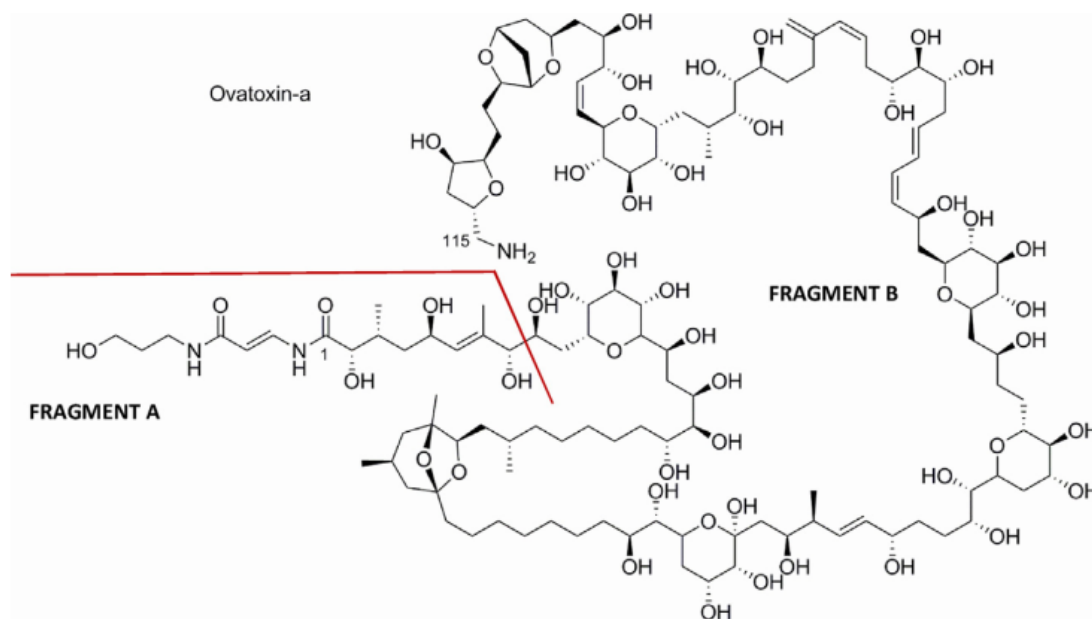


Figure 7 : Structure of the ovatoxin-a and its major fragments A and B.

2.3.3.8. Untargeted metabolomics approach data processing

Following UHPLC-UV-HRMS data acquisition, MS chromatogram (Base Peak chromatogram – BPC) were exported as line spectra and converted into the netCDF file format to process the data in centroid mode with XCMS (Smith et al., 2006) on R software (version 3.2.2.). The XCMS approach

involved several steps necessary to generate a final matrix: (1) Peak picking (peakwidth= c(2, 20), ppm=10) without threshold prefilter (Patti et al., 2012), (2) retention time correction (method="obiwarp"), (3) grouping (bw=10, minfrac=0.3, minsamp=1), (4) Fillpeaks using a parameter of "noise level" that sets the minimum intensity for a centroid data point to be considered as part of a peak (noise level = 10^3), and finally (5) report and data matrix generation (m/z / Retention time) that was exported into Microsoft Excel. Two successive filtering steps were applied to the matrix on R in order to suppress the data of high analytical variability: (i) variables are not considered if their mean intensities in the samples extracts is less than 10-fold the values obtained for the blank samples (culture medium extracts), and (ii) coefficient of variation in the intensity of the variables for the QC samples > 20 %. Each peak area was further normalized, mean-centered and finally log-transformed using on-line MetaboAnalyst 3.0 (Xia et al., 2015). Multivariate analysis was conducted (Principal components analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) and the discriminant PLS-DA was further chosen for further discussion and identification of the variable important for the discrimination. Variable Importance in Projection (VIP) are identified by PLS-DA together with the relative concentration of the corresponding metabolites for each group of samples under study.

2.3.3.9. Statistical analysis

The statistical significance of the variation of the various parameters measured (ovatoxin concentration, photosynthetic activity, cell abundance) between conditions was evaluated applying a Wilcoxon-Mann-Whitney test on the data set, using the R software.

Table 8 : List of the ovatoxins investigated in this study and their major ions.

Toxins	Elementary formulae	MW Da	[M+2H] ²⁺	[M+2H-H ₂ O] ²⁺	[M+3H-H ₂ O] ³⁺	A = [M-Fragment B-H ₂ O] ⁺
Ovatoxin-a	C ₁₂₉ H ₂₂₃ N ₃ O ₅₂	2646.48	1324.3	1315.3	877.2	327.19
Ovatoxin-b	C ₁₃₁ H ₂₂₇ N ₃ O ₅₃	2990.51	1346.3	1337.3	891.8	371.21
Ovatoxin-c	C ₁₃₁ H ₂₂₇ N ₃ O ₅₄	2706.51	1354.3	1345.3	897.2	371.21
Ovatoxin-d	C ₁₂₉ H ₂₂₃ N ₃ O ₅₃	2662.48	1332.3	1323.3	882.5	327.19
Ovatoxin-e	C ₁₂₉ H ₂₂₃ N ₃ O ₅₃	2662.48	1332.3	1323.3	882.5	343.18
Ovatoxin-f	C ₁₃₁ H ₂₂₇ N ₃ O ₅₂	2676.20	1332.3	1329.3	886.5	327.30
Ovatoxin-g	C ₁₂₉ H ₂₂₃ N ₃ O ₅₁	2630.49	1316.25	1307.2	871.8	327.19

2.4.1. Exometabolomes activity assessed by bioassays

The bioactivity of the exometabolomes (EX) of both *Ostreopsis cf. ovata* and *Licmophora paradoxa* was tested on the other species. A small aliquot of the EX (10% of the final volume) was added to the culture at early log phase and the in vivo fluorescence of the microalgae was measured after 24 h of incubation (Figure 8) as a representative measurement of the cells physiological state.

A significant decrease of the *in vivo* fluorescence ($F_v/F_m < 0.1$) of *L. paradoxa* was measured after 24 h in the presence of EX of *O. cf. ovata*. Interestingly, a decrease in the *in vivo* fluorescence was also measured for *Ostreopsis* cells after addition of the EX produced by *Licmophora* ($F_v/F_m < 0.430$).

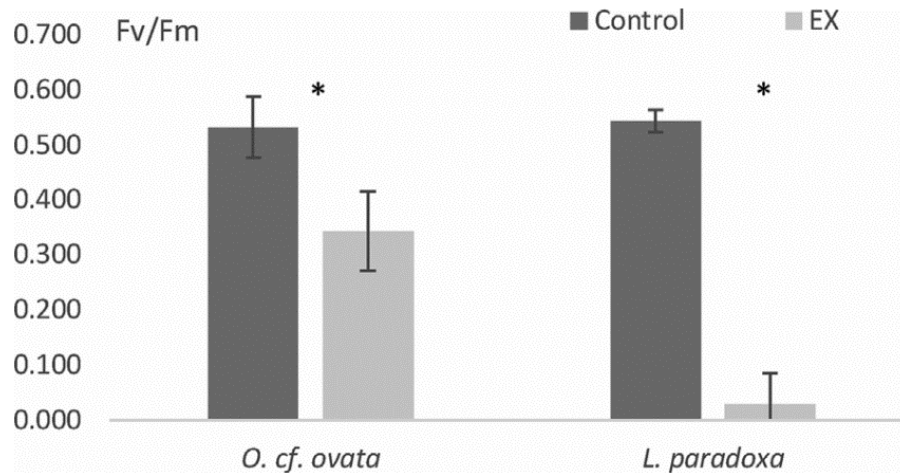


Figure 8 : Effect of the EX of (i) *L. paradoxa* onto the *in vivo* fluorescence (F_v/F_m) of *O. cf. ovata* (left) and (ii) *O. cf. ovata* onto *L. paradoxa* (right). Controls were performed with sterile media. Statistical significance was assessed using a Wilcoxon-Mann-Whitney test (* $p < 0.1$). errors bars correspond to standard error between samples ($n=4$).

2.4.2. Physiological effects of distant chemical interactions using co-cultures

The first results on the toxicity of each EX obtained from the bioassays guided the co-culture experiment set up. A distant deleterious allelopathic effect of *O. cf. ovata* onto *L. paradoxa* physiology was therefore expected in co-culture experiments. Interestingly, the foreseen effect was not observed while the presence of *L. paradoxa* in the second chamber affected the abundance and the physiological state of *O. cf. ovata* cells in the co-cultured chamber. The growth exponential phase lasted 10 and 7 days for *O. cf. ovata* and *L. paradoxa* respectively and remained stationary until the end of the experiment at day 13 (Figure 9 A and B). Both the cell abundance and the physiological state, assessed by the *in vivo* fluorescence, showed no significant differences between the mono-culture control and the co-culture for most of the duration of the experiment for *L. paradoxa* (Figure 9 B, D) except at day 10. On the contrary, *O. cf. ovata* growth was reduced throughout the experiment as shown by the significant decrease in both the cell abundance and the *in vivo* fluorescence at days 5, 12 and 13 (Figure 9 A and C). This effect was consistent with the decrease of the *in vivo* fluorescence of *O. cf. ovata* cells in presence of the EX of *Licmophora* observed in the bioassays. The diatom culture contains metabolite(s) that presents deleterious effects on *O. cf. ovata* photosynthetic apparatus. These metabolites can diffuse through the PVDF membrane whereas the toxic metabolites produced in *O. cf. ovata* cultures possibly did not diffuse through the separating PVDF membrane.

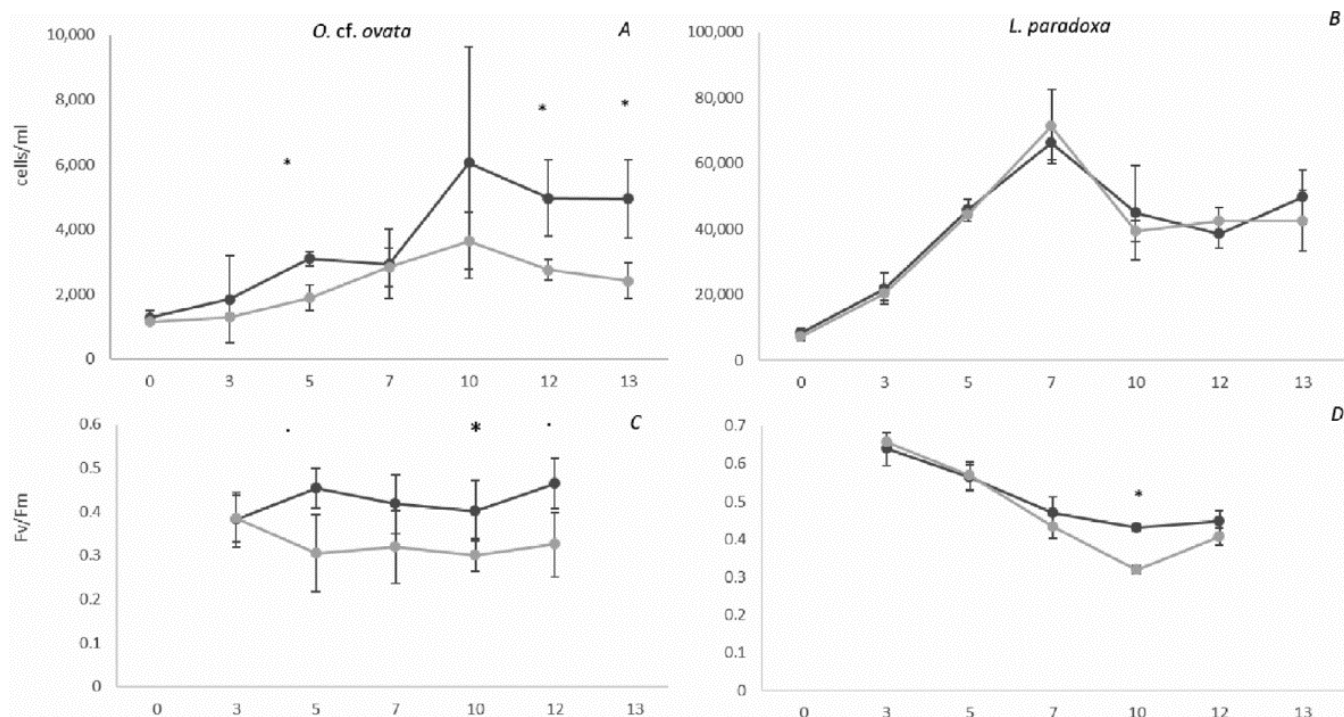


Figure 9 : Abundance of cells (cells/mL) and in vivo fluorescence (Fv/Fm ratio) of control (dark grey) and co-cultured (light grey) chambers for *O. cf. ovata* (A, C; left side) and *L. paradoxa* (B, D; right side). Statistical significance was assessed using a Wilcoxon-Mann-Witney test (* and · $p < 0.1$ and 0.2 respectively).

2.4.3. Ovatoxin content in EN and EX

The concentration of ovatoxins in *O. cf. ovata* cells (EN) was assessed in both the control and the co-cultured samples to investigate the influence of the presence of other species on the production of toxins. Using a palytoxin standard for mass spectrometry calibration, we evidenced that concentrations of ovatoxins in the control were quite similar to values published on other strains (18 against 15 pg/cell for 3 000 000 cells; Pezzolesi et al., 2012). Figure 10 EN shows a clear increase in the production of ovatoxins by *O. cf. ovata* cells when co-cultured with *L. paradoxa*, and this is especially intense for the ovatoxin-a, from 6.6 ± 0.5 to 17.8 ± 6.6 pg. cell⁻¹ eq. pltx (factor 2.7).

In the same manner, the toxin content in the EX followed a similar trend with an increase of all ovatoxins concentration in the medium of co-cultured samples (from 0.52 to 1.03 pg.cell⁻¹ eq pltx for ovatoxin-a) although these data were not significant due to high inter-samples variability (Figure 10 EX). The measurement of the toxin content in the EN and the EX further allowed an estimation of the percentage of toxins released by the cells in the medium (exudation) for both conditions, control and co-culture (Table 9). Interestingly, the presence of *L. paradoxa* did not enhance the exudation of ovatoxins in the medium despite the increased production in the EN (except for the ovatoxins d/e – Increase by a factor 2).

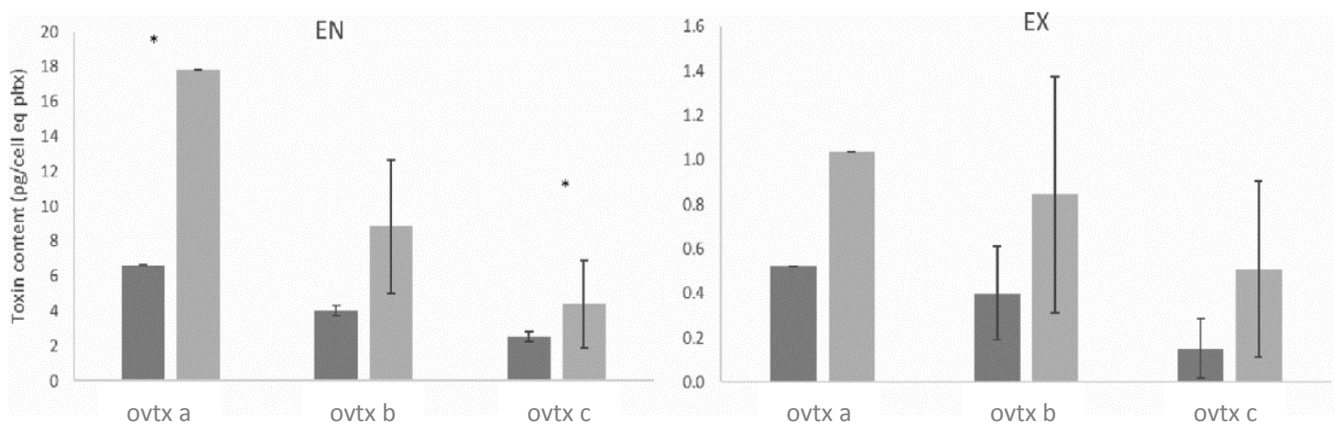


Figure 10 : Toxin content in the (EN) endometabolome and in the (EX) exometabolome normalized by the number of cells ; in controls= cultures (dark grey) and co-cultures (light grey). A palytoxin standard was used to obtain the calibration curve and the concentrations are therefore expressed as an equivalent palytoxin. Statistical significance was assessed using a Wilcoxon-Mann-Whitney test (*, $p < 0.1$). Error bars define the standard errors between samples (n=3).

2.4.4. Exchange of allelochemicals through the membrane

Ovatoxins – Since no deleterious effects of the EX of *O. cf. ovata* was observed on the development of *L. paradoxa* in co-cultures, an absence of diffusion through the PVDF membrane was considered. Figure 10 (part EX) reveals the presence of ovatoxins in the EX of all chambers containing *O. cf. ovata* (1.7 pg. cell⁻¹ eq pltx) whereas on the other side of the PVDF membrane of these co-cultures, no ovatoxins were quantifiable. Ovatoxins did not or just weakly diffuse through the PVDF membrane into the blank or co-culture chambers and we could show this absence of diffusion was not due to an adsorption on the PVDF membrane since no ovatoxins were detected in the PVDF extracts (data not shown).

Other allelochemicals from O. cf. ovata – Interestingly, the untargeted metabolomics approach revealed that other compounds of large molecular weight (2120 Da) also present in *O. cf. ovata* cells were exuded and then diffused through the PVDF membrane (Table in Figure 11A). Indeed, the diatom culture EX contains those compounds when co-cultured with the dinoflagellate (Figure 11A). The three ions at m/z 1046.0720; 1065.0463 and 1080.0515 were identified as discharged adducts of the monocharged $[M+H]^+=2121.1523$: $[M+H+K]^{2+}=1080.07615$, $[M+2Na-2H_2O]^{2+}=1065.07615$ and $[M+2Ca-6H_2O]^{2+}=1046.07615$. It is worth noting that fragmentation experiments were performed on those ions using a collision energy of 40 and 70 eV but no fragmentation was evidenced. Therefore, the compounds diffusing through the membrane do not share the major fragments found for the compounds belonging to the ovatoxin family (327.1923; 343.1861 and 371.2174) and these compounds clearly differ for the part A of the molecule.

Allelochemicals from L. paradoxa – Likewise, the untargeted metabolomics approach revealed the production of metabolites by the diatoms' culture that were able to diffuse through the PVDF membrane (as shown by the presence of several ions listed in the table of Figure 11B for EX of *O. cf. ovata* when co-cultured with the *L. paradoxa*). Those ions showed a mid-polarity (Rt 6.8 min) and a molecular formula was proposed for each of them by the software Data Analysis® and could correspond to small oxylipins (C₁₁, C₁₅ and C₁₈). About 20% of the concentration of those compounds detected in the EX of the diatom culture were retrieved on the PVDF membrane. Interestingly, those ions were not found in the EN of *L. paradoxa*, suggesting that either those compounds are exuded but not stored by the diatom or they have a bacterial origin.

Table 9 : Percentage of exudation (ratio EN/EX of ovatoxin concentration) of all detected toxins in the control and in the co-culture chambers that contained *Ostreopsis* cultures.

	Exudation (%)			
	Control		Co-culture	
	Mean	STDV	Mean	STDV
p-pltx	<LD	<LD	<LD	<LD
ovtxa	7.7	5.0	5.7	2.1
ovtxb	9.5	4.6	9.3	2.4
ovtxc	<LD	<LD	<LD	<LD
ovtxd/e	5.9	3.5	11.9	2.4

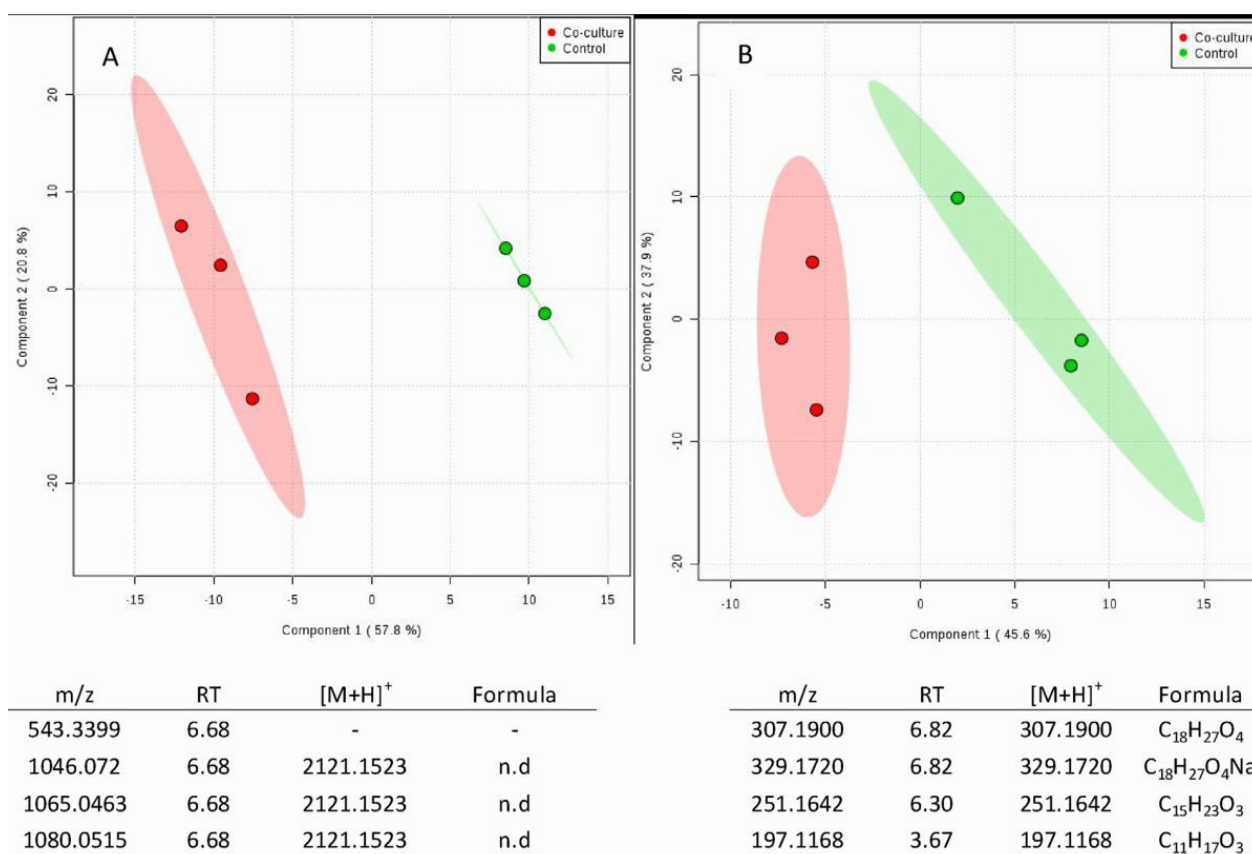


Figure 11 : PLS-DA plots obtained from the untargeted metabolomics analysis on the content of the EX of the chambers containing (A) the diatom *L. paradoxa* and (B) the dinoflagellate *O. cf. ovata*. The ions showed in the tables are the VIP over-expressed in the co-cultured chambers and correspond to compounds secreted by either *O. cf. ovata* (A) and *L. paradoxa* (B) cultures.

2.5. Discussion

The allelopathic activity of the toxic *O. cf. ovata* on the microalgal benthic assemblage has been poorly studied so far (García-Portela et al., 2016; Monti and Cecchin, 2012). In the present study, the diatom *L. paradoxa* was tested for its interaction with *O. cf. ovata* since these microalgae occupy the same ecological niche in the northwestern Mediterranean Sea and the diatom abundance decreases when the dinoflagellate blooms (Lemée, comm. pers). The experimental design allowed the exclusion of any abiotic factors favoring one species over the other on the base of any abiotic limitation, e.g. light, nutrients availability.

2.5.1. *Ostreopsis cf. ovata* chemical interactions may need cell-cell contact

A weak allelopathic activity of *O. cf. ovata* on the benthic dinoflagellate *Coolia monotis* has been observed by testing fresh exudates devoid of cells (García-Portela et al., 2016; Monti and Cecchin, 2012). Conducting similar experiments on the benthic diatom *L. paradoxa* in log phase (e.g. bioassays), the exudate of *O. cf. ovata* clearly impaired its photosystem II (PS II) in less than 24 h, suggesting a stress induced by putative allelochemicals. Allelopathy may be used by *O. cf. ovata* to outcompete other microalgal species as observed for *Karenia brevis* (Kubanek et al., 2005; Prince et al., 2008) or *Microcystis aeruginosa* (Dunker et al., 2017). However, co-occurring species can have a different response to allelochemicals (Kubanek et al., 2005; Poulson-Ellestad et al., 2014; Prince et al., 2008) and more investigation should be undertaken using various benthic microalgae in order to obtain a full comprehension of allelopathic activity of *O. cf. ovata*.

Interestingly, the second set of experiments using co-culture devices did not confirm the allelopathic character of *O. cf. ovata*. The experimental set up prevented the contact between cells of the two species but allowed the diffusion of compounds from one species to the other. As a result, neither the growth nor the PS II of *L. paradoxa* was impaired throughout this 13-days experiment by the presence of *O. cf. ovata*. The potential for exchange of the allelochemicals through PVDF membrane-separated culture chambers is thus not sufficient for an allelopathic control of *O. cf. ovata* onto *L. paradoxa*. This unexpected result indicates the inhibitory compounds exuded by *O. cf. ovata* are not diffused through the filtering membrane and are suspected to remain in the close vicinity of the cells. Indeed, in bioassays, the exudates were obtained by a soft centrifugation and filtration of the cultures. The allelochemicals, if adsorbed onto the cells or the mucus, may have been extracted with the aqueous phase. This would increase the concentration of their free-dissolved form in the exudate, leading to the observed toxicity. In the co-culture experiment, the gentle homogenization of the co-culture chambers performed three times a day might not have been sufficient to dissolve the active compounds from the cells or the mucus precluding from any diffusion through the PVDF membrane. Therefore, contact interaction between cells is most likely needed to impair the growth of the diatom *L. paradoxa*. The existence of aggregates (e.g. mucus) produced by *O. cf. ovata* in which competitors could be maintained at a close distance is a strong indication towards this conclusion. Similar contact interactions were already observed for cyanobacteria (Dunker et al., 2017) or dinoflagellates (Fernández-Herrera et al., 2016; Kubanek et al., 2005; Tillmann, 2003) and is supported by simple mathematical models (Jonsson et al., 2009).

2.5.2. Ovatoxins may be involved in *Ostreopsis cf. ovata* allelochemistry

Although ovatoxins synthesized by *O. cf. ovata* (Ciminiello et al., 2012a) might be good candidates as allelochemicals, no experimental result has supported this hypothesis so far (García-Portela et al., 2016; Giussani et al., 2015). It is worth noting the absence of toxicity of the exudate found by Giussani et al. (2015) was linked to an absence of ovatoxins in the 0.2 µm filtered exudate. Our findings were slightly different with low levels of ovatoxin-a found in the EX (<2 pg.cell⁻¹ eq pltx), using a different protocol for chemical extraction. Therefore, the involvement of the ovatoxins in the deleterious effects observed on the diatom cells cannot be ruled out at this stage. From this perspective, the absence of allelopathic effect of *O. cf. ovata* onto *L. paradoxa* in the co-culture chambers was actually a good opportunity to test ovatoxins as the compounds responsible for the growth impairment observed in the bioassays. Indeed, the barely detectable concentrations of ovatoxins in the diatoms' EX when co-cultured with *O. cf. ovata* indicated a weak potential of diffusion of the ovatoxins far from the secreting cells. The reason for the absence of diffusion of the ovatoxins through the membrane is likely due to their physico-chemical properties. Ovatoxins are amphiphilic compounds (Botana, 2014) that can be stored in the mucus (Giussani et al., 2015). Any adsorption of ovatoxins on the PVDF membrane hampered their diffusion and several studies have shown that complete exchange of substances (heptadienal, dimethylsulfonopropionate and Microcystin-LR) through the 0.2 µm PVDF membrane would happen by diffusion within 24 h (Dunker et al., 2017; Paul et al., 2013). In conclusion, the absence of diffusion of ovatoxins associated to healthy diatom cells in co-culture experiments suggests that if ovatoxins are involved in growth impairment of competitors, it would most likely be through contact interactions.

The question whether toxins from microalgae act as distance or contact allelochemicals remains controversial since experimental evidences showed on one hand some toxins, for instance karlotoxin-1 and -2 (*K. veneficum*; Van Wagoner et al., 2008), are involved in prey capture (Sheng et al., 2010), allelopathy (Adolf et al., 2006) and mechanistic interactions with other phytoplankton species (Deeds and Place, 2006). On the other hand, the exudation of other toxins such as brevetoxins (*K. brevis*, Kubanek et al., 2005) are unlikely involved in growth control of co-occurring microalgae (Poulson-Ellestad et al., 2014; Prince et al., 2008). Finally, exudates of *K. brevis* were shown to stimulate the growth of several of its diatoms competitors (Poulson-Ellestad et al., 2014).

Interestingly, other organic compounds were secreted by *O. cf. ovata* cells and diffused through the PVDF membrane as revealed by the untargeted metabolomics approach. Those putative allelochemicals are assumed to be adducts of one single compound having a molecular weight of 2120 Da, highlighting the potential for *O. cf. ovata* to produce other large molecules than ovatoxins. Indeed, the fragmentation experiment did not allow to establish a link between this unidentified compound biosynthesized by *O. cf. ovata* and the ovatoxins family since the ovatoxins' fragment-A (Figure 7,

Brissard et al., 2015; Ciminiello et al., 2008, 2012a) was not evidenced, and no other internal fragment matched (Brissard et al., 2015; Garcia-Altarets et al., 2015). Thus, the combination of the two experiments underlines the urgent need for a characterization of both the endo- and the exo-metabolome of *O. cf. ovata* in order to better understand the role of its specialized metabolome in chemical interactions.

2.5.3. Allelochemistry of *Licmophora paradoxa*

Unexpectedly, the exudates of *L. paradoxa* cultures also slightly but significantly inhibited the *O. cf. ovata*'s PSII in the bioassays. Some allelochemicals exuded by the diatom culture inhibited the growth of the dinoflagellate. Interestingly the co-cultures revealed similar effects on the development of *O. cf. ovata* since both its growth and PSII were modified in the presence of the diatom culture although contact between cells was not allowed. A diffusion of chemicals affecting the development of the dinoflagellate cells supports the existence of a distant allelopathic effect by *L. paradoxa* culture on *O. cf. ovata*. This result is in accordance with the very recent findings of Pichierri et al., (2017) who showed a growth inhibiting effect of several diatoms filtrates onto the DNA content of *O. cf. ovata*. The identity of the compound (s) involved in this interaction has not been investigated so far although an untargeted metabolomics analysis suggests a role of several compounds ($C_{18}H_{27}O_4$, $C_{15}H_{23}O_3$, $C_{11}H_{17}O_3$) enriched in the EX of *O. cf. ovata* when co-cultured with the diatoms. Nevertheless, there is no clue so far whether those compounds can be held liable for the impairment of the dinoflagellate growth. Recent research on diatoms exo-metabolome showed that compounds belonging to the polyunsaturated fatty acids (PUFAs) as well as the polyunsaturated aldehydes (PUAs) families are, in some cases, responsible for allelopathy (Ianora et al., 2011; Pohnert, 2005). Wichard et al. (2005) evaluated over 71 diatom studied species, and 38% are PUA-producers. Although *L. paradoxa* species are not reported in their study, they may also synthesize one or several of those compounds. The chemical markers highlighted by the metabolomics analysis show similar molecular composition to the PUFAs and PUAs involved in diatoms allelochemistry (see Pohnert, 2005 for a review). In particular, C_{16} , C_{17} and C_{18} fatty acids were shown to be biosynthetic precursors of bioactive short-chain aldehydes and oxylipins (Ackman et al., 1968; Domergue et al., 2003; Pohnert, 2005). The extraction procedure of the EX employed in our study precluded the detection of any potential aldehydes, known to be highly reactive, but would have allowed the detection of fatty acids and oxylipins. A control of *O. cf. ovata* growth by PUAs or PUFAs is thus highly plausible since a recent study shows the addition of three PUAs (2E, 4E-decadienal, 2E, 4E-octadienal and 2E, 4E-heptadienal) in *O. cf. ovata* cultures inhibited the PS II after 48 to 72 h of exposure (Pichierri et al., 2016). Yet, the concentrations tested by the authors of this study were 5 to 10 times higher than levels encountered *in situ* (Ribalet et al., 2014). Since no thorough identification of the allelochemical(s) inhibiting the growth of *O. cf. ovata* was

attempted, its synthesis by the diatom *L. paradoxa* cannot be secured and a potential bacterial origin can also be considered. Algicidal bacteria are widely distributed in the marine environment and can exert a control on the growth and the lysis of microalgae (Meyer et al., 2017 for a review). Diatoms blooms were shown to provide habitat for algicidal bacteria (Park et al., 2010) and a control of dinoflagellate growth by algicidal compounds has often been reported (Park et al., 2010; Pokrzywinski et al., 2012; Roth et al., 2008). Future research should be directed towards the understanding of the potential role of algicidal bacteria in the chemical inhibition of *O. cf. ovata* growth by the culture of *L. paradoxa*, using axenic cultures.

2.6. Conclusion

The chemical interactions between *O. cf. ovata* and one of its competitors observed with these laboratory experiments give some insights on the mechanisms that may occur in the field. The detection of the presence of a competitor clearly stimulated the production of ovatoxins by *O. cf. ovata* most likely by activating the corresponding biosynthetic pathway. Therefore, a chemical control performed by the toxins of *O. cf. ovata* on its competitors is plausible and may impact the species succession during its bloom. A debate is currently ongoing to understand whether bloom initiation of HABs species could be led by allelopathy (Jonsson et al., 2009). The latest authors estimate that at the beginning of a bloom, cell densities are too low to concentrate enough infochemicals and that cell-cell interactions offer an alternative transmission for allelopathic compounds, and may therefore allow chemical compounds such as toxins to control bloom initiation. The results obtained in the present study support this hypothesis, cell-cell contact or a close interaction is most likely needed to strongly impair the growth of competitor species. This hypothesis is particularly relevant for benthic microalgae species that accumulate on the substrate and would therefore concentrate their toxins nearby. Nevertheless, the nature of the *O. cf. ovata* allelochemicals and their role in shaping microalgae communities still remain to be addressed. Further work should concentrate on identifying the allelochemicals as well as defining their mode of action.

A control of the competitor growth appeared to be intense at a sensitive growth stage (early log phase) of *L. paradoxa* cell development, and more research is needed to evaluate an eventual control on other growth stages. In order to verify the hypothesis of a chemical control on species succession, waterborne exudates should be sampled at different phases of the bloom (early log, log and stationary phases) and tested against several co-occurring competitor species since effects could differ among species (García-Portela et al., 2016; Monti and Cecchin, 2012). Additionally, cell-cell chemical interaction between *O. cf. ovata* and its competitors should be investigated taking into account the difficulty of such a detection with the available chemical imaging.

2.7. Acknowledgments

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3. Synthèse des résultats

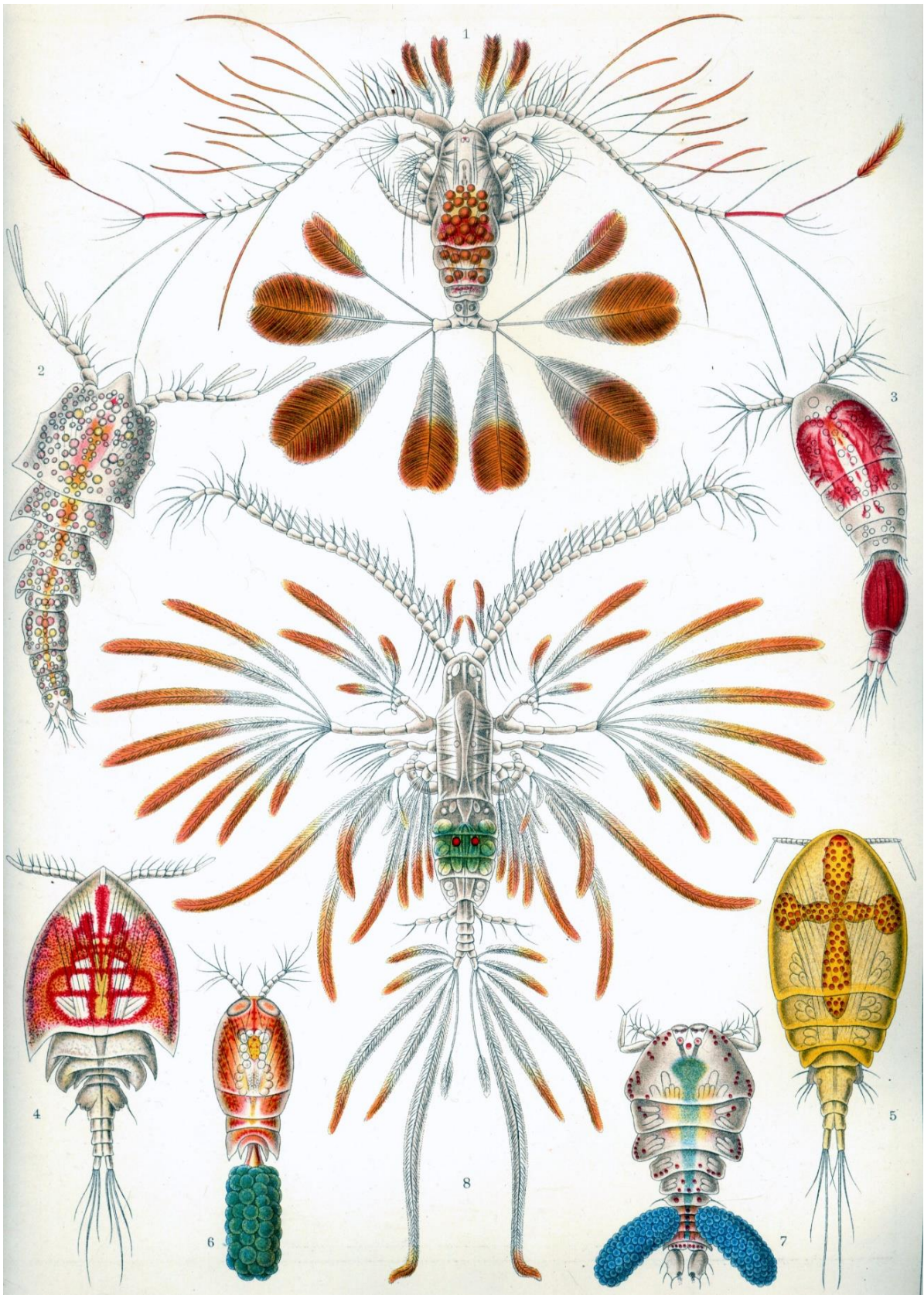
La diatomée est inhibée par le filtrat cellulaire d'*Ostreopsis cf. ovata* ; toutefois, aucun effet néfaste n'est observé, pour la diatomée, lors des co-cultures. La production d'OVTXs par *O. cf. ovata* est pourtant avérée (par étude métabolomique ciblée) mais il semblerait qu'en raison de leurs propriétés intrinsèques, les OVTXs ne traversent pas la membrane utilisée dans les systèmes de co-culture suggérant ainsi une toxicité d'*O. cf. ovata* par contact. Ceci confirme également l'implication des toxines produites par *O. cf. ovata* dans les relations allélopathiques qu'il entretient avec son environnement.

Cette étude a également permis de mettre en évidence, par l'utilisation d'une approche métabolomique non ciblée, la production de nouveaux composés par *O. cf. ovata*. Ces composés n'appartiennent pas à la famille des OVTXs mais sont impliqués dans les relations allélopathiques que ce dinoflagellé entretient avec son environnement.

Enfin, la croissance et l'activité photosynthétique du PSII d'*O. cf. ovata* ont été affectés par la présence de la diatomée. Une augmentation de la production de toxines est également notée. Ces effets sont certainement liés à la production, par la diatomée, des nouveaux composés identifiés dans cette étude.

Cette étude met en évidence la complexité des interactions qu'*O. cf. ovata* entretient avec son environnement et la méconnaissance des mécanismes associés.

Chapitre 3. Interactions entre *Ostreopsis* cf. *ovata* et consommateurs primaires : cas du copépode harpacticoïde *Sarsamphiascus* cf. *propinquus*



Copepoda. Plate of Wilhelm Giesbrecht (1892)

1. Présentation de l'étude

Une étude réalisée *in situ* en Méditerranée Nord Occidentale a mis en évidence une diminution significative du nombre de nauplii de copépodes benthiques lors des efflorescences d'*Ostreopsis cf. ovata* (Guidi-Guilvard et al., 2012). Les auteurs ont suggéré un impact de ce dinoflagellé sur les copépodes benthiques, amenant à un changement des communautés méiobenthiques.

L'objectif de la présente étude est de vérifier, en laboratoire, l'impact d'*Ostreopsis cf. ovata* sur les copépodes benthiques en étudiant différents paramètres tels que la survie, l'ingestion, ou la reproduction. Pour cela, le copépode benthique *Sarsamphiascus cf. propinquus*, a été isolé en rade de Villefranche-sur-Mer depuis des macroalgues sur lesquelles *O. cf. ovata*, a été sélectionné comme modèle d'étude. Maintenu en culture en laboratoire, ce copépode a ensuite été exposé à différentes concentrations d'*O. cf. ovata* afin d'étudier la toxicité aigüe d'*O. cf. ovata* sur cette espèce. L'ingestion des cellules de dinoflagellé a été vérifiée à travers des observations microscopiques et l'estimation de la production de pelotes fécales. L'impact des métabolites produits par *O. cf. ovata*, sur la reproduction de ce copépode, a été étudié via la mesure des taux de fécondité et de fertilité.

2. Effects of the toxic dinoflagellate *Ostreopsis cf. ovata* on survival, feeding and reproduction of a phytal harpacticoid copepod

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Effects of the toxic dinoflagellate *Ostreopsis cf. ovata* on survival, feeding and reproduction of a phytal harpacticoid copepod

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Key words: Benthic HABs, *Ostreopsis cf. ovata*, chemical ecology, meiobenthic copepods, reprotoxicity.

2.1. Abstract

Harmful algal blooms are a source of increasing concern within the health, economic and ecological sectors. In the Mediterranean Sea, severe blooms of the benthic dinoflagellate *Ostreopsis cf. ovata* have been occurring since the beginning of the century, causing human intoxications by inhalation of bio-aerosols or direct contact with cells. The toxicity of this dinoflagellate is attributed to the presence of palytoxin and several of its analogs called ovatoxins, palytoxin being one of the most potent marine toxins. While mass mortalities of marine invertebrates have already been reported in relation with *O. cf. ovata* blooms, the toxic effects of this dinoflagellate on benthic organisms is still poorly documented. In the present study, laboratory experiments were performed on a meiobenthic copepod (*Sarsamphiascus cf. propinquus*), which naturally lives on macrophytes in close contact to *O. cf. ovata*, in order to assess its potential toxic effects on mortality, fecal pellet production (as a proxy of feeding), as well as fecundity and fertility ratios. Both, *O. cf. ovata* as well as a non-toxic competitive diatom (*Licmophora paradoxa*), were used as food in the experiments. Regarding acute toxicity evaluation, this copepod proved to be the most tolerant organism to *O. cf. ovata* reported to date. Nevertheless, its fecundity and fertility ratios were lower when fed with the toxic dinoflagellate, indicating a possible reprotoxic effect. Moreover, although fecal pellet production decreased significantly when the copepod was fed with a mono-diet of *O. cf. ovata*, epifluorescence microscopy observations revealed the presence of the toxic cells inside the digestive track, hence suggesting that these primary grazers could be a vector of toxins through the marine food web.

2.2. Introduction

Over the past decades, the occurrence of Harmful Algal Blooms (HABs) has been increasing in frequency, intensity and geographic distribution (Berdalet et al., 2017; Glibert et al., 2005). These blooms negatively impact human health and wellbeing, by affecting coastal ecosystem services (Berdalet et al., 2015) as well as marine organisms and ecosystems (Dolah et al., 2001). In this regard, the strong geographical expansion of the toxic dinoflagellate *Ostreopsis cf. ovata* in the Mediterranean Sea constitutes an emerging problem (Parsons et al., 2012). This dinoflagellate has long been regarded as a potential vector involved in ciguatera fish poisoning (Glaziou and Legrand, 1994; Hallegraeff, 1993), and it has more recently been shown to produce palytoxin (PLTX) and palytoxin-like compounds (Ciminiello et al., 2006) named ovatoxins (OVTXs) a potential cause of toxic bio-aerosols causing human respiratory illnesses (Ciminiello et al., 2014). *Ostreopsis cf. ovata* is a benthic dinoflagellate usually described as epiphytic on macroalgae and seagrasses growing on rocky shallow seabeds (Mangialajo et al., 2008; Totti et al., 2010) but has also been reported to have a planktonic phase (Vila et al., 2016).

Several studies on invertebrate mass mortalities during *Ostreopsis cf. ovata* blooms (Granéli et al., 2002; Shears and Ross, 2009) suggest that this dinoflagellate may be harmful to benthic organisms living nearby even if its effects may be confounded with other stress factors such as pollution or hypo-/anoxic conditions (Shears and Ross, 2009; Shears and Ross, 2010). The toxic effect of *O. cf. ovata* has previously been investigated on several organisms using ecotoxicological bioassays. For instance, the jellyfish *Aurelia sp.*, has been shown to be particularly sensitive at the ephyra (or larval) stage (Giussani et al., 2016) and the mussel *Mytilus galloprovincialis* reacted to *O. cf. ovata* exposures by modifying immunological, histological and oxidative levels (Gorbi et al., 2013). Also, fertilization and early development of the sea urchin *Lytechinus variegatus* were affected by *O. cf. ovata* (Neves et al., 2018). Moreover, a decrease of more than 98.5% of gill cell viability was observed in response to *O. cf. ovata* exposure (Verma et al., 2016). Even though meiofauna living on macrophytes play important roles in benthic biochemical and ecological processes, little is known on the effects of this toxic microalga on the surrounding community. Only one *in situ* study has previously investigated the impact of *O. cf. ovata* on phytal meiofauna, and results showed a severe decrease in the number of nauplii during the toxic blooms, suggesting a reprotoxic effect of the dinoflagellate on harpacticoid copepods (Guidi-Guilvard et al., 2012).

The aim of the present study was to provide new insights on the potential effect of the toxic benthic dinoflagellate *Ostreopsis cf. ovata* on meiofauna, and more specifically on benthic copepods. A harpacticoid copepod belonging to the genus *Sarsamphiascus* (formerly *Amphiascus*) was isolated from the natural environment where *O. cf. ovata* summer blooms occur with densities reaching $4 \cdot 10^6$ cells.gFW⁻¹ (Cohu et al., 2013) and was here used as a model organism. Another copepod species

belonging to the same genus has already been widely used as a model in toxicity tests (Bejarano and Chandler, 2003; Cary et al., 2004; Chandler et al., 2004). This organism was shown to be relevant for ecotoxicological assays due to their small size, short life cycle, simplicity to maintain and manipulate in laboratory conditions and sensitivity to a wide array of toxic substances. In the present laboratory study, the selected copepod was exposed to ecologically relevant concentrations of the dinoflagellate *O. cf. ovata* (Accoroni et al., 2012; Cochu et al., 2013) as well as *Licmophora paradoxa*, a diatom frequently found associated with *O. cf. ovata* in benthic assemblages (Lemée, personal communication), which will here be used as a control. The effects of *O. cf. ovata* were investigated by recording three biological parameters of the copepod: survival, food uptake (assessed through the number of fecal pellets produced) and reproduction (fertility and fecundity ratios). The toxicity of the strain used in these experiments was evaluated using *Artemia franciscana* toxicity bio-assay and confirmed by UHPLC-HRMS quantification of ovatoxins.

2.3. Materials and Method

2.3.1. Algal cultures

The dinoflagellate *Ostreopsis cf. ovata* (MCCV 054) and the diatom *Licmophora paradoxa* (MCCV 033) used as a control microalga were isolated from macroalgal samples collected at the Rochambeau site (Bay of Villefranche-sur-mer, N-W Mediterranean, 43°41'35.64"N-7°18'31.54"E). The MCCV is the Mediterranean Culture Collection of Villefranche, where strains are maintained and available for the scientific community. Both strains were cultured in 150 mL flasks containing L1 medium (Guillard and Hargraves, 1993) prepared with autoclaved aged and 0.2 µm filtered seawater, adjusted to a salinity of 38. All the cultures were maintained at 24°C, under a 14:10 light/dark cycle (light intensity 250 µmol.m⁻².s⁻¹). Cells of *O. cf. ovata* used for the experiments were collected from the cultures during the exponential phase. They were counted in 12 mL aliquots (triplicates) fixed with acidic lugol (4% v/v) using a liquid particle counter (HIAC/Royco 9703, Pacific Scientific Instruments) following a size range of 2-80 µm (Stramski et al., 2002).

Cell diameter range of *O. cf. ovata* and *L. paradoxa* in culture were 20-40 µm and 15-20 µm, respectively. Biovolumes were calculated assuming *O. cf. ovata* (1) had the shape of a cone with a half sphere, and *L. paradoxa* (2) was a truncated cone, using the following equations (Olenina et al., 2006):

$$(1) V = \frac{\pi}{12} \times h \times D^2 \quad (2) V = \frac{\pi}{12} \times (d_1^2 + d_1d_2 + d_2^2)$$

where V = volume, h = height, D = diameter, d₁ = large diameter, d₂ = small diameter. The biovolume ratio between the two microalgae was 1:6, i.e. *O. cf. ovata* (average biovolume = 17 360 µm³) was 6 times larger than *L. paradoxa* (average biovolume = 2 968 µm³). These biovolumes were calculated using 15 cells for each strain.

2.3.2. Toxicity of the strain

2.3.2.1. *Artemia franciscana* toxicity bioassay

The level of toxicity of the *O. cf. ovata* strain used for this study was checked by performing a standard *Artemia franciscana* test (Faimali et al., 2012; Neves et al., 2017). The high sensitivity of *Artemia* to toxic substances makes it suitable for ecotoxicological assessment (Kalčíková et al., 2012; Nunes et al., 2006). Such bio-assays, described as simple, inexpensive and convenient, can be used to test the toxicity of harmful algal strains (Neves et al., 2017). Cysts of *A. franciscana* (Ocean Nutrition Sep-art) maintained at 4°C in the dark, were incubated in 2 L of filtered seawater (salinity 38), at 20°C with vigorous and continuous aeration until reaching the most sensitive development stages (Stages 2-3, Kerster and Schaeffer, 1983). The larvae were collected using a glass pipette and phototaxis and further transferred to 6-well plates. Five larvae were placed in each well, using one 6-well plate per experimental condition. A total of 4 well-plates were used to investigate the effects of the following cell concentrations of *O. cf. ovata*: 4, 40, 400 and 4000 cells.mL⁻¹.

The ratio number of algal cells: number of target animals was then between 20:5 to 20 000:5. The number of dead larvae was estimated after 2, 4, 24 and 48 h exposure times. Larvae were considered dead when there was no reaction to strong light or to glass pipette aspiration or when a shift to white coloration of the body was observed. LC₅₀ and LT₅₀ values were calculated, corresponding to the concentration which induced the mortality of 50% of the organisms and the lethal time (in hours) to reach 50% mortality, respectively.

2.3.2.2. UHPLC-HRMS analyses

The toxicity of the strain was confirmed using chemical analyses. *Ostreopsis cf. ovata* cells were grown in 300 mL flasks at previously described conditions and harvested at day 10. The culture was further centrifuged at 600 g during 10 min at ambient temperature. The cell pellet was flash-frozen using liquid nitrogen and stored at -80 °C until extraction. The metabolites were extracted using 4 mL of MeOH/H₂O (80:20; v:v) and sonicating during 5 min in a cooled ultra-sonic bath. The extracts were centrifuged at 2500 g during 10 min at ambient temperature and the supernatants were transferred into 20 mL vials. These steps (MeOH, ultra-sonic bath and centrifugation) were repeated 3 times. The resulting organomethanolic extracts were evaporated using a stream of nitrogen avoiding dryness; a volume of 500 µL of DMSO was added in each extract and the remaining H₂O was fully evaporated. All samples were therefore stored in 100% DMSO (500 µL) at -20°C until UHPLC-HRMS analysis.

Ovatoxins quantification by UHPLC-UV-HRMS were performed using an Agilent 1290 system (Agilent Technologies, USA) equipped with a diode array detector and coupled to an Agilent 6540 Qtof mass spectrometer (Agilent Technologies, USA) by the injection of 10µL on T3 column (Acquity UPLC HSS T3 1.8µm, 2.1mm x 100mm, Waters). Separation was achieved using a linear elution gradient of

H₂O:MeOH (80:20, v:v)/MeOH with 0.1mM of ammonium formate and 0.1% formic acid from 90:10 (v:v, isocratic from 0 to 2mins) to 0:100 (v:v, isocratic from 12 to 13 mins) with a 0.4 mL.min⁻¹ flow rate. UV detection was set at 210, 233 and 263 nm. Ions detections was recorded in positive mode (ESI +) in the range 60 -3000 Da. Collision energies (CE) of 30, 70 and 110 eV were applied to confirm the presence of ovatoxins moieties (Brissard et al., 2014; Ciminiello et al., 2012a). The spectrometer analyzer parameters were set as follows: nebulizer sheath gas, N₂ (35 psig); drying gas, N₂ (11 L.min⁻¹); Gas Temperature, 300°C; capillary, 4.129 µA; Vaporizer/Sheat Gas Temp, 350°C.

Ovatoxins quantification was performed by extracting and integrating all tri-charged ions (from 858 to 910 m/z) from the base peak chromatogram. The concentration of ovatoxins is given in palytoxin equivalent as commercially available standard of palytoxin (Wako Chemicals GmbH, Neuss, Germany) was used to perform a calibration curve, assuming their ionization pattern is similar.

2.3.3. The model copepod

The harpacticoid copepod used in the experiments thrives in the shallow rocky shore of the Marinières site (Bay of Villefranche-sur-Mer, N-W Mediterranean, 43°42'21.51"N – 7°19'07.44"E) where it was collected in 2012 using a WP2 net towed over the macroalgal cover. In the laboratory, samples were maintained in 10 L tanks together with a planktonic copepod (*Acartia clausi*) in 0.2-µm filtered aged seawater (salinity 38) at 22°C, in the dark to avoid phototactism, and fed three times a week with a mixture of the microalgae *Dunaliella salina* (MCCV 20) and *Tisochrysis lutea* (CCAP 927/14). The copepod belongs to the family Miraciidae Dana, 1946 (formerly Diosaccidae), and is part of the *varians*-Group. Within this group, the genus *Amphiascus*, renamed *Sarsamphiascus* by Huys (2009), is well known for taxonomic discrepancies and extreme difficulty in species identification (Hicks, 1971; Wells, 2007). In the present study, the species name remains uncertain due to inter-individual variation in the parapodial setation. However, among the possible *varians*-Group species, only *Amphiascus propinquus* had been previously reported in areas close to the Bay of Villefranche-sur-mer (i.e. the Italian Ligurian shore, Ceccherelli, personal communication). For all these reasons, this species was named *Sarsamphiascus cf. propinquus* (Sars, 1906) in our study.

2.3.4. Experimental setup

All experiments were run in 6-well plates, each well containing 4 mL of autoclaved aged and 0.2-µm filtered seawater (salinity 38), at 24°C, in the dark. One milliliter of microalgae at the appropriate cell concentration was added to the wells as food, except in the no-food controls which received instead 1mL of clean culture medium. The content of each well was renewed every 48 hours and wells were checked daily under a binocular microscope (ZEISS, SteREO Discovery V12).

Experiments involving the copepod *S. cf. propinquus* started with adults and late copepodites. Prior to the experiments, individuals were collected from the culture tanks, sorted with an elongated Pasteur pipette, flushed twice in seawater and transferred to the 6-well plates. They were left for 2 days without food to allow gut clearance, except in the reproduction experiments where the food was added from the start. At the end of some of the experiments, copepods were fixed with formalin (4% v/v) to determine sex and overall size (from the tip of the cephalosome, excluding the rostrum, to the end of the last body somite, excluding the caudal rami) using a NIKON AZ100 microscope coupled to a Digital Imaging System equipped with the NIS-Elements software (Nikon Instruments Inc., New-York , U.S.A.).

2.3.5. Acute toxicity of *Ostreopsis cf. ovata* on Copepods

To estimate and compare the level of toxicity of *O. cf. ovata*, the copepods were exposed to five increasing cell concentrations (500, 1 000, 2 000, 4 000 and 20 000 cells.mL⁻¹) and one no-food control in six-well plates, as previously described for *Artemia* bioassay. The test involved 72 individuals (12 per concentration and no-food control, 1 per well) and lasted 9 days. The ratio of algal cells/ number of target animals was between 2500:1 to 100 000:1. Mortality (as defined above for *Artemia* bio-assay) was daily recorded in order to calculate LC₅₀ and LT₅₀.

2.3.6. Impact of *Ostreopsis cf. ovata* on copepod survival and food uptake

To investigate potential toxic effects of *O. cf. ovata* cells, mono- and mixed diets were fed to the copepod. Five different treatments were applied: (i) *O. cf. ovata* at a concentration of 100 cells.mL⁻¹ reflecting the realistic bloom alert threshold measured in the natural environment (Lemée et al., 2012; Tichadou et al., 2010) ; (ii) *L. paradoxa* at a concentration of 600 cells.mL⁻¹ corresponding to the microalgae supplied in treatment (i) in terms of bio-volumes ; (iii) a mixed diet of *O. cf. ovata* and *L. paradoxa* (i.e. treatments (i) and (ii) combined) to investigate if the presence of the two microalgae could change the response of the copepods; (iv) the mixed diet (iii) at half concentrations (i.e. *O. cf. ovata* at 50 cells.mL⁻¹ + *L. paradoxa* at 300 cells.mL⁻¹) ; (v) a no-food treatment. To study both mortality and ingestion of *S. cf. propinquus*, two experiments were performed during 10 days and 14 days, each involving 150 individuals (30 per treatment, 1 per well). Mortality (as previously defined) was assessed daily, while feeding (assessed through the number of fecal pellets produced by alive copepods ; Souza-santos et al., 1995) every 48 h, at the time of medium renewal. Copepods that had been in contact with *O. cf. ovata* alone were observed under an epifluorescence inverted microscope (Axio Scope.A1, UV excitation, red fluorescence) to check for the presence of *O. cf. ovata* cells in the digestive track.

2.3.7. Impact of *Ostreopsis cf. ovata* on reproductive performances of Copepods

The potential effects of *O. cf. ovata* on the reproduction capacity of *S. cf. propinquus* were assessed through 3 identical experiments, which ran for 7 days with only mono-diets. The treatments were the same as (i), (ii) and (v) in the previous experiments, i.e. *O. cf. ovata* at 100 cells/mL⁻¹, *L. paradoxa* at 600 cells/mL⁻¹ and a no-food control. For each experiment, 120 males and females were first equally distributed in 3 glass crystallizing dishes and acclimatized to the corresponding treatment for 72h. Then, copepods were randomly transferred to 6-well plates (10 individuals per well to facilitate fecundation). The formation of egg sacs was checked every day to estimate fecundity ratios, i.e. number of ovigerous females to total number of females. Ovigerous females were isolated immediately after egg sacs were observed, but were still exposed to algal treatments described above. Hatching was monitored daily by counting the number of nauplii. Fertility ratios, i.e. number of nauplii to number of ovigerous females, were subsequently calculated, assuming that egg mortality and cannibalism were negligible. Indeed, we assumed that no egg mortality occurred only in control condition because the effect of the toxins on egg mortality was not tested. It is noteworthy that no treatment was used to induce reproduction in this experiment. At the end of the experiments, sex and size of all adult individuals were determined. Body length (Mean ± Standard Deviation) of adult *S. cf. propinquus* reared in the laboratory and used in the experiments was 479 ± 65 µm (n=68) for females and 407 ± 43 µm (n = 79) for males. No mortality was observed in this experiment since it only lasted 7 days and with very low concentrations of *O. cf. ovata*.

2.3.8. Statistical analyses

Mean fecal pellet production (number of pellets.cop⁻¹) at a given time was calculated only for the copepods that had survived at that given time. Mean egestion rate (number of pellets.cop⁻¹.day⁻¹) for a given treatment and experiment was the slope of the regression line between the corresponding mean fecal pellet production values and time. The R package ecotoxicology was used to calculate median lethal time (LT₅₀) and concentration (LC₅₀). Note that median lethal times were only calculated for the second experiment since a mortality of 100% of the copepods is required to estimate these values.

Kruskal-Wallis tests were used to assess the influence of *O. cf. ovata* on egestion; a Dunn's post hoc test was used *a posteriori* to identify which experimental treatment differed from the others when the Kruskal-Wallis test showed significant differences (p<0.05). Kaplan Meier curves and a log-rank test were applied to evaluate the influence of *O. cf. ovata* on mortality of *S. cf. propinquus*. The Fisher Exact Test was used to assess the potential toxicity of *O. cf. ovata* on the reproduction performances of the copepod. All tests were performed using the PAST software (Hammer et al., 2001).

2.4. Results

2.4.1. Quantification of the toxins

The *Ostreopsis cf. ovata* strain used in the present study (MCCV 054) was shown to produce 44 ± 17 pg PLTX eq/cell at the end of its exponential phase (day 10).

2.4.2. Acute toxicity of the *Ostreopsis cf. ovata* on *Artemia franciscana*

Mortality of *Artemia franciscana* larvae in the control conditions (without *Ostreopsis cf. ovata*) remained below 10% even after 48 hours of experiment (Figure 12), following standard *Artemia* toxicity tests which is less than 10% of mortality in the control.

Results showed that *O. cf. ovata* induced mortalities of *A. franciscana* larvae and values increased rapidly with exposure time and concentration of the dinoflagellate. Two hours exposure at the lowest concentration (4 cells.mL^{-1}) were sufficient to impact 13% of the larvae and more than 70% were dead at the highest concentration ($4\ 000 \text{ cells.mL}^{-1}$). After 4 hours of exposure, larval mortality almost reached 50% at the lowest concentration of microalgae, and more than 86% at the highest concentration. After 48 hours of exposure, irrespective of the cell concentration tested, all the larvae died while more than 80% survived in the no-food control. Standard measures of toxicity showed a median lethal concentration (LC_{50}) below 4 cells.mL^{-1} after a 48 hour exposure, and a median lethal time (LT_{50}) of 1.3 hours when exposed to the highest cell concentration ($4\ 000 \text{ cells.mL}^{-1}$).

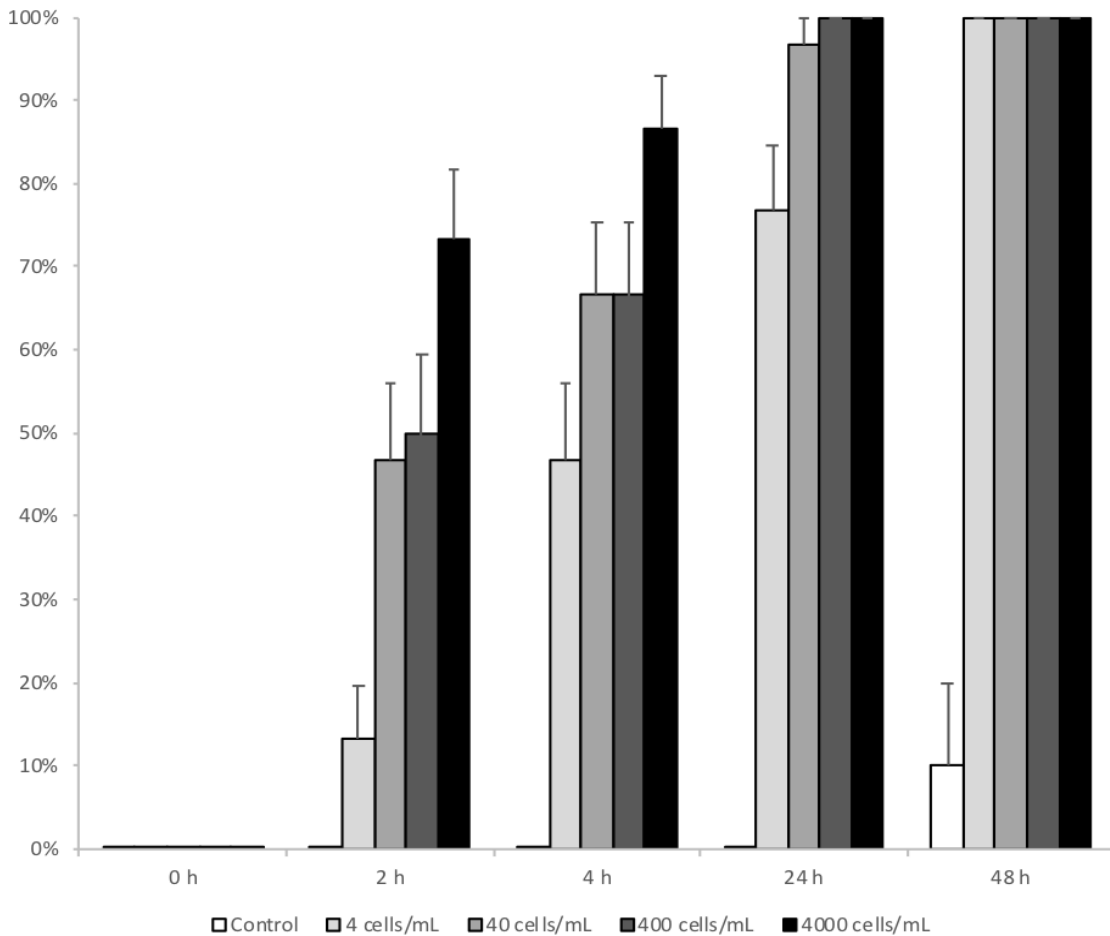


Figure 12 : Mortality (Means \pm SE, n=6) of *Artemia franciscana* larvae after different exposure times (hours) and for increasing concentrations of *Ostreopsis cf. ovata*. The ratio number of algal cells: number of target animals is between 20:5 to 20 000:5.

2.4.3. Acute toxicity of *Ostreopsis cf. ovata* on Copepods

Ostreopsis cf. ovata exposures induced mortality of *Sarsamphiascus cf. propinquus* and the impact increased with exposure time and cell concentration (Figure 13). However, results were highly variable and remained relatively low irrespective of the microalgal concentration tested. Even after 9 days of exposure at the highest cell concentration, mortality did not exceed 50% (on average), while it reached more than 58% in the no-food control. However mortality was higher at low concentration (i.e. below 1 000 cells.mL⁻¹) than at medium concentration (i.e. between 1 000 cells.mL⁻¹ to 4 000 cells.mL⁻¹). Indeed after 9 days of experiment, 40% of the copepods died at 500 cells.mL⁻¹ although only 20% died at 4 000 cells.mL⁻¹. Median lethal concentration (LC₅₀) calculated after an exposure time of 48 hours was more than 20 000 cells.mL⁻¹, and half lethal time (LT₅₀) for the highest cell concentration (20 000 cells.mL⁻¹) was 120 hours. This latter value was lower compared to that found in the no-food control, i.e. LT₅₀ (no-food) = 192 h.

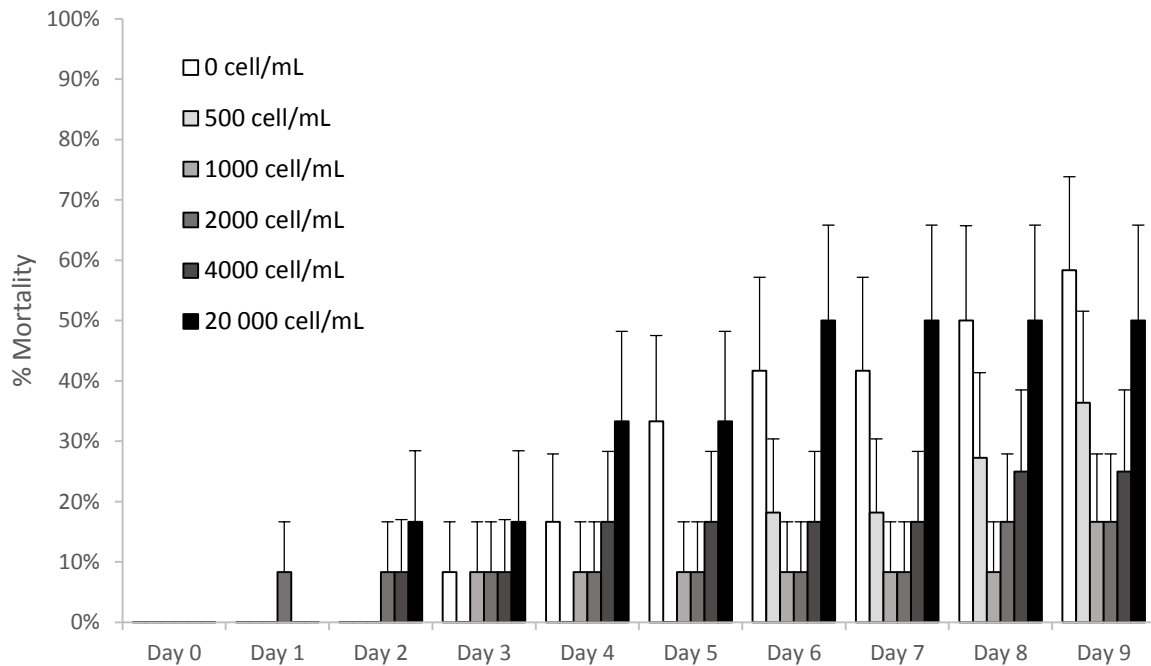


Figure 13 : Mortality (Means \pm SE, n=6) of the copepod *Sarsamphiascus cf. propinquus* after different exposure times (days) and for increasing concentrations of *Ostreopsis cf. ovata*. The ratio of algal cells: number of target animals is this time between 2500:1 to 100 000:1.

2.4.4. Impact of *Ostreopsis cf. ovata* on food intake of Copepods

Fecal pellet production (or egestion) was used as a proxy to estimate food intake. In both the 10-day and 14-day experiments, the number of fecal pellets increased with time in the four feeding treatments, except in the no food treatment (Figure 14). The Kruskal-Wallis analysis of variance showed that mean values differed significantly between treatments ($p < 0.001$, for the two experiments). In the absence of food, egestion was extremely low, and ceased shortly after the start of the experiments. Only 3 and 6 fecal pellets were collected in total from the 10-day and 14-day controls, respectively. When the copepods were fed with *O. cf. ovata* alone, fecal pellet production first increased sharply, and slowed down after a few days. A total of 189 and 45 fecal pellets were collected from the 10-day and 14-day experiments with mean egestion rates reaching 0.62 and 0.19 pellets.copepod⁻¹.day⁻¹, respectively.

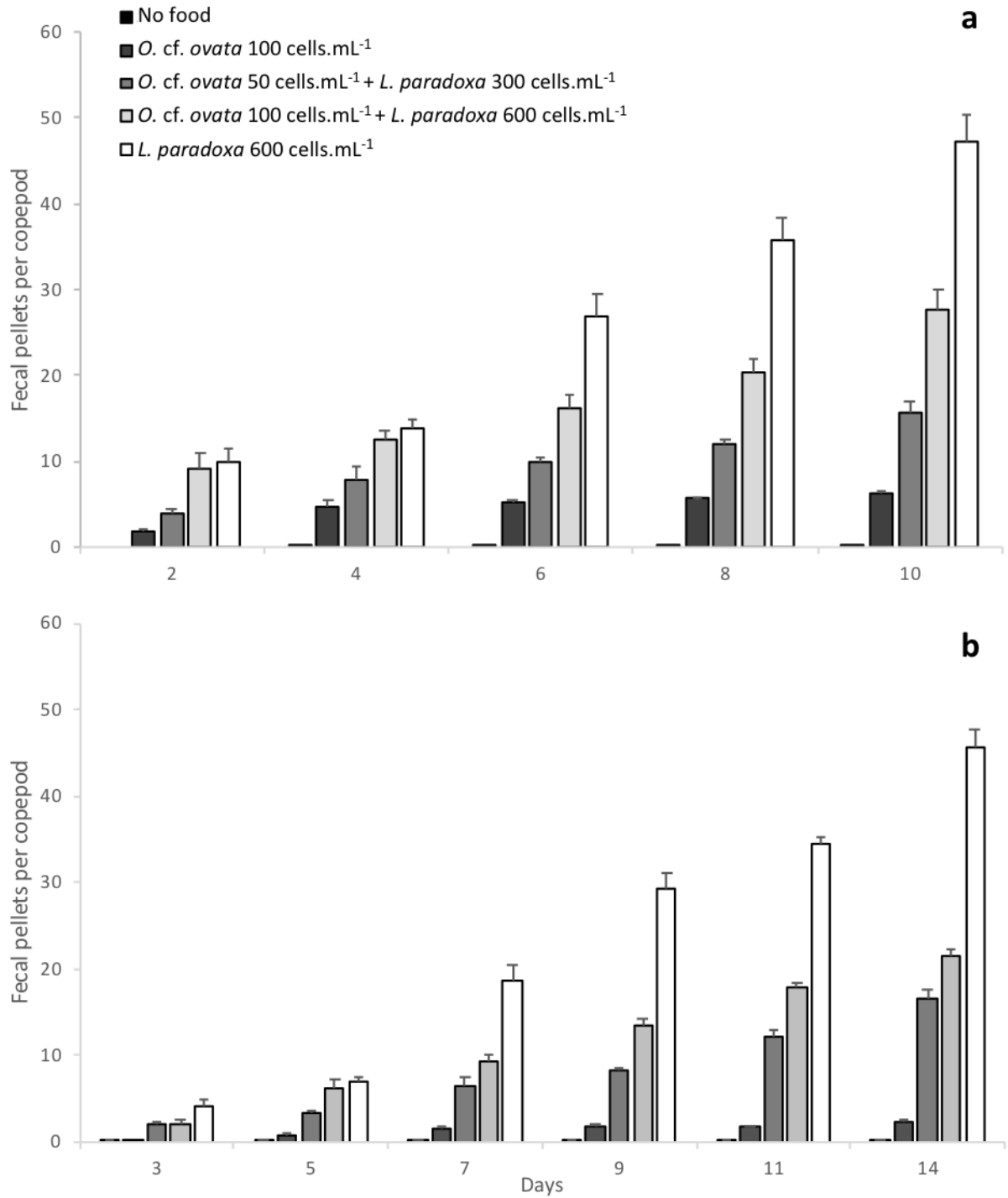


Figure 14 : Fecal pellet production (Mean \pm SE, n=6) of the copepod *Sarsamphiascus cf. propinquus* exposed to different feeding treatments and no-food treatment as a function of time. Number of fecal pellets were cumulated over time. Two identical experiments were performed during 10 days (1) and 14 days (2). Letters formulate significant differences: groups that have no common letter differ significantly.

The Dunn's post hoc test showed that the mean pellet production measured in the *O. cf. ovata* 14-day experiment was not significantly different from the corresponding no-food control, but was significantly different from measurements in the 10-day experiment. Mean fecal pellet production was significantly different between all treatments (including controls), except for the above stated treatment. It was also significantly different between the *O. cf. ovata* fed as mono-diet and the half concentration of both microalgae mixed-diet as well as between both mixed diets. The highest fecal pellet production values were measured with the diatom mono-diet. Egestion increased linearly with time ($\alpha < 0.005$) with a mean production rate of 5.1 and 4.0 pellets.copepod⁻¹.day⁻¹ in the 10-day and 14-day experiments, respectively. When the diatom was supplemented together with *O. cf. ovata* in the food (mixed diets), copepod egestion followed the same trend, but mean egestion rates were lower. With the full concentration of both microalgae in the mixed diet, copepods produced on average 2.5 and 3.8 pellets.copepod⁻¹.day⁻¹, while with the half-mixed diets the mean pellet production decreased to 1.6 and 1.4 pellets.copepod⁻¹.day⁻¹ in the 10-day and 14-day experiments, respectively.

Copepods fed with *O. cf. ovata* alone (mono-diet) were observed under an epifluorescence microscope and images revealed an intense fluorescence of their digestive tracks due to the autofluorescence of chlorophyll indicating the presence of dinoflagellate chloroplast (Figure 15).



Figure 15 : The copepod *Sarsamphiascus cf. propinquus* observed under epifluorescence microscopy (excitation in UV, fluorescence in red; left) and light (middle) after having been fed with *Ostreopsis cf. ovata*. The autofluorescence of chlorophyll is visualized inside the digestive track. *Ostreopsis cf. ovata* cells (Light microscopy; right) also appear attached to the body and the caudal rami.

2.4.5. Impact of *Ostreopsis cf. ovata* on survival of Copepods

Results obtained in the 10-day and 14-day experiments showed similar trends (Figure 16). Mortality of *S. cf. propinquus* increased with the duration of the experiment, but the magnitude of this increase differed significantly between treatments (log rank test, $p < 0.001$ for the two experiments).

The highest mortalities occurred in the absence of food, while when *O. cf. ovata* alone was fed to the copepods (mono-diet), mortalities were less pronounced. Feeding a mixed diet by introducing the diatom *L. paradoxa* with *O. cf. ovata* in the food, reduced mortalities. Finally, when the diatom alone was used as food, mortality along time was almost null and only slightly increased towards the end of the experiments. The log-rank test showed that mortality associated to the diatom mono-diet did not significantly differ from mortality measured when copepods were fed with the mixed diet (iii) (i.e. *O. cf. ovata* at 100 cells.mL⁻¹ + *L. paradoxa* at 600 cells.mL⁻¹). On the other hand, mortality values with *O. cf. ovata* differed significantly from mortality values observed in all other treatments. Moreover, mortality due to the absence of food differed significantly from mortality observed in all the other treatments. In other words, *O. cf. ovata* alone offered to the copepods and the absence of food, both, strongly affected their survival.

However, mortality associated to *O. cf. ovata* in the food always remained lower compared to starvation, excepted during the first 7 days. This difference is reflected in the median lethal time values, i.e. LT₅₀ under starvation was 9 days, while LT₅₀ with *O. cf. ovata* (at 100 cells.mL⁻¹) was 11 days. When the copepods were fed with *O. cf. ovata* alone at 100 cells.mL⁻¹, mortality was significantly different from results obtained with mixed diets. For example, after 14 days, ca. 67% of the copepods died with the *O. cf. ovata* mono-diet, while they were only ca. 13% when fed with the same concentration of *O. cf. ovata* supplemented with the diatom at 600 cells.mL⁻¹. However, when reducing the cell concentrations of both micro-algae by two fold in the mixed diet (i.e. *O. cf. ovata* at 50 cells.mL⁻¹ + *L. paradoxa* at 300 cells.mL⁻¹), copepod mortality increased to ca. 37%. Finally, when the diatom was fed alone to the copepods, mortality values remained below 10%.

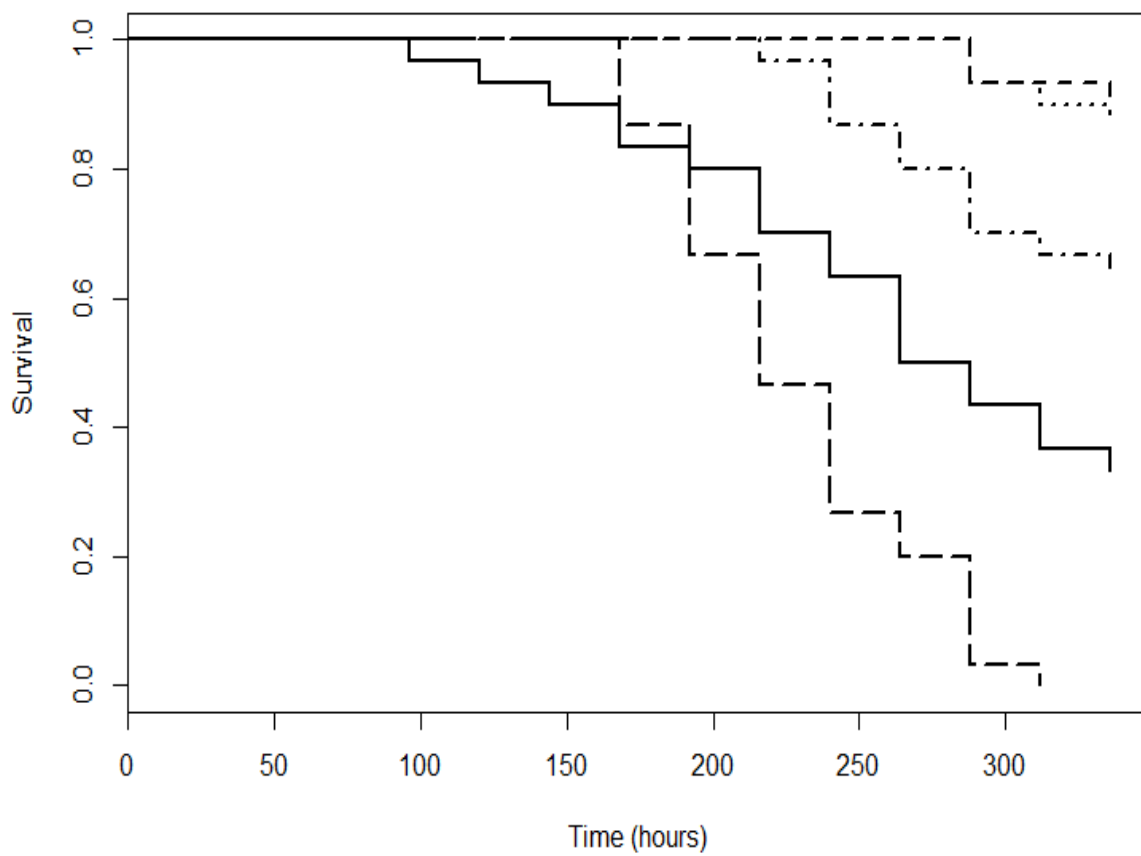
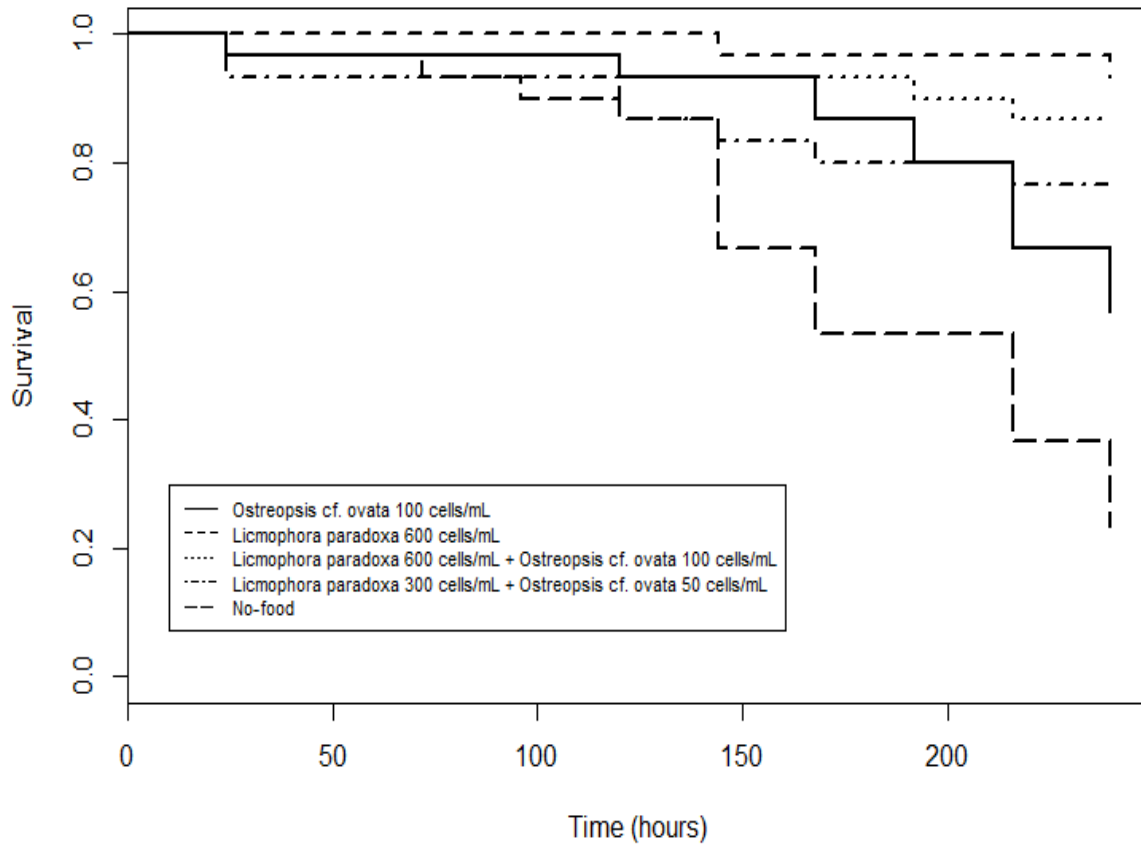


Figure 16 : Kaplan-Meier survival analysis of the copepod *Sarsamphiascus cf. propinquus* exposed to different feeding treatments and no-food treatment as a function of time. Two identical experiments were performed during 10 days (1) and 14 days (2). Letters formulate significant differences: groups that have no common letter differ significantly ($p < 0.05$).

2.4.6. Impact of *Ostreopsis cf. ovata* on reproductive performances of Copepods

Depending on the experiment, when the copepods were fed with the diatom *L. paradoxa*, 55 to 88% of the females were gravid (Table 10). On the other hand, when *O. cf. ovata* was used as food, only 13 to 18% of the females were gravid, and in the absence of food (starvation) gravid females did not exceed 10%. The Fisher test showed that fecundity ratios obtained with the *O. cf. ovata* mono-diet were actually not significantly different from values in the no-food controls, but significantly lower than with the diatom mono-diet (Table 11).

Table 10 : Fecundity and fertility ratios of *S. cf. propinquus* exposed to different food treatments (two different diets and a no-food control) assessed through 3 identical experiments which ran for 7 days. Experiments started off with 30 adults and late copepodites of unknown sex. Actual number of females, resulting ovigerous females and nauplii are indicated.

	Treatments	Females	Ovigerous females	Fecundity Ratio	Total nauplii	Fertility ratio
Experiment 1	<i>O. cf. ovata</i>	11	2	0.18	2	1.0
	<i>L. paradoxa</i>	26	23	0.88	210	9.1
	No food	16	0	0	0	0
Experiment 2	<i>O. cf. ovata</i>	14	2	0.14	3	1.5
	<i>L. paradoxa</i>	18	10	0.55	88	8.8
	No food	10	1	0.10	0	0
Experiment 3	<i>O. cf. ovata</i>	15	2	0.13	11	5.5
	<i>L. paradoxa</i>	13	9	0.69	90	10.0
	No food	17	1	0.06	5	5.0

Depending on the experiment, between 88 and 210 nauplii hatched in the presence of the diatom *L. paradoxa* (Table 10). Only 2 to 11 nauplii hatched in the presence of the dinoflagellate *O. cf. ovata*, and between 0 and 5 nauplii hatched in the absence of food. More specifically, each gravid female produced on average 9.3 nauplii with the diatom mono-diet, 2.7 nauplii with the *O. cf. ovata* mono-diet, and 1.7 nauplii when not fed. Here again, the Fisher test showed no significant difference in the fertility ratios derived from the two latter treatments (Table 11). Moreover, the number of nauplii per ovigerous female was significantly lower when the copepods were exposed to *O. cf. ovata* compared to copepods fed *L. paradoxa*. Irrespective of the experimental treatment, all the nauplii that hatched were viable, at least until the end of the experimental period.

Table 11 : P-values of the Fisher exact test for the fecundity (a) and fertility ratios (b) of *Sarsamphiascus* cf. *propinquus* exposed to different feeding treatments and no-food treatment. Significant results are evidenced in bold; *, **, *** corresponding respectively to $p < 0.05$, $p < 0.01$ and $p < 0.001$.

A	<i>O. cf. ovata</i> / <i>L. paradoxa</i>	<i>O. cf. ovata</i> / No food	<i>L. paradoxa</i> / No food
Experiment 1	7.91 x 10⁻⁵ ***	0.20	6.90 x 10⁻⁸ ***
Experiment 2	2.76 x 10⁻² *	1	4.17 x 10⁻² *
Experiment 3	5.58 x 10⁻⁵ ***	0.60	9.85 x 10⁻⁴ ***

B	<i>O. cf. ovata</i> / <i>L. paradoxa</i>	<i>O. cf. ovata</i> / No food	<i>L. paradoxa</i> / No food
Experiment 1	1.18 x 10⁻¹⁰ ***	0.21	3.35 x 10⁻¹⁶ ***
Experiment 2	4.82 x 10⁻¹³ ***	0.54	2.64 x 10⁻⁹ ***
Experiment 3	3.06 x 10⁻⁷ ***	0.23	5.35 x 10⁻¹⁰ ***

2.5. Discussion

The physiological state of the copepod *Sarsamphiascus* cf. *propinquus* was altered when exposed to the dinoflagellate *Ostreopsis* cf. *ovata*. Although this copepod appeared as very resistant to the toxic dinoflagellate during acute toxicity evaluation tests, changes in fecal pellet production as well as reduced fecundity and fertility indexes were observed.

2.5.1. Acute toxicity of *Ostreopsis* cf. *ovata*

The toxicity of the dinoflagellate strain used in this study was estimated by a simple ecotoxicological assay using *Artemia franciscana* larvae and the content in ovatoxins was further assessed by UHPLC-UV-HRMS. Using a palytoxin standard for calibration, we estimated that the ovatoxin content of the dinoflagellate strain used in this study was comparable to other Mediterranean strains (Brissard et al., 2014), although more enriched than previously assessed for this same strain (18 pg PLTX eq/cell, Ternon et al., 2018). This enrichment can be easily explained by the protocol for cell extraction and data analysis that differed from the previous study.

The *Artemia* larvae were highly sensitive to *O. cf. ovata* with a half lethal concentration (LC₅₀) below 4 cells.mL⁻¹ and a median lethal time of 2 hours at a 4 000 cells.mL⁻¹ cell concentration. These crustacean larvae have previously shown high sensitivity towards other Mediterranean strains of *O. cf. ovata* (Faimali et al., 2012; Pezzolesi et al., 2012) with median lethal concentrations varying from 4 to 8 cells.mL⁻¹. The results obtained in the present study were consistent with previous studies undertaken on other toxic dinoflagellates such as *Gambierdiscus excentricus*, *Prorocentrum lima* (Neves et al., 2017) and *Ostreopsis siamensis* (Rhodes et al., 2002). The extreme sensitivity exhibited by the *Artemia* larvae in the present study confirms that *Artemia* is a suitable model for assessing the toxicity of *O. cf. ovata*.

The toxicity of *O. cf. ovata* has previously been investigated on benthic and pelagic model organisms. Regarding crustaceans, previous studies have shown clear differences between species. For instance, the isopod *Sphaeroma serratum* exhibited lower acute toxicity compared to the amphipod *Corophium insidiosum* (Prato et al., 2011). Our study highlighted (1) a higher mortality of the copepod *S. cf. propinquus* at low concentration due to a starvation. At medium concentration, the mortality was the lowest suggesting an optimal balance between food needs and toxicity effects due to *O. cf. ovata*; (2) a strong resistance of *S. cf. propinquus*, with a median lethal concentration (LC₅₀), reaching more than 20 000 cells.mL⁻¹ after 48 hours of exposure. Indeed, *S. cf. propinquus* was at least 80 times more tolerant compared to *Tigriopus fulvus*, for which the LC₅₀ after a 48 hour exposure was 250 cells.mL⁻¹ (Faimali et al., 2012). Such differences could be explained by variable environmental conditions experienced by each species (Prato et al., 2011) or strains used for toxicity assays. We suggest that *S. cf. propinquus* might have adapted to the toxicity of *O. cf. ovata* due to the natural co-occurrence of both organisms. Resistance of grazers facing toxic dinoflagellates has already been reported for calanoid copepod populations that have frequently experienced blooms of the highly toxic *Alexandrium spp.* These copepods were reported to be more resistant to toxic blooms compared to naive populations and had a relatively better fitness in the presence of the toxic dinoflagellates (Colin and Dam, 2005). The difference of sensitivity to *O. cf. ovata* exposure observed between the two benthic copepods could also be explained by the strains used for toxicity assays. Indeed, very few studies have tested the variability among strains (see Faimali et al., 2012; Giussani et al., 2016) and it was impossible to realise this test for technical constraints.

In some studies, the toxicity of *O. cf. ovata* was also evaluated by using lysed cells or cellular extracts. For exemple, Faimali et al., (2012) compared the effects of different states of *O. ovata* cultures (such as filtered and resuspended *O. ovata* cells, growth medium devoid of algal cells and sonicated cells) on crustacean and fish larvae and found a significant toxic effect only when the whole algal cells were physically present. However, it is difficult to compare such treatments as little is known about released PLTX and OVTXs toxins which are released, their solubility as well as their stability in seawater. Moreover, the lack of harmonization between extraction procedures applied within studies also make comparisons difficult. It is extremely difficult to identify which chemical cues induced these toxic effects.

2.5.2. Was *Ostreopsis cf. ovata* ingested by *Sarsamphiascus cf. propinquus*?

Although fecal pellet production is rarely used to evaluate the food uptake in copepods (Souza-santos et al., 1995), the method is simple, fast and quite satisfying when only estimates are needed. In our study, mean egestion rates was between 0.19 and 0.62 pellets.copepod⁻¹.day⁻¹ when copepods

were fed with *O. cf. ovata*. This suggests a true ingestion of the toxic dinoflagellate by *S. cf. propinquus* even if the egestion rates were significantly lower than those obtained with all other food treatments.

This ingestion was confirmed by epifluorescence microscopy observations showing the presence of algal chlorophyll in the gut content. The copepods even seemed to have obtained an energetic advantage since mortality rates were lower when they were fed with *O. cf. ovata* compared to the no-food control. Nevertheless, *O. cf. ovata* alone was not enough to sustain the energy requirements of the copepods as mortality rates were higher in the mono-diet experiment compared to the mixed diet.

2.5.3. Negative impacts of *Ostreopsis cf. ovata* on the copepod physiology

The food uptake results show a clear decrease of fecal pellet production when the copepods fed on *O. cf. ovata*. Significant differences of fecal pellet production observed when the diatom was offered in mono-diet and in mixed-diet clearly, show that the presence of *O. cf. ovata* is deleterious. This difference could be due to the higher bio-volume of *O. cf. ovata* (compared to the control diatom *L. paradoxa*) or to its toxicity. Copepods are known to actively select the size of their prey (Mullin, 1963). However, some studies suggest that prey size might not be the main factor controlling ingestion. For example, De Troch et al. (2006) showed that the harpacticoid copepod *Harpacticus obscurus* had no clear preference for small diatoms. In the present study, the difference in fecal pellet production by *S. cf. propinquus* between the feeding treatments could then rather be explained by the presence of toxic secondary metabolites produced by *O. cf. ovata* since mortality was also higher in the presence of *O. cf. ovata*. The addition of the toxic dinoflagellate to the diet clearly contributed to increase copepods mortality. It might hence be suggested that the copepod could avoid toxic cells by chemosensory detection of toxic cues. Some studies have previously shown the ability of pelagic copepods to select a non-toxic prey in a mixed algal culture including the toxic dinoflagellate *Alexandrium excavatum* (Turrieff et al., 1995). Such a selection capacity, as suggested for *S. cf. propinquus*, is highlighted by a difference in fecal pellet production between mixed- and mono-diets of the diatom *L. paradoxa*. Even if the concentration of the diatom was the same in both diets, the decrease of egestion might be due to an effort to select non-toxic preys, leading to a lower ingestion rate of the algal particles. In addition to the decrease in fecal pellet production of copepods fed *O. cf. ovata*, hatching success was also significantly lower. In fact, compared to the no-food treatment, the presence of the toxic dinoflagellate reduced the fecundity and fertility ratios by 4.7-fold and 3.5-fold, respectively. These results agree with *in situ* observations of Guidi-Guilvard et al (2012) who showed changes in the composition of the meiobenthic community when *O. cf. ovata* blooms occurred in summer 2008, with a 72% decrease in the number of nauplii, hence suggesting that the dinoflagellate affected benthic harpacticoid copepods reproduction. These impacts on reproduction are comparable

to results obtained in previous studies for planktonic copepod species, such as *Temora stylifera* fed *Prorocentrum micans*, *Gymnodinium sanguinium*, and *Gonyaulax polyedra*, (Ianora et al., 1999; Laabir et al., 2001), as well as *Calanus finmarchicus* (Roncalli et al., 2016) and *Calanus sinicus* (Liu and Wang, 2002) fed *Alexandrium fundyense* and *Alexandrium tamarense* respectively. The reprotoxicity of microalgae on copepods could have various origins. For *Temora stylifera* fed on *Prorocentrum micans*, the reduced fertilization was due to an effect on the sperm. Maternal effects or male age are excluded since hatching rates returned to normal when new males were introduced in the experiment (Ianora et al., 1999).

It is worth noting that, due to technical constraints, our results were obtained for only a single *O. cf. ovata* strain. These effects on survival and reproduction should be ascertained using other strains from different sites even if these effects of *O. cf. ovata* on reproduction have already been demonstrated on sea urchin (Migliaccio et al., 2016).

2.5.4. Were these negative effects due to toxins produced by *Ostreopsis cf. ovata*?

After ingestion of *O. cf. ovata* cells, the release of toxins could have caused a gradual physical incapacitation of the copepod leading to a reduced feeding efficiency. Indeed, during the first 7 days mortality rates were higher with *O. cf. ovata* than in the no-food control. Moreover, during the first 4 days of the “food uptake” experiments, there was no significant difference between ingestion when the diatom was offered alone or when it was mixed with *O. cf. ovata*, suggesting again that the ingestion decrease observed after the first 5 days could be due to an alteration of the copepod’s physiology due to chemical cues produced by the dinoflagellate. This is supported by a previous study by Sykes and Huntley (1987), reporting that ingestion of *Gonyaulax grindleyi* and *Karenia brevis* (ex. *Ptychodiscus brevis*) cells can cause physiological reactions such as regurgitation or elevated heart rate. Other studies have moreover demonstrated an induction of domoic acid production by *Pseudo-nitzschia sp.* exposed to herbivorous copepods (Lundholm et al., 2018) which could act as zooplankton grazing deterrents. The question arises about the role of toxins produced by *O. cf. ovata* in the bloom formation and maintenance by deterring copepods. The ability of *Alexandrium minutum* to increase its toxin production in response to the exposition to copepods has already been demonstrated as a way to facilitate its bloom formation by being more resistant to grazers (Selander et al., 2006). However, the question concerning the digestibility of *O. cf. ovata* arises. *Acartia clausi* fed with the dinoflagellate *Prorocentrum micans*, showed a longer gut transit time suggesting that the digestibility of these cells was difficult, probably because of the presence of cellulose-rich compounds in the theca hence requiring additional enzymes to break down these compounds (Tirelli and Mayzaud, 2005).

Very little information is available regarding the effects of PLTX and OVTXs on sperm viability or as antimetabolic compounds. Nonetheless, this impairment on reproductive ability caused by *O. cf.*

ovata has previously been described in two species of sea urchins. In *Paracentrotus lividus*, fertilization success and progeny development processes were both compromised (Migliaccio et al., 2016) and in *Lytechinus variegatus*, fertilization and early stage development were also affected (Neves et al., 2018). The mode of action of toxins (i.e. OVTXs) produced by *O. cf. ovata* is poorly documented. PLTX, for instance, is known as a potent inhibitor of sperm motility in several species including the sea urchin *Tripneustes gratilla* (Morton et al., 1982). However, PLTX represents only 8 % of the known toxins contained in Villefranche *O. cf. ovata* (Brissard et al., 2014) and OVTXs toxicity still needs to be evaluated.

Reprotoxicity could also be related to the inadequate nutritive values of the dinoflagellate. A previous study has shown that the number of copepod nauplii depends on the quality of the ingested food, suggesting that the biochemical composition of the toxic dinoflagellate *Cochlodinium polykrikoides* may significantly affect *Acartia omorii* reproduction (Kyoungsoon et al., 2003). Such a hypothesis needs to be explored in detail by performing an assessment of the reproductive impacts of *O. cf. ovata* on other copepods or meiobenthic organisms, to confirm if the reproductive effect was due to an inadequate or poor nutritive value or to the toxicity of *O. cf. ovata* secondary metabolites. Interaction between *O. cf. ovata* and its environment could also be mediated by the presence of an extracellular mucilage (Giussani et al., 2015). Indeed, this dinoflagellate produces an abundant mucilaginous matrix, which helps the cells to adhere to substrates. This mucus, which plays diverse roles such as to improve competition with other micro-organisms (for space and nutrients) or as a physical barrier against predators, is actually increasingly reported for dinoflagellates (Barone, 2007; Giussani et al., 2015). During the present study, we often observed an intense production of mucus when *O. cf. ovata* concentrations were high. This mucilage helped the algal cells to adhere to the body of the copepods, mostly on their furca. This could have compromised the copepod's movements, and hence their ability to find food, which furthermore could explain the clear decrease in fecal pellet production when the toxic dinoflagellate was used as food. It could also have altered fertilization (Gasparini et al., 2000).

2.6. Conclusion

This study highlights the high resistance of *S. cf. propinquus* to acute toxicity from *O. cf. ovata*, compared to other tested animals. The observed resistance can result from an acclimatization process developed by this copepod species after frequent exposures to the toxic algae.

The present study, together with the previous *in situ* analyses undertaken by Guidi-Guilvard et al (2012), clearly shows the impact of *O. cf. ovata* on the reproduction of benthic copepods, and more precisely on the fecundity and fertility ratios. These deleterious effects on reproduction and

consequently on population growth, will modify the composition of benthic communities and can also suggest the existence of chemical defence mechanisms exposed by *O. cf. ovata* as a survival strategy against grazers.

2.7. Acknowledgements

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3. Synthèse de l'étude

La toxicité de la souche *Ostreopsis cf. ovata* utilisée dans cette étude a été vérifiée suivant le protocole de test biologique mis en place au chapitre 6 et confirmée par des analyses en UHPLC-HRMS/MS.

Le copépode benthique *Sarsamphiascus cf. propinquus* est très résistant à la souche d'*O. cf. ovata* utilisée dans cette étude. Ce résultat suggère même l'existence potentielle de mécanisme de résistance voire d'acclimatation. L'ingestion des cellules d'*O. cf. ovata* a été démontrée via le comptage des pelotes fécales et vérifiée par des observations microscopiques en épifluorescence, même si les taux d'ingestion restent faibles. Ces faibles taux d'ingestion peuvent être expliqués par la taille des cellules ou la présence d'une thèque protégeant les cellules. Néanmoins, l'hypothèse privilégiée est plutôt la production de toxines qui pourrait à la fois expliquer les effets reprotoxiques et la diminution de la production de pelotes fécales.

L'effet néfaste des toxines produites par *O. cf. ovata* observé sur les taux de fécondité et de fertilité confirme les résultats obtenus *in situ* par Guidi-Guilvard et al. (2012) et pourrait amener à un changement, voire un déséquilibre des communautés méiobenthiques.

Chapitre 4. Migration verticale d'*Ostreopsis* cf. *ovata* et interactions avec les copépodes planctoniques

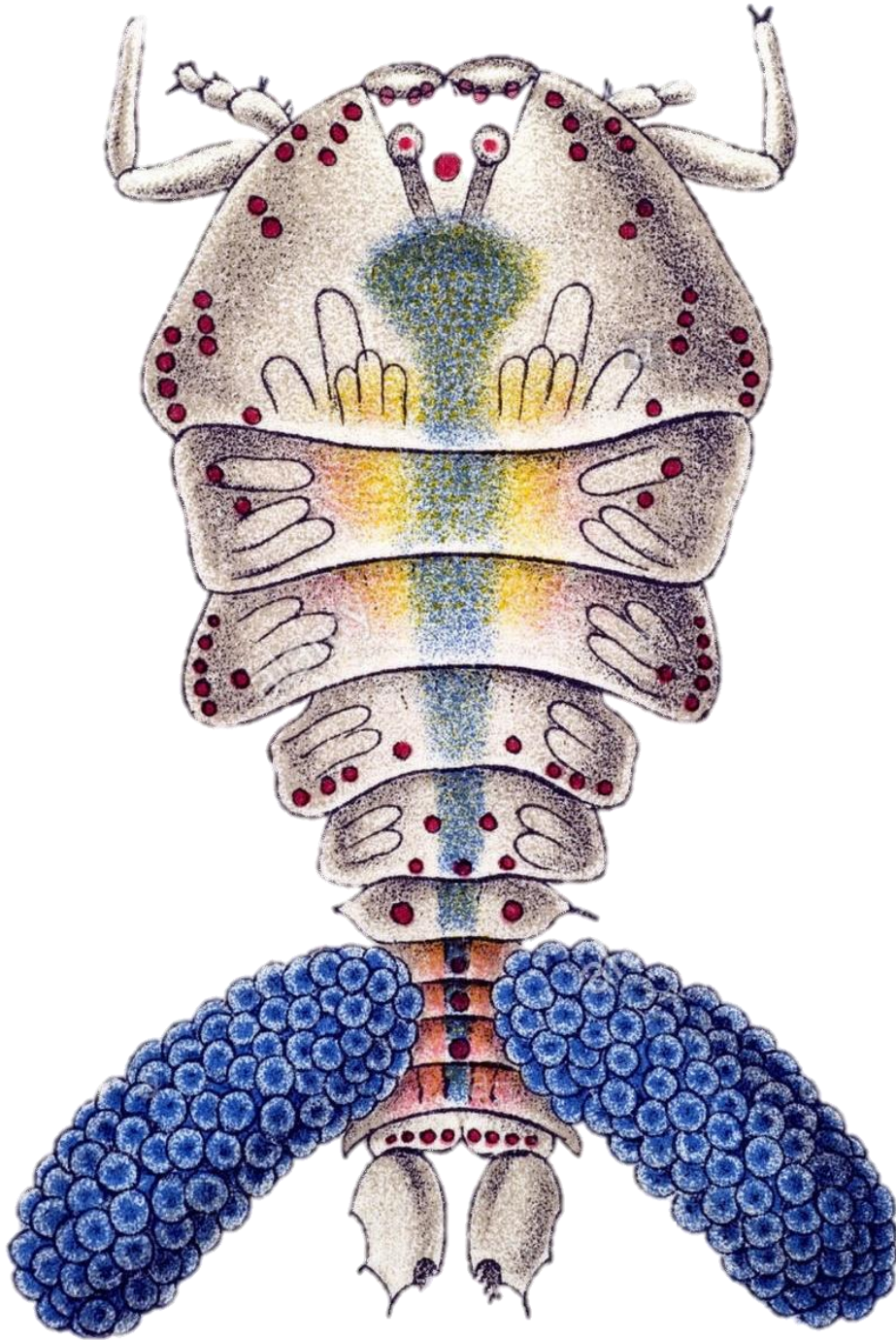


Illustration of *Sapphirina darwinii*. Plate of Wilhelm Giesbrecht (1892)

1. Présentation de l'étude

Ostreopsis cf. ovata est décrit comme un dinoflagellé benthique pourtant, l'étude de Mangialajo et al., (2011) a mis en évidence une corrélation entre les concentrations benthiques et planctoniques de ce dinoflagellé. Cette remise en suspension suggère des interactions potentielles entre les cellules d'*O. cf. ovata* et les organismes planctoniques

Nous nous sommes donc intéressés à la remise en suspension des cellules d'*O. cf. ovata* en réalisant un suivi des abondances à haute résolution temporelle. Pour cela, des prélèvements ont été réalisés *in situ*, à trois profondeurs différentes et ce toutes les 4 heures pendant 3 jours. La migration verticale des cellules d'*O. cf. ovata* ainsi quantifiée dans cette première partie, a permis d'évaluer la pertinence d'étudier les interactions entre *O. cf. ovata* et les organismes planctoniques, étude réalisée dans la deuxième partie de ce chapitre. Les espèces de copépodes planctoniques ciblées dans cette seconde partie sont *Acartia clausi*, *Temora stylifera* et *Lucicutia flavicornis*, trois espèces très fréquentes en Méditerranée. Ces espèces ont été exposées, en laboratoire, à différentes concentrations cellulaires d'*O. cf. ovata* afin de comparer leur sensibilité vis-à-vis de ce dinoflagellé toxique.

2. Etude des phases benthiques et planctoniques d'*Ostreopsis cf. ovata*

2.1. Introduction

Les différentes espèces d'*Ostreopsis* sont souvent décrites comme benthiques (Lenoir et al., 2004; Mangialajo et al., 2008; Penna et al., 2010) et les précédents chapitres de cette thèse se sont focalisés sur son impact sur d'autres organismes benthiques. Mais *Ostreopsis cf. ovata* est également décrit comme tychoplanctonique, c'est-à-dire occasionnellement planctonique : des cellules benthiques vont être remises en suspension dans la colonne d'eau et peuvent également former des agrégats à la surface, communément appelés fleurs d'eau (Carnicer et al., 2015a; Vila et al., 2016). Les mécanismes à l'origine de cette remise en suspension ne sont pas encore très clairs, même si le rôle de l'hydrodynamisme associé aux tempêtes ou à la brise thermique estivale, est souvent avancé (Mangialajo et al., 2011; Vila et al., 2016). Deux autres hypothèses sont actuellement discutées : (1) les bulles d'oxygènes produites durant la journée seraient séquestrées dans le mucilage exsudé par les microalgues benthiques et finirait par faire remonter les agrégats et (2) il existerait une migration verticale intrinsèque de cette espèce, selon un rythme nycthéral. A notre connaissance, ce phénomène de partage entre zones benthique et planctonique, des cellules d'*Ostreopsis cf. ovata*, n'a jamais été étudié ; la plupart des publications ne font qu'observer et quantifier les cellules dans chacun des compartiments.



Figure 17 : Site d'échantillonnage dans la rade de Villefranche au cours des étés 2018 et 2019.

Au cours des étés 2018 et 2019, les variations d'abondances benthiques et planctoniques d'*O. cf. ovata* ont été étudiées à haute résolution temporelle. Des prélèvements à trois profondeurs ont été réalisés toutes les 4 heures, jour et nuit pendant 72h, afin d'estimer les concentrations benthiques, planctoniques et de surface d'*O. cf. ovata*. Cette étude a été réalisée en collaboration avec David Velasquez, doctorant au Laboratoire du Biologie du Développement, qui s'intéresse à la reproduction asexuée d'*O. cf. ovata*, en utilisant des techniques de biologie cellulaire et de transcriptomique.

2.2. Matériels et Méthodes

2.2.1. Site d'échantillonnage

L'échantillonnage a été effectué dans une crique de la rade de Villefranche (lieu-dit Rochambeau) sur la côte méditerranéenne française (Figure 17 - 43°41'34.83" N and 7°18'31.66" E). Cette crique est connue pour présenter des efflorescences récurrentes d'*O. cf. ovata*, qui sont suivies depuis plus de 5 ans par l'équipe de R. Lemée (Sites A, B, C – Figure 17) et qui menacent périodiquement les activités récréatives pendant la saison estivale. La zone est caractérisée par une côte rocheuse abritée et protégée du vent thermique estival. Trois stations, séparées d'un mètre les unes des autres au niveau du site B (Figure 17), ont été sélectionnées en raison (i) de la facilité d'accès, même la nuit, (ii) de la qualité et de la quantité de macroalgues disponibles et (iii) du fait que qu'il s'agit déjà d'un site régulièrement échantillonné dans le cadre d'un suivi estival d'*O. cf. ovata* réalisé dans la baie de Villefranche-sur-mer.

2.2.2. Période d'échantillonnage

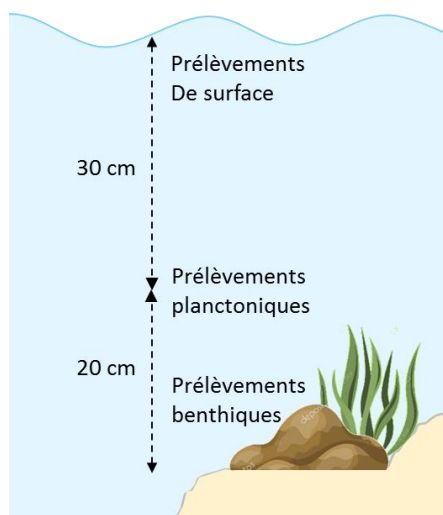


Figure 18 : Schéma représentant les différentes profondeurs échantillonnées.

L'échantillonnage s'est déroulé au cours de deux étés consécutifs. En 2018, les prélèvements ont été réalisés entre le 18 juillet à 8h et le 21 juillet 8h, soit juste après le pic de l'efflorescence d'*O. cf. ovata* sur ce site (Figure 19 a). En 2019, l'expérimentation a été réalisée au moment du pic de l'efflorescence soit entre le 5 juillet et le 8 juillet (Figure 19 b). Les prélèvements ont été réalisés toutes les 4 heures pendant 72h, à trois profondeurs (Figure 18) : (i) à 50 cm de profondeur, une macroalgue (poids frais environ $3,57 \pm 2,1$ g) a été prélevée dans un flacon de 250 mL, comme décrit par Cohu et al. (2013) afin d'estimer les concentrations benthiques d'*O. cf. ovata*. En 2018, les macroalgues prélevées (Figure 20) majoritairement (42%) furent *Dictyota sp.* (Lamouroux, 1809), mais *Padina*

pavonica (Linnaeus – Thivy 1960) et *Halopteris scoparia* (Linnaeus – Sauvageau, 1904) ont également été prélevées. En 2019, seule *Dictyota* sp. a été prélevée (Figure 20a); (ii) à 30 cm de profondeur, c'est à dire 20 cm au-dessus de la macroalgue citée précédemment, afin d'estimer les concentrations planctoniques ; et (iii) à la surface de l'eau afin de suivre l'apparition potentielle de fleurs d'eau. L'ordre des prélèvements est le suivant : surface, planctonique puis benthique, afin d'éviter toute remise en suspension pendant les prélèvements. La température de l'eau a été mesurée à chacun des prélèvements.

2.2.3. Traitement et comptages des échantillons

De retour au laboratoire, la salinité a été mesurée et les échantillons fixés au lugol acide (1 % v/v). Les échantillons planctoniques et de surface ont été conservés à 4°C jusqu'à leurs comptages, réalisés à l'aide d'un microscope inversé en utilisant la méthode d'Utermöhl et une colonne de sédimentation de 50 mL (Utermöhl, 1958). Les abondances planctoniques et de surface sont exprimées en nombre de cellules par mL. Les échantillons de macroalgues ont été vigoureusement agités afin de décrocher les cellules d'*O. cf. ovata* épiphytes de la macroalgue puis filtrés à travers un tamis de 500 µm pour séparer la macroalgue de l'eau de mer contenant les cellules d'*O. cf. ovata*. La macroalgue a été ensuite rincée à 2 reprises avec 100 mL d'eau de mer (filtrée sur 0.2 µm) et pesée. L'abondance des cellules épiphytes a été estimée à l'aide d'une lame de Sedgwick Rafter, qui permet de déterminer le nombre de cellules dans un volume de 1mL. L'abondance benthique est exprimée en nombre de cellules par gramme de poids frais de macroalgue (cell/gPF).

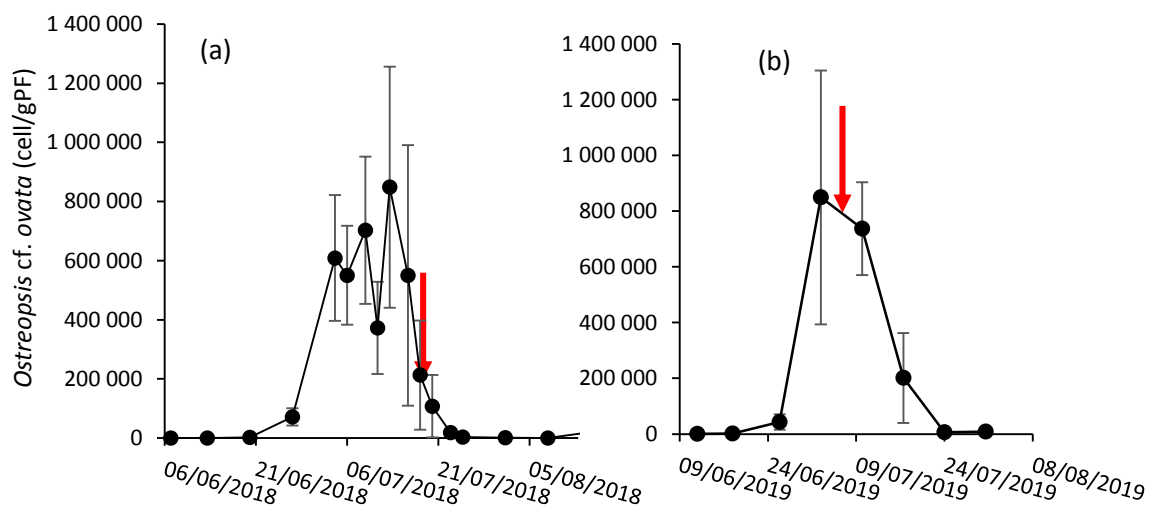


Figure 19 : Evolution de la concentration d'*Ostreopsis cf. ovata* à Rochambeau (Villefranche-sur-Mer, France) en 2018 (a) et 2019 (b). En 2018, la période d'échantillonnage à haute résolution temporelle s'est déroulée juste après le pic de l'efflorescence et au moment du pic de l'efflorescence en 2019 (flèches rouges).

Trois prélèvements de macroalgues supplémentaires ont été réalisés en 2019 pour des expérimentations en relation avec la reproduction asexuée de la microalgue (5g de macroalgues ont été prélevés sur le site le 5 juillet à 12h, 20h, et 4h). De retour en laboratoire, ces échantillons de macroalgues ont été agités pendant 10 secondes afin de décrocher les microalgues, et un sous-échantillon de 10 mL a été prélevé et fixé en paraformaldéhyde (PFA).

2.2.4. Taux de croissance apparent

Un taux de croissance apparent (taux résultant de la croissance, de la dispersion, de la prédation, de la mort cellulaire, etc...) d'*O. cf. ovata*, exprimé en nombre de cellules par heure (cellules.h⁻¹) a été calculé selon la formule suivante :

$$\text{Taux de croissance apparent} = \frac{\ln(\text{abondance cellulaire}_{t_1}) - \ln(\text{abondance cellulaire}_{t_0})}{t_1 - t_0}$$

Ce taux de croissance apparent a été calculé entre chacun des prélèvements (soit entre 8h et 12h par exemple).

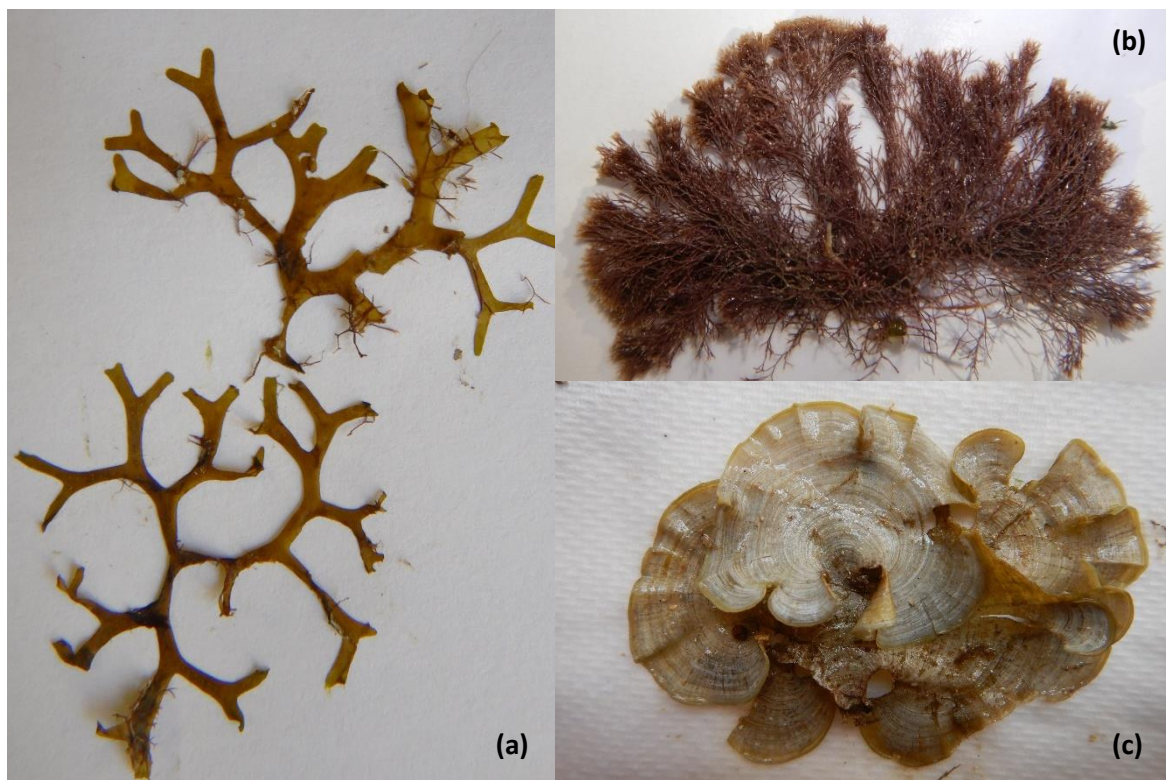


Figure 20 : Macroalgues échantillonnées en 2018 : (a) *Dyctiota* sp.(Lamouroux, 1809), (b) *Halopteris scoparia* (Linnaeus – Sauvageau, 1904) et (c) *Padina pavonica* (Linnaeus – Thivy 1960).

2.2.5. Analyse statistique

Le test de student a été réalisé afin de mettre en évidence des différences entre les taux de croissance apparents mesurés le jour et la nuit.

2.3. Résultats

2.3.1. Variations des abondances benthiques, planctoniques et de surface

L'abondance des cellules benthiques suit une tendance sinusoïdale (Figure 21 et 22) avec des abondances maximales en journée et minimales la nuit. En 2018, les abondances maximales benthiques sont reportées à 12h alors qu'elles se situent aux alentours de 8h en 2019. Le 19 juillet 2018, l'abondance cellulaire était de 44 269 cellules/gPF à 8h et atteint 136 338 cellules/gPF à 12h. L'abondance diminue ensuite pour atteindre 1 571 cellules/gPF à 20h. Une augmentation de l'abondance est observée à minuit et atteint 31 826 cellules/gPF avant de rediminuer à 7 961 cellules/gPF à 04h. Cette tendance sinusoïdale est également suivie par les concentrations cellulaires planctoniques et en surface aussi bien en 2018 qu'en 2019 et l'abondance maximale est plutôt atteinte aux alentours de 16h dans ces deux compartiments. La même tendance est suivie sur les deux années échantillonnées malgré des prélèvements réalisés à des périodes différentes de l'efflorescences.

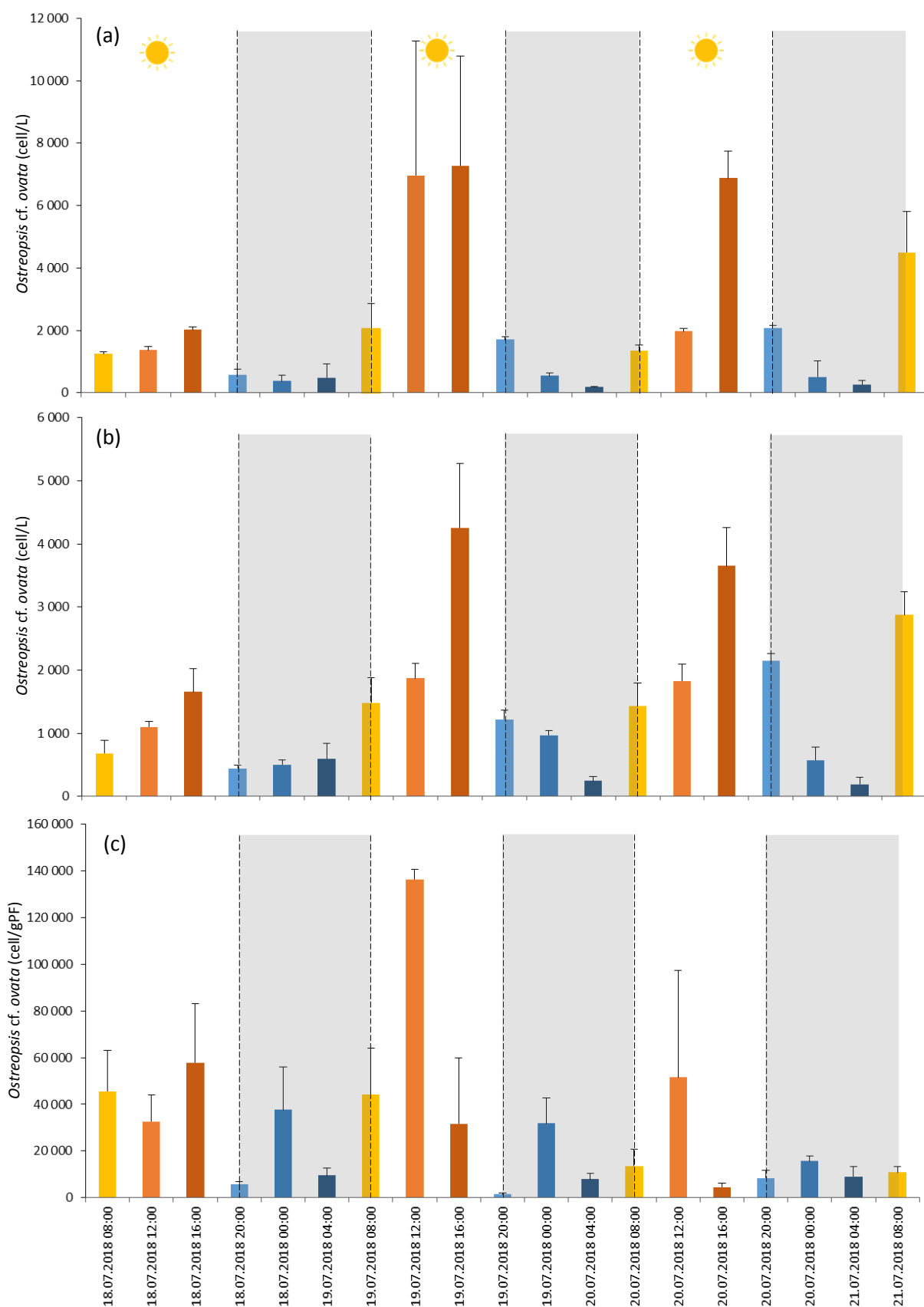


Figure 21 : Evolution de la concentration d'*Ostreopsis cf. ovata* en surface (a), planctonique (b) et benthique (c) au cours du temps en 2018. Les dégradés d'orange et de bleu représentent respectivement les abondances cellulaires obtenues la journée et la nuit.

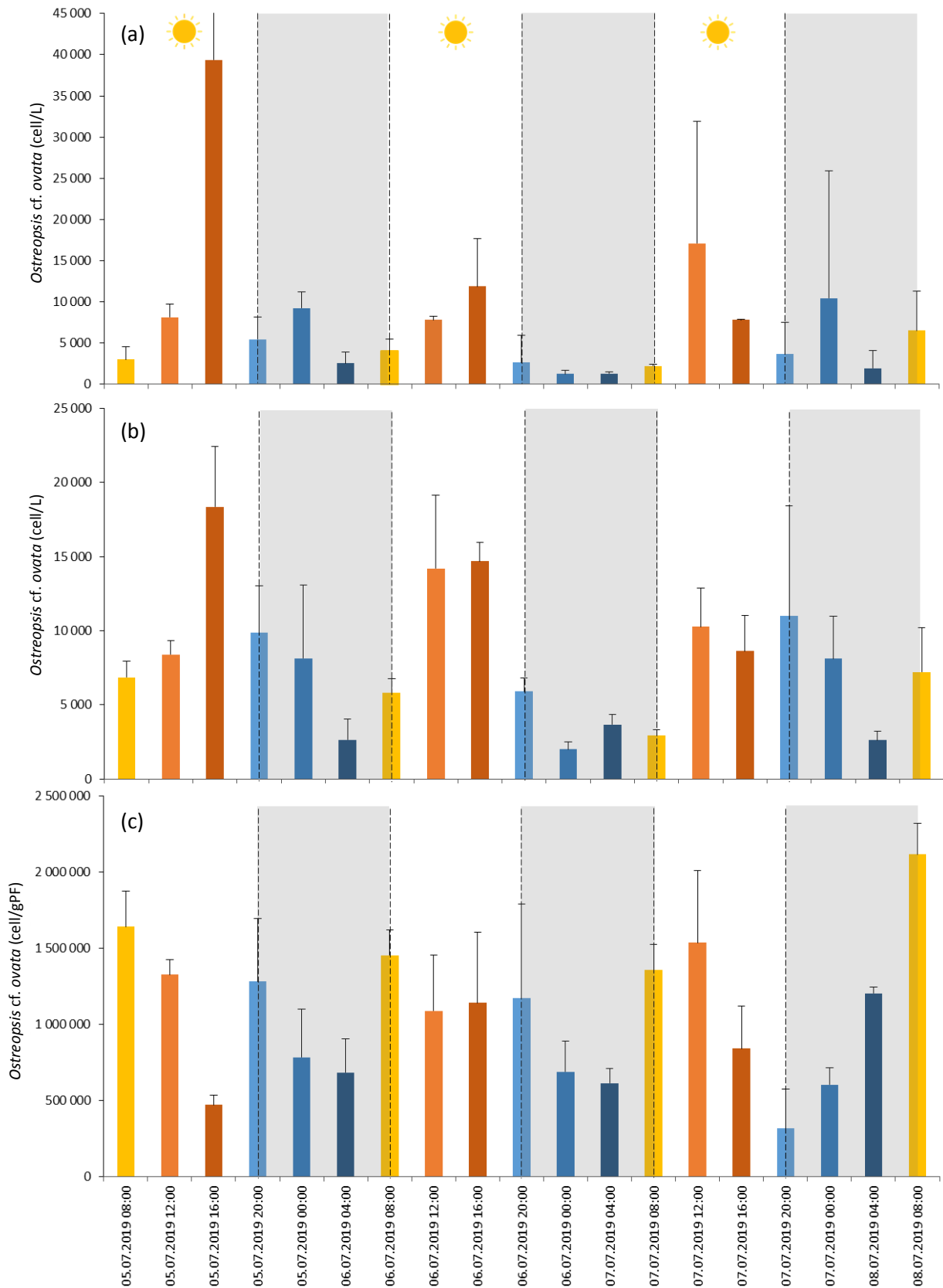


Figure 22 : Evolution de la concentration d'*Ostreopsis cf. ovata* en surface (a), planctonique (b) et benthique (c) au cours du temps en 2019. Les dégradés d'orange et de bleu représentent respectivement les abondances cellulaires obtenues la journée et la nuit.

2.3.2. Variations des abondances cellulaires à l'échelle d'une journée

Les abondances cellulaires ont également été moyennées par heure et par compartiments puis exprimées sous forme de proportions (Figure 23) afin d'obtenir les variations dans les différents compartiments pour une journée « type ». Une évolution cyclique est observée au cours de la journée : au niveau benthique, les abondances maximales sont atteintes à 12h et 8h en 2018 et 2019 respectivement. Les abondances maximales des cellules planctoniques et de surfaces sont obtenues simultanément mais plus tardivement, vers 16 h aussi bien en 2018 qu'en 2019.

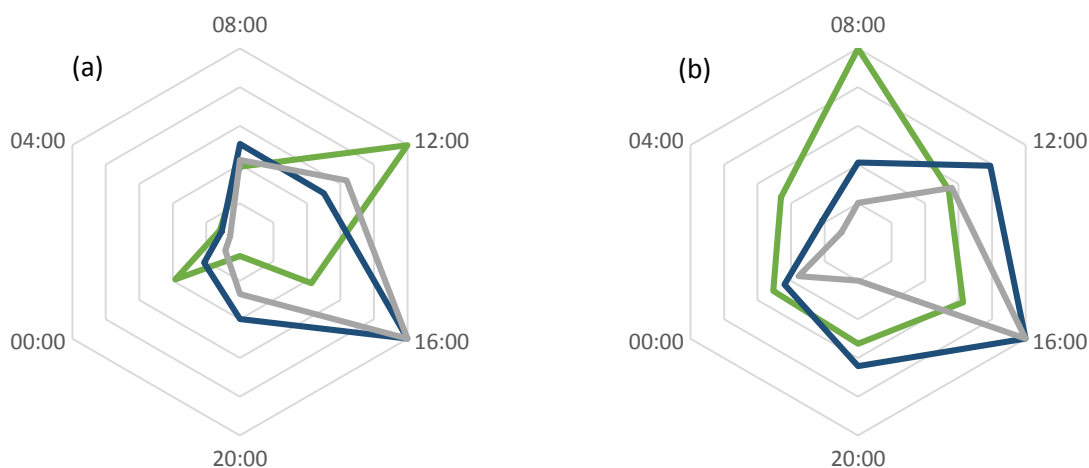


Figure 23 : Variation des abondances cellulaires d'*Ostreopsis cf. ovata* dans le benthos (vert), la colonne d'eau (bleu) et à la surface de l'eau (gris) en 2018 (a) et 2019 (b).

2.3.3. Taux de croissance apparent

Le taux de croissance apparent a été calculé pour toutes les profondeurs échantillonnées sur les deux années (Figure 24). Pour les cellules en surface et dans la colonne d'eau, les taux de croissance apparents sont significativement différents entre le jour et la nuit ($p < 0,01$) quelle que soit l'année étudiée. Ils sont toujours positifs la journée et négatifs la nuit. En 2018, en surface par exemple, les taux de croissance apparents sont compris entre 0,060 et 0,30 cellules.h⁻¹ la journée alors qu'ils sont compris entre -0,31 et -0,12 cellules.h⁻¹ la nuit.

Table 12 : Moyenne des taux de croissance apparents par profondeurs, période du jour et année.

		Surface	Colonne d'eau	Benthos
2018	Jour	0,28 ± 0,078	0,23 ± 0,068	0,043 ± 0,091
	Nuit	-0,23 ± 0,047	-0,19 ± 0,054	-0,10 ± 0,17
2019	Jour	0,20 ± 0,067	0,13 ± 0,046	0,036 ± 0,066
	Nuit	-0,18 ± 0,087	-0,13 ± 0,053	-0,025 ± 0,037

En moyenne, les taux de croissance apparents sont plus élevés en surface et dans la colonne d'eau (Tableau 12) aussi bien en 2018 qu'en 2019. Au niveau benthique, les taux de croissance apparents ne suivent pas cette même tendance et aucune différence significative n'est observée entre le jour et la nuit ($p > 0,05$). En 2019, les taux de croissance apparents sont très faibles voire nuls (ils sont compris entre -0,15 et 0,043) ; en 2018, ces taux restent également très faibles avec des variabilités très fortes.

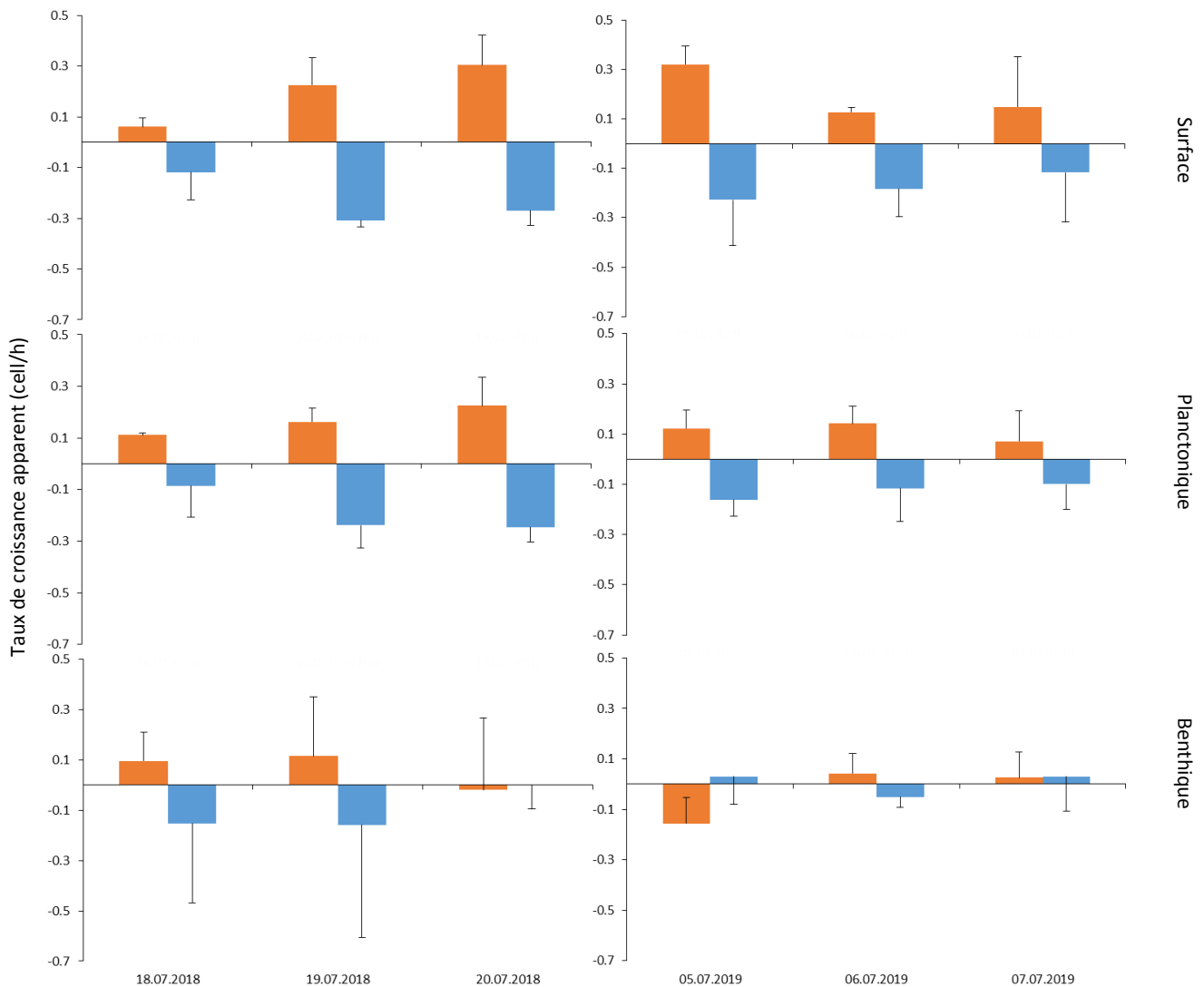


Figure 24 : Taux de croissance apparent des cellules d'*Ostreopsis cf. ovata* benthiques, planctoniques et de surface pour les années 2018 (gauche) et 2019 (droite).

2.4. Discussion

2.4.1. *Ostreopsis* cf. *ovata* est une microalgue tychoplanctonique

Ostreopsis spp. est décrite comme une microalgue benthique depuis la première description d'*Ostreopsis siamensis* dans le golfe de Siam par Schmidt en 1901. Pourtant plusieurs études ont reporté la présence de cellules d'*Ostreopsis* cf. *ovata* dans la colonne d'eau (Carnicer et al., 2015a; Mangialajo et al., 2011). Les mécanismes à l'origine de ces mouvements dans la colonne d'eau sont peu étudiés mais un rôle de la brise thermique et de son effet sur l'hydrodynamisme a déjà été avancé (Mangialajo et al., 2011; Vila et al., 2016). Dans cette étude, une variation cyclique des abondances benthiques a été observée au cours du temps, aussi bien lors du pic de l'efflorescence (année 2019) qu'à la fin de l'efflorescence (année 2018). Le maximum des abondances cellulaires est atteint le jour alors que les abondances minimales sont plutôt observées la nuit. Un décalage des abondances maximales est observé entre les trois compartiments : les abondances benthiques maximales sont atteintes en début de journée, entre 8h et 12h en 2019 et 2018 respectivement, alors que les abondances maximales sont atteintes plus tardivement dans la colonne d'eau et en surface (le plus souvent à 16h), suggérant une migration des cellules benthiques vers les compartiments planctoniques et de surface au cours de la journée. Cette migration est indépendante de la phase du bloom puisqu'elle est aussi bien observée à la fin de l'efflorescence en 2018, qu'au pic de celle-ci en 2019.

Le calcul des taux de croissance apparents moyens met en évidence des différences de taux de croissance fortes entre les compartiments. Pour les cellules planctoniques et de surface, les taux de croissance apparents sont les plus élevés et des différences significatives sont observées entre jour et nuit. Les taux de croissance sont positifs le jour alors qu'ils sont négatifs la nuit. Au niveau benthique, les taux de croissance sont très faibles, voire nuls et aucune différence significative n'est observée entre le jour et la nuit. Pourtant une nette augmentation des observations tôt dans la journée. Ces taux de croissance apparents faibles peuvent s'expliquer par une croissance et une division forte au début de la journée expliquant l'augmentation de l'abondance observée à 8h et 12h en 2019 et 2018 respectivement et une migration forte de ces cellules. Un grand nombre de cellules vont migrer vers les compartiments planctoniques et de surface expliquant (1) un faible taux de croissance apparent dans le compartiment benthique en journée puisque la migration des cellules est plus forte que la division cellulaire et (2) des abondances cellulaires qui augmentent au cours de la journée en surface et dans la colonne d'eau associées à des taux de croissance apparents positifs et forts la journée dans ces deux compartiments. Si cette hypothèse est avérée, elle sera facilement confirmée par les expérimentations de David Velasquez dont le but est de dénombrer le nombre de cellules en divisant à chacun des prélèvements via le marquage au Hoechst des noyaux. Une migration verticale intrinsèque a déjà été observée chez d'autres dinoflagellés toxiques planctoniques comme *Karenia brevis* (Schofield et al., 2006) ou *Gymnodinium catenatum* (Doblin et al., 2006), mais jamais décrite

pour les dinoflagellés benthiques. Cette étude permet de démontrer pour la première fois une migration des cellules d'*O. cf. ovata* au cours du jour et de la nuit, certainement en raison de la complexité des expérimentations et des mécanismes expliquant cette migration. Même si la migration est avérée, il est difficile de savoir si elle est active ou passive. L'hydrodynamisme associé à la brise thermique pourrait favoriser la remise en suspension des cellules benthiques dans la colonne d'eau. Mais d'autres mécanismes peuvent également intervenir à des échelles plus fines. La prolifération des cellules en profondeur engendre la production de fortes quantités de mucus, dans lequel des bulles d'oxygène provenant de la photosynthèse sont emprisonnées, pouvant favoriser la remise en suspension des cellules dans la colonne d'eau voire en surface. Toutefois, des mécanismes actifs peuvent également entrer en jeu comme c'est le cas pour *G. catenatum* dont la migration active vers le fond à la tombée de la nuit et vers la surface au début du jour, est liée à la prise de nitrate en profondeur la nuit (Doblin et al., 2006).

2.4.2. Variabilité des abondances cellulaires au cours du jour dans les différents compartiments : quels impacts pour les suivis sanitaires ?

Cette étude a démontré des variations importantes de l'abondance cellulaire d'*Ostreopsis cf. ovata* au cours de la journée aussi bien au niveau des cellules benthiques, planctoniques que des cellules en surface. Ceci aura donc des conséquences sur la qualité des suivis d'*O. cf. ovata* en mer Méditerranée et dans le monde. Malgré les efforts d'harmonisation des protocoles d'échantillonnage et de suivi en cours, il existe de très nombreuses sources de variations dans l'estimation des abondances d'*O. cf. ovata*. L'identification des cellules au microscope notamment est complexe, surtout quand différentes espèces co-occurrent et nécessite une grande expertise taxonomique (Battocchi et al., 2010) même si de nouvelles méthodes de comptages faisant intervenir des techniques de biologie moléculaire (qPCR - Battocchi et al., 2010; Casabianca et al., 2013) sont mises en place. Les variabilités spatiales à long terme (avec des blooms plus précoces en mer Ligure (Mangialajo et al., 2011; Vila et al., 2012) que sur la côte Catalane (Vila et al., 2016)) et à court terme des blooms notamment due aux variations dans la composition et la distribution des macroalgues dans le temps et l'espace constituent également une source de variabilité forte dans l'estimation des abondances. La diversité des méthodes de prélèvement utilisées (*vacuum apparatus* (Parsons et al., 2010), seringues (Abbate et al., 2012), substrats artificiels (Faust, 2009; Jauzein et al., 2016; Tester et al., 2014) ou macrophytes) sont également un facteur de variations, tout comme les différentes unités utilisées (de poids ou de surface - Jauzein et al., 2016). La migration des cellules d'*O. cf. ovata*, qu'elle soit active ou passive, complexifie encore les efforts d'harmonisation des données puisque selon l'heure des prélèvements, les concentrations obtenues sont différentes. Les seuils d'alerte français et italiens (Tichadou et al., 2010) sont basés sur les abondances planctoniques même si cela est critiqué

notamment en raison de la forte variabilité dans le temps et dans l'espace de ces concentrations (Lemée et al., 2012). Des prélèvements benthiques et planctoniques conjoints sont ainsi fortement indiqués pour obtenir une vision plus représentative des abondances cellulaires (Lemée et al., 2012; Vila et al., 2012). Concernant l'échantillonnage, les prélèvements sont régulièrement réalisés en début de journée, avant le début des activités récréatives. La variation des abondances planctoniques au cours de la journée amène à revoir les stratégies d'échantillonnage utilisées en intégrant ce nouveau paramètre.

2.5. Conclusion

Cette analyse a permis de mettre en évidence une variation importante des abondances cellulaires d'*Ostreopsis cf. ovata* dans les compartiments benthiques, planctoniques et de surface au cours de la journée, potentiellement en lien avec une migration verticale des cellules d'*O. cf. ovata*. Les prélèvements supplémentaires réalisés conjointement avec D. Velasquez pour la quantification des cellules en division sont toujours en cours d'analyse, mais apporteront des informations importantes quant à la période de division des cellules d'*O. cf. ovata*.

Ces variations d'abondances au cours de la journée sont à intégrer dans les stratégies d'échantillonnage d'*O. cf. ovata* car elles peuvent avoir un impact sanitaire prépondérant.

De part ces migrations, *O. cf. ovata* va donc pouvoir interagir avec les organismes planctoniques d'où la nécessité d'étudier l'impact de ce dinoflagellé sur les organismes planctoniques et notamment les copépodes.

Associée aux résultats obtenus par David Velasquez sur le cycle cellulaire d'*Ostreopsis cf. ovata*, cette partie de ma thèse fera l'objet d'une publication en cours de rédaction.

3. Impact d'*Ostreopsis cf. ovata* sur les copépodes planctoniques

3.1. Introduction

Ostreopsis cf. ovata n'est pas uniquement un organisme benthique. Les cellules de cette microalgue peuvent se retrouver en grande quantité dans la colonne d'eau et même en surface (cf. chapitre précédent). Il est par conséquent logique de penser qu'*O. cf. ovata* peut interagir sur les organismes planctoniques. Certaines études ont déjà montré un impact de cette algue sur des méduses et des larves d'oursins (Giussani et al., 2016; Migliaccio et al., 2016; Neves et al., 2018). Néanmoins, aucune étude ne s'est focalisée sur les copépodes planctoniques, alors qu'ils représentent une part importante de la biomasse du zooplancton, même en zone côtière (Christou, 1998).

Dans cette étude, nous nous sommes intéressés à l'impact (toxicité aiguë, évaluation de la mortalité) d'*O. cf. ovata* sur trois espèces de copépodes planctoniques prélevés en zone côtière, dans la rade de Villefranche, durant la période de développement d'*O. cf. ovata*. Il s'agit des espèces suivantes : *Acartia clausi*, *Temora stylifera* et *Lucicutia flavicornis*, espèces très fréquentes en mer méditerranée (Christou, 1998). Il est nécessaire de mieux comprendre l'impact d'*O. cf. ovata* sur les copépodes planctoniques car ces organismes sont considérés comme des vecteurs de transfert des toxines dans la chaîne alimentaire (Kozlowsky-Suzuki et al., 2006; Maneiro et al., 2000; Teegarden and Cembella, 1996). L'étude de Kozlowsky-Suzuki et al. (2006) a par exemple, mis en évidence une accumulation d'acide okadaïque produit par *Dinophysis* spp., par *Temora longicornis* et a quantifié un transport potentiel de 0,16 kg d'acide okadaïque quotidiennement vers les populations de sprat dans la Mer Baltique.

3.2. Matériels et méthodes

3.2.1. Échantillonnage des copépodes

Les copépodes ont été prélevés au niveau du site des Marinières (43°42'21.7"N 7°18'58.2"E – Villefranche-sur-Mer, France) à l'aide d'un filet WP2 de 200 µm de vide de maille, réalisé en sub-surface. De retour au laboratoire, les copépodes ont été séparés dans deux enceintes de culture de 20 L, placées dans des conditions contrôlées (température de l'eau de 24°C, salinité de 38 et obscurité) pendant 48h. Les prédateurs potentiels (ex : salpes, copépodes carnivores) ont été éliminés par une filtration douce à travers un tamis de 500 µm. Après cette acclimatation, les copépodes ont été triés et l'espèce majoritaire identifiée, soit *Lucicutia flavicornis* (Claus, 1863) le 21 août 2018, *Acartia clausi* (Giesbrecht, 1889) le 28 août 2018 et *Temora stylifera* (Dana, 1849) le 11 septembre 2018. Les copépodes ont été nourris avec un mélange de *Dunaliella salina* (Teodoresco, 1905 - MCCV 020) et *Tisochrysis lutea* (Bendif & Probert, 2013 - CCAP 927/14) pendant ces premières 48h d'acclimatation.

3.2.2. Culture cellulaire d'*Ostreopsis cf. ovata*

La souche d'*Ostreopsis cf. ovata* utilisée dans cette étude est la MCCV 054 dont le profil toxique est connu (cf chapitre 3) et conservée dans du milieu L1 préparé à partir d'eau de mer vieillie filtrée et autoclavée et dont la salinité est ajustée à 38. Cette souche est maintenue à 24°C suivant une photopériode 16:8 (lumière : obscurité).

3.2.3. Evaluation de la toxicité

Afin d'évaluer la toxicité d'*Ostreopsis cf. ovata* sur les trois espèces de copépodes, 8 conditions expérimentales correspondant à 7 concentrations différentes d'*O. cf. ovata* (2, 5, 10, 25, 50, 100 et 1000 cellules.mL⁻¹) ont été réalisées. Une condition contrôle a également été réalisée pour laquelle la concentration d'*O. cf. ovata* ajoutée est nulle. Cent vingt individus adultes (soit 15 individus par condition expérimentale) de chaque espèce ont été transférés à l'aide de pipettes pasteur dans des béchers remplis avec 20 mL d'eau de mer filtrée et autoclavée dont la salinité a été ajustée à 38. Les enceintes de culture (béchers de 20mL) utilisées dans cette expérimentation diffèrent de celles utilisées pour les copépodes benthiques (chapitre 3), car le maintien des copépodes planctoniques nécessite une certaine hauteur d'eau compte tenu de la nature de leurs mouvements). L'utilisation d'une roue à plancton a été envisagée mais les cultures d'*Ostreopsis cf. ovata* conservées en laboratoire supportent mal l'agitation (pavaux, comm. pers.), c'est pourquoi l'utilisation de béchers fixes, c'est-à-dire sans agitation, a été privilégiée. Les copépodes ainsi transférés dans les béchers ont été placés 48 h dans les mêmes incubateurs que ceux utilisés pour maintenir *O. cf. ovata* afin de les acclimater aux conditions utilisées durant l'expérimentation. Après cette seconde période d'acclimatation, les cellules d'*O. cf. ovata* ont été ajoutées afin d'obtenir les 7 concentrations finales suivantes : 2, 5, 10, 25, 50, 100 et 1000 cellules.mL⁻¹. La mortalité a été observée toutes les 2 heures pendant les 12 premières heures d'exposition puis après 24h et 48h d'exposition. Les copépodes sont considérés comme morts lorsqu'ils ne présentent plus aucune réaction à l'aspiration et à l'exposition la lumière pendant 10 secondes. La concentration létale médiane (ou LC₅₀) a été estimée comme la concentration correspondant à une mortalité de 50% de la population d'une espèce de copépodes à l'aide du package ecotoxicology disponible sous R (Gama, 2015).

3.2.4. Analyses statistiques

Un test de Kruskal-Wallis a été réalisé afin d'évaluer l'effet d'*O. cf. ovata* sur la mortalité des trois espèces de copépodes. En cas de différences significatives ($p < 0.05$), un test post-hoc de Dunn a été réalisé *a posteriori* pour identifier quelles conditions expérimentales étaient significativement différentes les unes des autres.

3.3. Résultats

La mortalité dans les conditions contrôles est supérieure à 10% : une mortalité de 13% est observée après respectivement 2h et 24h d'expérimentation pour *Lucicutia flavicornis* et *Acartia clausi* dans la condition contrôle (Figure 25). Selon les critères d'acceptabilité de ce test décrit par Faimali et al. (2012), les résultats obtenus sont difficilement exploitables. Néanmoins, *Ostreopsis cf. ovata* induit une mortalité des copépodes, quelle que soit l'espèce testée et cette mortalité augmente avec le temps et la concentration testée. Après 2 heures d'exposition de *Lucicutia flavicornis* à *O. cf. ovata*, la mortalité des copépodes atteint en effet 26,7% et 86,7% lorsqu'ils sont exposés respectivement à 5 et 1 000 cellules.mL⁻¹. La mortalité d'*Acartia clausi* est plus tardive : une mortalité de 13,4% et 73,3% des copépodes exposés à 5 et 1 000 cellules.mL⁻¹ est observée après 24h d'exposition.

Des différences significatives sont mises en évidence entre la condition contrôle et lorsque les copépodes sont exposés à 100 et 1000 cellules.mL⁻¹ d'*O. cf. ovata*, quelle que soit l'espèce de copépodes testée ($p < 0.05$).

Les LC₅₀ ont également été estimées après 24h d'exposition et sont de 1,15 cellules.mL⁻¹, 1,38 cellules.mL⁻¹ et 445,73 cellules.mL⁻¹ pour *Lucicutia flavicornis*, *Temora stylifera* et *Acartia clausi* respectivement.

3.4. Discussion

Cette étude a permis de tester la sensibilité de trois espèces de copépodes planctoniques *Acartia clausi*, *Temora stylifera* et *Lucicutia flavicornis*, prélevés en été dans rade de Villefranche-sur-Mer, et exposés à *Ostreopsis cf. ovata*. Les résultats obtenus montrent des mortalités importantes, même dans les conditions contrôle, mettant en évidence que le protocole expérimental utilisé dans cette étude est perfectible. La difficulté est de mettre en contact des organismes planctoniques et benthiques. Les études précédentes concernant des organismes planctoniques se sont portées sur l'exposition de larves de méduses et d'œufs d'oursins à *O. cf. ovata* (Giussani et al., 2016; Neves et al., 2018), par l'intermédiaire de boîtes de pétri et plaques multi-puits qui n'offrent pas de meilleures conditions expérimentales que celles utilisées dans notre étude. L'utilisation d'enceintes de culture plus grandes et/ou rotatives est probablement à envisager pour améliorer et diminuer les mortalités liées aux conditions expérimentales.

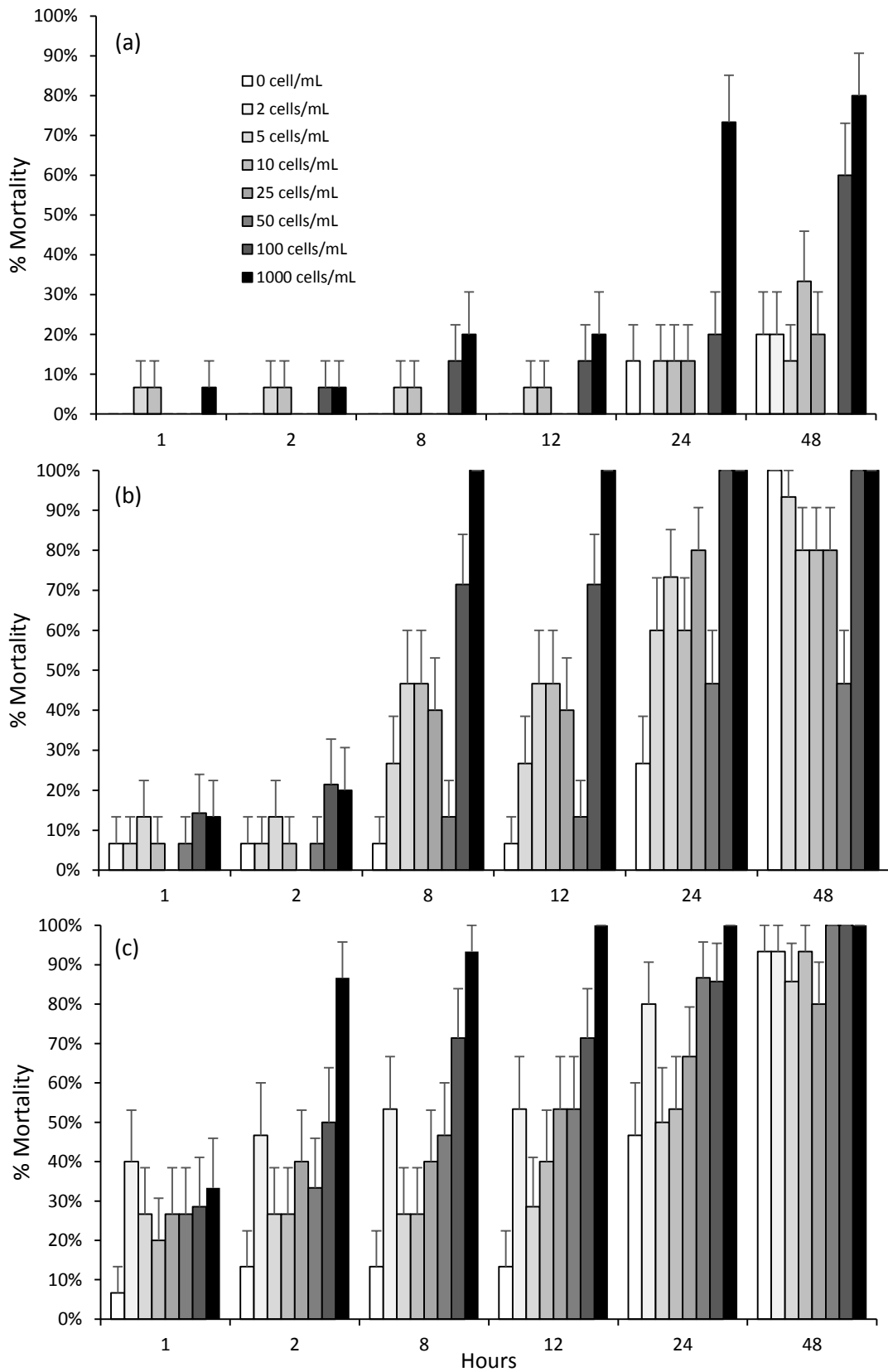


Figure 25 : Pourcentage de mortalité des copépodes planctoniques *Acartia clausi* (a), *Temora stylifera* (b) et *Lucicutia flavicornis* (c) exposés à différentes concentrations d'*Ostreopsis cf. ovata*.

Les trois espèces de copépodes testées montrent des sensibilités différentes à *O. cf. ovata* : *Acartia clausi* est la moins sensible des trois espèces de copépodes ($LC_{50} = 445,73 \text{ cellules.mL}^{-1}$). *Temora stylifera* et *Lucicutia flavicornis* sont très sensibles à la présence de ce dinoflagellé puisque 1,38 et 1,15 cellules.mL^{-1} sont suffisantes pour observer 50% de mortalité après 24h de présence du dinoflagellé. La mortalité d'*A. clausi* est plus tardive et sa moindre sensibilité est confirmée par le calcul des LC_{50} (24h) qui est près de 450 fois supérieures à celle de *L. flavicornis* et *T. stylifera*. Les trois espèces de copépodes planctoniques testées sont plus sensibles que le copépode benthique *Sarsamphiascus cf. propinquus* (cf. chapitre 3 de cette thèse). Ils sont également plus sensibles que *Tigriopus fulvus*, un autre copépode benthique dont la LC_{50} (24h) est comprise entre 1486 cellules.mL^{-1} et des valeurs supérieures à 4 000 cellules.mL^{-1} (Faimali et al., 2012; Giussani et al., 2016). Ces différences pourraient s'expliquer par le développement de mécanismes adaptatifs plus efficaces chez les espèces potentiellement plus exposées (comme les copépodes benthiques) tandis que les copépodes pourraient opter pour une stratégie de fuite afin d'éviter toute exposition à *O. cf. ovata*. Il a déjà été démontré qu'en réponse à des efflorescences de *Phaeocystis globosa*, *Temora longicornis* est capable d'adapter son comportement natatoire en réalisant des sauts plus petits et plus complexes, favorisant sa survie et à sa prise de nourriture en période d'efflorescence de cette Prymnésiophycées (Seuront and Vincent, 2008). Néanmoins, des processus d'acclimatation ont déjà été montré pour d'autres espèces de copépodes planctoniques exposés au dinoflagellé toxique *Alexandrium fundyense* (Colin and Dam, 2005). La population d'*Acartia hudsonica* originaire du Maine, qui a vécu des efflorescences récurrentes d'*Alexandrium* spp. depuis des décennies présentaient, lors d'une exposition à *A. fundyense* en laboratoire, une meilleure croissance somatique, des tailles plus importantes ainsi qu'une production d'œuf et une survie supérieure à la population d'*A. hudsonica* de New Jersey, qui n'avait jamais été exposée aux efflorescences d'*Alexandrium* spp. Cette adaptation des populations locales locale a été confirmée par des expériences de sélection génétique en laboratoire montrant notamment une augmentation de l'ingestion et la production d'œufs après 3 générations de copépodes naïfs maintenus en élevage avec un régime alimentaire à base de *A. fundyense*.

La réponse des copépodes exposés à des algues toxiques est également espèce dépendante puisque l'ingestion d'algues toxiques n'est pas néfaste pour certains copépodes planctoniques (Turner, 2014). C'est le cas d'*A. clausi* et *Temora longicornis* qui se nourrissent respectivement de *Pseudo-nitzschia multiseriis* (Maneiro et al., 2005) et *Dinophysis norvegica* et *Dinophysis acuta* (Kozlowsky-Suzuki et al., 2006) sans présenter aucun effet néfaste. Pourtant, des effets négatifs sont observés lorsque *A. clausi* se nourrit d'*Alexandrium tamarense* (Wohlrab et al., 2010).

3.5. Conclusion

La portée des résultats est malheureusement limitée par les difficultés méthodologiques induites par la mise en contact d'organismes planctoniques avec un dinoflagellé toxique considéré comme tychoplanctonique. Toutefois, les résultats montrent clairement que la sensibilité des copépodes à *Ostreopsis cf. ovata* est espèce dépendante et que les copépodes planctoniques sont beaucoup plus sensibles que les copépodes benthiques. Le développement de mécanismes adaptatifs en raison d'une exposition plus importante et plus fréquente des copépodes benthiques, pourrait expliquer ces observations.

4. Synthèse des résultats

Longtemps décrite comme benthique, cette étude a pourtant permis de décrire le caractère tychoplanctonique d'*Ostreopsis cf. ovata*. Des variations fortes d'abondance cellulaires ont été mises en évidence au cours de la journée, aussi bien pour les concentrations benthiques, planctoniques que de surface, suggérant une potentielle migration verticale d'*O. cf. ovata*. Ces variations d'abondances cellulaires au cours de la journée sont à intégrer dans les stratégies d'échantillonnage de ce dinoflagellé en particulier pour les suivis sanitaires.

La remise en suspension d'*O. cf. ovata* confirme également la nécessité de mieux comprendre l'impact de ce dinoflagellé sur les espèces vivant dans la colonne d'eau et notamment les copépodes planctoniques. Les trois espèces testées dans cette étude présentent des sensibilités différentes vis-à-vis d'*O. cf. ovata* mais demeurent beaucoup plus sensibles que les copépodes benthiques. Des études supplémentaires sont nécessaires afin de savoir si cette plus forte sensibilité est due (1) à la capacité de fuite des copépodes planctoniques et ainsi l'absence de processus d'acclimatation à cette algue ou (2) à des processus de résistance inhérents aux copépodes benthiques.

Chapitre 5. Impacts d'*Ostreopsis cf. ovata* sur la
macrofaune herbivore : cas de l'oursin adulte
Paracentrotus lividus



Echinoidea. Plate from Ernst Haeckel's "Kunstformen der Natur" (1904)

1. Présentation de l'étude

En Méditerranée, l'oursin comestible *Paracentrotus lividus* est considéré comme un des principaux macroherbivores avec la Saupe (poisson ; *Sarpa salpa*). En se nourrissant des macroalgues qui constituent un substrat privilégié pour *Ostreopsis cf. ovata*, ces oursins vont être en contact direct avec ce dinoflagellé toxique. Des études antérieures ont reporté (i) des épisodes de mortalités de masse des oursins au cours des efflorescences d'*Ostreopsis* spp., notamment en Nouvelle Zélande et (ii) une accumulation des toxines au niveau du tube digestif jusqu'à 15 fois supérieures aux limites préconisées par l'Agence Européenne de Sécurité des Aliments, confirmant ainsi la pertinence de ce modèle, aussi bien d'un point de vue écosystémique que d'un point de vue de santé publique.

Afin d'étudier les effets d'*O. cf. ovata* sur les oursins adultes de l'espèce *Paracentrotus lividus*, des études *in situ* réalisées au cours de la période d'efflorescence d'*O. cf. ovata* ont été couplées à des études en milieu contrôlé, en laboratoire. Les paramètres utilisés pour estimer les potentiels effets de ce dinoflagellé sont le temps de retournement, un paramètre comportemental couramment utilisé pour estimer l'état de santé des oursins, le taux d'ingestion, ainsi que le rapport gonado-somatique.

2. Introduction

Au cours des quinze dernières années, une intensification mondiale des efflorescences d'*Ostreopsis* sp. a été reportée (Mangialajo et al., 2011). Même s'il est difficile de savoir si ce phénomène est directement lié à une augmentation réelle du nombre d'événements, ou uniquement lié à un effort d'échantillonnage plus important, le nombre de cas d'hospitalisations dû à une intoxication à *Ostreopsis cf. ovata* a considérablement augmenté (Durando et al., 2007; Gallitelli et al., 2005). Plus de 200 personnes ont ainsi été hospitalisées à plusieurs reprises en Espagne, en Algérie et en Italie entre 2001 et 2006 suite à une exposition à *O. cf. ovata* (Brescianini et al., 2006; Illoul et al., 2012; Vila et al., 2008). Les intoxications, probablement liées à l'aérosolisation des cellules d'*O. cf. ovata* (Ciminiello et al., 2014; Scalco et al., 2012), provoquent des irritations cutanées et oculaires, des nausées, des diarrhées et des problèmes respiratoires. Les efflorescences d'*O. cf. ovata* sont également liées à des mortalités fortes d'organismes benthiques, comme c'est le cas en Nouvelle Zélande, en Italie et en Espagne, où des mortalités accrues d'invertébrés (dont des échinodermes) ont été reportées (Ciminiello et al., 2006; Shears and Ross, 2009; Totti et al., 2010; Vale and Ares, 2007).

Dans ce travail, l'effet d'*Ostreopsis cf. ovata* sur l'oursin *Paracentrotus lividus* (Lamarck, 1816), l'un des principaux macroherbivore en Méditerranée (Ruitton et al., 2000), a été étudié. Cette espèce est comestible et largement pêchée en Méditerranée (Lawrence, 2013). Elle est également une espèce clef, en constituant le brouteur dominant des zones benthiques peu profondes de la Mer Méditerranée (Ruitton et al., 2000), zone de prédilection pour le développement du dinoflagellé *O. cf. ovata*. Des études antérieures ont montré une forte accumulation des toxines produites par *O. cf. ovata* par cette espèce d'oursin (Amzil et al., 2012; Biré et al., 2015, 2013; Brissard et al., 2014; Milandri et al., 2010) : jusque $423 \mu\text{g eq. PLTX.kg}^{-1}$ ont été mesurés dans le tube digestif, soit des taux 15 fois supérieurs aux limites préconisées par l'Agence Européenne de Sécurité des Aliments (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009). Les études réalisées montrent que les toxines sont uniquement retrouvées au sein tube digestif, mais pas au niveau des gonades, normalement les seules parties consommées par l'Homme. Des études *in vitro* ont mis en évidence un impact important des toxines produites par *O. cf. ovata* sur la fécondation et les premiers stades larvaires de ce macroherbivore (Caroppo and Pagliara, 2011; Pagliara and Caroppo, 2012). Neves et al. (2018) ont montré une diminution significative de la fécondation ainsi qu'une augmentation des malformations des larves issues des gamètes exposés aux toxines d'*O. cf. ovata*. L'étude menée par Migliaccio et al. (2016) a montré un effet néfaste des toxines produites par *O. cf. ovata* sur la fécondation et le développement larvaire jusque 8 mois après l'exposition des adultes à une efflorescence de ce dinoflagellé. Toutefois, aucune étude ne s'est penchée sur l'impact de ce dinoflagellé sur la physiologie de l'oursin adulte, alors qu'il est potentiellement soumis à des abondances très fortes d'*O. cf. ovata*, pouvant atteindre

plus de 2 541 000 cellules par gramme de poids frais d'algue (Mangialajo et al., 2008). Plusieurs études ont pourtant déjà mis en évidence une forte mortalité d'organismes benthiques, et notamment d'oursins adultes, au cours d'efflorescences d'*O. cf. ovata* (Accoroni et al., 2011; Illoul et al., 2012; , ainsi qu'une perte des épines, indicateur d'un stress vécu par ces oursins (Sansoni et al., 2003; Shears and Ross, 2009). Le lien entre ces épisodes de mortalité de masse et les efflorescences d'*O. cf. ovata* n'a jamais été démontré. Il est aujourd'hui difficile de savoir si ces mortalités de masse sont dues uniquement à la toxicité des composés produit par *O. cf. ovata*, ou aux conditions anoxiques/hypoxiques générées indirectement par la respiration bactérienne dégradant l'abondante biomasse d'*O. cf. ovata*.

Afin d'étudier les effets directs de ce dinoflagellé sur les oursins, deux types d'expérimentation, *in situ* et en laboratoire, ont été réalisées. Un suivi *in situ* des oursins a tout d'abord été réalisé, au niveau de deux sites localisés dans la baie de Villefranche, où les concentrations d'*O. cf. ovata* sont totalement différentes : le Lido où les concentrations sont très faibles, voire nulles, et Rochambeau, où les concentrations peuvent atteindre 1 665 873 cellules par gramme de poids frais d'algues. Au cours de ce suivi, le temps de retournement, le rapport gonado-somatique des oursins ainsi que la concentration d'*O. cf. ovata* au cours des été 2017 et 2018 ont été étudiés. L'impact d'*O. cf. ovata* sur les oursins a également été étudié en laboratoire, en conditions contrôlées, en exposant les oursins à des concentrations réalistes d'*O. cf. ovata*, ajoutées à la nourriture artificielle préparée pour maintenir les oursins en aquarium.

Cette étude combine donc des méthodes d'études *in situ* et d'autres en conditions totalement contrôlées afin de suivre la réponse des oursins adultes à l'exposition saisonnière au dinoflagellé toxiques *Ostreopsis cf. ovata*.

3. Matériels et Méthodes

3.1. Expérimentations *in situ*

Deux sites ont été choisis pour étudier l'effet d'*Ostreopsis cf. ovata* sur l'oursins *Paracentrotus lividus* (Figure 26) : le site de Rochambeau (43°41'35.64"N - 7°18'31.54"E, Villefranche-sur-Mer, France), un site peu profond où de fortes concentrations d'*Ostreopsis cf. ovata* sont reportées depuis une dizaine d'année l'été (Lemée, comm. pers.), ainsi que le site du Lido (43°41'36.3"N - 7°19'26.1"E, Saint-Jean-Cap-Ferrat, France) où les concentrations d'*O. cf. ovata* sont au contraire faibles, voire nulles, et constituant de cette manière un site témoin. Un suivi spécifique des concentrations de ce dinoflagellé a été effectué au niveau de ces deux sites, au cours des années 2017 et 2018 (soit du 14 juin au 13 août 2017 et du 9 juin au 23 août 2018). Des échantillons de macroalgues ont été prélevés à 2 m de profondeur (au lieu de 0,5 m de profondeur réalisé lors d'un suivi classique) afin d'estimer la

concentration d'*O. cf. ovata* à la profondeur à laquelle les oursins ont été prélevés. Les macroalgues les plus abondantes à 2 m de profondeurs sont respectivement *Halopteris scoparia* (Linnaeus – Sauvageau, 1904) et *Dictyota* sp. (Lamouroux, 1809) à Rochambeau et au Lido, en 2017 et 2018. L'abondance des cellules épibenthiques d'*O. cf. ovata* a alors été déterminée après fixation des échantillons de macroalgues au lugol et séparation suivant le protocole établi par Cohu et al. (2013). Dix oursins adultes sauvages (diamètre du test = à $4,65 \pm 0.2$ cm) ont été respectivement choisis de manière hebdomadaire à 2 m de profondeur sur les sites de Rochambeau et au site du Lido. Le temps de retournement et la taille ont été mesurés, dans un premier temps, avant de prélever ces oursins afin d'estimer le rapport gonado-somatique dès le retour au laboratoire. A noter que ce rapport gonado-somatique n'a été déterminé que pour l'année 2017.

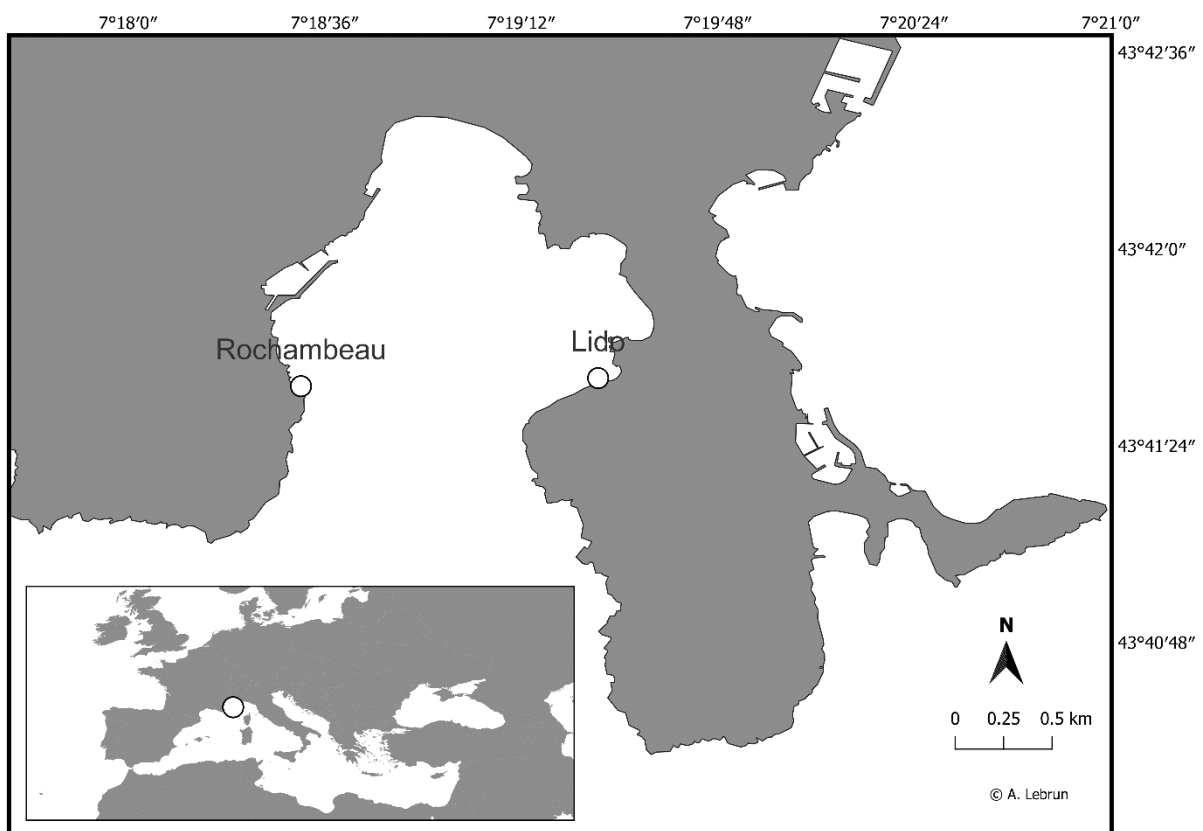


Figure 26 : Sites d'échantillonnage dans la rade de Villefranche au cours des étés 2017 et 2018

3.2. Expérimentations en laboratoire

Pour l'expérimentation en laboratoire, trente oursins adultes sauvages (diamètre $4,9 \pm 0,05$ cm) ont été prélevés au niveau du site de la Pointe Malalongue ($43^{\circ}40'27.1''N - 7^{\circ}19'35.3''E$, Saint-Jean-Cap-Ferrat, France) à 20 m de profondeur afin d'éviter que les oursins n'aient déjà été exposés à *Ostreopsis cf. ovata*. Les oursins ainsi échantillonnés ont alors été transférés dans des aquariums de 20 L à raison d'un oursin par aquarium. La température de l'eau de mer, renouvelée en continue (système ouvert), était comprise entre 24 et 26,5°, la salinité ajustée à 40 et une photopériode de 16:8

(Lumière/obscurité). Une arrivée d'air permettait une homogénéisation continue des paramètres physico-chimiques des aquariums (Figure 27 a). En raison de la potentielle aérosolisation des toxines d'*O. cf. ovata*, il nous était impossible de nourrir les oursins avec des macroalgues couvertes d'*O. cf. ovata* prélevées directement *in situ*. Les oursins ont donc été nourris avec une nourriture artificielle préparée à base d'un mélange de spiruline (7g – GreenSea, France), de paillettes pour poissons (30g – Tetra, Allemagne) et d'un œuf cuit (Shpigel et al., 2004 - Figure 27 c). L'agar liquide (60°C - 17g - Fisher Scientific, Angleterre) est ensuite ajouté et le mélange obtenu est coulé dans des boîtes de pétri (diamètre de 55 mm) tarés afin de connaître la quantité de nourriture préparée et ensuite ingérée par les oursins. Les 30 oursins ont alors été nourris pendant une semaine avec cette nourriture artificielle avant d'être divisé en trois groupes correspondant à trois conditions expérimentales : 10 oursins ont été nourris avec la nourriture artificielle à laquelle ont été ajoutés des concentrations d'*O. cf. ovata* réalistes i.e. des concentrations atteintes naturellement par *O. cf. ovata* en période d'efflorescences (Cohu et al., 2013; Mangialajo et al., 2011; Vila et al., 2008), soit d'abord 500 000 cellules.g⁻¹, puis 1 000 000 cellules.g⁻¹, avant de redescendre à 500 000 cellules.g⁻¹ (Figure 27 b). La souche d'*O. cf. ovata* (MCCV 054) utilisée dans cette étude, provient de la collection de culture de Villefranche (Mediterranean Culture Collection of Villefranche – MCCV - <http://www.embrc-france.fr/fr/our-services/supply-biological-resources/microorganisms/microorganismes-unicellulaires>). Elle a été cultivée en laboratoire à 24°C dans du milieu L1 (Andersen, 2005) dont la salinité est ajustée à 38 et avec une photopériode de 12h/8h (Lumière/obscurité) et utilisée pour enrichir la nourriture artificielle en *O. cf. ovata*. La toxicité de cette souche a été vérifiée via l'utilisation du test biologique mis en place dans le chapitre 6 ainsi que par l'utilisation de méthode de chimie analytique (UHPLC-HR-MS/MS ; voir résultats page 6 de cette thèse). Un autre lot de 10 oursins a été nourris avec la même nourriture artificielle à laquelle la diatomée *Licmophora paradoxa* (MCCV 033) a été ajoutée. Cette diatomée, co-occurente avec *O. cf. ovata* (Ternon et al., 2018), est également cultivée en laboratoire dans les mêmes conditions d'*O. cf. ovata*. Les concentrations de *L. paradoxa* sont équivalentes à celle d'*O. cf. ovata* en termes de biovolume (comme estimé dans le chapitre 3) soit d'abord 1 000 000 cellules.g⁻¹ puis 2 000 000 cellules.g⁻¹ et enfin 1 000 000 cellules.g⁻¹. Enfin, les 10 derniers oursins ont été nourris avec la nourriture artificielle sans aucun ajout. Après trois semaines nourris de cette façon, les 30 oursins ont tous été à nouveau nourris avec une nourriture artificielle sans ajout (Figure 27b).

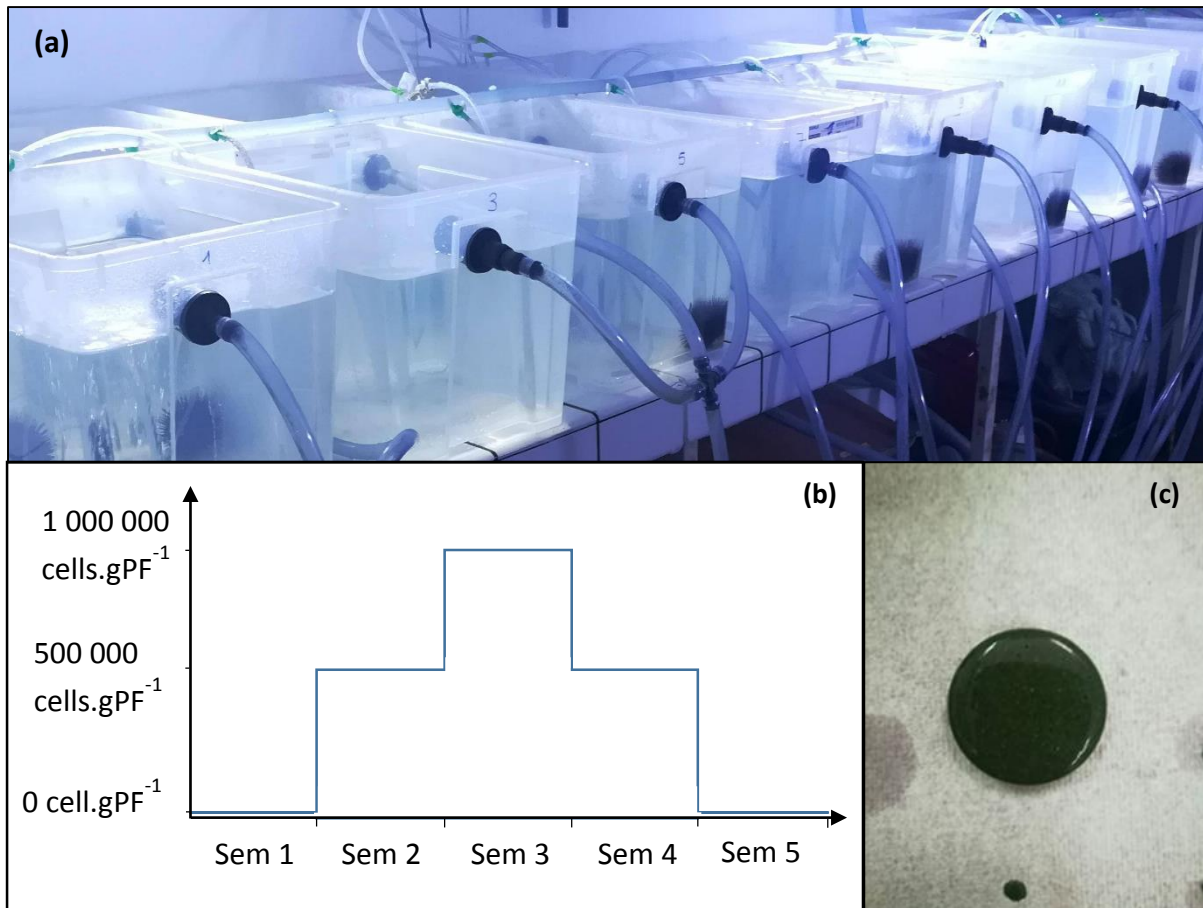


Figure 27 : Installation utilisée pour maintenir les oursins pour l'expérimentation en laboratoire (a). Les oursins ont été exposés à des concentrations variables d'*Ostreopsis cf. ovata* (b) incorporées à la nourriture artificielle préparée en laboratoire pour maintenir les oursins (c).

La température de l'eau ainsi que la salinité ont été mesurées quotidiennement et les aquariums ont été nettoyés tous les 2 jours afin de limiter l'ingestion des fèces par les oursins. Lors de ces nettoyages, la nourriture artificielle restante a été récupérée puis pesée afin d'estimer les quantités ingérées. Le temps de retournement a été mesuré une fois par semaine, à chaque changement de condition. A la fin de l'expérimentation, soit après 5 semaines, les oursins ont été sacrifiés afin de mesurer le rapport gonado-somatique.

3.3. Paramètres d'études

Afin d'étudier l'effet d'*O. cf. ovata* sur l'état de stress des oursins, le temps de retournement des oursins a été mesuré. Cet indicateur est régulièrement utilisé chez ces échinodermes puisqu'il reflète le bon fonctionnement du système neuromusculaire régulant la locomotion (Bayed et al., 2005; Percy, 1973) et a déjà été utilisé dans différentes études pour mettre en évidence un stress en réponse à des conditions environnementales variables (Lawrence and Cowell, 1996) ou la présence de

macroalgues toxiques comme *Caulerpa taxifolia* (Boudouresque et al., 1996). Cette mesure a été réalisée en retournant un oursin sur un carrelage de 1 000 cm², situé entre 2 et 3 m de profondeur pour éviter l'effet des vagues. Le temps de retournement est le temps nécessaire pour qu'un oursin, mis sur sa face aborale, reprenne sa position normale. Le rapport gonado-somatique a également été calculé de la manière suivante :

$$\text{Rapport Gonado – somatique} = \frac{\text{Poids des gonades sèches}^{(a)}}{\text{Diamètre de l'oursin sans épine}^3} * 100$$

^(a) Les gonades ont été séchées pendant 24h dans une étuve à 60°C.

Le taux d'ingestion des oursins (uniquement pour l'expérimentation en aquarium) a également été mesuré en estimant la quantité de nourriture artificielle ingérée par jour et par oursin.

3.4. Analyse statistique

Le test de Kruskal-Wallis a été utilisé pour évaluer l'influence d'*Ostreopsis cf. ovata* sur les temps de retournement, l'ingestion et le rapport gonado-somatique des oursins.

4. Résultats

4.1. Efflorescences d'*Ostreopsis cf. ovata* en 2017 et 2018

Lors de l'étude *in situ* des oursins, un suivi des abondances cellulaires d'*Ostreopsis cf. ovata* à 2 m de profondeur (soit la profondeur à laquelle les oursins ont été échantillonnés) a été réalisé, en plus de celui classiquement réalisé à 0.5 m de profondeur lors des suivis à but sanitaire réalisés chaque année au site de Rochambeau (Figure 28a et b). Les efflorescences de 2017 et 2018 à Rochambeau sont différentes en terme de périodicité, d'intensité et de durée. Les concentrations atteintes en 2017 à 0.5 m de profondeur sont plus faibles (maximum de 457 267 cellules par gramme de poids frais de macroalgues ou cellules/gPF, contre 848 561 cellules/gPF en 2018). A 2 m de profondeur, les abondances cellulaires atteintes au pic de l'efflorescence sont similaires pour les deux années échantillonnées (respectivement 357 510 cellules/gPF et 284 701 cellules/gPF en 2017 et 2018). L'efflorescence à 2 m de profondeur est plus précoce en 2017 avec un maximum de concentrations atteint le 27 juin en 2017 et un déclin le 11 juillet, alors que le pic est atteint plus tardivement en 2018 (le 11 juillet), avec un déclin le 25 juillet. Au site du Lido, les concentrations sont très faibles, que ce soit en 2017 ou en 2018, et ne dépasse pas 66 002 cellules/gPF.

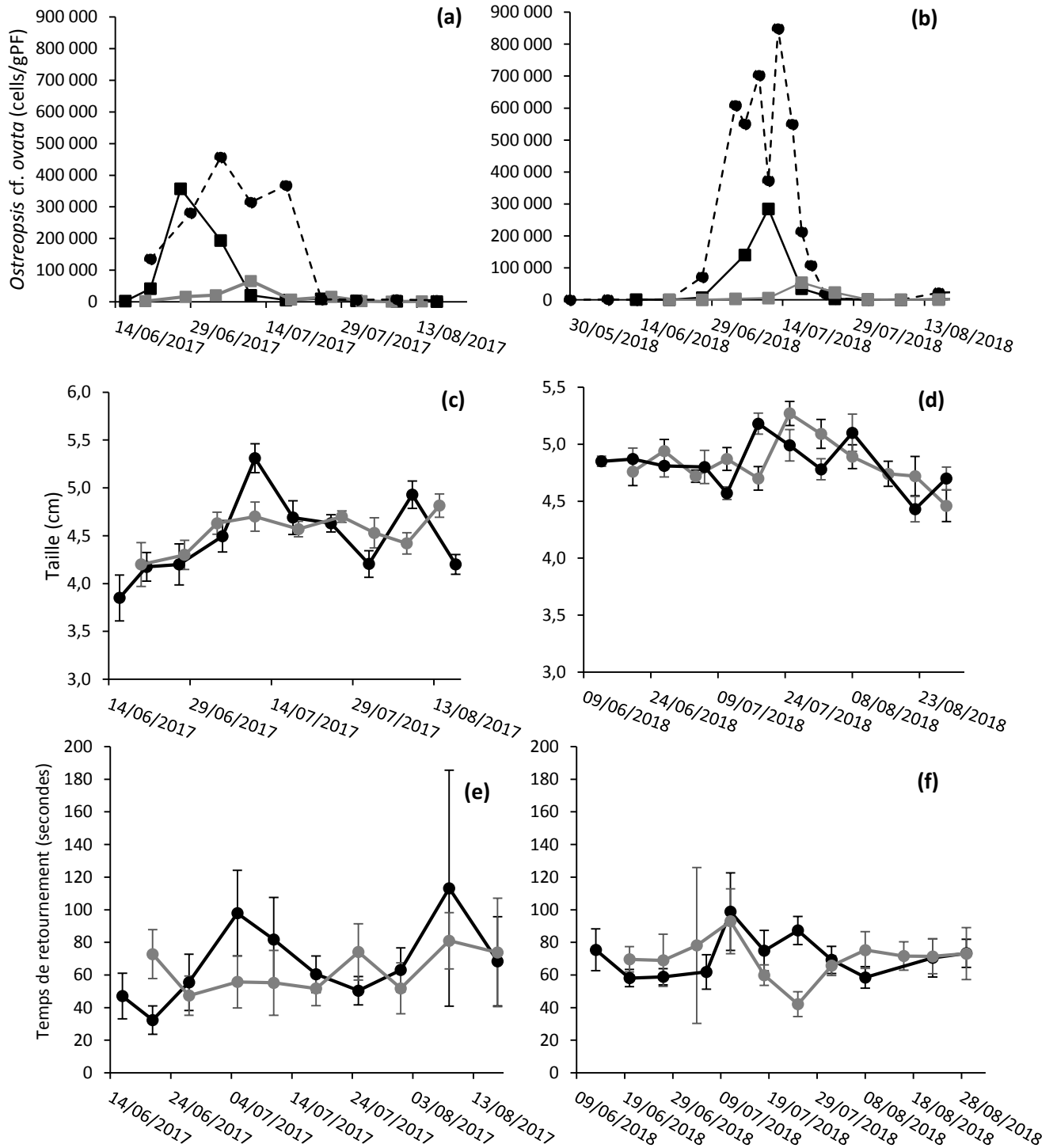


Figure 28 : Concentration cellulaire d'*Ostreopsis cf. ovata* au site de Rochambeau (en noir) à 0.5 m (ligne pointillée) et 2 m de profondeur (ligne pleine) et du Lido à 2 m de profondeur (ligne pleine grise) en 2017 (a) et 2018 (b). L'évolution de la taille des oursins *Paracentrotus lividus* (Moyenne \pm erreur standard ; n=10) prélevés à Rochambeau (ligne noire) et au Lido (ligne grise) au cours des étés 2017 (c) et 2018 (d) est également représentée. Le temps de retournement (moyenne \pm erreur standard ; n=10) des oursins (*Paracentrotus lividus*) mesurées *in situ* en 2017 (e) et 2018 (f) au niveau du site de Rochambeau (lignes noires) et du Lido (lignes grises) est également figuré.

4.2. Evolution du taux d'ingestion des oursins exposés à *Ostreopsis cf. ovata*

Lorsque les oursins sont nourris avec la nourriture artificielle enrichie en cellules d'*Ostreopsis cf. ovata*, le taux d'ingestion de $0,68 \text{ g.jour}^{-1}$ ($\pm 0,10$), alors que les oursins nourris avec la nourriture enrichie en diatomées ou non enrichie présentent respectivement un taux d'ingestion de $0,61$ ($\pm 0,13$) et $0,60$ ($\pm 0,14$) g. jour^{-1} . Aucune différence significative d'ingestion n'est observée entre ces conditions expérimentales ($p < 0,05$).

4.3. Mesure de la taille des oursins

Une augmentation de taille de 10% en cinq semaines est observée pour tous les oursins maintenus en laboratoire ($p < 0,05$). Néanmoins, aucune différence de taille significative n'est observée pour les oursins dont la nourriture a été enrichie en *Ostreopsis cf. ovata*, ainsi que pour les oursins nourris avec la nourriture artificielle enrichie en diatomées ou non enrichie ($p < 0,05$).

Les paramètres étudiés, soit le temps de retournement et le rapport gonado-somatique, pouvant varier avec la taille, la taille des oursins prélevés *in situ* a également été mesurée (Figure 28c et d). Aucune évolution significative de la taille n'est observée au cours de l'été : les oursins prélevés au cours de étés 2017 et 2018 ne présentent pas de différence significative de taille, quel que soit le site étudié. La taille moyenne des oursins est de respectivement $4,5 \pm 0,4$ et $4,5 \pm 0,2$ à Rochambeau et au Lido.

4.4. Impact d'*Ostreopsis cf. ovata* sur le temps de retournement des oursins

Le temps de retournement des oursins maintenus en laboratoire et nourris avec une nourriture artificielle enrichie ou non en *O. cf. ovata* a été mesuré deux fois par semaine durant les 5 semaines d'expérimentation. Une diminution des temps de retournement de 20 et 44 % a été observé pour les oursins nourris respectivement avec la nourriture artificielle enrichie en *O. cf. ovata* et en diatomée. Le temps de retournement des oursins nourris avec la nourriture sans ajout est constant tout au long de l'expérimentation et aucune différence significative ne peut être mise en évidence entre les différentes conditions expérimentales (Figure 29).

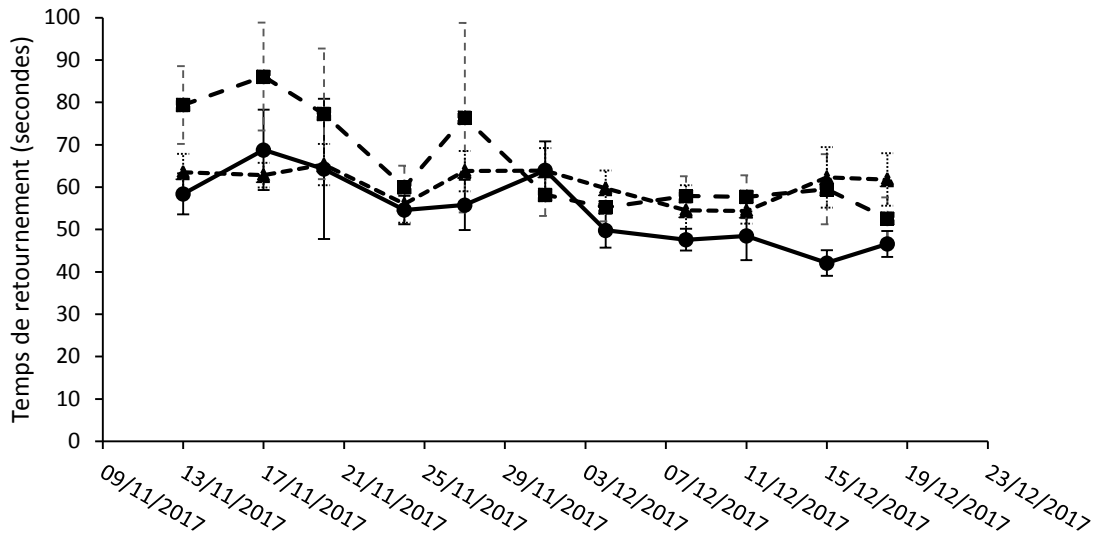


Figure 29 : Evolution du temps de retournement des oursins maintenus en laboratoire et nourris avec une nourriture artificielle préparée e laboratoire enrichie en *Ostreopsis cf. ovata* —●—, en diatomées *Licmophora paradoxa* —■— ou non enrichie —▲—

Le temps de retournement a également été mesuré *in situ* sur des oursins prélevés à Rochambeau, donc exposés à de fortes abondances d'*O. cf. ovata*, et au Lido, peu exposés à *O. cf. ovata*. Les oursins à Rochambeau sont exposés à une abondance cellulaire de 350 000 cellules/gPF en 2017, mais aucune différence significative n'est observée avant, après ou pendant le bloom d'*O. cf. ovata* (Figure 28e). De la même manière, aucune différence n'est observée au niveau des temps de retournement des oursins prélevés à Rochambeau ou au Lido en 2017 (Figure 28e). Pour l'été 2018, les résultats sont similaires : aucune différence significative du temps de retournement n'est observée entre les oursins exposés ou non à *O. cf. ovata* (Figure 28f).

4.5. Impact sur le rapport gonado-somatique

A la fin des 5 semaines d'expérimentation en laboratoire, le rapport gonado-somatique des 30 oursins utilisés dans cette expérimentation a été mesuré. Aucune différence significative ne peut alors être mise en évidence entre les trois conditions expérimentales : lorsque les oursins sont nourris avec la nourriture artificielle enrichie en *O. cf. ovata*, le rapport gonado-somatique moyen est de 0,44 ($\pm 0,05$) alors qu'il est de respectivement 0,35 ($\pm 0,05$) et 0,45 ($\pm 0,06$) lorsque la nourriture est enrichie en diatomée ou non enrichie. Lors du suivi *in situ* des oursins en 2017, le rapport gonado-somatique des oursins a également été mesuré (Figure 30). L'évolution de ce rapport est similaire pour les oursins prélevés à Rochambeau et ceux prélevés au Lido. Il semble y avoir un cycle dans les deux sites, avec

des variations allant d'environ 4 à 7.5, mais ce cycle n'est pas en relation avec les abondances d'*O. cf. ovata*.

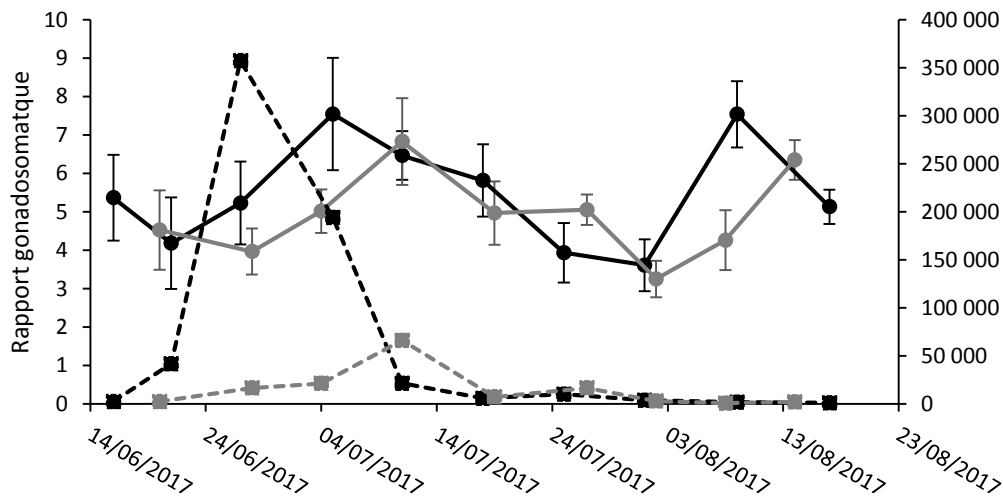


Figure 30 : Evolution du rapport gonado-somatique (Moyenne \pm erreur standard ; n= 10) des oursins (*Paracentrotus lividus*) mesuré *in situ* aux sites de Rochambeau (ligne noire) et du Lido (ligne grise) lors de l'été 2017. Les concentration cellulaires d'*Ostreopsis cf. ovata* à Rochambeau (tirets noirs) et au Lido (tirets gris) sont également représentées.

5. Discussion

5.1. Absence d'effet d'*Ostreopsis cf. ovata* sur le comportement des oursins

Le comportement est souvent étudié afin d'estimer l'état de santé des échinodermes (Dumont et al., 2007; Percy, 1973). Parmi ces indicateurs comportementaux, le temps de retournement est couramment étudié puisqu'il est en relation avec la coordination du système neuromusculaire en réponse à un changement d'orientation (Kleitman, 1941) et reflète, par exemple, la capacité d'un oursin à survivre, une fois déplacés par un prédateur (Percy, 1973). Ce paramètre a déjà été utilisé pour estimer l'état de santé d'oursins exposés à divers stress tels que des polluants chimiques (Böttger et al., 2001), des variations de salinité ou de température (Brothers and McClintock, 2015; Lawrence, 1975), de pCO₂ (Challener and McClintock, 2013) ou encore des toxines (Canty et al., 2009). Cet indicateur a donc été utilisé dans le cadre de la présente étude afin d'estimer l'effet toxique potentiel des cellules d'*O. cf. ovata* sur les oursins adultes. Aucune influence de ce dinoflagellé toxique n'a pu être en évidence, aussi bien *in situ* qu'en laboratoire. La première hypothèse pouvant expliquer cette absence d'effet serait que ce paramètre comportemental n'est pas suffisamment sensible pour mettre en évidence un effet néfaste des cellules d'*O. cf. ovata*. Cet indicateur a déjà mis en évidence une

toxicité de la macroalgue *Caulerpa taxifolia* sur la même espèce d'oursins adultes (Boudouresque et al., 1996), avec des temps de retournement des oursins dépassant 30 minutes. Ces effets sont observés après une exposition de plus de 70 jours, alors que le temps de retournement est uniquement multiplié par 2, en moyenne, après 20 à 40 jours d'exposition à *C. taxifolia*. Cette dernière expérience suggère qu'un temps assez long est nécessaire avant de mettre en évidence un effet sur le temps de retournement des oursins. Lors de la présente étude, ce temps est volontairement réduit à un mois, pour être en accord avec la réalité écologique des floraisons d'*O. cf. ovata*. En mer Méditerranée, au niveau de la rade de Villefranche-sur-Mer, *O. cf. ovata* n'atteint des abondances importantes qu'en été et pour des périodes variant de 3 semaines à 1 mois, soit équivalentes des durées testées dans cette étude. Les premières cellules sont souvent observées à la fin juin et diminuent très fortement début août (Cohu et al., 2011), même si une efflorescence tardive peut se produire en automne (Pavaux, com. pers.). Tester de fortes abondances d'*O. cf. ovata* sur des périodes plus longues permettrait potentiellement d'observer un effet toxique chronique d'*O. cf. ovata*, mais cela n'aurait aucune réalité écologique vis-à-vis des conditions rencontrées dans la baie de Villefranche-sur-mer. Dans d'autres régions de Méditerranée, la durée des efflorescences est différente. C'est le cas à Sant Andreu de Llavaneres, sur la côte catalane de l'Espagne, où les efflorescences d'*O. cf. ovata* débutent plutôt fin juin et se terminent fin septembre et durent ainsi près de 4 mois (Vila et al., 2016).

Les concentrations d'*O. cf. ovata* choisies dans cette étude font référence à des concentrations cellulaires reportées *in situ* (au niveau du site de Rochambeau, Villefranche-sur-Mer), pour garder une certaine réalité écologique c'est-à-dire des concentrations cellulaires comprises entre 500 000 et 1 000 000 cells/gPF. Néanmoins, des concentrations bien plus fortes sont observées dans d'autres régions méditerranéennes : c'est le cas à Gênes où les abondances cellulaires pouvaient atteindre $2\,541\,000 \pm 588\,000$ cell/gPF le 27 juillet 2006. L'absence de toxicité d'*O. cf. ovata* sur les oursins adultes pourrait donc également être due à l'exposition à des concentrations d'*O. cf. ovata*, réalistes vis-à-vis des conditions observées à Rochambeau, mais insuffisantes pour observer un effet toxique.

La seconde hypothèse pouvant être émise pour expliquer les résultats obtenus est l'absence d'effet toxique des cellules d'*O. cf. ovata* pour les oursins adultes ; les mortalités de masse ainsi observées en Nouvelle Zélande et en Méditerranée (Shears and Ross, 2009; Totti et al., 2010; Vale and Ares, 2007) pourraient être plutôt dues à des phénomènes d'anoxie et/ou d'hypoxie des milieux. Des études précédentes ont en effet montré que les oursins sont capables contenir de grandes quantités d'*O. cf. ovata* sans effet léthal. Plusieurs études ont montré que les oursins avaient des quantités de toxines supérieures à $400 \mu\text{g eq PLTX}/\text{kg}^{-1}$ sans effet mortel (Amzil et al., 2012; Biré et al., 2015, 2013; Brissard et al., 2014; Milandri et al., 2010). Il est également important de noter que ces toxines sont localisées uniquement dans le système digestif et que les quantités augmentent puis diminuent, en suivant le cycle de l'efflorescence d'*O. cf. ovata* (Amzil et al., 2012). Les oursins adultes sont donc

capables de résister à des concentrations très fortes d'*O. cf. ovata* et les toxines ne sont *a priori* pas stockées puisqu'elles ne sont pas retrouvées dans les gonades, organe ayant un double rôle de stockage et de reproduction (Lawrence, 1975). Cette diminution de la concentration de toxines au cours du temps suggère (i) une nouvelle fois que les oursins n'accumulent pas les phycotoxines et que les quantités mesurées ne reflètent que la quantité de microalgues ingérées, ou (ii) qu'il existe un mécanisme de détoxification rapide. Des mécanismes de détoxification sont déjà connus chez les oursins et impliquent un certain nombre de gènes tels que les métallothionéine 6 (MT6) ou la catalase (CAT), dont l'expression est régulée lorsque les larves d'oursins sont exposées aux dérivés aldéhydes tératogènes produits par les diatomées (Varrella et al., 2014).

Les résultats obtenus dans cette étude suggèrent une absence d'effets néfastes d'*O. cf. ovata* sur le temps de retournement des oursins adultes. Des études complémentaires sont néanmoins nécessaires afin de vérifier si des floraisons plus longues d'*O. cf. ovata* (comme celles observées en Espagne) ou des concentrations plus fortes, pourraient avoir un effet sur le comportement des oursins adultes.

5.2. Impact d'*Ostreopsis cf. ovata* sur le taux d'ingestion de *Paracentrotus lividus*

Les taux d'ingestion mesurés en laboratoire ne montrent aucun impact de la présence *O. cf. ovata* dans la nourriture. En ingérant des macroalgues sur lesquelles se développent *O. cf. ovata*, les oursins peuvent être exposés à des concentrations supérieures à 900 000 cellules/gPF. Mais très peu d'études se sont intéressées à l'impact d'*O. cf. ovata* sur les oursins adultes (alors qu'ils sont comestibles ; Addis et al., 2009), la plupart des études étant focalisées sur les stades larvaires (Caroppo and Pagliara, 2011; Migliaccio et al., 2016; Neves et al., 2018; Pagliara and Caroppo, 2012; Privitera et al., 2012).

Plusieurs paramètres intrinsèques aux macroalgues peuvent agir sur leurs taux d'ingestion par les oursins (Cook and Kelly, 2007) : la forme des macroalgues les rendant plus ou moins facile à être ingérées par les oursins, leur qualité nutritionnelle, leur défense mécanique (en particulier la calcification) et enfin leur défense chimique, en relation avec la présence de métabolites secondaires plus ou moins abondants et efficaces. Il est important de noter qu'à notre connaissance, aucune publication traitant de la défense chimique des macroalgues vis-à-vis des oursins ne fait mention des potentiels métabolites secondaires des épiphytes de ces macroalgues. La présente étude ne montre pas d'impact des métabolites secondaires d'*Ostreopsis cf. ovata*, mais il est important de prendre en compte ce paramètre.

5.3. Impact d'*Ostreopsis cf. ovata* sur le rapport gonado-somatique

L'évolution du rapport gonado-somatique est similaire pour tous les oursins prélevés *in situ*, qu'ils aient été exposés ou non à *O. cf. ovata*. Ces résultats sont confirmés par les expérimentations en laboratoire au cours desquelles aucune différence n'a pu être mise en évidence entre les rapports gonado-somatiques des oursins nourris avec *O. cf. ovata* ou non. Des études précédentes ont reporté des effets reprotoxiques de ce dinoflagellé chez l'oursin. Une diminution significative du succès de fécondation ainsi que le développement de larves anormales a été mis en évidence lorsque les œufs et gamètes d'oursins sont exposés à *O. cf. ovata* directement (Caroppo and Pagliara, 2011; Neves et al., 2018; Pagliara and Caroppo, 2012). Cet effet reprotoxique a également été observé chez des adultes exposés à *O. cf. ovata* : le succès de reproduction est de 47 % jusque 5 mois après l'exposition (Migliaccio et al., 2016). Ces études se sont focalisées sur la qualité des gamètes formées et non sur la quantité. Plus précisément, ces études ont montré que les gamètes produits étaient moins fertiles mais aucune ne s'est focalisées sur la quantité de gamètes produites, qui est en lien direct avec le rapport gonado-somatique. Ceci pourrait donc expliquer les divergences de résultats obtenus entre notre étude et les études précédentes qui se sont plutôt intéressées au succès de reproduction.

6. Conclusion

Les résultats obtenus dans cette étude n'ont pas montré de lien entre la toxicité liée aux efflorescences d'*Ostreopsis cf. ovata* en Mer Méditerranée et les épisodes de mortalité de masse d'oursins. Aucune variation du temps de retournement, des taux d'ingestion et du rapport gonado-somatique n'a pu être mise en évidence par les expériences en laboratoire couplées aux études *in situ*. Ces résultats sont en accord avec l'étude *in situ* de Blanfuné et al. (2012) qui reporte l'absence de lien entre la diminution de densité des oursins et les efflorescences d'*O. cf. ovata* à Gênes (Italie), à Villefranche-sur-Mer et à Nice (France). La diminution de la densité des oursins a plutôt été reliée à des variabilités naturelles ou aux activités de pêche existant en France. Les résultats obtenus par Shears and Ross (2009) ont pourtant reporté une diminution de 60 % de la densité des oursins pendant les efflorescences d'*Ostreopsis siamensis* en Nouvelle Zélande. Ces mortalités pourraient s'expliquer par une combinaison de facteurs environnementaux (augmentation des températures, anoxie, pollution anthropogénique) qui fragiliseraient les oursins, les rendant plus vulnérables à de fortes concentrations d'*Ostreopsis* spp.

Les concentrations et les durées d'exposition utilisées dans cette étude reflètent les conditions observées *in situ* dans la rade de Villefranche-sur-Mer, mais sont peut-être inappropriés pour observer un effet néfaste sur les oursins adultes. Il pourrait être intéressant d'étudier des expositions plus

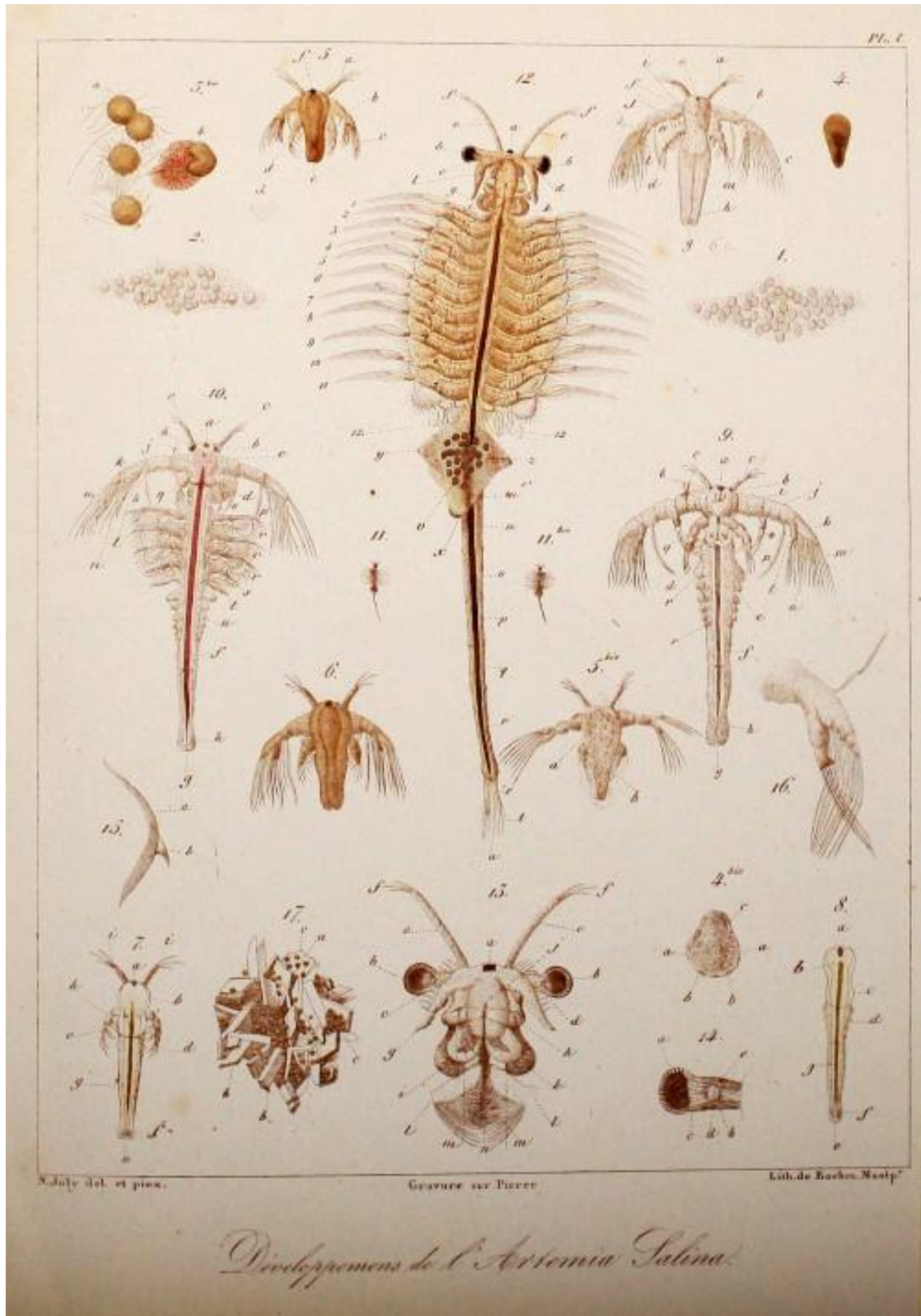
longues, avec de plus fortes concentrations d'*Ostreopsis* cf. *ovata*, en se référant par exemple aux efflorescences se produisant en Espagne.

Enfin, même si cette expérience n'a pas montré d'effet des métabolites secondaires d'*Ostreopsis* cf. *ovata* sur les adultes de *Paracentrotus lividus*, il est important de noter que les épiphytes pourraient avoir un effet sur la consommation des macroalgues par les oursins, alors que ce paramètre n'est jamais considéré dans les publications traitant des préférences alimentaires de ces échinodermes.

7. Synthèse de l'étude

Les résultats obtenus dans cette étude, couplant des approches *in situ* et en laboratoire, n'ont pas permis d'établir de liens entre les efflorescences d'*Ostreopsis cf. ovata* et des changements dans la physiologie et le comportement des oursins adultes. Ces résultats sont en contradiction avec ceux obtenus avec l'étude *in situ* de (Shears and Ross, 2009) identifiant un lien entre le pourcentage de recouvrement des macroalgues par *Ostreopsis siamensis* et le déclin des populations d'oursins adultes. L'absence d'effets néfastes lors de l'exposition des oursins adultes à *O. cf. ovata* peut s'expliquer par une exposition potentiellement trop courte à des concentrations trop faibles pour observer des effets toxiques. Dans l'état actuel des connaissances, et des résultats obtenus dans cette étude, l'hypothèse de l'hypoxie et/ou de l'anoxie comme cause de mortalité semble à privilégier.

Chapitre 6. Mise au point d'un test biologique rapide,
simple et pertinent pour évaluer la toxicité d'*Ostreopsis*
cf. *ovata*



Artemia salina. Plate from Nicolas Joly (1840)

1. Présentation de l'étude

La toxicité des cellules d'*Ostreopsis* cf. *ovata* est souvent évaluée par deux méthodes majeures : (i) les méthodes biologiques comme le « *Mouse Bioassay* » (MBA) ou le « *Hemolysis Neutralization Assay* » (HNA) et (ii) les techniques de chimie analytiques (HPLC-HR MS/MS). Les méthodes biologiques posent des préoccupations éthiques et politiques et des problèmes de mise en œuvre ; les techniques de chimie analytique présentent également des désavantages en raison de la complexité des analyses et de leurs coûts mais également des équipements et compétences nécessaires à ces analyses.

Au cours de mon travail de thèse, j'ai donc mis au point un test biologique rapide pour tester simplement et de manière précise la toxicité de différentes souches d'*Ostreopsis* spp. Ce test repose sur la sensibilité des larves d'*Artemia franciscana*, qui est un modèle couramment utilisé pour tester la toxicité de pesticides et d'autres contaminants chimiques et biologiques. Pour cela, la toxicité de différentes souches d'*O.* cf. *ovata* a été évaluée, ainsi que les variations de toxicité inhérentes au cycle de croissance cellulaires. Le protocole ainsi mis en place a ensuite été appliqué à un suivi *in situ* de la toxicité des cellules d'*O.* cf. *ovata*. Les résultats de toxicité obtenus par ce test ont été validés par des mesures quantitatives réalisées à la plateforme HPLC-HRMS/MS du laboratoire de Marine Biodiscovery de Galway.

2. Reliable and handful monitoring of *Ostreopsis* sp. toxicity using an *Artemia* sp. bioassay

L'article qui suit sera soumis prochainement à *Marine Environmental Research*.

Reliable and handful monitoring of *Ostreopsis* sp. toxicity using an *Artemia* sp. bioassay

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2.1. Abstract

Blooms of the benthic toxic dinoflagellate *Ostreopsis* sp. have increased in intensity and frequency in the Mediterranean Sea during the past decades. These blooms are linked to adverse effects on Human health (skin and eye irritations, fever, headaches, breath difficulties) by direct contact with contaminated seawater or bio aerosols and deleterious effects on marine fauna. HPLC-MS analysis are commonly used to quantify the toxicity of *Ostreopsis* cells although these analyses are time-consuming and expensive. Hence there is a need to find a relevant ecotoxicity test to set toxic threshold during *Ostreopsis* blooms. The crustacean species *Artemia* is widely used to test chemical and biological contaminants and thus could be reasonably used to test *Ostreopsis* toxicity. The relevance of this was first assessed by investigating the variability of the toxic effect among *Ostreopsis* strains and throughout the dinoflagellate life cycle, in combination with chemical analysis of the toxin content performed by UHPLC-HRMS highlighting toxicity variations among strains and growth stages. Testing the toxicity of *Ostreopsis*' endometabolome, including pure palytoxin and ovatoxins, revealed that these toxins are not the only toxic compounds to *Artemia franciscana*, indicating that several toxic compounds synthesized by *Ostreopsis* sp. still remain to be identified. To allow transferring the bioassay from lab to *in situ* monitoring, the toxicity of the whole benthic microalgal consortium was tested during a natural bloom of *Ostreopsis* cf. *ovata*. The results obtained highlight the accuracy and

sensitivity of *Artemia franciscana* to assess the toxicity of blooms of *Ostreopsis sp.* and may also be relevant for other toxic species.

2.2. Introduction

Ostreopsis sp. blooms become more intense in temperate areas and regularly occur in the Mediterranean Sea during summer (Accoroni et al., 2011; Accoroni and Totti, 2016; Battocchi et al., 2010; Mangialajo et al., 2011, 2008; Totti et al., 2010; Vila et al., 2001). In these regions, *Ostreopsis cf. ovata* is well known to cause adverse effects on human health (Durando et al., 2007; Gallitelli et al., 2005; Kermarec et al., 2008; Tichadou et al., 2010; Vila et al., 2016) associated to hospitalization of hundreds of people, like in Genova in 2005 (Brescianini et al., 2006) and in Algeria in 2009 (Illoul et al., 2012). Toxic effects on vertebrates and invertebrates have also been documented (Accoroni et al., 2016b; Blanfuné et al., 2012; Faimali et al., 2012; Prato et al., 2011; Privitera et al., 2012): reprotoxicity on benthic copepods (Guidi-Guilvard et al., 2012; Pavaux et al., 2019) and sea urchins (Migliaccio et al., 2016; Pagliara and Caroppo, 2012), as well as harmful effects on the polychaete *Dinophylus gyrociliatus* and the jellyfish *Aurelia sp.* (Privitera et al., 2012; Simonini et al., 2011). The toxicity of *Ostreopsis cf. ovata* is being attributed to the production of palytoxin (PLTX) and its analogs (named ovatoxins, OVTX-a, -b, -c, -d, -e, -f, -g; Ciminiello et al., 2012a, 2012b, 2010), known to be strong Na^+/K^+ ATPase pump inhibitors (Poli et al., 2018; Rouzair-Dubois and Dubois, 1990).

Due to these sanitary impacts, the monitoring of *Ostreopsis cf. ovata* toxicity has been the focus of most research the past decades (Battocchi et al., 2010; Casabianca et al., 2014, 2013). Analytical chemistry (HPLC-MS) constitutes the most widespread approach to monitor OVTX concentrations in the ecosystem (Ciminiello et al., 2006; Pierce and Kirkpatrick, 2001), despite its various constrains: (1) cutting edge facilities (HPLC-HRMS) and skills are needed; (2) relatively high quantification limit (2.5 μ g PLTX/mL is reached); (3) involve time- and solvent-consuming analysis. Therefore, there is an urgent need to develop an efficient (i.e rapid, simple, inexpensive and sensitive) bioassay to allow assessing the toxicity of *Ostreopsis* strains, and further improve the early detection of toxic events in concerned areas.

In the early 70s, the brine shrimp *Artemia sp.* was proposed as a bio-indicator for chemical contamination (Michael et al., 1956; Vanhaecke et al., 1981) owing to its sensitivity to sodium lauryl sulfate and various insecticides. Up to now *Artemia sp.* has been widely used to test fungal toxins (Harwig and Scott, 1971), plant extract toxicity (Meyer et al., 1982), heavy metals (Martínez et al., 1999; Sarabia et al., 2008), cyanobacteria toxins (Jaki et al., 1999; Tokodi et al., 2018) and pesticides (Comeche et al., 2017; Gambardella et al., 2018; Jawahar et al., 2018). *Artemia sp.* was also shown to be useful for marine natural products screening as shown by the work of Carballo et al. (2002) who

compared the cytotoxicity of the specialized metabolites produced by 20 marine organisms (mostly invertebrates) against 2 human cell lines (lung carcinoma A-549 et colon carcinoma HT29). Therefore, *Artemia franciscana* bioassay may constitute an excellent way to assess the toxicity of *Ostreopsis* blooms.

In this context, a protocol investigating the variability of the toxicity between *Ostreopsis*' strains and throughout its life cycle was first set up using living cells of two Mediterranean strains (*O. cf. ovata* and *O. fattorussoi*). As a second step, the compounds responsible for *Ostreopsis* toxicity were seek using chemical extracts of *O. cf. ovata* cells and pure PLTX and OVTX. Finally, the implemented bioassay was successfully used to monitor a natural bloom of *O. cf. ovata* in 2018.

2.3. Materials and Methods

2.3.1. Bioassays

Four experiments were performed that will be referred as Experiment 1 to 5 hereinafter. Experiment 1 investigates the variability of the toxicity among species and strains of *Ostreopsis* at a given stage of growth, using two french *O. cf. ovata* and two Lebanese *O. fattorussoi* strains; Experiment 2 follows the toxicity throughout the growth of *O. cf. ovata*; Experiment 3 investigates the nature of the toxic compound using cell extracts and pure OVTX and PLTX; Experiment 4 evaluates *A. franciscana* as a tool for biomonitoring the toxicity of a natural bloom.

2.3.2. Micro-algal culture

The four strains used in this study were obtained from the Mediterranean Culture Collection of Villefranche (MCCV, <http://www.embrc-france.fr/fr/our-services/supply-biological-resources/microorganisms/microorganismes-unicellulaires>). *Ostreopsis cf. ovata* (MCCV 054 and MCCV 055) were collected at the Rochambeau site in 2014 (Bay of Villefranche-sur-Mer, N-W Mediterranean, 43°41'35.64"N-7°18'31.54"E) and *Ostreopsis fattorussoi* (MCCV 057 and MCCV 058) were isolated in 2015 from Bat 14 site (Lebanon, Batroun, 34°15'9.0"N-35°39'41.3"E). All microalgae are grown in L1 and L1/2 medium (Andersen, 2005) prepared with autoclaved aged and filtered seawater, adjusted to a salinity of 38. Cultures are maintained at 24°C, under a 14:10 light/dark cycle with a light intensity of 250 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$.

2.3.3. Taxonomic Molecular Identification by a PCR Based Assay

A volume of 5 mL of each MCCV clonal culture was centrifuged for 6 min at 2000 rpm and 4°C. The supernatant was removed, and the cell pellet was resuspended with 1 mL of sterile water (autoclaved MilliQ) and centrifuged again for 2 min to remove salt and media. The cell pellet was kept

and homogenized with 50 µL of sterile water and a fraction of 10 µL of was further transferred to 0.2 µL polymerase chain reaction (PCR) tubes. Then, PCR tubes were stored at -20°C until further analysis. Approximately 300 base pairs of the internal transcribed spacer region (ITS1-5.8s-ITS2) rDNA were amplified by PCR using the primer 329-F (5'-GTGAACCTGCRGAAGGATCA-3') which is the inverse complementary sequence of the universal eukaryote primer 329-R (Moon-van der Staay et al., 2001) and the designed primer D1R-R (5'-TATGCTTAAAATTCAGCAGGT-3') which is the inverse complementary sequence of the primer DIR-F (Scholin et al., 1994). A mix of 1µl of each primer, 1 µl of dNTP at 10mM, 5 µl of 10X Advantage 2 PCR Buffer, 31µl of sterile water and 1µl of 50X Advantage 2 Taq polymerase (Clontech) was added in each PCR tube containing the cell pellet. The PCR was performed using a MasterGradient thermocycler (Eppendorf) with the following conditions: one initial denaturation at 94°C for 10 min followed by 35 cycles each consisting of 1 min at 94°C, 1 min at 53°C, 1 min at 68°C and a final elongation for 10 min at 68°C. The PCR products were purified using QIAGEN MinElute PCR Purification kit according to the recommendations. After sequencing by Genewiz, sequences were treated using BioEdit software and were compared to NCBI database using BLASTn tool to ensure the taxonomy of the strains.

2.3.4. Cells sampling

2.3.4.1. Cultures

All strains used in Experiment 1, 2 and 3 were grown in triplicates, in 150-mL and 300-mL flasks, respectively, at an initial cell concentration of 1000 cell.mL⁻¹. A volume of 12 mL was sampled and fixed with acidic lugol (4% v/v) for cell counting at day 10 in Experiment 1 (150 mL-flasks) and every other day during 14 days in Experiment 2 (300-mL flasks). Cell counting was performed in triplicates using a liquid particle counter (HIAC/Royco 9703, Pacific Scientific Instruments) following a size range of 2-80 µm (Stramski et al., 2002). Aliquots of living cells from each flasks were subsequently diluted using L1 medium to a final volume of 20 mL and a cell concentration of 20 000 cell.mL⁻¹ to perform *Artemia* bioassays.

2.3.4.2. In situ cells

Living cells used in Experiment 4 were sampled on artificial substrates (Jauzein et al., 2016) during summer 2018 (27th of June to 23rd of August) to cover the bloom of *Ostreopsis cf. ovata*. Three screens composed by a rigid frame and a 1 mm mesh were deposited in the morning at a depth of 50 cm depth at 3 sampling sites, separated from each other by few meters. After 24 hours, the screens were carefully collected in 1 L plastic bottles containing seawater and gently homogenized to resuspend all microalgae. For each screen, a volume of 20 mL was subsampled in triplicates fixed with acidified lugol (4% v/v) and observed under microscope using Sedgewick rafter counting chamber to

measure both the abundance and the diversity. After counting, dilutions were applied to each sample obtained from the screens to reach a final concentration of 20 000 cells.mL⁻¹ to perform *Artemia* bioassays. It is noteworthy that these samples are natural samples and therefore contain the whole benthic micro-algal assemblage, including *Ostreopsis* cells.

2.3.5. Obtention of *Ostreopsis* extracts, fractions and pure ovatoxins

A volume of 1.4 L of a culture of *Ostreopsis* that aged 10 days was centrifuged at 600 g at 21°C for 10 min. The supernatant was discarded and the cell pellet flash frozen using liquid nitrogen to stop the cells metabolism, and further stored at -20°C before being freeze dried. An amount of 85 mg of dried cells was extracted using 4 ml of MeOH:H₂O (80:20) in an ultrasonic bath for 10 min. The extract was centrifuged at 1000 g during 10 min and the supernatant was collected and transferred into a glass vial. The procedure was repeated two more times to obtain 12 mL of extract. The extract was then evaporated under a gentle flow of N₂ at 35°C until full evaporation of the MeOH (leaving about 2-3 ml of water). The aqueous extract was deposited on a C18 SPE cartridge (500 mg, Supelco) previously washed with MeOH (6 mL) and equilibrated with MilliQ-water (6 mL). Before elution, the cartridge was rinsed with 6 mL of MilliQ-water. A step gradient elution was thus applied to the cartridge, from H₂O:MeOH (100:0) to H₂O:MeOH (0:100), using steps of 20% and volumes of 6 mL. An additional elution with 6 mL of acetonitrile (ACN 100%) was performed, yielding seven fractions (F1 to F7, from the most polar to apolar metabolites). Aliquots of 100 µl sampled from each fraction were evaporated to dryness under a N₂ stream (35°C) to assess their respective weight. A volume of 1 mL of dimethylsulfoxide (DMSO) was added to the remaining fractions before evaporating other volatile organic solvents (MeOH, ACN) and water. Based on the fractions weight, their volume of solubilisation were further adjusted using DMSO to reach a concentration of 3 mg.mL⁻¹. All fractions were stored at -20°C until further bioassays and chemical analysis.

A purified pool of ovatoxins was obtained in the frame of the OCEAN15 project, from IFREMER partner (Amzil group), following a protocol previously published (Brissard et al., 2015). Briefly, MeOH 50% raw extracts were obtained from 75 g of *O. cf. ovata* (strain MCCV 054) and fractionated by size-exclusion chromatography (Sephadex LH20). Methanolic fractions containing OVTXs were pooled, concentrated and purified by semi-preparative chromatography (Uptisphere C18-TF, 250 mm × 4.6 mm, 5 µm; λ= 233 and 266 nm). We decided to purify a pool of OVTXs as the separation of all analogues is highly challenging. The final pool was concentrated in DMSO and contained a mixture of OVTX-a (61 %), -b (25 %), -c (3.4 %), -d (5.3 %), -e (3.9 %) and -f (1.1 %) at 7.8 mg/mL, based on LC-MS/MS quantification. As important losses may arise from evaporation to dryness (Brissard et al., 2015), the purity has not been assessed. In the meantime, pure palytoxin was purchased from Wako Chemicals (GmbH, Neuss, Germany). All toxins were solubilized in DMSO at a concentration of 12

µg/mL that would allow an addition of 5 µl for the *Artemia* bioassay without exceeding 0.1% of DMSO in final volume of seawater.

2.3.6. Experimental bioassays

0.15 g of dehydrated *Artemia franciscana* cysts were hatched in 1 L of filtered and aged sea water at a salinity of 38, at 20°C under a continuous light of 250 µmol.m⁻².s⁻¹ intensity and a continuous and strong aeration. After 40 hours of incubation, all larvae moulted to metanauplii stage (2-3 stages), considered as the most sensitive stages to be used in ecotoxicological bioassay (Nunes et al., 2006; Sorgeloos et al., 1978). For each experiments (1 to 4), *Artemia* larvae were isolated from empty cysts using a Pasteur Pipette and transferred in 6-well plates (5 larvae per well); each well containing 4 mL of 0.2 µm filtered and aged sea-water adjusted to salinity 38 and kept at a temperature of 24°C (except for Experiments 3 that contained 5 mL of seawater). All tests were performed using 30 metanauplii larvae per condition. Experiments 1, 2 and 4 were conducted using the four following cellular concentrations: 4, 40, 400 and 4000 cells.mL⁻¹. Experiment 3 was performed using four concentrations of ovatoxins and palytoxin (0.012, 0.12, 1.2 and 12 µg/mL) corresponding to theoretical concentrations that would be reached with 0.4, 4, 40 and 400 cells.mL⁻¹ of *Ostreopsis* cells (Brissard et al., 2014; Ciminiello et al., 2012b; Nascimento et al., 2012a; Pistocchi et al., 2011). Also, all seven chemical fractions obtained from cell pellet were added in the wells at a final concentration of 10 µg/ml.

Artemia franciscana larvae's mortality was measured every hour during 12 hours, then at 24 and 48 h exposure times (only at 24 h for chemical fraction, Experiment 3). Larvae were considered dead when there was no movement during 10 seconds and no reaction to strong light. A negative control was also performed for all experiments, using seawater + fresh L1 medium, 80: 20 (v:v) for Experiments 1, 2 and 4 and 0.1% DMSO (final volume) for Experiments 3. Bioassays are considered as acceptable when mortalities rate in the control condition are lower than 10% as described in Faimali et al. (2012).

2.3.7. Toxin profiles

2.3.7.1. Cell extraction

To assess the variability of the toxin content (i) among strains and species, and (ii) throughout the growth phase, chemical extractions of MCCV 054, 055, 057 and 058 were performed. For inter-strain variability, each strain was inoculated with 1000 cell.mL⁻¹ in triplicates 50-mL flasks and grown during ten days before harvesting the whole culture. For growth-dependent variability, the MCCV 055 was inoculated at 1000 cell.mL⁻¹ in twenty-one 300-mL flasks and grown for fourteen days. Every other day, three flasks were sacrificed for cell abundance (subsample of 1.2 mL) and chemical extraction (about 300 mL). Samples for cell abundance were fixed and counted as previously described.

All flasks content (50 mL and 300 mL) were transferred to 50 mL falcon tubes before being centrifuged at 600 g at ambient temperature, for 10 min. After the supernatant being removed, the cell pellets were flash frozen using liquid nitrogen to stop any metabolic reaction, and further stored at -20°C. A volume of 4 mL of MeOH: H₂O (80:20, v/v) was poured in the falcon tubes before they were being placed in ultra-sonic bath for 10 min, while cooling in an ice batch. The falcon tubes were then again centrifuged at 2500 g for 10 min at ambient temperature and the supernatants were transferred in 20 mL vials.

These steps (organic extraction, ultra-sonic bath and centrifugation) were repeated 2 more times and the resulting extracts were evaporated under a gentle stream of N₂ until full evaporation of the MeOH. To avoid full dryness of the samples, 500 µl of DMSO was then added to the extracts and the remaining H₂O further evaporated. All extracts were stored at -20°C until UHPLC-HRMS analysis.

2.3.7.2. UHPLC-HRMS

UHPLC-UV-HRMS experiments were performed using an Agilent 1290 system (Agilent Technologies, USA) equipped with a diode array detector and coupled to an Agilent 6540 Qtof mass spectrometer (Agilent Technologies, USA). Mass spectrum detection was carried out in positive mode (ESI +) between 60 to 3000 Da. 10 µL of each samples were injected and separated on a T3 column (Acquity UPLC HSS T3 1.8 µm, 2.1 mm x 100 mm, Waters) with a linear elution gradient of H₂O:MeOH (80:20, v:v)/MeOH with 0.1 mM of ammonium formate and 0.1% formic acid from 90:10 (v:v, isocratic from 0 to 2 mins) to 0:100 (v:v, isocratic from 12 to 13 mins) at a 0.4 mL.min⁻¹ flow rate. Collision energies (CE) of 30, 70 and 110 eV were applied to confirm the presence of OVTXs. UV detection was set at 210, 233 and 263 nm. The spectrometer analyzer parameters were set as follow: nebulizer sheath gas, N₂ (35 psig); drying gas, N₂ (11 L.min⁻¹); Gas Temperature, 300°C; capillary, 4.129 µA; Vaporizer/Sheat Gas Temp, 350°C.

The quantification of the OVTXs in the samples was done using commercial PLTX as a reference, considering identical ionization of these compounds. A calibration curve for PLTX was set using concentrations between 1 and 10 mg.mL⁻¹, using the tricharged adduct [M+3H-4H₂O]³⁺ for quantification. This same adduct was used to quantify the OVTX a in all samples.

2.3.8. Statistical analysis

The statistical significance of the mortality variation between the different experimental conditions tested was evaluated using Kruskal-Wallis analyses. Dunn's post hoc tests were used *a posteriori* to identify which experimental conditions differed from the others when significant differences were showed by the Kruskal-Wallis test (p<0.05). These tests were realized using the PAST

software (Hammer et al., 2001) The R package ecotoxicology was used to assess the median lethal concentration (LC₅₀) at 24 and 48 hours of exposition.

2.4. Results

2.4.1. Molecular analysis

PCR amplification based assay of the strains using genus and species-specific primers confirmed the morphological analysis. The sequences obtained after PCR analysis showed a perfect alignment between the strains MCCV 054/MCCV 055 and fully matched (100% of confidence) *Ostreopsis cf. ovata* (E-value: 2e-157, accession number: MG551865.1). Similarly, the strains MCCV 057/MCCV 058 were identified as *Ostreopsis fattorussoi* (level of confidence 100%, E-value: 1e-129, accession number: LT220222.1 ; Accoroni et al. 2016).

2.4.2. Inter-strain variability of the toxicity - Experiment 1

Mortality rates in controls were lower than 10% throughout the experiment, meeting the acceptability threshold of this bioassay as defined by Faimali et al. (2012). Mortality was significantly higher (p-value <0.05) in presence of all *Ostreopsis* cells suggesting all strains are toxic to *Artemia franciscana* larvae, whilst slight variability owed to the time of exposure, the cell concentration and the nature of the strain used were found (Figure 31).

A positive correlation between mortality and exposure time (p < 0.001) was observed, and twelve hours of exposure was sufficient to induce significant mortality of *A. franciscana* (p < 0.05). Mortality reached its maximum after 48 hours (96,6%) for all strains and concentrations tested. Several concentrations of *Ostreopsis* cells were tested to explore their effects on *A. franciscana*. Despite inter-strains variability, a dose-dependent response is observed with a strong increase of mortality until a plateau of 96% of mortality was reached at 400 cells.mL⁻¹ at all exposure time and for most strains.

Inter-strains variability was mostly observed for low exposure times (12 and 24 hours) and low cell concentrations (4 to 40 cell.mL⁻¹). Looking at a concentration of *Ostreopsis* cells of 4 cell.mL⁻¹, the toxicity of the strains can be ranked as following: MCCV 054<055=057<058 (Cf. LC_{50-24h} values – Table 13). By increasing the concentration of *Ostreopsis* cells, this distribution is smoothed and no significant differences are observed between strains for 400 and 4000 cell.mL⁻¹ (except MCCV 058 that shows the most toxic behaviour, 96,6 %).

Interestingly, the toxin content determined by UHPLC-HRMS did not support the toxicity pattern highlighted by the *Artemia* bioassay since no ovatoxins were found in neither MCCV 057 nor 058. On the other hand, an amount of 18.0 (± 4.9) and 23.7 (± 9.2) pg PLTX eq/cell were measured for

MCCV 054 and MCCV 055, respectively, therefore matching the level of toxicity highlighted in the *Artemia* bioassay.

Table 13 : LC₅₀ values and confidence limits in cells.mL⁻¹ obtained exposing *Artemia franciscana* larvae to different strains of *Ostreopsis* sp. and dissolved toxins.

Treatment	LC _{50-24h} (cells.mL ⁻¹)	LC _{50-48h} (cells.mL ⁻¹)
MCCV 054	81.96 (± 63.33)	Nan
MCCV 055	0.25 (± 0.90)	Nan
MCCV 057	0.0012 (± 0.00033)	Nan
MCCV 058	0.023 (± 0.039)	Nan
OVTX	5.96 (± 2.12)	0.42 (± 0.092)
PLTX	48.42 (± 14.23)	0.42 (± 0.085)
Living cells	9.01 (± 3.30)	1.16 (± 0.40)

2.4.3. *Ostreopsis* cf. *ovata* toxicity throughout growth - Experiment 2

After inoculation, *Ostreopsis* cf. *ovata* (MCCV 055) showed a linear and exponential growth during nine days, corresponding to an increase of cells concentration by a factor 2, up to 6 621 cells.mL⁻¹ at day 10 (growth rate of 0.24 day⁻¹), before declining on day 14 (3 637 cells.mL⁻¹; Figure 32). Meanwhile, the toxin content quickly stabilized around 10 pg eq. PLTX/cell between day 4 and 10, followed by a decrease towards the end of the experiment (Figure 32). The high concentrations obtained at day 0 were likely influenced by the fact that the cells that were extracted had been sampled in a culture that aged 10 days.

Interestingly, the variability of the toxin content did not reflect the pattern of mortality observed for *Artemia franciscana* (Figure 33 a, b and c). No clear trend emerged from the mortality measured after 12 hours of exposure, although it increased with cell concentrations. Likewise, at high cell concentration of *Ostreopsis* (40 and 400 cell.mL⁻¹) and for an exposure time of 48 hours, mortality varied little (Figure 33b – 6c ; p>0.05) and was close to 100 % during the whole duration of the experiment, precluding drawing any relationship between mortality and the growth phase.

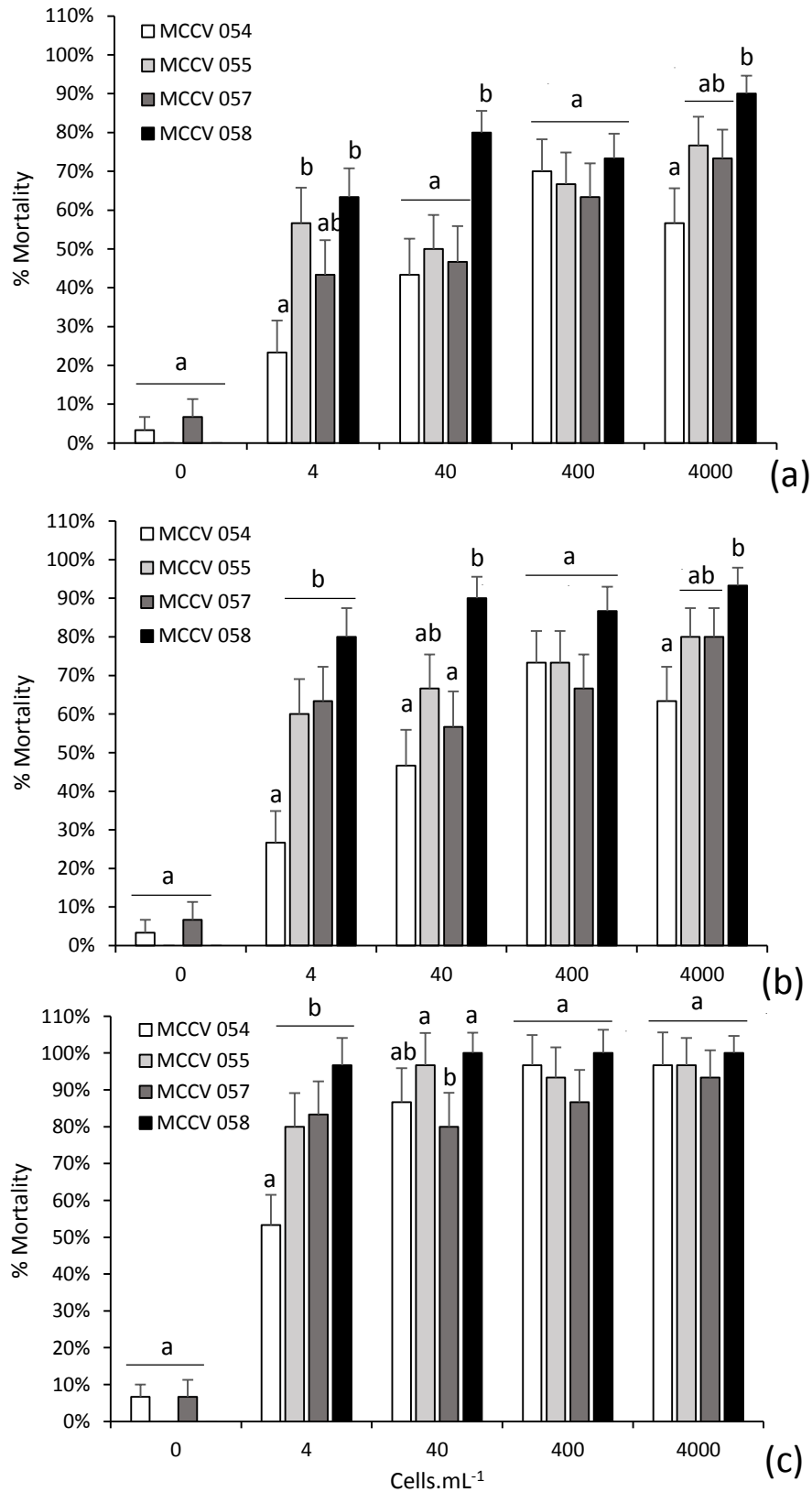


Figure 31 : Percentage of mortality (Mean ± ES) after 12 h (a), 24 h (b) and 48 h (c) of *Artemia franciscana* larvae exposed to various concentrations of Mediterranean strains of *Ostreopsis cf. ovata* (MCCV 054 and 055) and *Ostreopsis fattorussoi* (MCCV 057 and 058).

Only an exposure time of 24 and 48 hours at low concentrations of *Ostreopsis* cells (4 cell.mL⁻¹) allowed to draw a pattern of mortality for *Artemia* larvae that followed both the cellular growth and the toxin content (Figure 33A) with a minimum between days 2 and 4 (10% and 43 % for 24h and 48h respectively, 0.40 pg eq. PLTX/cell, 1436 cells.mL⁻¹) and a maximum reached at day 10 (26% and 83% for 24h and 48h, respectively, 13.1 pg eq. PLTX/cell, 6389 cells.mL⁻¹).

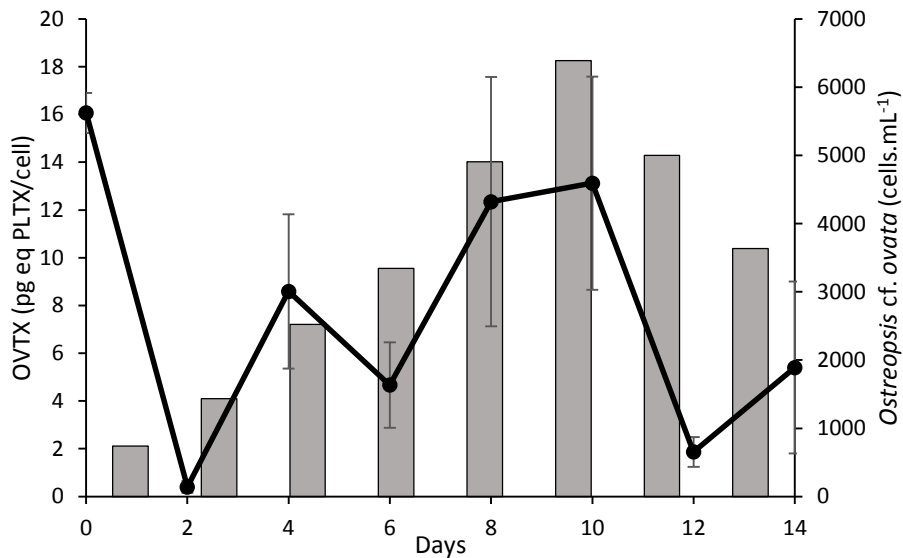


Figure 32 : Toxin concentration (Mean \pm ES) in pg eq. PLTX/cell (black line) depending on the growth phase of *Ostreopsis* cf. *ovata* (MCCV 055 – grey bars).

2.4.4. Toxic metabolite responsible for *Artemia* mortality – Experiments 3

The nature of the toxic metabolite(s) responsible for the toxicity of *Ostreopsis* cells on *Artemia* was further investigated comparing the toxic behaviour of (i) living *Ostreopsis* cells, (ii) chemical fractions obtained from *Ostreopsis* cells extracts, (iii) purified OVTX, and (iv) commercial PLTX. Interestingly, living cells induced an earlier response than equivalent amount of dissolved PLTX and OVTX (mortality of 46%, 13% and 13%, respectively after 12h of exposure at a concentration of 400 cells.mL⁻¹), although these differences tend to be reduced with increasing exposure time (Figure 36). After 48h, mortality is significantly higher with the dissolved toxins (100 % for both OVTXs and PLTX and 76,7 % for living cells at a cellular concentration of 4 cells.mL⁻¹), as also confirmed by the LC₅₀ values given in Table 13.

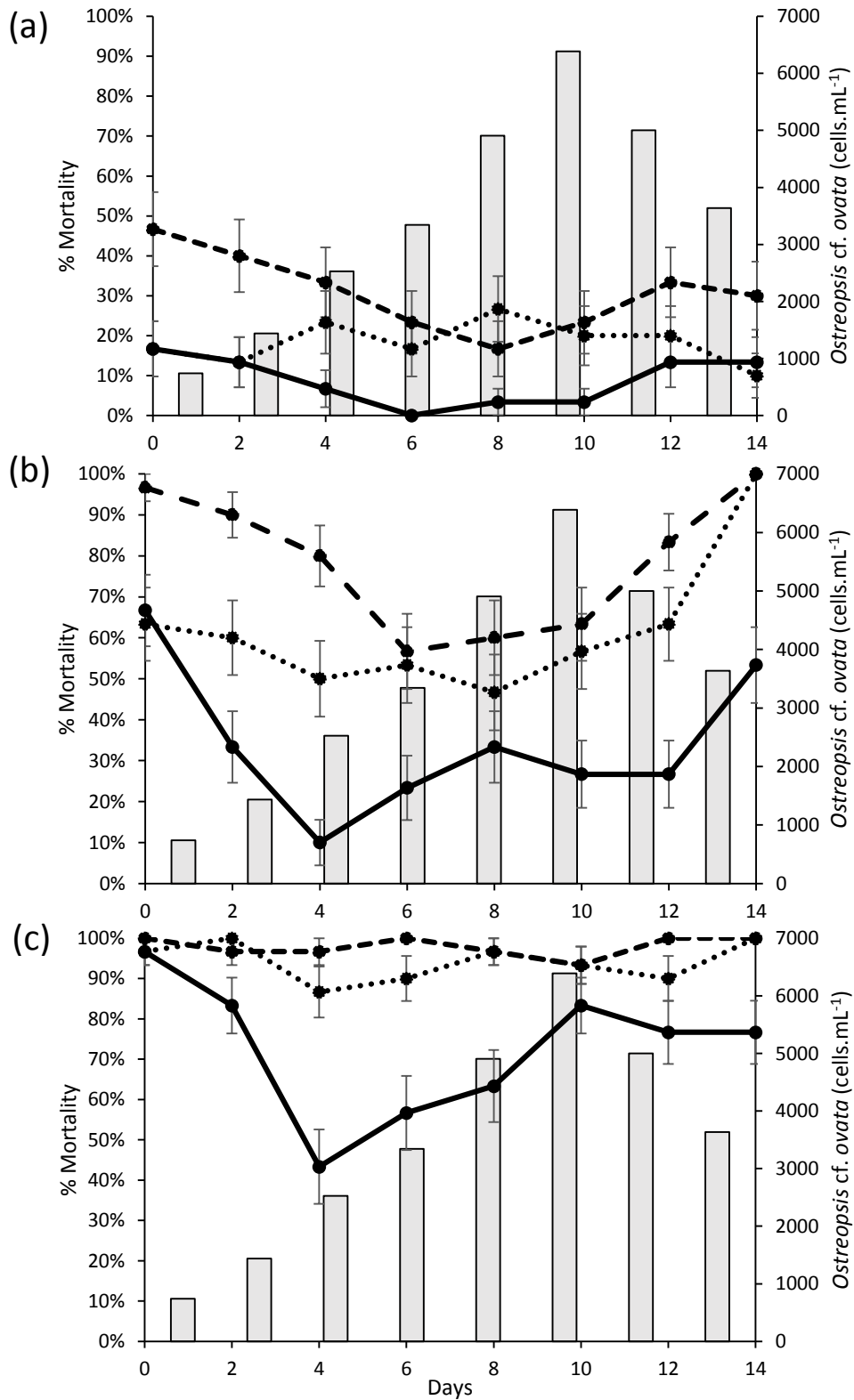


Figure 33 : Percentage of mortality (Mean ± ES) of *Artemia franciscana* larvae exposed to 4 (black lines), 40 (dotted lines) and 400 cells.mL⁻¹ (dashed lines) of *Ostreopsis cf. ovata* (MCCV 055) after (a) 12 hours, (b) 24 hours and (c) 48 hours exposure time. The growth curve of *O. cf. ovata* (MCCV 055) cultured in 300mL flasks is also represented in grey bars.

To test whether the OVTX and PLTX are the only toxic metabolites produced by *Ostreopsis*, the activity of seven fractions obtained from the separation of the various metabolites synthesized by the dinoflagellate, was tested over 24 hours on *Artemia* larvae (Figure 34). No toxicity was found for fractions 1, 2 and 3 whereas all fractions from 4 to 7 were toxic showing mortalities of 13%, 30%, 33% and 71% for fractions 6, 4, 7 and 5, respectively. The analysis of the chemical content of each fraction by UHPLC-HRMS revealed that only fractions 5 and 6 contained detectable amounts of OVTX (29 and 7.5 µg, respectively), that yield a final concentration in the wells of 305 and 15 ng eq. PLTX.mL⁻¹, respectively. Taken altogether, these results indicate that *Artemia* larvae are sensitive to amount of OVTX and PLTX as low as 12 ng.ml⁻¹ but also to other compounds produced by *Ostreopsis*.

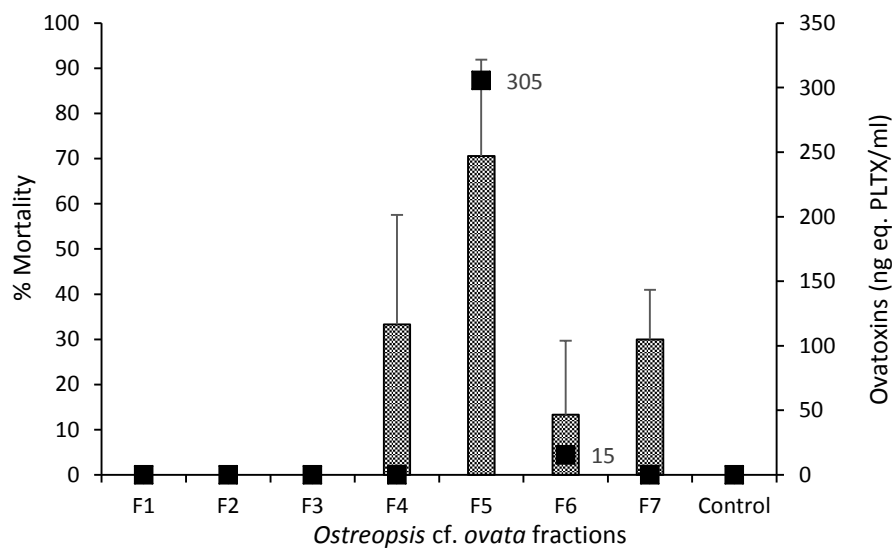


Figure 34 : Mortality (Mean ± ES) of *Artemia franciscana* larvae after being exposed 24 hours to fractions of *Ostreopsis* cf. *ovata* obtained from MCCV 054 extracts. The concentration of all fractions was previously adjusted to 3 mg.ml⁻¹ using DMSO and MilliQ. The polarity of the metabolites decreases from Fraction 1 to 7 (F1-F7). The content of OVTXs in each fraction is also represented (black squares).

2.4.5. Application to *in situ* monitoring - Experiment 4

Since *Artemia franciscana* larvae mortality was shown to be a good proxy for the toxicity of *Ostreopsis* cells from lab cultures, the application of the *Artemia* bioassay to *in situ* monitoring of *Ostreopsis* cf. *ovata* blooms was further investigated. Cells sampled *in situ* were tested for their toxicity throughout the bloom, in July 2018. The bloom of *Ostreopsis* cf. *ovata* lasted a month (June 27th to July 24th), with a peak the 13rd of July. The cell concentration increased from 100 000 cells.mL⁻¹ to 800 000 cells.mL⁻¹ before decreasing to 8000 cells.mL⁻¹ at the end of the bloom, showing no other increase before the end of August (Figure 35).

Based on the results obtained from experiments 1 and 2, we decided to only monitor the mortality of *Artemia* larvae after a 24-hours exposure to *Ostreopsis* cells (at a cellular concentration of 4 cells.mL⁻¹). The mortality of *A. franciscana* correlated the concentration of *O. cf. ovata* ($r=-0.6448$, $p<0.05$) at all cell concentrations tested (Figure 35). The mortality of *Artemia* larvae exhibited a pattern that followed the bloom of *O. cf. ovata*, with a minimum at the end of the bloom (between 10 to 25 % after the 3rd of August) and a maximum at the peak of the bloom (87% on the 14th of July – Figure 35). Interestingly, an increase of mortality was observed in August while low abundance of *Ostreopsis* was detected (from 8th of August to 22th of August; Figure 35). A quick survey of the diversity of the microalgae showed that several species of diatoms (*Licmophora paradoxa*, *Naviculaceae sp.* and *Cylindrotheca closterium*) were blooming at that time (data not shown).

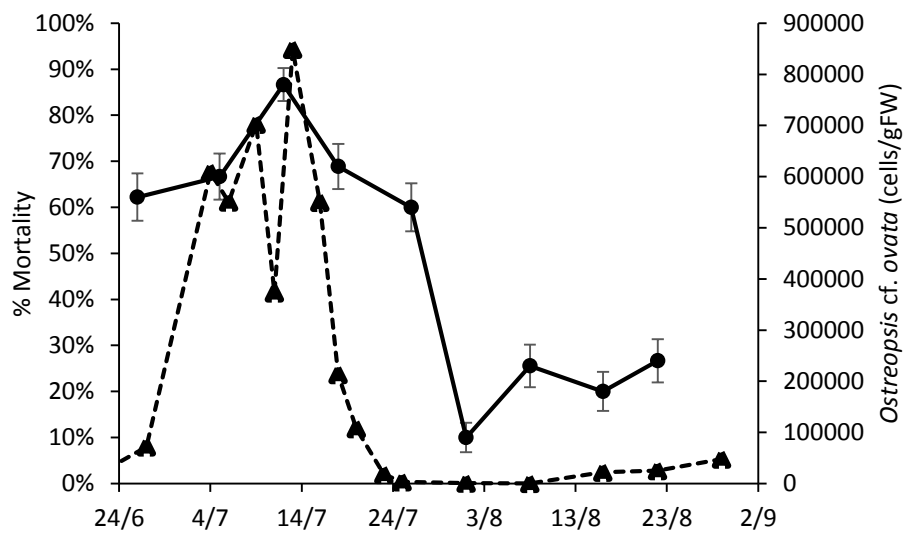


Figure 35 : Mortality (Mean ± ES) of *Artemia franciscana* after 24 hours exposition to 4 cells.mL⁻¹. Evolution of cellular concentration of *Ostreopsis cf. ovata* on artificial substrates is also represented in dotted line.

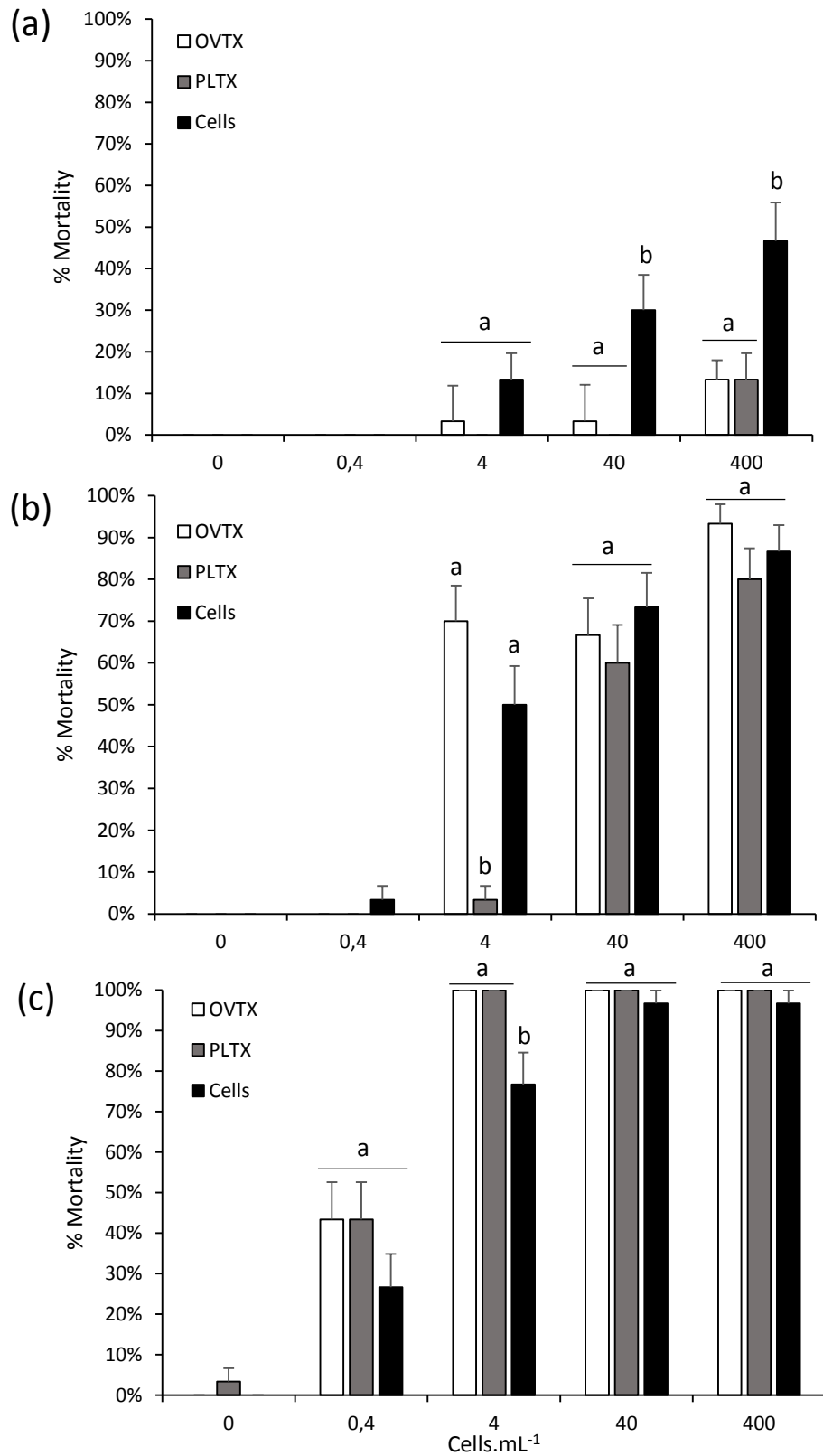


Figure 36 : Mortality (Mean ± ES) after 12 h (a), 24 h (b) and 48 h (c) of *Artemia franciscana* larvae exposed to increased concentrations of dissolved PLTX and OVTX (from 0.012, 0.12, 1.2 and 12 µg/mL)

and living cells at various cell-equivalent concentrations. Letters formulate significant differences: groups that have no common letter differ significantly ($p < 0.05$).

2.5. Discussion

2.5.1. Validating *Artemia franciscana* as a relevant model to evaluate *Ostreopsis* sp. toxicity

To ensure monitoring of Harmful Algal Blooms and consumer protection, the usefulness of biological assays has been long recognized for toxicity determination of samples (Botana, 2014). For instance, mouse bioassay (MBA) has been widely used as primary tool for HABs monitoring, notably for shellfish toxicity (Delaney, 1984). This biological assay however presents a lack of sensitivity, is prone to interferences with various metals and salts resulting in false positives and negatives, and often raises major ethical and political concerns (McCulloch et al., 1989; Wiberg and Stephenson, 1961). Hence developing more sensitive and ethic bioassays is currently of high importance. The crustacean species *Artemia* is very useful to evaluate the toxicity of diverse biological and chemical contaminants (Michael et al., 1956; Nunes et al., 2006; Vanhaecke et al., 1981) and its relevance for assessing the toxicity of *Ostreopsis* cells was successfully investigated in this study. First, the replicability of this bioassay was satisfactory with coefficients of variation between 0 and 6.1%, therefore demonstrating the excellent replicability of this test. The significant mortality of the *Artemia* larvae induced by the two *Ostreopsis* species (*ovata* and *fatturosoi*) and the four strains (MCCV 054, 055, 057 and 058) is in accordance with previous results obtained with *Ostreopsis siamensis* and *ovata*, isolated from New Zealand (strains CAWD 73, 74, 75 and 96) and the Marche Region (Italy – Strain OOAN0601), respectively (Pezzolesi et al., 2012; Rhodes et al., 2000), supporting the suitability of this bioassay to monitor the toxicity of any *Ostreopsis* species from any origin.

The *Artemia* larvae were shown to be extremely sensitive to the presence of *Ostreopsis* cells since concentrations as low as 0.4 cell. mL⁻¹ induced significant mortality (Experiment 3). In the context of *in situ* biomonitoring, this sensitivity could greatly facilitate an early detection of blooms. Nevertheless, it should be considered that differences in toxicity exist according to the growth stage of the dinoflagellate (Experiment 2), following a pattern that correlated the cellular growth: toxicity was maximal at the end of the exponential phase (43%, day 10) and minimal at the beginning of the growth (83%, day 3). Thus, for standardization aspects, our data suggest that the toxicity of newly isolated strains/species should be tested using cells that reached the end of their exponential phase (~10 days after inoculation).

Several exposure time (12, 24 and 48 hours) and cell concentrations (0.4, 4, 40, 400 and 4000 cell.mL⁻¹) were tested in Experiments 1, 2 and 3 to evaluate the best conditions that must be observed to run the *Artemia* bioassay. The data obtained suggest that exposure lower than 12 hours is not

relevant whereas exposure over 24 hours provides the best accuracy (Experiment 1 and 2) and allows determining the LC₅₀ values. Also, cell concentrations ranging from 0.4 to 400 cell.mL⁻¹ gives the best results (Experiment 1 and 3).

However, results obtained in the experiment 3 highlighted earlier action of living cells compared to dissolved toxins that could go unnoticed with only one check. Standardized conditions that should be used to run an *Artemia* bioassay in the context of evaluating the toxicity of an *Ostreopsis* species are summarized in the Appendices 1 and 2 of the supplemental information.

2.5.2. Compounds leading to *Ostreopsis* sp. toxicity

Despite no OVTX was quantified, the *fattorussoi* species was surprisingly highly toxic to the *Artemia* larvae (93,3%), suggesting the production of toxic compounds by this *Ostreopsis* species (Experiment 1). Although the production of OVTXs by *O. fattorussoi* has been already reported (Accoroni et al., 2016a) the experimental conditions used in this study did not provide any quantification of OVTXs. This suggest the variability of toxin quantity among this specie (already documented for *O. cf. ovata* (Accoroni et al., 2011; Brissard et al., 2014; Ciminiello et al., 2012b; Hwang et al., 2013) and the potential production of other toxic compounds. Indeed, several fractions of the cells of the *ovata* species were also shown to be toxic although devoid of OVTX (e.g. F4 and F7, Experiment 3), and supporting the production of unknown toxic metabolites by *Ostreopsis cf. ovata*. Historically, the identification of the toxic compounds produced by the Mediterranean *Ostreopsis* species were undertaken by LC-MS, searching for known metabolites (Ciminiello et al., 2006, 2008 and 2011). Other metabolites have been identified in *Ostreopsis* species (*O. heptagona*: Accoroni et al., 2016; *O. labens*: Rhodes et al., 2002; *O. cf. ovata*: Ternon et al., 2018), underlining the utility of bio-guided fractionation and isolation approach used in classic natural product chemistry. The OVTX are nevertheless likely to be the most toxic metabolites produced by *O. cf. ovata* given the high mortality reached when testing the most OVTX-concentrated fraction (F5). The activity of both OVTX and PLTX was further confirmed by testing purified compounds on *Artemia* larvae using theoretical concentrations that would be carried by 0.4, 4, 40 and 400 cells.mL⁻¹. This dose-dependent experiment highlighted the extreme sensitivity of the *Artemia* larvae to the presence of both OVTX and PLTX since concentrations as low as 12 pg induced 100% mortality. Therefore, the *Artemia* bioassay allows the detection of the OVTX 230 000 times more sensitive than UHPLC-HRMS, that could greatly help the early detection of toxic events.

Despite a high similarity in their molecular formulae, PLTX and OVTX have been previously shown to display different toxic activity on HaCaT cell lines and rats (Pelin et al., 2016; Poli et al., 2018), which is contradictory with the results obtained at all concentrations and exposure times (80 and 93%, respectively) with the *Artemia* larvae. Interestingly, living cells induced a higher mortality than pure

compounds at low exposure times (12 and 24 hours), confirming the toxic role of other metabolites produced by *O. cf. ovata* highlighted by the fractions' activity. Several metabolites of high molecular weight displaying the same ionization pattern than OVTX (tri-charged ions) were shown to be released by *O. cf. ovata* in the medium when co-cultured with the competitive diatom *Licmophora paradoxa* (Ternon et al. 2018), although they were not toxic for the diatom. Indeed, living cells are ingested by *A. franciscana* (as previously described by Faimali et al., 2012) although dissolved toxins affected the larvae by direct contact. However, this difference of toxicity could also be explained by the production of other toxic metabolites by *Ostreopsis* sp. also suggested by all the experiments of this study. Taken altogether, these results highlight the complementarity of using the *Artemia* assay together with chemical analysis since they promote a rapid and highly sensitive screening of toxic fractions before an identification of the toxic metabolites.

2.5.3. Application to *in situ* monitoring of *Ostreopsis cf. ovata*

The efficiency of the *Artemia* bioassay to monitor a real bloom of *Ostreopsis cf. ovata* was investigated in 2018 at the Rochambeau site (Villefranche-sur-mer, France). Consistently with the results obtained *in vitro*, the *Artemia* mortality was closely correlated with the concentration of *Ostreopsis* cells. Although the toxin content was not analyzed during this experiment, previous monitoring showed that the OVTX concentrations is maximal at the peak of the bloom at this site (e.g. highest cell concentration; Gémin et al., in prep.). Various toxin profiles have been obtained in other Mediterranean areas, suggesting a variability in the species/strains and environmental parameters, that could easily be highlighted using the *Artemia* bioassay. Indeed, this simple monitoring tool is relevant to evaluate the toxicity provoked by the OVTX and other metabolites the *Ostreopsis* cells produce in the field. Interestingly, a slight increase of mortality of *Artemia* larvae was measured in August while *Ostreopsis* exhibited low cell abundance. Other benthic microalgae could also induce a mortality of *Artemia* larvae such as other dinoflagellates. Indeed, chemical extracts of *Prorocentrum* sp. were shown to induce 100 % mortality of *Artemia* whereas no mortality was observed testing diatoms chemical extracts (*Naviculacae arenaria*, *Licmophora paradoxa*, Ternon, pers. data).

The sensitivity of this test, its speed, its reproducibility, its ease of use and its low cost are major assets for routine evaluation of the toxicity of *Ostreopsis* sp. in the field, and potentially of other toxic dinoflagellates, but also as a tool to investigate the presence of toxins in microalgae using bio-guided fractionation approach. This would greatly facilitate the implementation of this bioassay for the assessment of HABs species toxicity by city halls, water and coastal managers. One potential limitation of the *Artemia* bioassay is the information it provides is only qualitative and not specific.

2.6. Acknowledgement

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Appendix 1: Hatching conditions used in all the toxicity experiments

Incubation	Glassware	Media	Volume	Light	Temperature	Salinity	pH	Oxygen	G cysts	Food
40 hours	Erlenmeyer 500 ml	Filtered autoclaved sea water	300 mL	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	20°C	38		Continuous aeration	0.15 g	No food

Appendix 2: Experimental conditions for toxicity assay

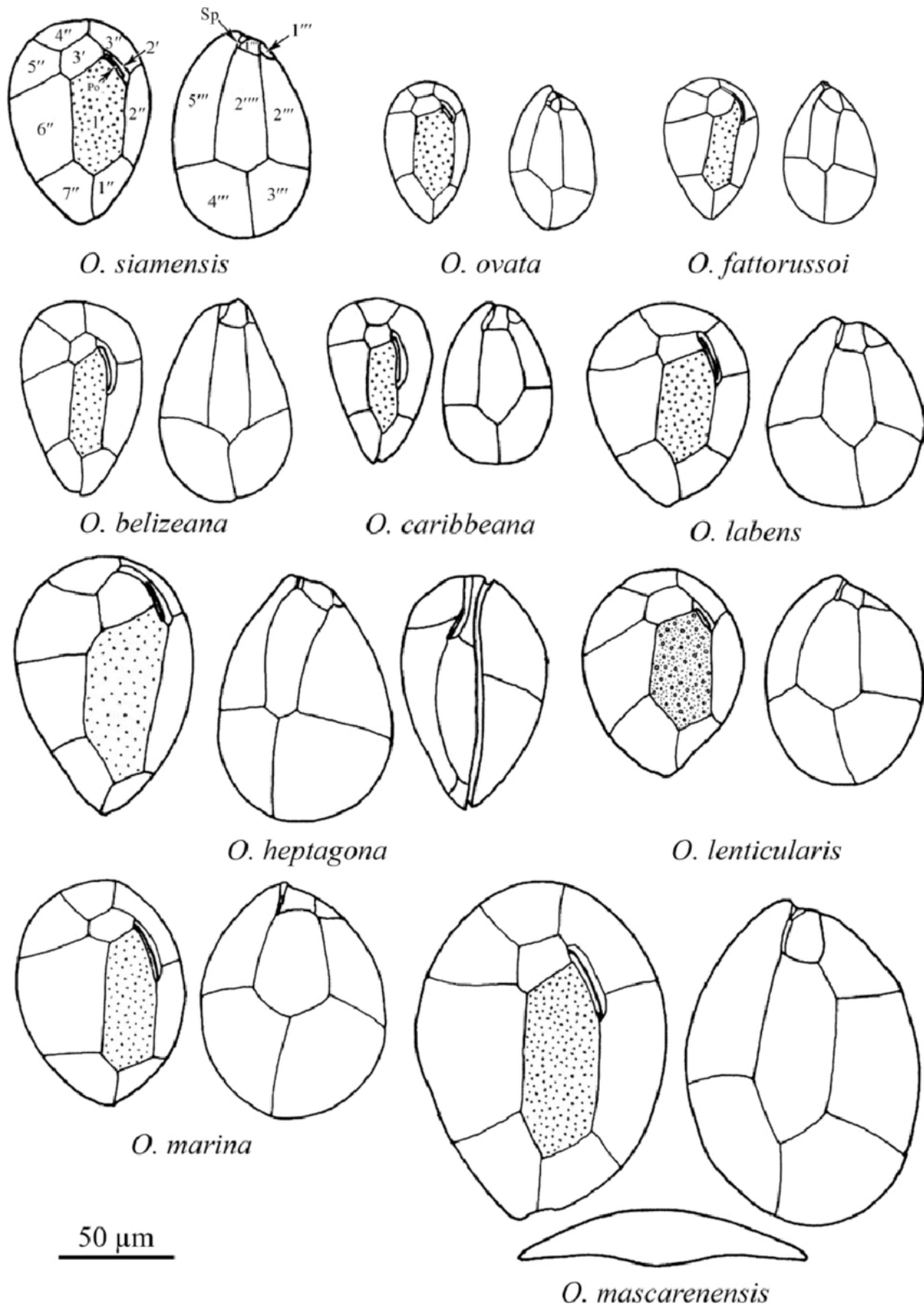
	Experiment 1: strain comparison	Experiment 2: Growth vs toxicity	Experiment 3: PLTX/OVTX/living cells	Experiment In situ bloom
Experimental treatment	MCCV 054/055/057/058 4, 40, 400, 4000 cells $\cdot\text{mL}^{-1}$	MCCV 055 every 2 days during 14 days	PLTX/OVTX/MCCV055 living cells	In situ <i>Ostreopsis cf. ovata</i> cells / 1 toxicity assay per week
Time exposition	48 hours	48 hours	48 hours	48 hours
Time observation	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time	0,1,2,3,4,5,6,7,8,9,10,11,12,24,48 hour exposition time
Temperature	24°C	24°C	24°C	24°C
Light	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$
Salinity	38	38	38	38
Glassware	6 well plates	6 well plates	6 well plates	6 well plates
Replicates	30 (5 larvae per well / 6 wells per experimental condition)	30 (5 larvae per well / 6 wells per experimental condition)	30 (5 larvae per well / 6 wells per experimental condition)	30 (5 larvae per well / 6 wells per experimental condition)
Medium	Filtered and autoclaved sea water + cells culture (80:20, v:v)	Filtered and autoclaved sea water + cells culture (80:20, v:v)	Filtered and autoclaved sea water + cells culture (80:20, v:v)	Filtered and autoclaved sea water + cells culture (80:20, v:v)
Volume	5 ml	5 mL	5 mL	5 mL
Control	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)	Filtered and autoclaved sea water + fresh L1 medium (80:20, v:v)
Quality Control	Mortality < 10%	Mortality < 10%	Mortality < 10%	Mortality < 10%
Parameters calculated	LC _{50-24h} LC _{50-48h}	LC _{50-24h} LC _{50-48h}	LC _{50-24h} LC _{50-48h}	LC _{50-24h} LC _{50-48h}

3. Synthèse de l'étude

Les résultats ont montré la pertinence de ce modèle biologique pour évaluer la toxicité d'*Ostreopsis* spp. La production de métabolites secondaires inconnus mais toxiques, à la fois par *O. cf. ovata* et par *O. fattorussoi*, a été mise en évidence par l'utilisation de ce test, soulignant la sensibilité de ce modèle biologique. Ceci met également en évidence l'utilité du fractionnement bio-guidé et suggère un couplage de ce test biologique aux méthodes de chimie analytiques pour l'identification de nouveaux composés bio-actifs chez *Ostreopsis* spp.

L'application de ce test biologique au suivi *in situ* confirme la pertinence de ce modèle pour évaluer la toxicité des efflorescences d'*O. cf. ovata* voire même d'autres espèces toxiques.

Chapitre 7. Discussion et perspectives



Une grande partie de mes résultats de thèse a déjà été discutée dans les publications ou les chapitres en français de mon manuscrit. J'ai souhaité ici confronter mes résultats avec des considérations actuelles en relation avec l'écologie chimique marine, comme la localisation des toxines et l'impact de cette localisation sur leurs effets, le transfert possible de ces métabolites dans les réseaux trophiques et les facteurs contrôlant la production de toxines. Je terminerai par une conclusion sur le rôle écologique des métabolites secondaires d'*Ostreopsis cf. ovata* et présenterai quelques voies de recherche afin de poursuivre ce travail.

1. Impacts d'*Ostreopsis cf. ovata* sur les écosystèmes méditerranéens

De nombreuses études ont déjà décrit l'impact néfaste des efflorescences d'*Ostreopsis cf. ovata* sur les écosystèmes (Del Favero et al., 2012; Mangialajo et al., 2011; Parsons et al., 2012). Ces efflorescences vont impacter les écosystèmes à tous les niveaux trophiques puisque de fortes concentrations de toxines ont été quantifiées aussi bien chez des organismes herbivores qu'omnivores, ainsi que chez certains carnivores se nourrissant majoritairement de crustacés et de mollusques (Biré et al., 2015, 2013). Ces quantités dépassent parfois même le seuil de 30 $\mu\text{g}\cdot\text{kg}^{-1}$ établi par l'Agence européenne de sécurité des Aliments (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009) comme c'est le cas de l'oursin *Paracentrotus lividus* qui peut accumuler jusqu'à 108 $\mu\text{g}\cdot\text{kg}^{-1}$ d'équivalent palytoxine (Amzil et al., 2012; Biré et al., 2015, 2013). La présence de ces toxines dans des organismes dont les régimes alimentaires diffèrent, soulève la question du transfert des toxines dans les réseaux trophiques supérieurs ainsi que le risque de contamination des populations humaines via l'ingestion d'organismes contaminés.

Les efflorescences d'*Ostreopsis cf. ovata* affectent également les communautés benthiques et notamment le polychaete *Dinophilus gyrociliatus* (Simonini et al., 2011) ou les bivalves filtreurs comme la moule *Mytilus edulis* (Aligizaki et al., 2008; Amzil et al., 2012; Gorbi et al., 2013, 2012; Milandri et al., 2010; Rhodes et al., 2002). Des mortalités de masse d'oursins ont été décrites, notamment en Nouvelle Zélande et en mer Méditerranée (Illoul et al., 2012; Shears and Ross, 2009; Totti et al., 2010). Néanmoins, le rôle des toxines (ou autres métabolites secondaires) produites par *O. cf. ovata* dans ces épisodes de mortalité de masse, est encore sujet à controverse car les conditions anoxiques et/ou hypoxiques générées par ces efflorescences pourraient également expliquer ce phénomène.

Les organismes adultes ne sont pas les seuls à être impactés par ces toxines puisque de nombreuses études ont décrit des effets néfastes pour la fécondation mais également pour les premiers stades de développement embryonnaires et larvaires (Caroppo and Pagliara, 2011; Giussani et al., 2016; Migliaccio et al., 2016; Neves et al., 2018; Pagliara and Caroppo, 2012).

Enfin, ces efflorescences n'affectent pas seulement les écosystèmes à l'échelle individuelle mais également à l'échelle des communautés. Des changements au niveau des communautés méiobenthiques ont, par exemple, été décrits par Guidi-Guilvard et al. (2012).

1.1. *Ostreopsis cf. ovata* : toxicité aigüe vs toxicité chronique ?

L'une des premières interrogations soulevées face aux efflorescences d'*O. cf. ovata* est de savoir si ces efflorescences sont à considérer comme des phénomènes de toxicité chronique ou aigüe. En raison de la durée de ces efflorescences en rade de Villefranche (généralement un mois, en juillet - Cohu et al., 2011), ces épisodes pourraient être considérés comme des épisodes de toxicité aigüe, c'est-à-dire provoquant une exposition rapide à de fortes concentrations qui pourrait engendrer des réponses biochimiques et cellulaires pouvant conduire à un changement physiologique ou comportemental (Landsberg, 2002). Lorsque l'on considère la capacité de certains copépodes planctoniques à sélectionner leur nourriture, on peut effectivement penser à un effet aigüe. C'est le cas de l'espèce *Acartia tonsa* qui, exposée à *Karenia brevis*, va modifier son comportement de broutage (Hong et al., 2012). Cette capacité de sélection a également été observée pour le copépode benthique *Sarsamphiascus cf. propinquus* testé au cours de cette thèse (chapitre 3). Les copépodes planctoniques exposés à *O. cf. ovata* (chapitre 4) étaient très sensibles à la présence d'*O. cf. ovata* et une mortalité de 100% est observée en moins de 48h. La notion de seuil apparaît : un effet mortel intervient à partir d'un certain seuil (dépendant de la sensibilité de l'organisme testé) mais en dessous de ce seuil, on observe uniquement des réponses comportementales, physiologiques voire pathologiques (Cembella, 2003; Landsberg, 2002).

La récurrence annuelle des efflorescences, au site de Rochambeau (Villefranche-sur-mer), laisse présager l'existence d'un effet chronique. Aujourd'hui, il existe peu d'études sur les effets à long terme des algues toxiques sur la faune, que ce soit en termes d'acclimatation, c'est-à-dire de plasticité phénotypique, ou d'adaptation (qui conceptuellement est le résultat de la sélection naturelle d'individus selon leurs traits génétiques (Edholm, 1966; Mazess, 1975; Meier et al., 2011). L'étude de sélection génétique de Colin and Dam (2005) a reporté l'existence de mécanismes adaptatifs au sein d'un élevage d'*Acartia hudsonica* naïfs exposés chroniquement à *Alexandrium fundyense* permettant une augmentation de l'ingestion et de la production d'œufs après 3 générations. A long terme, cette exposition à *O. cf. ovata* pourrait augmenter la sensibilité de la faune aux infections virales et bactériennes, favoriser l'immunosuppression ou le développement d'embryons anormaux (Landsberg, 2002). L'étude de Guidi-Guilvard et al. (2012) a noté une diminution significative du nombre de nauplii de copépodes benthiques lors des efflorescences d'*O. cf. ovata* qui pourrait s'expliquer par une

diminution des indices de fécondité et de fertilité (Pavaux et al., 2019), voire par un développement embryonnaire anormal.

Ce travail de thèse s'est focalisé sur les effets aigus liés à des expositions courtes (variant de 48h à 1 mois) à de fortes concentrations. La réponse des copépodes benthiques (chapitre 3) mais également l'absence de réponse des oursins exposés à *O. cf. ovata* (chapitre 5) soulignent la nécessité d'étudier à long terme, les effets d'une exposition chronique aux efflorescences d'*O. cf. ovata*.

1.2. Facteurs contrôlant la production de toxines

Ostreopsis cf. ovata et les dinoflagellés en général, présentent une plus faible affinité d'absorption des nutriments que les diatomées, et ont dû mettre en place des adaptations majeures pour faire face à ce désavantage écologique. Il s'agit par exemple de la mixotrophie, l'allélopathie et la défense contre les prédateurs (Smayda, 1997b). La production de toxines peut ainsi faire partie des avantages évolutifs adoptés par les dinoflagellés pour devenir plus compétitifs. Le rôle des toxines dans le cycle de vie d'*O. cf. ovata*, et des autres microalgues toxiques en général, reste pourtant encore peu décrit. L'influence des facteurs environnementaux sur la production de toxines a été mise en évidence chez les dinoflagellés toxiques (Granéli and Flynn, 2006) comme c'est le cas notamment pour *Alexandrium minutum* (Hwang and Lu, 2000). Chez *O. cf. ovata*, les facteurs environnementaux influencent également la production de toxines (Pistocchi et al., 2011) : une diminution de la production de toxines de 53% et de 40% est en effet observée lors de limitations respectives en azote et phosphore (Vanucci et al., 2012b).

Aucune étude ne s'est focalisée sur l'impact des relations biotiques sur la production de toxines par *O. cf. ovata*. Chez *Pseudo-nitschia*, la production d'acide domoïque est pourtant augmentée par l'exposition à des prédateurs (Bergkvist et al., 2008; Lundholm et al., 2018; Selander et al., 2006). Cette augmentation est décrite comme une réponse à la présence de prédateurs et à la sécrétion de signaux chimiques par ces prédateurs. Cette réponse spécifique permet ainsi d'éviter d'allouer de l'énergie à la défense lorsque cela est inutile (Karban, 2011). Dans le cadre de notre étude (chapitre 2), une augmentation de la production de toxines par *O. cf. ovata* a été mise en évidence en présence d'une diatomée compétitrice (Ternon et al., 2018). Ceci confirme le contrôle de la production des toxines par les relations biotiques. Cette production serait ainsi induite par la présence de prédateurs et/ou compétiteurs, afin de minimiser les coûts métaboliques générés par la production de ces composés (Pohnert, 2000). Ces mécanismes restent cependant encore peu étudiés et l'analyse de cette stratégie dynamique pourrait contribuer à une compréhension plus précise des relations qu'*O. cf. ovata* entretient avec son environnement.

1.3. Localisation des toxines produites par *Ostreopsis cf. ovata*

La toxicité des souches d'*O. cf. ovata* utilisées au cours de cette étude a été vérifiée par la mise en place d'un test biologique faisant intervenir *Artemia franciscana* (chapitre 6). Ce test permet d'évaluer qualitativement et de manière simple et rapide, la toxicité des souches d'*O. cf. ovata*. Le profil toxinique de cette souche a également été déterminé de manière quantitative par des méthodes chimiques faisant intervenir les techniques de chromatographie liquide à haute résolution (UHPLC-HRMS/MS - chapitre 3 et 6). Ces deux méthodes (biologiques et chimiques) ont permis de mettre en évidence la présence de toxines intracellulaires (endométabolome) mais également leur excrétion (exométabolome). Les toxines produites par *O. cf. ovata* vont ainsi pouvoir agir selon différentes voies d'exposition, en étant intracellulaires ou excrétées à l'extérieur de manière active ou via la lyse cellulaire.

1.3.1. Toxines intracellulaires et leurs impacts

La présence de toxines intracellulaires, au niveau du cytoplasme (Honsell et al., 2011), a déjà été démontrée par immunolocalisation via l'utilisation d'anticorps antipalytoxine. Au cours de mes travaux de thèse, les toxines appartenant à l'endométabolome (soit les toxines à l'intérieur des cellules) ont été quantifiées (chapitre 2). Les concentrations mesurées varient entre 18,0 et 23,7 pg eq. PLTX/cell et sont en accord avec les valeurs précédemment publiées par Brissard et al. (2014) et Pezzolesi et al. (2012). Les organismes peuvent donc être exposés directement à ces toxines en les ingérant (que ce soit par prédation directe ou par filtration). Les copépodes benthiques testés dans cette étude sont directement exposés à ces toxines puisque la présence de cellules d'*O. cf. ovata* a été observée dans leur tractus digestif par microscopie à épifluorescence (chapitre 3). *Artemia salina* est également capable d'ingérer ce dinoflagellé. En effet, la présence d'*O. cf. ovata* dans le système digestif a été démontrée par l'utilisation de la microscopie confocale (Faimali et al., 2012). L'ingestion des cellules d'*O. cf. ovata* a également été démontrée pour différents organismes filtreurs tels que les moules et les éponges par la quantification des toxines produites par *O. cf. ovata* dans le système digestif de ces organismes. Enfin, les protistes hétérotrophes et notamment *Gyrodinium dominans*, *Gyrodinium moestrupli*, *Oxyrrhis marina* et *Polykrikos kofoidii*, sont également capables d'ingérer les cellules d'*O. cf. ovata* (Yoo et al., 2015). Les conséquences liées à l'ingestion des cellules d'*O. cf. ovata* diffèrent selon les organismes. L'ingestion de ces cellules semble sans effet pour ces protistes alors qu'elle est mortelle *Artemia franciscana*.

Très peu d'études se sont focalisées sur les kystes d'*O. cf. ovata*. Les études sur le sujet se sont surtout intéressées à la description de ces kystes (Accoroni et al., 2014; Bravo et al., 2012) et à leur processus de germination (Accoroni et al., 2015a). Pourtant des études réalisées sur les kystes

d'*Alexandrium* sp. ont mis en évidence la toxicité de ceux-ci (Dale and Yentsch, 1978). Les kystes en dormance pendant plusieurs mois sont considérés comme 10 fois plus toxiques que les cellules végétatives et peuvent même être 1000 fois plus toxiques quelques heures après leur formation. Ceci suggère une nouvelle voie d'exposition jamais envisagées pour *O. cf. ovata*.

Une capacité de sélection des proies a été mise en évidence chez le copépode benthique *S. cf. propinquus* (chapitre 3) et a déjà été reportée pour des copépodes planctoniques exposés à d'autres algues toxiques (Teegarden et al., 2001). Ceci suggère que ces brouteurs évitent d'ingérer ces cellules car ils sont capables de détecter des signaux qui vont dissuader le broutage (Colin and Dam, 2005) ou encore que ces cellules sont indigestes (Breier and Buskey, 2007). Pourtant de nombreux copépodes ingèrent ces microalgues toxiques sans conséquence apparente sur leur physiologie ou leur comportement (Turner, 2014).

1.3.2. Toxines libérées activement dans l'eau et leurs impacts

L'étude réalisée dans le chapitre 2 a permis de quantifier les concentrations de toxines excrétées. Les quantités retrouvées dans l'exométabolome varient entre 0,52 et 1,03 pg eq. PLTX/cellules, et sont inférieures à celle quantifiées dans l'endométabolome dans les mêmes conditions expérimentales. Les mécanismes d'excrétion des toxines sont encore peu connus chez *O. cf. ovata* même si le rôle des tricocystes et mucocystes a été avancé (Honsell et al., 2013). La libération des toxines a déjà été décrite chez d'autres dinoflagellés tels que *Dinophysis acuta* et quantifiée à 90% (Nielsen et al., 2013) alors qu'elle est estimée à moins de 10% chez *O. cf. ovata*. Les faibles quantités de toxines retrouvées dans l'exométabolome peut s'expliquer par une concentration des toxines excrétées dans le mucus comme suggéré par l'étude des relations allélopathiques entre *O. cf. ovata* et une diatomée co-occurrence présentée au chapitre 2. En effet, cette étude n'a mis en évidence aucun effet toxique d'*O. cf. ovata* lors de l'absence de contact entre les deux compétiteurs. Cette séquestration des toxines dans le mucus permettrait (i) d'éviter les phénomènes de dilution des toxines dans le milieu et (ii) d'agir directement sur les compétiteurs et prédateurs d'*O. cf. ovata*. Le rôle du mucus dans la prédation a déjà été avancé pour *O. cf. ovata* et d'autres dinoflagellés toxiques. Très peu d'études se sont intéressées au rôle écologique de ce mucus, notamment en raison de la difficulté à séparer les cellules d'*O. cf. ovata* de leur mucus.

Les toxines libérées dans l'eau vont impacter les organismes par contact, mais également par ingestion. C'est le cas des copépodes, qui, en plus d'être exposés aux toxines intracellulaires, vont également être exposés aux toxines exudées, tout comme les organismes filtreurs. Le rôle des toxines pourrait ainsi être de limiter le broutage par des prédateurs potentiels. Ces propriétés dissuasives ont déjà été décrites pour d'autres dinoflagellés toxiques comme *Karlodinium veneficum* (Waggett et al.,

2008), *Gymnodinium catenatum* (Costa et al., 2012) ou *Alexandrium minutum* (Guisande et al., 2002) même si elles n'agissent pas chez tous les copépodes (Turner, 2014). Ceci indique clairement l'existence de phénomènes de chémoréception par lesquels les copépodes sont capables de détecter des signaux chimiques produits par les algues toxiques. Ces propriétés dissuasives peuvent constituer un mécanisme favorisant l'installation et le maintien des efflorescences d'algues toxiques. Le rôle des toxines dans ces phénomènes de dissuasion a souvent été avancé mais aucune étude ne confirme que ce soit les toxines qui aient ces propriétés dissuasives. L'étude métabolomique réalisée au cours mes travaux de thèse (chapitre 2) a mis en évidence la production d'un grand nombre de métabolites par *O. cf. ovata* dont les propriétés biologiques ne sont pas encore connues. En effet, le métabolome complet d'*O. cf. ovata* est encore peu connu et aucune étude ne s'est encore intéressée aux réponses métaboliques d'*O. cf. ovata* en la présence de prédateurs.

L'excrétion des toxines va également avoir un rôle important dans les relations allélopathiques (chapitre 2). Ces interactions sont dépendantes des propriétés physicochimiques des toxines qui vont déterminer la stabilité de ces composés, une fois libérés. Ces toxines relarguées vont également avoir un rôle prépondérant dans les relations allélopathiques, en empêchant le développement d'autres microalgues compétitrices pour la lumière, l'espace ou les nutriments par exemple. Toutefois, le rôle des toxines comme agent allélopathique est encore sujet à débat dans la communauté scientifique. Certaines études montrent le rôle prédominant de ces toxines dans les relations que les microalgues entretiennent avec leur environnement comme c'est le cas pour *Karlodinium micrum* par exemple (Adolf et al., 2006; Deeds and Place, 2006) alors que d'autres études montrent que les brevetoxines produites par *Karenia brevis* n'ont aucun effet sur les algues co-occurrentes (Poulson-Ellestad et al., 2014; Prince et al., 2008) voire même qu'elles favorisent la croissance d'algues compétitrices (Poulson-Ellestad et al., 2014). L'étude des relations allélopathiques qu'*O. cf. ovata* entretient avec son environnement que nous avons réalisée (Chapitre 2 - Ternon et al., 2018) suggère également que ce rôle allélopathique pourrait être assuré par d'autres composés que les ovatoxines. L'analyse métabolomique a mis en évidence la production d'un composé organique à haut poids moléculaire (2120 Da), indépendant de la famille des ovatoxines, lors d'un contact avec une micro-algue compétitive. Ceci souligne la nécessité d'étudier ces relations allélopathiques notamment pour leurs rôles dans le maintien des efflorescences d'algues toxiques. La méta-analyse réalisée par Jonsson et al. (2009) a démontré que la formation des efflorescences d'algues toxiques n'était pas déterminée par les relations allélopathiques mais que celles-ci favorisent leur maintien.

Le rôle des toxines dans les relations qu'*O. cf. ovata* entretient avec la sphère microbienne est également peu étudié. Les bactéries vivent en relation étroite avec ce dinoflagellé et sont indispensables à sa croissance, c'est pourquoi il est aussi difficile de maintenir en *O. cf. ovata* en culture axénique (Pavaux, com. pers.). Les communautés bactériennes sont probablement importantes au sein

de la matrice mucilagineuse produite par *O. cf. ovata*. Les seules études existantes à ce sujet ont décrit les principales communautés bactériennes associées à *O. cf. ovata* et la variation de celles-ci au cours de la croissance cellulaire du dinoflagellé (Guidi et al., 2018; Vanucci et al., 2016). Les interactions existantes sont encore mal connues chez *O. cf. ovata* même si des relations de mutualisme ont déjà été démontrées pour d'autres algues néfastes comme les cyanobactéries (Paerl et al., 2001). Ces relations permettent entre autre la dominance de ces cyanobactéries par l'échange de métabolites, de facteurs de croissance ou un rôle détoxifiant des bactéries appartenant à cette phycosphère.

Les toxines peuvent également jouer un rôle dans la lutte contre les infections parasitaires et virales. Aujourd'hui, très peu de d'informations sont disponibles à ce sujet si ce n'est que la présence d'*O. cf. ovata* est propice à la présence des bactéries du genre *Vibrio* (Bellés-Garulera et al., 2016). La présence de parasites chez *O. cf. ovata* n'a pas encore été démontrée, alors que cette infection est considérée comme une manière de limiter la prolifération d'autres dinoflagellés toxiques (Lu et al., 2014, 2016).

1.3.3. Toxines libérées suite à la lyse cellulaire

Des conditions environnementales stressantes telles que des changements de salinité ou des courants forts, mais également la sénescence cellulaire et le déclin d'une efflorescence, peuvent amener à la lyse cellulaire induisant un relargage des toxines dans le milieu. Ce relargage de toxines peut également avoir un impact sur les écosystèmes notamment en phase de déclin des efflorescences au cours desquelles les quantités libérées pourront être très importantes. Certaines méthodes de mitigation ont été proposées et consistaient à contenir les efflorescences en entraînant la lyse cellulaire (Sun et al., 2004b, 2004a). Toutefois ces méthodes engendrent la dispersion des toxines dans le milieu. Les débris formés par la lyse des cellules fournissent un substrat à de nombreuses bactéries et virus qui peuvent impacter les écosystèmes et l'homme en favorisant par exemple la dispersion du virus du genre *Vibrio* (Bellés-Garulera et al., 2016).

Cette libération de toxines extracellulaires peut également influencer la communication intraspécifique. Même si ces mécanismes sont encore peu étudiés chez *O. cf. ovata*, certaines études ont mis en évidence le rôle prépondérant des signaux chimiques libérés lors de la lyse cellulaire dans la communication intraspécifique (Griffin et al., 2019). L'étude de Senft-Batoh et al. (2015) p

1.3.4. Aérosolisation

L'aérosolisation des cellules d'*O. cf. ovata* est à l'origine de nombreux épisodes de contamination amenant à l'hospitalisation de plusieurs centaines de personnes à cause de l'apparition de symptômes de type allergiques, comme des nausées ou des irritations cutanées et oculaires (Illoul

et al., 2012; Vila et al., 2016). La présence de ces composés dans les aérosols est certainement due à la présence d'*O. cf. ovata* et des toxines dans la couche supérieure des eaux de surface. La présence des algues dans les aérosols a été vérifiée par qPCR (Casabianca et al., 2014, 2013) et par des méthodes de chimie analytiques qui ont permis de quantifier 2,4 pg d'ovatoxines par litre d'air (Ciminiello et al., 2014). L'aérosolisation est déjà connue pour d'autres algues toxiques (Backer and McGillicuddy, 2006; Cheng et al., 2005; Pierce et al., 2003). Les conditions météorologiques ont un rôle important dans les processus d'aérosolisation (Sialve et al., 2015). La pluie ainsi que la force, la direction et la vitesse du vent permettent une dispersion importante des cellules aérosolisées ; la température et l'humidité ont une incidence sur le temps des cellules passé dans l'atmosphère avant leur dépôt. Mais outre ces variables météorologiques et les processus physiques associés, la structure de la communauté microbienne planctonique peut influencer la qualité et la quantité des bioaérosols émis. En effet, les espèces, la taille, la forme, l'organisation cellulaire et l'adhésion des particules (poussières, sels ou matrices organiques) peuvent favoriser une dispersion aérienne des microalgues (Sialve et al., 2015). L'aérosolisation des cellules d'*O. cf. ovata* est souvent décrite comme la méthode de contamination humaine la plus probable dans les régions tempérées (Ciminiello et al., 2014). La dangerosité liée à ce phénomène est également l'échelle spatiale que peut atteindre ces intoxications.

1.4. Transfert des toxines dans le réseau trophique

1.4.1. Transfert direct

L'ingestion des cellules d'*O. cf. ovata* engendre un transfert des toxines dans l'organisme qui s'en nourrit ; c'est le cas de l'oursin *Paracentrotus lividus* (Lamarck, 1816) qui, en ingérant les macroalgues sur lesquelles se développent *O. cf. ovata*, accumule de fortes quantités de toxines pouvant atteindre 423 $\mu\text{g.kg}^{-1}$ (Brissard et al., 2014), soit des valeurs 14 fois supérieures aux limites préconisées par l'Agence européenne de sécurité des Aliments (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009). Des quantités de toxines variant de 230 $\mu\text{g.kg}^{-1}$ à 392 $\mu\text{g.kg}^{-1}$ ont respectivement été quantifiées chez la saupe *Sarpa salpa* (Linnaeus, 1758) et le mulot *Mugil cephalus* (Linnaeus, 1758), deux poissons herbivores qui se nourrissent respectivement de macroalgues servant de substrat à *O. cf. ovata* et des fleurs d'eau formées à la surface (Biré et al., 2015, 2013). Les cellules d'*O. cf. ovata* sont également ingérées par des organismes filtreurs tels que les éponges ou les bivalves qui accumulent de fortes concentrations d'*O. cf. ovata* (chapitre 1).

Les toxines s'accumulent quasi exclusivement au niveau des tissus digestifs (Biré et al., 2015) et la présence de toxines dans d'autres tissus, comme c'est le cas pour les céphalopodes, reste une exception. Chez les oursins, les toxines sont quantifiées au niveau du système digestif uniquement, ce qui pourrait expliquer pourquoi, malgré les fortes concentrations retrouvées, aucun cas d'intoxication

humaine, suite à l'ingestion d'oursins, n'a été décrit (Biré et al., 2013). Il est difficile de savoir s'il existe un réel stockage des toxines car ces quantifications concernent le système digestif entier et les toxines quantifiées ne pourraient être que celles retrouvées dans les cellules algales présentes dans le contenu du tube digestif (pré-fèces) et donc excrétées immédiatement, sans période de stockage. Néanmoins, toutes les études ayant quantifiées les toxines d'*O. cf. ovata* dans différents organismes marins (Amzil et al., 2012; Biré et al., 2015, 2013; Brissard et al., 2014) montrent une accumulation préférentielle des toxines dans le tube digestif, à l'exception du céphalopode *Octopus vulgaris*. L'éviscération peut ainsi constituer une méthode efficace pour protéger les consommateurs contre l'exposition aux toxines produites par *O. cf. ovata*. Des études supplémentaires restent nécessaires pour étudier les taux de dépuración et ainsi la détermination des cinétiques de détoxification afin de permettre la mise en place des processus de remédiation pour réduire le niveau de toxines dans les organismes marins comestibles. Le problème de l'oursin est que, même si sa pêche est interdite l'été c'est-à-dire pendant les périodes d'efflorescences d'*O. cf. ovata*, le braconnage et les habitudes alimentaires locales (ingestion à la fois des gonades et du contenu du système digestif) peuvent entraîner des intoxications alimentaires.

1.4.2. Transfert indirect

Le transfert des toxines produites par *O. cf. ovata* dans les réseaux trophiques est encore mal connu, bien que ce soit la manière la plus commune d'intoxication des plus hauts niveaux trophiques (Landsberg, 2002). Les études réalisées par Biré et al. (2015, 2013) ont montré la présence de fortes quantités de toxines dans des organismes omnivores voire carnivores tels que le céphalopode et suggèrent un transfert des toxines vers les niveaux trophiques supérieurs. Ce transfert de toxines est déjà connu pour d'autres dinoflagellés toxiques notamment pour les espèces du genre *Gambierdiscus* (Ledreux et al., 2014; Yogi et al., 2011). Le transfert des ciguatoxines dans le réseau trophique s'accompagne d'une biotransformation de celles-ci, conduisant notamment à une oxydation des toxines (Ikehara et al., 2017). La quantification des toxines produites par *O. cf. ovata* dans les organismes marins est souvent réalisée via une analyse ciblée, c'est-à-dire une recherche des toxines déjà connues. Une analyse non ciblée des métabolites est donc à envisager au vu de l'existence possible de phénomène de biotransformation des toxines au sein du réseau trophique.

Le zooplancton constitue un élément essentiel des réseaux trophiques marins notamment par le transfert d'énergie vers les niveaux trophiques supérieurs. En se nourrissant sur des algues toxiques, il devient un vecteur de transfert des toxines dans les réseaux trophiques (Engström-Öst et al., 2002; Lehtiniemi et al., 2002; Teegarden and Cembella, 1996; Tester et al., 2000). Il a ainsi été démontré que le copépode planctonique *Calanus finmarchicus* est le vecteur principal de transfert de l'acide

domoïque dans les réseaux trophiques (Leandro et al., 2010). La principale difficulté dans l'observation de ce transfert réside en la capacité de détection de faibles quantités de toxines dans les organismes planctoniques qui est rendue possible par les avancées récentes dans les techniques analytiques telles que l'HPLC (Tester et al., 2000). Le copépode benthique *Sarsamphiascus* cf. *propinquus* en se nourrissant des cellules d'*O. cf. ovata* (retrouvées par épifluorescence dans son tractus digestif - Pavaux et al., 2019), peut ainsi devenir un vecteur de transfert des toxines produites par *O. cf. ovata* dans le réseau trophique puisqu'il constitue une source de nourriture pour les larves de poissons ou les crustacés, par exemple. De la même manière, certains poissons comme la saupe (*Sarpa salpa*) qui se nourrit des macroalgues sur lesquelles se développent *O. cf. ovata*, ou les mulets (*Mugil cephalus*) qui se nourrissent directement des fleurs d'eau d'*O. cf. ovata* à la surface, peuvent constituer des vecteurs de transfert des toxines produites par *O. cf. ovata*. Néanmoins, les quantités de toxines mesurées dans les prédateurs en fin de chaîne alimentaire, comme les murènes (*Muraena helena*) prélevées directement dans des zones de proliférations d'*O. cf. ovata*, ne sont pas détectables (Biré et al., 2015).

2. Perturbation des écosystèmes suite aux efflorescences d'*Ostreopsis* cf. *ovata*

En parallèle de l'effet néfaste reporté sur la santé et les activités humaines, les efflorescences d'algues nuisibles peuvent également perturber la structure et le fonctionnement des écosystèmes (Michaloudi et al., 2009). On parle même de « Ecosystem Disruptive Algal Blooms » soit d'efflorescences algales qui vont perturber les écosystèmes (Sunda et al., 2006). Ces efflorescences d'algues toxiques et/ou néfastes, vont diminuer le taux de broutage par les herbivores aussi bien benthiques que planctoniques, et vont ainsi perturber le transfert de matières vers les niveaux trophiques supérieurs, tout en diminuant le recyclage des nutriments. Se pose alors la question de comment les écosystèmes vont s'adapter à ces efflorescences qui tendent à augmenter dans un contexte de changement climatique (Paerl and Huisman, 2008) ? Ces phénomènes sont aujourd'hui peu étudiés dans le domaine des algues toxiques.

2.1. Sélection de la nourriture

Une des premières manières de s'adapter à ces efflorescences et de lutter contre les effets toxiques potentiels est la capacité de sélectionner sa nourriture. Cette capacité de sélection de la nourriture a déjà été mise en évidence pour certaines espèces de copépodes (Cowles et al., 1988; Donaghay and Small, 1979; Schultz and Kiorboe, 2009). L'étude de Witt et al. (2019) a par ailleurs mis en évidence un changement des communautés zooplanctoniques en période d'efflorescence de

Prymnesium parvum : l'abondance des rotifères (omnivores non sélectifs) diminuait de 28% alors que l'abondance des copépodes augmentait de 25% en période d'efflorescence de cette haptophyte.

Dans le cadre de notre étude, le copépode benthique *Sarsamphiascus* cf. *propinquus* est capable de sélectionner la diatomée non toxique dans une nourriture mixte composée de cellules d'*O. cf. ovata*. (Chapitre 3 - Pavaux et al., 2019). Néanmoins, cette capacité de sélection peut avoir un impact négatif à plus long terme. En se nourrissant sur d'autres microalgues, les brouteurs facilitent la domination des communautés phytoplanctoniques par *O. cf. ovata* (Ger et al., 2019).

2.2. Fuite

Les copépodes planctoniques ont montré une sensibilité aux toxines produites par *O. cf. ovata* plus forte que les copépodes benthiques (chapitre 4). Cette différence de sensibilité pourrait s'expliquer par la capacité de fuite des copépodes planctoniques lors des efflorescences d'*O. cf. ovata*. Des copépodes planctoniques comme *Temora longicornis* sont en effet capables d'adapter leurs comportements natatoires en réalisant des sauts plus petits et plus complexes, favorisant la prise de nourriture en période d'efflorescence de *Phaeocystis globosa* et donc leur survie (Seuront and Vincent, 2008). Les copépodes planctoniques pourraient ainsi s'enfuir et ne plus être exposés à *O. cf. ovata* contrairement aux copépodes benthiques qui ne peuvent pas fuir les fortes concentrations auxquelles ils sont exposés.

2.3. Production de composés toxiques

La dernière stratégie qui peut être mise en place est la production de composés potentiellement toxiques qui vont diminuer la croissance, voire la toxicité d'*O. cf. ovata*. Les diatomées sont par exemple connues pour produire des aldéhydes polyinsaturés (PUAs - Wichard et al., 2005). Ces métabolites secondaires cytotoxiques, également appelés oxylipines, sont le résultat de la décomposition d'acides gras insaturés, dont les PUAs constituent le groupe le plus étudié (Miralto et al., 1999). Ils sont notamment décrits comme ayant des effets négatifs sur la reproduction et le développement des invertébrés marins comme les copépodes, les oursins ou les étoiles de mer (Caldwell, 2009; Ianora et al., 2004; Lauritano et al., 2012; Romano et al., 2010). Ils semblent également avoir un effet sur *O. cf. ovata* en diminuant son efficacité photosynthétique et en augmentant le nombre de cellules anormales (Pichierri et al., 2017). Dans le cadre de notre étude, la diatomée benthique *Licmophora paradoxa* exposée à *O. cf. ovata* était également capable de produire des métabolites secondaires toxiques pour le dinoflagellé (Chapitre 2).

3. Bilan sur le rôle écologique des toxines produites par *Ostreopsis cf. ovata*

D'un point de vue évolutif, le rôle écologique des toxines est encore mal connu. Cependant, compte tenu du coût métabolique élevé de leur production supportée par les cellules, il est logique de présumer que les toxines ont un rôle écologique essentiel. Initialement, ces toxines étaient décrites comme ayant un rôle prépondérant dans la physiologie des cellules, rôle potentiellement aujourd'hui perdu (Carmichael, 1994). Au vu du coût métabolique lié aux réactions enzymatiques complexes impliquées dans leur production, il semble plus probable que ces toxines aient conservé une fonction réelle dans la physiologie et l'écologie des algues toxiques (Leflaive and Ten-Hage, 2007). Une autre hypothèse est l'utilisation des toxines comme une défense contre les prédateurs en constituant des composés toxiques dissuasifs. Notre étude a en effet montré des effets reprotoxiques des toxines produites par *O. cf. ovata* sur les copépodes benthiques (Chapitre 3) et un effet mortel sur les copépodes planctoniques (Chapitre 4). Ces effets sont également observés pour d'autres dinoflagellés toxiques comme *Karlodinium veneficum* dont les karlotoxines dissuadent son broutage par *Acartia tonsa* (Waggett et al., 2008). La toxicité des composés produits par *O. cf. ovata* induit même une sélection de la nourriture en faveur d'autres algues non toxiques. Ceci va aussi favoriser indirectement le maintien des efflorescences d'*O. cf. ovata* en permettant la dominance de ce dinoflagellé dans les assemblages de microalgues (Ger et al., 2019). Ceci confirme le rôle écologique des toxines dans le maintien des efflorescences d'*O. cf. ovata* en diminuant les effets de prédation. Néanmoins, il est difficile de savoir si ces effets sont uniquement dus aux toxines libérées dans le milieu ou si les toxines présentes dans l'endométabolome jouent également ce rôle. La plupart des études concernant l'écologie chimique se focalise en effet uniquement sur les toxines libérées dans le milieu. Très peu prennent en compte l'effet des toxines présentes dans les cellules, alors qu'elles peuvent jouer un rôle déterminant dans la communication chimique.

Une question subsiste : peut-on considérer les toxines comme des agents allélopathiques ? Certaines études ont montré que les toxines peuvent constituer des agents allélopathiques : les microcystines purifiées de la cyanobactérie *Microcystis aeruginosa* inhibent par exemple la croissance de plusieurs algues vertes et cyanobactéries (Singh et al., 2001). Chez *O. cf. ovata*, le rôle des toxines dans les relations allélopathiques est encore méconnu. L'étude menée dans le cadre de cette thèse suggère que les toxines peuvent avoir une influence dans les relations allélopathiques mais uniquement lors d'un contact entre *O. cf. ovata* et son compétiteur (chapitre 2). Néanmoins, cette étude a également reporté la production d'un autre composé majoritaire par *O. cf. ovata*, n'appartenant pas à la famille des ovatoxines, qui pourrait potentiellement être impliqué dans les relations allélopathiques. De nombreux auteurs excluent un rôle allélopathique des toxines car de fortes concentrations de toxines sont parfois nécessaires pour observer un effet néfaste. Cependant,

la différence de toxicité entre les extraits cellulaires et rapport aux toxines purifiées (en faveur des extraits cellulaires) suggère une action synergique des toxines avec d'autres métabolites secondaires expliquant ainsi une toxicité à des concentrations plus faibles (Leflaive and Ten-Hage, 2007).

Il est difficile de conclure sur le rôle écologique des toxines produites par *O. cf. ovata*. Elles semblent jouer un rôle important dans la dissuasion de la prédation mais il reste difficile de savoir si elles rentrent en compte dans les relations allélopathiques. Néanmoins, la production de ces toxines est affectée par les paramètres biotiques et notamment par la présence de compétiteurs, ce qui laisse présager un rôle des toxines dans les interactions biotiques qu'*O. cf. ovata* entretient avec son environnement.

4. Synthèse et perspectives de recherche sur l'écologie chimique d'*Ostreopsis cf. ovata*

Ce travail de thèse combinant suivi *in situ*, approche expérimentale et chimie analytique, a permis d'étoffer les connaissances se rapportant à l'écologie chimique d'*Ostreopsis cf. ovata*.

Le premier résultat majeur concerne les différences de sensibilité des copépodes exposés à *O. cf. ovata*. Cette étude est la première à décrire les effets combinés d'*O. cf. ovata* sur l'ingestion, la mortalité et la reproduction des copépodes. Elle suggère l'existence de phénomènes d'adaptation/acclimatation à la présence d'*O. cf. ovata* pour le copépode harpacticoïde *Sarsamphiascus cf. propinquus*. Même si des phénomènes d'adaptation ont déjà été reportés pour d'autres espèces de copépodes planctoniques exposées à d'autres dinoflagellés toxiques (Colin and Dam, 2005), cette hypothèse nécessite plus d'investigations et notamment l'exposition de copépodes benthiques naïfs, c'est-à-dire jamais exposés, à *O. cf. ovata*. La comparaison de la réponse et de la sensibilité de ces copépodes naïfs aux réponses des copépodes benthiques (forte résistance) et planctoniques (forte sensibilité) permettrait de savoir si la résistance de *S. cf. propinquus* est potentiellement due à l'existence de phénomène d'acclimatation ou à des processus de résistance inhérents aux copépodes benthiques, voire à cette espèce. Il pourrait également être intéressant de coupler cette étude à des expériences d'adaptation à long terme. En exposant à de faibles concentrations ces copépodes naïfs, une éventuelle évolution de l'ingestion, de la mortalité voire des taux de fécondité et de fertilité pourrait être observée et validerait l'existence de phénomène d'adaptation des copépodes aux efflorescences d'*O. cf. ovata*. Ces processus d'adaptation menant à une résistance des brouteurs d'*O. cf. ovata* sont prépondérants car ils peuvent constituer un moyen de rétroaction des copépodes pouvant conduire à un contrôle de la prolifération des efflorescences d'*O. cf. ovata*. Il est également nécessaire d'évaluer le transfert éventuel des toxines produites par *O. cf. ovata* dans le réseau trophique. Les seules études existantes sur le sujet ont quantifié de fortes concentrations de toxines dans des organismes omnivores et carnivores mais aucune étude ne

démontre un véritable transfert des toxines alors que cela pourrait avoir des conséquences sanitaires importantes. De plus, il faut considérer que les copépodes peuvent constituer un vecteur de transfert important des toxines dans les niveaux trophiques supérieurs.

Ces travaux ont également souligné les méconnaissances du métabolome d'*O. cf. ovata*. Ce travail a en effet permis de mettre en évidence (i) la production de nouveaux composés toxiques indépendants de la famille des OVTXs jamais décrits dans la littérature (Chapitres 2 et 6) et (ii) la production de nombreux autres métabolites secondaires dont le rôle dans les relations allélopathiques qu'*O. cf. ovata* entretient avec son environnement restent à investiguer (Chapitre 2). Une meilleure connaissance de ces métabolites pourrait aider à mieux comprendre les successions phytoplanctoniques et ainsi l'importance de ces mécanismes dans le maintien et l'apparition des efflorescences d'*O. cf. ovata*. Il existe un véritable essor des techniques « omics », notamment en écologie chimique car ils permettent la découverte, sans *a priori*, de nouvelles molécules actives, ainsi que l'identification des interactions chimiques entre les organismes (Kuhlisch and Pohnert, 2015; Prince and Pohnert, 2010). Il serait donc être intéressant de combiner ces technologies notamment les approches de transcriptomique et de métabolomique pour accélérer les découvertes concernant le métabolome et le transcriptome d'*O. cf. ovata*, potentiellement impliqués dans les réactions biotiques.

Aussi il serait également intéressant d'étudier les conséquences des interactions *O. cf. ovata*/copépodes sur la physiologie et le comportement du dinoflagellé toxique. Des études précédentes ont montré que l'exposition de différentes espèces d'algues toxiques à des copépodes ou uniquement à l'eau de mer utilisée pour l'élevage de copépodes, engendrait notamment un changement du profil toxinique (Bergkvist et al., 2008; Lundholm et al., 2018; Selander et al., 2006). Ces résultats suggèrent que la présence des copépodes et la pression de broutage seraient synonymes de stress pour l'algue toxique.

Dans le cadre de ces travaux de thèse, l'impact d'*O. cf. ovata* sur les oursins adultes a également été investigué à travers des approches expérimentales en laboratoire et des suivis *in situ* (Chapitre 5). Dans les conditions expérimentales utilisées, l'exposition d'oursins adultes à des concentrations réalistes d'*O. cf. ovata* n'a eu aucun effet néfaste visible sur le temps de retournement, l'ingestion ni même le rapport gonado-somatique. Dans l'état actuel des connaissances, l'hypothèse de l'hypoxie et /ou de l'anoxie est à privilégier pour expliquer les mortalités de masse précédemment décrites.

Enfin la migration verticale des cellules d'*O. cf. ovata* a été décrite dans ces travaux (Chapitre 4). Sans pouvoir déterminer si cette migration est due à des rythmes endogènes ou des paramètres extérieurs comme l'hydrodynamisme, cette étude a permis de démontrer un transfert des cellules et l'existence de variations des abondances cellulaires à l'échelle de la journée. Ces informations sont cruciales dans la mise en place de protocole de suivi *in situ* de ce dinoflagellé toxique.

En conclusion, la plupart des études s'intéressant aux facteurs expliquant la formation, le maintien et le déclin des efflorescences d'*O. cf. ovata* se sont focalisées sur les paramètres abiotiques. Les résultats de ces études sont souvent contradictoires et aucun paramètre abiotique ne semble être prépondérant dans la formation des efflorescences d'*O. cf. ovata*. La complexité et l'omniprésence des relations biotiques dans le cycle de vie d'*O. cf. ovata* (Figure 37) suggèrent que ces relations biotiques peuvent avoir un rôle crucial dans la formation, le maintien voire même le déclin des efflorescences d'*O. cf. ovata* en Méditerranée nord occidentale. De nombreux points sont encore à explorer comme le transfert des toxines dans la chaîne trophique, les relations intraspécifiques, les relations de parasitisme ainsi que le métabolome d'*O. cf. ovata* pour mieux comprendre les interactions que ce dinoflagellé entretient avec son environnement et comment elles sont impliquées dans le contrôle et la formation des efflorescences de ce dinoflagellé.

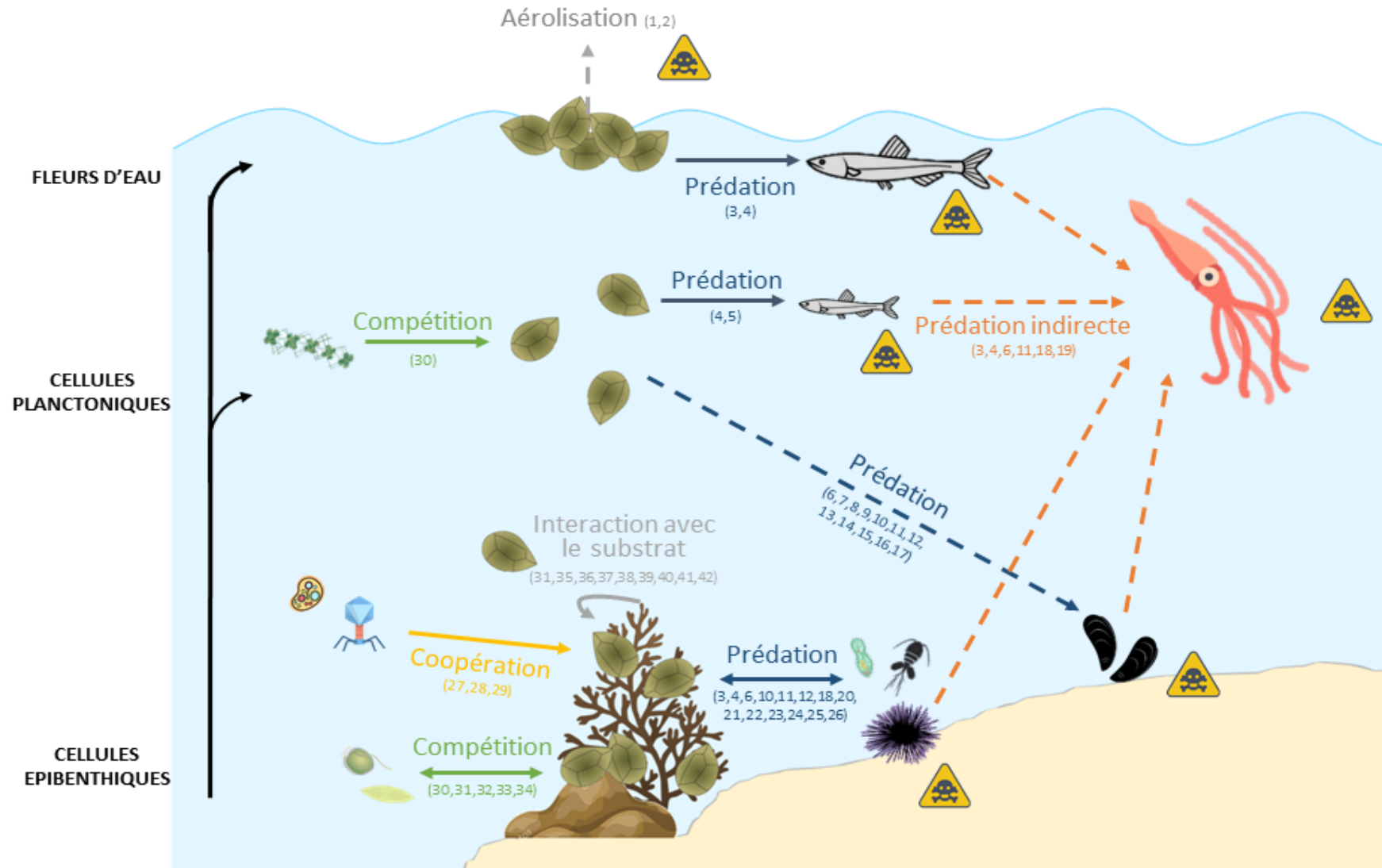


Figure 37 : Schéma représentant l'ensemble des relations biotiques (connues dans la littérature) qu'*Ostreopsis cf. ovata* entretient avec son environnement. Les flèches en traits entiers représentent les interactions directes, celles en tirets représentent les interactions indirectes. Les références bibliographiques associées sont précisées sous la forme de chiffres (référéncées ci-dessous). Le symbole triangulaire jaune représente les voies d'intoxication humaines possibles aux toxines produites par *Ostreopsis cf. ovata*.

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The use of stable isotopes to measure the ingestion rate of potentially toxic benthic dinoflagellates by harpacticoid copepods

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Keywords: *Ostreopsis cf. ovata*, *Amphidinium cf. carterae*, grazing rate, isotope, meiofauna, food web

Abstract

Phycotoxins synthesized by benthic dinoflagellates are known to bioaccumulate in macrofauna and hence represent a risk for the human health. However, the presence of toxins synthesized by benthic dinoflagellates in smaller marine organisms, compared to macrofauna, have not been considered despite the fact that such organisms have an important ecological role in the benthic food web. This present study quantified, for the first time, the trophic relationship between benthic dinoflagellates and meiofauna by using stable isotope enriched dinoflagellates during ingestion experiments. Results showed that harpacticoid copepods did not distinguish potentially toxic cells of *Ostreopsis cf. ovata* from the non-toxic cells of *Amphidinium cf. carterae*, even when another food resource, such as diatoms (e.g. *Odontella* sp.), was provided (Kruskal Wallis test, $p > 0.05$). These results suggest that i) meiobenthic organisms are liable to introduce phycotoxins synthesized by benthic dinoflagellates in the food web and ii) meiofauna could consequently concentrate these toxins and thereby contaminate their predators.

Toxin content of *Ostreopsis cf. ovata* depends on bloom phases, depth and macroalgae in the NW Mediterranean Sea

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Keywords: Dinoflagellate, Benthic HABs, *Ostreopsis cf. ovata*, Ovatoxins, Mediterranean Sea.

Abstract

For the last fifteen years, blooms of the genus *Ostreopsis* have been reported more frequently and with higher abundances in the Mediterranean area where *Ostreopsis cf. ovata* is known to produce ovatoxins (OVTXs), structural analogues of palytoxin which is one of the most potent non-polymeric toxin. However, the production of OVTXs is poorly characterized *in situ*. The present study therefore focused on toxin content and profile depending on the bloom phases during summer 2017 in Villefranche-sur-Mer, France (NW Mediterranean Sea), the depth (from 0.5 to 5 m) and three macroalgae substrates of this epiphytic dinoflagellate (*Padina pavonica*, *Dictyota* spp. and *Halopteris scoparia*). Ovatoxin quantification of all samples was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The bloom started at the end of June and declined mid-July confirming the typical seasonal pattern in the NW Mediterranean Sea area. The peak was observed the 10th of July with 1.8×10^6 cells/g FW and 1.7×10^4 cells/L for benthic and planktonic cells, respectively. Total toxin content of cells, collected using artificial substrates, increased during the exponential and stationary growth phases. After reaching a maximum concentration of 9.2 pg/cell the 18th of July, toxin concentration decreased and remained stable from the 25th of July until the end of the monitoring. Concerning depth, a decreasing trend of the abundance and of the associated total toxin content was noted. Finally, the decreasing order of maximal epiphytic concentration of *O. cf. ovata* was: *Dictyota* spp. (8.3×10^5 cells/g FW), *H. scoparia* (3.1×10^5 cells/g FW) and *P. pavonica* (1.6×10^5 cells/g FW). Interestingly, the highest OVTX quota was obtained in the cells present on *Halopteris scoparia*, then on *Dictyota* spp. and *Padina pavonica*. This suggested that the nature of the macroalgae substrate influenced both growth and toxin production of *O. cf. ovata* and further work will be required to understand the underlying mechanisms (e.g. competition for nutrition, pH, allelopathic interaction). However, the toxin profiles (i.e. the proportion of each ovatoxin analogue) was not affected by either the phase of the bloom, the depth or the macroalgae and the use of artificial substrate.

Abstract

Toxic microalgae are known to produce a large panel of secondary metabolites whose effect on the environment remains so far poorly understood. To better understand this ecological problematic, this thesis aimed to decipher the chemical ecology of the benthic, toxic dinoflagellate *Ostreopsis cf. ovata* in the Northern Occidental Mediterranean Sea.

First, we realized a monitoring of *O. cf. ovata* abundances on biotic substrates (macroalgae) and in the water column to confirm the tychoplanktonic lifestyle of *O. cf. ovata*, meaning that this dinoflagellate alternatively switches between benthic and planktonic phase within a single day. Because of these daily changes, it thus impacts both benthic and planktonic ecosystems.

In order to study the interactions between *O. cf. ovata* and its environment during both benthic and planktonic phases, we evaluated its impact on survival, growth, and metabolome of various biologic models, including competitors (diatoms), several direct (benthic and planktonic copepods) and indirect (sea urchins) predators during *in situ* and laboratory assays. Both *in situ* and laboratory monitoring could not reveal any effect of *O. cf. ovata* on the physiology and behavior of sea urchins, suggesting that the invertebrate mass mortality episodes that have been previously documented rather result of the hypoxia linked to *O. cf. ovata* blooms. By contrast, our results showed that *O. cf. ovata* inhibits the growth of competitor diatoms, while the later in return inhibits photosynthetic efficiency and growth of *O. cf. ovata*. Finally, our results exhibited species-related effects on copepods, with most importantly reprotoxic effects on benthic species.

To further analyze toxic effects of *O. cf. ovata* strains during blooms in a faster, easier and steadier way, we then adapted to this dinoflagellate an *Artemia franciscana* based toxicity assay.

Finally, this work was complemented by a metabolomic approach, which allowed us to identify the nature of the secondary metabolites produced by *O. cf. ovata*. Among these metabolites, toxins are likely to take part in the biological interactions mentioned above. In addition, our results further suggested that other metabolites of unknown nature also contribute to the chemical ecology of this species.

In sum, our work demonstrates that *O. cf. ovata* is a valuable biological model to study chemical ecology of toxic marine microalgae, because of its frequent blooms, its tychoplanktonic lifestyle as well as the great diversity of secondary metabolites it produces.

Key words: *Ostreopsis cf. ovata*, dinoflagellates, toxic microalgae, chemical marine ecology, allopathy, Mediterranean Sea.

Résumé

Les microalgues toxiques sont connues pour produire un grand nombre de métabolites, dont les effets sur l'environnement demeurent relativement méconnus à ce jour. Dans ce contexte, le travail présenté ici s'est focalisé sur l'écologie chimique du dinoflagellé benthique toxique *Ostreopsis cf. ovata* en Méditerranée nord occidentale.

Dans un premier temps, un suivi de l'abondance cellulaire d'*O. cf. ovata* sur les substrats biotiques (macroalgues) ainsi que dans la colonne d'eau, a été réalisé à différentes échelles de temps et d'espace, permettant de confirmer la nature tychoplanctonique de ce dinoflagellé. En raison de ces migrations journalières, il va donc impacter aussi bien les écosystèmes benthiques que planctoniques.

Afin d'étudier les interactions qu'*O. cf. ovata* entretient avec son environnement au cours de ces différentes phases, nous avons évalué l'effet d'*O. cf. ovata* sur la survie, la croissance et le métabolisme secondaire de différents modèles biologiques incluant un compétiteur (diatomée) et plusieurs espèces de prédateurs directs (copépodes benthiques et planctoniques) et indirects (oursins) au cours d'analyses *in situ* et en laboratoire. Les suivis réalisés *in situ* et les expériences en laboratoire n'ont pas permis de mettre en évidence un effet néfaste d'*O. cf. ovata* sur la physiologie et le comportement des oursins, suggérant que c'est l'hypoxie engendrée par les efflorescences d'*O. cf. ovata* qui est le facteur à l'origine des mortalités de masse d'invertébrés décrites en milieu naturel. En revanche, nos résultats montrent qu'*O. cf. ovata* impacte négativement la croissance des diatomées compétitrices, tandis que ces dernières inhibent en retour sa croissance et son efficacité photosynthétique. Concernant les interactions avec les copépodes, nos résultats montrent une réponse espèce-dépendante, avec en particulier un effet reprotoxique sur les copépodes benthiques.

Afin d'analyser plus rapidement, facilement et de façon reproductible la toxicité des différentes souches d'*O. cf. ovata* ainsi que l'évolution de cette toxicité au cours d'une efflorescence, nous avons ensuite adapté à ce dinoflagellé un test de toxicité utilisant le crustacé *Artemia franciscana*.

Enfin, ces expérimentations ont été couplées à une approche métabolomique qui a permis d'étudier la nature des métabolites secondaires produits par *O. cf. ovata*. Parmi ces métabolites, les toxines semblent participer aux interactions biotiques mises en évidence au cours des expériences décrites précédemment. En outre, nos résultats suggèrent que d'autres métabolites, dont la nature n'est pas encore connue, contribuent également à l'écologie chimique de cette espèce.

En conclusion, notre travail montre qu'*O. cf. ovata*, par la récurrence de ses efflorescences, sa nature tychoplanctonique, l'abondance et la diversité des métabolites secondaires qu'il produit, s'avère être un excellent modèle pour étudier l'écologie chimique des microalgues marines toxiques.

Mots clefs : *Ostreopsis cf. ovata*, dinoflagellés, microalgues nuisibles, écologie chimique marine, allélopathie, Méditerranée.