



HAL
open science

Impacts of *Karenia brevis* on bivalve reproduction and early life history

Anne Rolton

► **To cite this version:**

Anne Rolton. Impacts of *Karenia brevis* on bivalve reproduction and early life history. Reproductive Biology. Université de Bretagne occidentale - Brest, 2015. English. NNT : 2015BRES0002. tel-02271917

HAL Id: tel-02271917

<https://theses.hal.science/tel-02271917>

Submitted on 27 Aug 2019

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

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

UBO

université de bretagne
occidentale



THÈSE / UNIVERSITÉ DE BRETAGNE OCCIDENTALE

sous le sceau de l'Université européenne de Bretagne

pour obtenir le titre de

DOCTEUR DE L'UNIVERSITÉ DE BRETAGNE OCCIDENTALE

Mention : Biologie Marine

École Doctorale des Sciences de la Mer

présentée par

Anne ROLTON

Préparée au Laboratoire des Sciences de la Mer,
Institut Universitaire Européen de la Mer

IMPACTS OF *KARENIA* *BREVIS*, ON BIVALVE REPRODUCTION AND EARLY LIFE HISTORY

Thèse soutenue le 20/01/2015

devant le jury composé de :

Philippe SOUDANT

Directeur de Recherche, CNRS / Directeur de thèse

Aswani VOLETY

Professeur, University of North Carolina Wilmington / Directeur scientifique

Philipp HESS

Chargé de Recherche (HDR), Ifremer / Rapporteur

Gary WIKFORS

Directeur de Recherche, NOAA / Rapporteur

Hélène HEGARET

Chargé de Recherche, CNRS / Examineur

Leila BASTI

Chargé de Recherche, Fisheries Research Agency, Japan/
Examineur

Laure GUILLOU, PRESIDENT

CNRS Team Leader/ Examineur



For my husband Julien
and our incredibly supportive parents
Thank you

Acknowledgements

Thank you to every single person who has helped me throughout my Ph.D.

Firstly, my thesis director, Philippe Soudant: I am very lucky to have had you as my supervisor. Every time you came to Florida you made time for me; from the 'quick' flow cytometry assays (that took forever) to trawling over hundreds of graphs. You have encouraged and guided me from start to finish. Thank you also to you and your family for welcoming us into your home.

My thesis scientific director, Aswani Volety, without whom this would never have happened! You went out of your way to bring me to the U.S and presented me with this opportunity. You've sent me to conferences, workshops and training and pushed me for posters and presentations from the very beginning of this project.

To my committee members: H  l  ne H  garet and Caroline Fabioux. Thank you for the review of manuscripts, feedback on presentations and finally, looking out for us when we arrived at UBO. Your advice has been so important. Mike Parsons; thank you for help with stats, help in the lab and help with all my questions! Richard Pierce: thank you for your positive attitude and encouragement throughout the Ph.D. You got me started on the right foot with all the papers you sent and, introduced me to your awesome team: Michael Henry, Patricia Blum and Val Polubok. You all welcomed me when I came to Mote and I thank you all for sending *K. brevis* cultures (whenever they were asked for!), and for the endless analysis of samples!

To the members of my jury: Leila Basti, Laure Guillou, H  l  ne H  garet, Phillip Hess and Gary Wikfors. Thank you for your invaluable comments and for coming from so far away.

Thank you to Sandra Shumway and Monica Bricelj for your expert guidance on presentations, posters and papers. Your feedback was always very fast, even when I sent you things at the last minute....

Inke Sunila and Jim Winstead: thank you for the histopathology training and always being available to help. Inke: your generosity in time, expertise and hospitality (cocktails) has made learning this subject so enjoyable. I hope we will be able to work together in the future, and I look forward to purchasing your long-awaited cook book.

Ludovic Donaghy and Nelly LeGo  c: thank you for your guidance and training on the flow cytometers; for help sampling clams and oysters; transporting samples to and from campus, running samples...sometimes well into the night and, for talking the time to explain what on earth it all meant!

Win Everham thank you for taking time to discuss stats...at length...when you were busy. Your strength, encouragement and kind words throughout the Ph.D went a long way. I hope we will catch up soon....either over beers or in the swamp.

Fu Lin Chu, thank you for all of your encouragement....and amazing cooking!

From THE Vester field station: Brooke Black, Jeff Devine, Molly Rybovich, Vaiola Osne, Ga  lle Richard, Kelsey McEachern, Patricia Goodman, Audrey Barbe, Rheannon Ketover, Ian Campbell, Maud Baudequin, Marine Remize, Nicole Martin, Chelsea Miley and Bob Wasno.

Big up the Marine Lab Crew! Lesli Haynes, Katie McFarland, Lindsay Castret, Andy Griffith, David Segal and Bob Halstead. From the first day when we hand-ground what felt like a million clam samples under nitrogen....I knew this was going to be epic. You have all helped me out countless times. THANK YOU for showing me how to use the equipment at the field station and on campus, teaching me endless protocols and lab techniques, and all your help organizing, collecting, shucking, counting, homogenizing, weighing, cleaning....but mostly, thank you for your patience and laughs.

For helping order, ship, book, rent, apply, etc: Tim Bryant (rock on), Christal Niemeyer, Elizabeth Bondu and Sébastien Hervé.

Jay Leverone, Mark Poli, Dan Baden, Leanne Flewelling and Ann Abraham: thank you for answering my questions by email so quickly. Diane Blackwood, from FWC for providing *K. brevis* count and water quality data and; Leslie Sturmer and Tony Heebe for provision clams and answering my many, many questions.

Thank you for all of the support from my friends and family along the way and, to the new friends made at FGCU, UBO and far beyond as a result of this project.

Finally, thank you to my husband Julien, without whom I would not have got this far. You kept me grounded, encouraged and supported.....all whilst going through exactly the same thing yourself! We didn't do this by half...two PhD's, moving across an ocean a couple of times, a once-in-a-lifetime road trip and....getting married. Whatever the future brings, I know we will be able to face it together.

Impacts of *Karenia brevis* on bivalve reproduction
and early life history

Contents

Abstract.....	1
Résumé.....	2
Abbreviations.....	3

Introduction

Part 1: Introduction to *Karenia brevis* and its effects:

1.1 Harmful algal blooms	9
1.2 Life cycle and growth of <i>Karenia brevis</i>	12
1.3 Toxins	14
1.4 Blooms	15
1.5.1 Effects: Neurotoxic shellfish poisoning	16
1.5.2 Mortalities	17
1.5.3 Community structure	18
1.5.4 Sublethal effects and trophic transfer	18

Part 2: Bivalves and *Karenia brevis*

2.1 Importance	20
2.2. Distribution	20
2.3 Filter feeding	20
2.4 Life cycle	22
2.5 Reproduction	24
2.6 Encounters with <i>Karenia brevis</i>	25
2.7 Brevetoxin accumulation	25
2.8 Sensitivity	27
2.9 Lethal and sublethal effects	28
2.10 Summary	30

Part 3: Objectives and Rationale

3.1 Objectives	31
3.2 Rationale	32

References	33
------------	----

Chapter 1: Short and long-term effects of *Karenia brevis* exposure on *Crassostrea virginica*: Toxin accumulation and histopathological responses in adults and effects on gametogenesis, gamete and larval quality.....51

Chapter 2: Effects of *Karenia brevis* exposure on adult *Mercenaria mercenaria*: Histopathological and cellular responses in adults and effects on gamete and larval quality.....95

Chapter 3: Transfer of brevetoxin to the gametes of eastern oysters (*Crassostrea virginica*) and northern quahogs (= hard clam, *Mercenaria mercenaria*) following field exposure to *Karenia brevis*.....125

Chapter 4: Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*.....165

Chapter 5: Susceptibility of gametes and embryos of the eastern oyster, *Crassostrea virginica*, to *Karenia brevis* and its toxins175

General Discussion

1. Introduction	187
2. Adults	
2.1 Toxin accumulation	188
2.2 Physiological effects	190
2.2.1 Feeding processes	193
2.3 Reproductive effects	194
3. Early stages	199
4. Summary	202
5. Implications	203
6. Additional considerations	204
7. Conclusion	205
References	206

Publications and communications.....213

Abstract

Harmful algal blooms (HAB) have important economic, environmental and human health impacts worldwide. The most prevalent of these HAB species in the Gulf of Mexico (GoM) is the toxic dinoflagellate, *Karenia brevis*. Brevetoxins (PbTx) produced by *K. brevis* can have severe effects, causing marine mammal, bird and fish kills and, neurotoxic shellfish poisoning (NSP) in humans. Many important shellfish species are affected by *K. brevis* yet, effects of this alga on the commercially and environmentally important hard clam (*Mercenaria mercenaria*) and eastern oyster (*Crassostrea virginica*) are poorly understood. Blooms of *K. brevis* typically last for several months and bivalves may therefore be exposed to *K. brevis* for prolonged periods, covering times of reproduction and spawning.

The aims of this project were to i) determine the chronic effects of controlled laboratory exposure and field exposure of *K. brevis* on the reproductive and related physiological processes of adult *M. mercenaria* and *C. virginica* and the quality of the offspring that were subsequently produced and ii) to determine the effects of *K. brevis* exposure on gamete, embryo and larval development in these species.

Clams and oysters accumulated high levels of PbTx following exposure to *K. brevis*, which may indicate a degree of 'insensitivity' to PbTx, yet, effects of *K. brevis* exposure on both reproductive and physiological parameters of these species were recorded. Brevetoxin was present in both sperm and oocytes from *K. brevis* exposed adults of both species. This maternal transfer of PbTx to the offspring via the oocytes, may have resulted in the significant negative effects recorded on larval development, but only up to the end of the lecithotrophic phase (in which larvae utilize lipid reserves laid down from the mother).

The similar dose-dependent negative effects caused by exposure of gamete and early life stages to different cell preparations of *K. brevis* suggests that other toxic compounds in addition to PbTx may be involved in toxicity. Larvae exposed from gamete and early embryonic stages responded similarly in terms of sensitivity suggesting that the majority of negative effects may occur during embryonic divisions.

Hard clams and eastern oysters are more susceptible to *K. brevis* exposure than previously realized. Recurrent and prolonged exposure to high bloom concentrations may result in significant multiple stressor effects on adult and early life stages which could cause bottle necks on recruitment and population dynamics of these species. Consideration of other biotic and abiotic stressors in addition to bloom exposure should be given in order to fully understand the effects of this alga on bivalves. The identification of other bioactive compounds is also required to determine the proportion of negative effects attributable to *K. brevis*/ PbTx/ other bioactives.

Improved understanding of these effects may help with *K. brevis* bloom modeling, allowing predictions of both acute and chronic impacts of blooms on the fecundity, recruitment and therefore sustainability of bivalve populations in SW Florida and other affected areas of the GoM. Results may also enable more informed decisions on suitable sites for bivalve fisheries/ aquaculture as well as informed decisions on restoration and enhancement of wild bivalve stocks to be made.

Résumé

Les efflorescences d'algues toxiques (HAB) peuvent avoir des effets sur la santé humaine, et ont des impacts économiques et environnementaux dans le monde entier. L'espèce de HAB la plus répandue dans le golfe du Mexique (GoM) est le dinoflagellé toxique, *Karenia brevis*. Les brevétoxines (PbTx) produites par *K. brevis* peuvent avoir des effets graves, provoquant des épisodes de mortalité de mammifères marins, d'oiseaux et de poissons, mais aussi l'intoxication neurotoxique (NSP) chez l'homme. Même si de nombreuses espèces de coquillages sont affectées par *K. brevis*, les effets de cette algue sur des espèces écologiquement et économiquement importantes telles que le « clam » (*Mercenaria mercenaria*) et l'huître Américaine (*Crassostrea virginica*) sont encore mal compris. Les efflorescences de *K. brevis* peuvent durer plusieurs mois. Par conséquent, les bivalves pourraient être exposés à *K. brevis* pendant de longues périodes, coïncidant avec les saisons de reproduction et de ponte.

Les objectifs de ce projet étaient i) de déterminer les effets chroniques d'une exposition contrôlée en laboratoire et d'une exposition naturelle de terrain à *K. brevis*, sur la reproduction des adultes de *M. mercenaria* et *C. virginica*, et sur la qualité de la descendance ainsi produite et ii) de déterminer les effets directs d'une exposition de *K. brevis* sur les gamètes, les embryons et le développement larvaire de ces espèces.

Les « clams » et les huîtres ont accumulé des niveaux élevés de PbTx après exposition à *K. brevis*, indiquant un degré « d'insensibilité » à PbTx, même si les paramètres physiologiques de la reproduction de ces deux espèces ont été affectés. La brevétoxine était présente à la fois dans le sperme et les ovocytes des adultes des deux espèces exposées à *K. brevis*. Un transfert maternel de PbTx à la descendance *via* les ovocytes, peut avoir entraîné les effets négatifs significatifs observés lors du développement larvaire, mais seulement jusqu'à la fin de la phase lécithotrophe (au cours duquel les larves utilisent les réserves lipidiques de l'ovocyte).

La similarité des effets négatifs et dose-dépendants causés par l'exposition de gamètes et de jeunes stades de vie à différentes préparations de cellules de *K. brevis* suggère que d'autres composés toxiques, en plus de PbTx, pourraient être impliqués dans la toxicité. Les larves exposées à partir du stade de gamète ou du stade embryonnaire ont répondu de manière similaire en termes de sensibilité suggérant que la majorité des effets négatifs se produisent au cours des divisions embryonnaires.

Le « clam » et l'huître américaine sont plus sensibles à *K. brevis* que ce qui est généralement admis. Les effets résultant de stress multiples sur les adultes et jeunes stades de vie, combinés à une exposition quasi-annuelle aux efflorescences de *K. brevis*, pourraient engendrer des perturbations significatives en termes de recrutement et de dynamique des populations de ces espèces, et pourraient donc avoir des répercussions environnementales et économiques plus larges. D'autres études sont nécessaires pour identifier les composés bioactifs et déterminer la proportion des effets négatifs attribuables à *K. brevis* / PbTx / ou autres composés bioactifs.

Une meilleure compréhension de ces effets pourrait faciliter la modélisation des efflorescences de *K. brevis*, ce qui permettrait de prédire les impacts aigus et chroniques d'efflorescences sur la fécondité, le recrutement et donc la résilience des populations de bivalves dans le SO de la Floride et d'autres régions touchées du GoM. Ces résultats pourraient également aider à la prise de décisions plus scientifiquement fondées sur le choix de sites appropriés pour la pêche / l'aquaculture de bivalves ainsi que sur les méthodes à envisager pour la restauration et l'amélioration des stocks de bivalves sauvages à entreprendre.

Abbreviations

ANOVA: Analysis of variance

ASP: Amnesic shellfish poisoning

AU: Arbitrary units

AZA: Azaspiracid

AZP: Azaspiracid shellfish poisoning

BTX: Brevetoxin

Ca²⁺: Calcium ion

CCMP: Center for Culture of Marine Phytoplankton

CFP: Ciguatera Fish Poisoning

CHNEP: Charlotte Harbour National Estuary Program

CI: Condition index

Cl: Chloride

CTX: Ciguatoxin

CT: Connective tissue

DA: Domoic acid

DCFH: Dichloro-dihydro-fluorescein

DCFH-DA: Dichloro-dihydro-fluorescein diacetate

DDT: Dichlorodiphenyltrichloroethane

DO: Dissolved oxygen

DSP: Diarrhetic Shellfish Poisoning

DTX: Dinophysistoxins

ELISA: Enzyme-linked immunosorbent assay

EPS: Extracellular polymeric substances

Introduction

FGCU: Florida Gulf Coast University

FL: Florida

FSW: 0.1 μm carbon filtered and UV sterilized sea water

FWC: Florida Fish and Wildlife Conservation Commission

GoM: Gulf of Mexico

GS: Gonadal stage

GTX: Gonyautoxins and analogs of paralytic shellfish toxins

GYM: Gymnodimine

IMW: International Mussel Watch Program

JC-10: J-aggregate-forming delocalized lipophilic cation, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide

K: Kidney

K^+ : Potassium ion

LC-MS/MS: Liquid-chromatography, mass-spectrometry

MML: The Mote Marine Laboratory

MMP: Mitochondrial membrane potential

MTX: Maitotoxin

MU: Mouse units

Na^+ : Sodium ion

NCMA: National center for Marine Algae and Microbiota

NMFS: National Marine Fisheries Service

NSP: Neurotoxic Shellfish Poisoning

NSSP: National Shellfish Sanitation Program

Introduction

OA: Okadaic acid

PbTx: Brevetoxin

PbTx- CA: Brevetoxin- carboxylic acid

PF: Post- fertilization

PKS: Polyketide synthase

PLTX: Palytoxin

PST: Paralytic shellfish toxins

PSP: Paralytic Shellfish Poisoning

PTX: Pectenotoxins

ROS: Reactive oxygen species

ScTX: Scaritoxin

SD: Standard deviation

SE: Standard error

SL: Shell length

SPX: Spirolides

STX: Saxitoxin

SW FL: South west Florida

TTX: Tetrodotoxin

USDOA: United States Department of Agriculture

VSSC: Voltage- sensitive sodium channel

YTX: Yessotoxin

Introduction

Introduction

Part 1: Introduction to *Karenia brevis* and its effects

1.1 Harmful algal blooms

Harmful algal blooms (HAB) have negative effects on co-occurring organisms due to the production of toxins, physical contact, and/or increased biomass (Anderson et al., 2002; Landsberg, 2002). These negative environmental impacts are often accompanied by devastating economic losses caused by the closure of fishing and shellfish harvest areas and a drop in recreation and tourism activities to affected areas (Alcock et al., 2007; Hoagland and Scatasa, 2006; Jensen, 1975; Larkin et al., 2003; Matsuyama and Shumway, 2009; Shumway, 1990; Steidinger, 2009). An apparent global increase in the occurrence, severity and expansion of HAB and, identification of new HAB species, have highlighted the need for increased research in this area (Hallegraeff, 1993; Glasgow et al., 2001; Otero et al., 2010; Poirier et al., 2007; Shumway, 1990). Of the ~5000 known marine phytoplankton species, ~300 can form HAB and ~40 of these HAB species, most of which are dinoflagellates, can produce potent toxins (Table 1, Hallegraeff, 1993; Smayda, 1997). The fragile, unarmored dinoflagellate, *Karenia brevis*, is one such species (Figure 1), producing brevetoxin (PbTx), an intracellular lipid soluble biotoxin which causes permanent activation of voltage-sensitive sodium channels (VSSC) in nerve cells, and so disrupts nervous transmission in animals (Table 1, Gawley et al., 1995; Poli et al., 1986).

Table 1: A summary of major biotoxins produced by marine algae, their geographical range and, effects on humans and co-occurring organisms.

Toxin	Class of chemical	Mode of action	Algal species	Geographical range	Effects	Reference
Azspiracids (AZA) and analogues	Lipophilic linear and macrocyclic non-azotated polyethers	Neurotoxin: K ⁺ channel inhibitor	<i>Protoperidinium crassipes</i> , <i>Azadinium spinosum</i>	Global	Azspiracid shellfish poisoning (AZP), cytotoxic.	Twiner et al., 2005, 2012
Brevetoxin (PbTx)	Lipophilic transfused polyethers	Neurotoxin: Na ⁺ channel activator. Pulmonary and enzyme regulatory systems (?)	<i>Karenia brevis</i> , <i>K. concordia</i> , <i>Chattonella verruculosa</i>	Gulf of Mexico, south east U.S., New Zealand, Caribbean (?)	Neurotoxic shellfish poisoning (NSP), marine mammal, seabird, fish and shellfish mortalities.	Baden et al., 2005 ; Bourdelais et al., 2002; Davis, 1948; Landsberg, 2002; Steidinger et al., 2008a
Diarrhetic shellfish toxins (DST): Okadaic acid (OA) and Dinophysistoxins (DTX)	Lipophilic linear and macrocyclic non-azotated polyethers.	Enterotoxin (primarily) (OA): serine/threonine protein phosphatase inhibitor. OA and DTX: secretion and gene transcription of nerve growth factors	<i>Dinophysis</i> spp., <i>Prorocentrum</i> spp.	Temperate: Europe, Japan, North America and tropical areas	Diarrhetic shellfish poisoning (DSP), tumour promotor (?).	Bialojan et al., 1988; Franchini et al., 2010; Pshenichkin and Wise, 1995; Rossini and Hess, 2010
Domoic Acid (DA) and analogues	Hydrophilic amino acid-like compounds	Neurotoxin: glutamate receptor activator	<i>Pseudo-nitzschia</i> spp.	North America, Japan, Europe	Amnesic shellfish poisoning (ASP), mortalities of marine birds and mammals, stress of some bivalve species.	Lefebvre and Robertson, 2010; Lelong et al., 2012
Gambiertoxin [Ciguatoxin (CTX),	CTX and ScTX: Lipophilic transfused	Neurotoxin: (CTX) Na ⁺ channel activator,	<i>Gambierdiscus toxicus</i> ,	Gulf of Mexico, Hawaii,	Ciguatera fish poisoning (CFP),	Bienfang et al., 2008; Landsberg, 2002

Introduction

Maitotoxin (MTX), Scaritoxin (ScTX), Palytoxin (PLTX)]	polyethers. MTX and PLTX: hydrophilic polyethers	(MTX): Ca ²⁺ channel activator	<i>Ostreopsis</i> spp	Caribbean, Australia	behavioural changes in crustaceans and fish, fish kills (?).	
Paralytic shellfish toxins (PST): Saxitoxin (STX) and analogues (GTX)	Hydrophilic purine derivatives	Neurotoxin: Na ⁺ channel inhibitor	<i>Alexandrium</i> spp., <i>Gymnodinium</i> spp.	Global: tropical and temperate	Paralytic shellfish poisoning (PSP), marine mammal, seabird, fish and shellfish mortalities.	Bricelj et al., 2005; Landsberg, 2002; Shumway, 1990
Pectenotoxins (PTX)	Lipophilic polyether- lactones	Neurotoxin: selective binding to the membrane Na ⁺ -K ⁺ ATPase,	<i>Dinophysis acuta</i> , <i>D. fortii</i>	Temperate.	Lethal to mice, hepatotoxic, tumor promotor	Draisci et al., 1996; Deeds and Schwartz, 2010; Vale et al, 2006.
Spirolides (SPX), Gymnodimine (GYM), Prorocentrolide, Pinnatoxin	Lipophilic cyclic imines	Neurotoxin: antagonism of nicotinic acetylcholine receptors	<i>Alexandrium ostenfeldii</i> (SPX), <i>Karenia selliformis</i> (GYM)	Global temperate areas.	Lethal to mice, GYM: fish mortalities, Oyster larvae mortalities.	Seki et al., 1995; Steidinger et al., 2008a ; Munday et al, 2004; Bourne et al, 2010; Hellyer et al., 2011
Yessotoxins (YTX)	Lipophilic transfused polyethers	Unknown: phosphodiesterase activator ?	<i>Lingolodinium polyedrum</i> , <i>Gonyaulax spinifera</i> , <i>Prorocentrum reticulatum</i>	Japan, California, South Africa.	cytotoxic	Franchini et al, 2004a,b; Pérez-Gómez et al, 2006; Vinagre et al, 2003 ; Tubaro et al, 2010

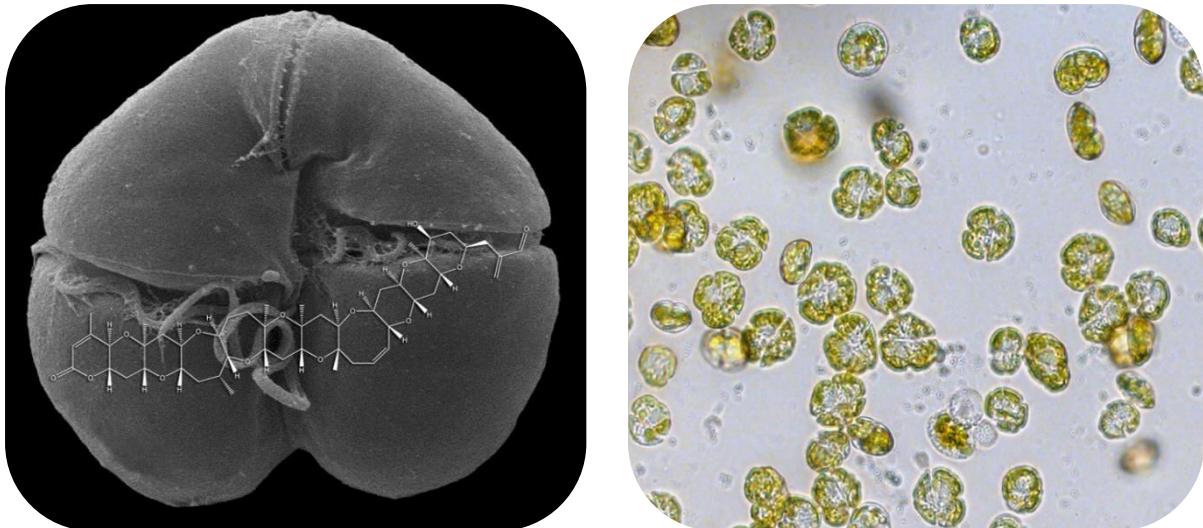


Figure 1: Left: Scanning electron micrograph of *Karenia brevis* with the superimposed chemical structure of PbTx-2 (picture: Florida Fish and Wildlife Conservation Commission; chemical structures: Chris Miles, Norwegian Veterinary Institute, University of Oslo). Right: Pleomorphic *K. brevis* cells in a laboratory culture (Manasota Key Isolate, mean spherical diameter: 35 μ m, picture: Anne Rolton).

1.2 Lifecycle and growth of *Karenia brevis*

The lifecycle of *Karenia brevis* is not yet fully understood, although it reproduces predominantly asexually by binary fission (Figure 2, Persson et al., 2013; Walker, 1982; Wilson, 1967). Sexual reproduction occurs via the production of gametes that fuse to produce a planozygote (Figure 2), however, the range of morphologies that occur in a clonal culture of *K. brevis* (Figure 1 and 2) make identifying different life cycle stages difficult (Persson et al., 2013; Walker, 1982). It is unknown if *K. brevis* produces true resting cysts in the same way as other toxic dinoflagellates (i.e. *Alexandrium* sp.), which lay dormant in the sediment between blooms (Mendoza et al., 2008; Steidinger, 1975; Wilson, 1967). A recent study by Persson et al. (2013) however, found no evidence of true resting cysts [cells with a thick cell wall, colourless cytoplasm and accumulation body formed from sexual reproduction] either in laboratory cultures or in sediments from areas frequented by *K. brevis* blooms. In the field, *K. brevis* has been recorded in water between 7- 33°C and at salinities of 5- 45, although optimal conditions for growth are typically between 22- 28°C and salinities near 30 (Brand et al., 2012; Magaña and Villareal, 2006; Vargo, 2009; Maier Brown et al., 2006; Dortch et al., 1998). Under these optimal growth conditions, cultures of *K. brevis* progress through several growth phases: lag, exponential, stationary and decline. It is during the stationary phase, in which the cell

population maintains itself and no longer increases that the brevetoxin content in the culture is highest as increasing numbers of *K. brevis* cells lyse, releasing intracellular PbTx into the culture medium (Roth et al., 2007). Brevetoxin content varies widely according to environmental conditions (Errera et al., 2010; Errera and Campbell, 2011; Maier Brown et al., 2006) and even between clonal isolates (Baden and Tomas, 1988; Lekan and Tomas, 2010; Loret et al., 2002). Concentrations ranging from 5- 126 pg PbTx cell⁻¹ have been recorded (Greene et al., 2000; Haubois et al., 2009; Lekan and Tomas, 2010; VanDolah et al., 2009). Brevetoxin is produced in the cell by the polyketide synthase (PKS) pathway and as toxins have so far not been associated with any organelles, are thought to be dissolved in the cytoplasm (Baden et al., 2005; Richard Pierce, personal communication).

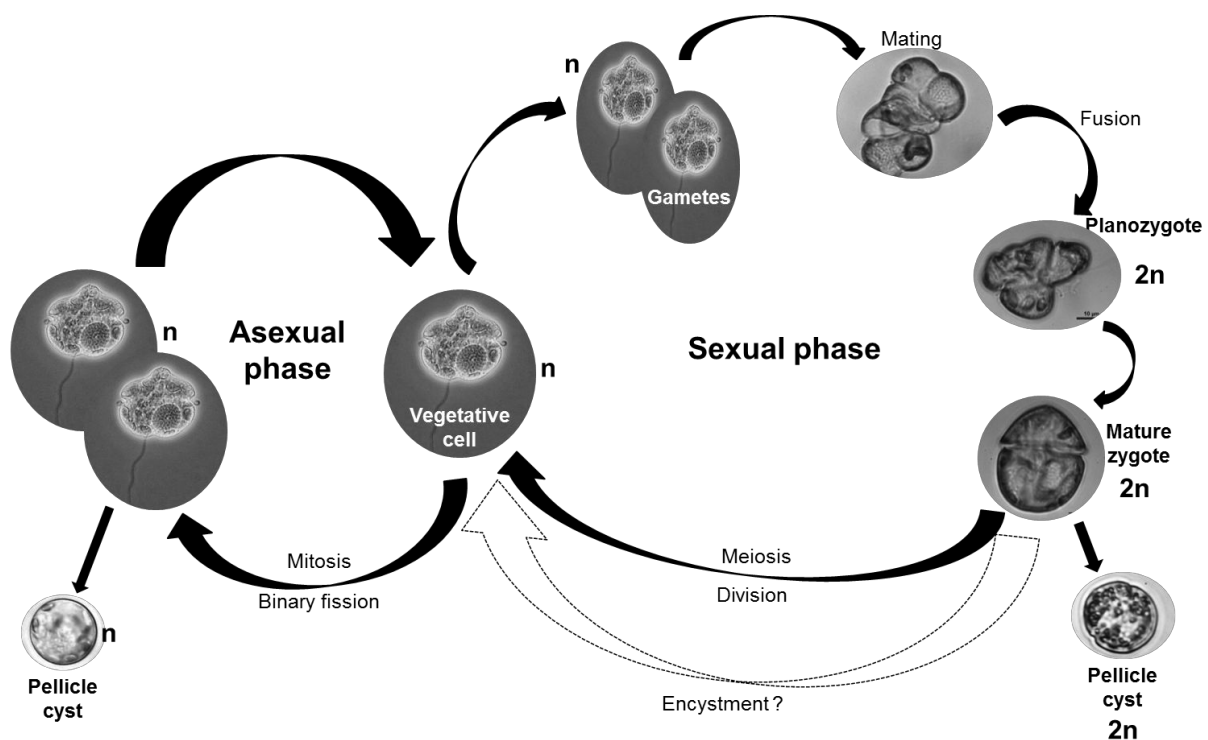


Figure 2: The life cycle of *Karenia brevis*, showing the asexual phase of reproduction by which cells predominantly divide and also the sexual phase, in which gametes are formed and fuse to form zygotes. N.B. the wide range of morphologies of cells at different life stages. Although temporary cysts may be formed from asexual reproduction, a true resting cyst from the sexual phase has not been found. Visible nucleus, chloroplasts and thin cell wall of the pellicle cyst formed during sexual reproduction is not a true resting cyst. Pictures from Persson et al., 2013 and FWC.

1.3 Toxins

Two non-polar, non-charged parent brevetoxins, PbTx-1 and PbTx-2, have been identified in cultures of *K. brevis*: PbTx-1 is considered more potent however; PbTx-2 (Figure 1) is much more abundant (Baden and Tomas, 1988; Landsberg, 2002; Shimizu et al., 1986). From these parent toxins, so far thirteen PbTx derivatives have been identified in *K. brevis* cultures, each of which has a specific toxicity correlated to the binding affinity on the VSSC (Baden et al., 2005). Isolated and identified analogs of PbTx-1 and PbTx-2 in blooms and laboratory cultures of *K. brevis* include; hydrolyzed and oxidized forms of PbTx-1 and analogs PbTx-7 and PbTx-10 (from PbTx-1) and; hydrolyzed and oxidized forms of PbTx-2 and analogs PbTx-3, -5, -6 and -9 (from PbTx-2) (Abraham et al., 2006). Brevenal, a short chain polyether compound which binds competitively with brevetoxin at site 5 of the VSSC and blocks the effects of PbTx, has also been identified in *K. brevis* cultures, and is thought to influence the potency of *K. brevis* blooms (Bourdelais et al., 2004, 2005). As with total brevetoxin content, the profile of PbTx parent toxins and derivatives varies with culture age, environmental conditions and between strains (Lekan and Tomas, 2010). The profile of these toxins also varies in relation to the location in and around the *K. brevis* cell. Parent toxins PbTx-1 and -2 and brevenal were found to be present almost exclusively within the *K. brevis* cell whereas derivatives PbTx-3, -6 and -9 were present in the surrounding culture medium (Lekan and Tomas, 2010).

Brevetoxin may also affect pulmonary systems and cathepsin catabolic enzymes as well as interfering with nervous transmission, (Abraham et al., 2005; Sudaranam et al., 1992; Baden et al., 2005). In addition to PbTx, other chemically uncharacterized and often unstable polar compounds that can be allelopathic, ichthyotoxic and/or hemolytic are also produced by *K. brevis* (Kubanek et al., 2005, 2007; Marshall et al., 2005; Neely and Campbell, 2006; Prince et al., 2010; Sipler et al., 2014; Tatters et al., 2010). Thus, multiple biotoxins and antagonists produced by *K. brevis* may interact with at least 3 receptors in the nervous, pulmonary and enzymatic regulatory systems of animals (Baden et al., 2005). Blooms of this species can therefore have severe and complex effects on the surrounding environment; causing impacts such as; animal kills, human illness and altered habitat and community structure (Fleming et al., 2011; Flewelling et al., 2005; Kirkpatrick et al., 2004; Meyer et al., 2014; Steidinger et al., 2008a; Turner and Tester, 1997).

1.4 Blooms

Cells of *K. brevis* (formally *Ptychodiscus brevis* and *Gymnodinium breve*) are found primarily in the Gulf of Mexico (GoM), where they are ubiquitous below 1 cell mL^{-1} concentrations (Finucane, 1964). Occasionally blooms are transported along the east coast of the U.S. and there are unconfirmed reports of cells in the Caribbean (Figure 3; Lackey, 1956; Tester et al., 1989).

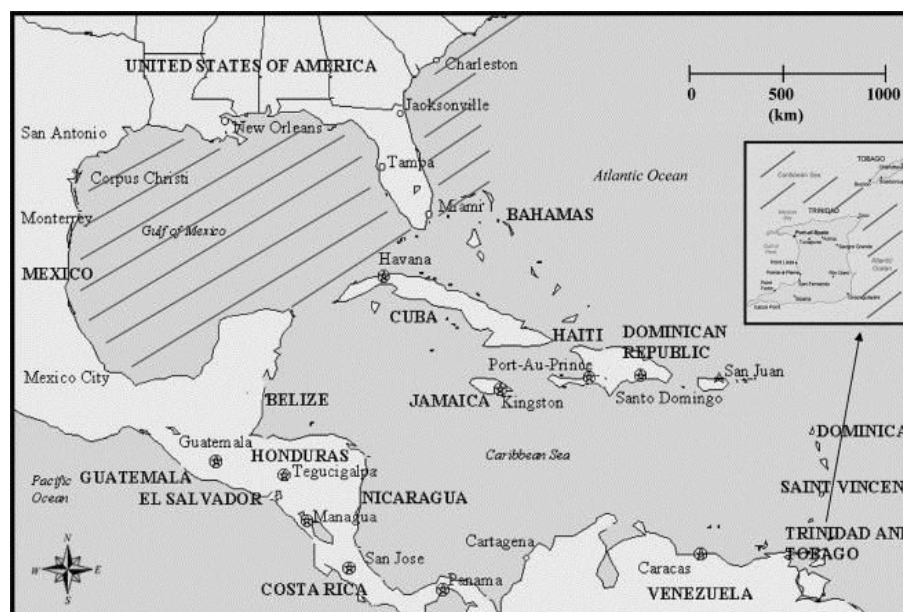


Figure 3: Known distribution (diagonal lines) for *Karenia brevis* in the Gulf of Mexico and North Atlantic. It has also been documented from Jamaica (Steidinger et al., 2009).

Near-annual blooms of *K. brevis* ($> 1 \text{ cell mL}^{-1}$ to $> 10^4 \text{ cells mL}^{-1}$) in the eastern GoM represent one of the most predictable global HAB events; however, the formation of blooms in this area is not fully understood (Heil et al., 2014; Heil and Steidinger, 2009; Steidinger 2009; Tester and Steidinger, 1997). Blooms are thought to develop as a result of upwelling in offshore shelf waters, typically in the late summer and autumn, where they increase in biomass and are often moved by currents/ winds to near-shore areas (Heil et al., 2014; Steidinger et al., 2008a; Stumpf et al., 1998, 2008; Weisberg et al., 2009). Once moved inshore, cells may reach very high concentrations ($> 10^5 \text{ cells mL}^{-1}$), persist for long periods (> 18 months), and cover enormous areas ($> 25,000 \text{ km}^2$) (Florida Fish and Wildlife Conservation Commission, FWC, 2013, monitoring data; Geesey and Tester, 1993; Gunter et al., 1948; Heil and Steidinger, 2009; Hu et al., 2006; Rounsefell and

Nelson, 1966; Steidinger, 2009; Steidinger et al., 2008a). It is upon reaching these near-shore/ shallow estuary areas that blooms arguably exert most negative effects. In addition to providing economic, cultural and environmental benefits to human communities, these near-shore/ estuary areas are fundamental life-support services upon which many marine organisms depend (Costanza et al., 1997; Daily et al., 1997; Heck et al., 1997; Micheli and Peterson, 1999; Sheridan, 1992). Nutrient inputs from various natural (e.g. nitrogen fixing *Trichodesmium* blooms) or anthropogenic sources (e.g. increased human activities) may lengthen the time and intensity of a bloom (Brand and Compton 2007; Bronk et al., 2004; Geesey and Tester, 1993; Mulholland et al., 2004, 2006; Steidinger et al., 2008). The toxicity of each bloom is also unique; the concentration of *K. brevis* cells, age of the bloom and biochemical differences in the strain of *K. brevis* all determine the amount of toxin produced and so the magnitude of negative effects (Baden and Tomas, 1988; Bourdelais et al., 2004; Pierce, 1986; Pierce et al., 2008; Roszell et al., 1990).

1.5 Effects

1.5.1 Neurotoxic shellfish poisoning

Blooms of *K. brevis* may cause neurotoxic shellfish poisoning (NSP) in humans, a rare and so far non-fatal condition typically caused by ingestion of brevetoxic filter feeding bivalve molluscs. The neurological and gastrointestinal symptoms of this poisoning include; reversal of hot and cold sensations, nausea, vomiting, diarrhoea, paraesthesia, cramps, bronchoconstriction, paralysis, seizures and coma (Poli et al., 2000; Watkins et al., 2008). NSP is not restricted to the GoM region, cases have also been recorded along the south east coast of the U.S. and even in New Zealand (in New Zealand the causative organism was thought to be a similar *Karenia* species, *K. concordia*) (Chang et al., 2006; Haywood et al 2004; Steidinger et al., 2008; Tester et al., 1989). Non-filter feeding molluscs, such as whelks, and fish can also accumulate levels of PbTx toxic to humans, highlighting that NSP can also be caused by ingestion of species other than filter feeding bivalves (Poli et al., 2000; Naar et al., 2007).

1.5.2 Mortalities

Perhaps the most well-known and well reported impacts of *K. brevis* blooms are the significant mortalities of marine mammals, birds, fish and invertebrates, caused by exposure to toxic material through ingestion or as an aerosol (Flewelling et al., 2005; Gunter et al., 1948; Landsberg, 2002; Plakas and Dickey, 2010; Quick and Henderson, 1974; Shen et al., 2010; Steidinger and Ingle, 1972). The primary route of exposure for marine mammals and sea birds is thought to be via ingestion of toxic fish and shellfish that have been washed up on the shore or are floating in the sea (Forrester et al., 1977; Geraci, 1989; van Deventer et al., 2012). Lysing of the fragile *K. brevis* cells as they reach near-shore areas and are broken open by wave action also releases brevetoxin as an aerosol (Pierce et al., 1990, 2001). Breathing this toxic aerosol can cause respiratory irritation in humans and air breathing marine animals such as the Florida manatee, *Trichechus manatus latirostris* (Bossart et al., 1998; Pierce et al., 1986, 1990; Music et al., 1973).

Fish kills may occur when brevetoxin is absorbed across gill membranes (following lysis of the fragile *K. brevis* cell and release of the intracellular toxin), or from ingestion of toxic organisms (Abbott et al., 1975; Baden and Mende, 1982; Landsberg 2002; Naar et al., 2007; Shi et al., 2012; Steidinger et al., 1973; Quick and Henderson, 1974; Tester et al., 2000). Bloom concentrations as low as 100 cells mL⁻¹ have been linked to fish kills (Finucane, 1964; Morton and Burklew, 1969; Starr, 1958; Steidinger and Ingle 1972; Steidinger 2009).

Severe invertebrate mortalities have also been reported and linked to *K. brevis* blooms (Gunter et al., 1947, 1948; Jefferson et al., 1879; Simon and Dauer, 1972; Summerson and Peterson, 1990; Taylor, 1917; Tiffany and Heyl, 1978). There have been mixed reports of the effects of *K. brevis* exposure on the same bivalve species. For example; Jefferson et al. (1879) and Gunter et al. (1947, 1948) reported mortalities in the eastern oyster, yet; Taylor (1917) and Steidinger and Ingle (1972) reported no effect on eastern oysters during historic *K. brevis* blooms which caused severe mortalities of other organisms such as fish, marine birds and mammals. These observations highlight the importance of reporting cell concentrations and other environmental data to differentiate between effects from *K. brevis*/ associated toxins and those attributable to other negative environmental factors which may be

associated with blooms. For example; at least 5 other *Karenia* species have been found to co-occur in blooms of *K. brevis*, including *K. mikimotoi* and *K. selliformis* (Heil and Steidinger 2009; Steidinger et al., 1998, 2008b). These two algal species are known to produce other biotoxins. Gymnodimine (GYM), produced by *K. selliformis*, has been linked with fish kills (Table 1, Arzul et al., 1995; Gentien and Arzul, 1990; Seki et al., 1995; Steidinger et al., 2008a). In addition, the high biomass that sometimes accompanies blooms, may add mucilage and anoxia to the unfavorable environmental conditions (Brand et al., 2012).

1.5.3 Community structure

Blooms which result in these simultaneous lethal effects on marine organisms are only one aspect of *K. brevis* exposure. They also directly and indirectly alter the structure of the entire community. Declines in the recruitment of fish and bivalve species following blooms of *K. brevis* were observed by Flaherty and Landsberg (2011) and Summerson and Peterson (1990) respectively. Heil et al. (2004) and Meyer et al. (2014) observed changes in the microbial community structure during a bloom of *K. brevis* due to nutrient cycling by bacteria and mixotrophic grazing of *K. brevis* on bacteria. Finally, a long lasting (> 1 year) bloom altered the entire nekton community structure (Flaherty and Landsberg, 2011), highlighting that long-lasting blooms, even at low concentrations can cause a cascade of lethal and sublethal changes (Landsberg, 2002).

1.5.4 Sublethal effects and trophic transfer

Sublethal impacts may lead to an overall reduction in the fitness of an individual. Sublethal effects such as reduced feeding (Leverone et al., 2007), reproduction (Kubanek et al., 2007), development (Leverone et al., 2006) and physiological changes (Leverone, 2007) and; increased pathologies and impaired immune function (Bossart et al., 1998), have also been recorded in response to *K. brevis* exposure (Landsberg, 2002).

In addition, brevetoxin is fairly stable in the environment and can be accumulated and/ or transferred by organisms at many trophic levels, thus even in the absence of a bloom, negative effects may continue (Bricelj et al., 2012; Fire et al., 2007; Flewelling et al., 2005, 2010; Landsberg et al., 2009; Naar et al., 2007; Steidinger et

al., 2008; Tester et al., 2000). For example, a lag time between *K. brevis* blooms and manatee mortalities was attributed to the consumption of still brevetoxic filter feeding organisms living on seagrass and PbTx associated directly with seagrass on which manatees feed (Flewelling et al., 2005; Landsberg and Steidinger, 1998). Poli et al. (2000) and Pierce et al. (2004) reported trophic transfer of PbTx from the filter-feeding bivalve, *Mercenaria mercenaria*, firstly to the whelk, *Busycon contrarium*, and then to humans (resulting in NSP).

The possible lethal and sublethal effects of exposure of bivalves to *K. brevis* and its associated toxins has been studied very little. Along the south west Florida (SW FL) coast, where *K. brevis* blooms are especially common, environmentally and economically important, bivalve species such as the eastern oyster (*Crassostrea virginica*) and the hard clam (= northern quahog, *Mercenaria mercenaria*) may be affected more than is currently realized. Several aspects of *C. virginica* and *M. mercenaria* physiology and life history mean encounters with *K. brevis* blooms are frequent. For these reasons they were chosen as study organisms in the current project.

Part 2: Bivalves and *Karenia brevis*

2.1 Importance

Bivalve molluscs filter large amounts of algae and other particles from the water column and so provide vital ecosystem services such as clearing the water of particles (allowing increased light penetration for aquatic vegetation), nutrient cycling and, provision of secondary habitat in shallow estuaries (sensu Burkholder and Shumway, 2012; Dame, 1993; Newell, 2004; Wells, 1961). Bivalves also have important economic value. For example; eastern oyster (*Crassostrea virginica*) fisheries and hard clam (*Mercenaria mercenaria*) production contribute markedly to the economies of states bordering the GoM; notably in Florida. In 2012, landings of market size oysters on the Gulf coast of Florida alone were estimated at 1.5×10^3 tonnes, worth over \$9.9 million (NMFS, 2012). In 2005, 4×10^4 tonnes of food size hard clams worth over \$60 million were produced in the U.S., primarily in Virginia, Florida and Connecticut (United States Department of Agriculture (US DOA), 2005).

2.2 Distribution

The native ranges of *C. virginica* and *M. mercenaria* covers the temperate and sub-tropical eastern seaboard of north America; from the Gulf of St Lawrence, Canada, to the Gulf of Mexico (Palmer, 1927; Andrews, 1971; Abbott, 1974; Carlton and Mann, 1996; Yonge, 1960), although intentional and accidental introduction has expanded the range of these species throughout north America, Europe and Asia (Hanna, 1966; Heppell, 1961; Ruesink et al., 2005; Zhang et al., 2003). Both species live in near-shore and shallow estuary areas, plentiful along the Gulf coast of Florida.

2.3 Filter-feeding

The process of filter feeding is similar for both species. Water containing algal cells and other particles is pumped over the gills and cells are passed to the labial palps (Figure 4). Particle selection may occur at the palps, where algae (and other particles including dissolved organic matter, sediment and bacteria, Newell and Jordan, 1983) are either ingested through the mouth as slurry, or are rejected (Figure 4, Ward et al., 1994). Both bivalve species exhibit particle selection based on factors such as the size, shape, structure and compounds associated with the algal

cell and, concentration of the particles in the water (including algae and sediment) (Tammes and Dral, 1955; Newell et al., 1989; Shumway and Cucci, 1987; Ward and Shumway, 2004). Bivalves may reject particles either without incorporation in pseudofeces, or more often as pseudofeces (Bricelj et al., 1998; Hégaret et al., 2007; Shumway et al., 1985; Shumway and Cucci 1987, Ward et al., 1997, Ward and Shumway, 2004).

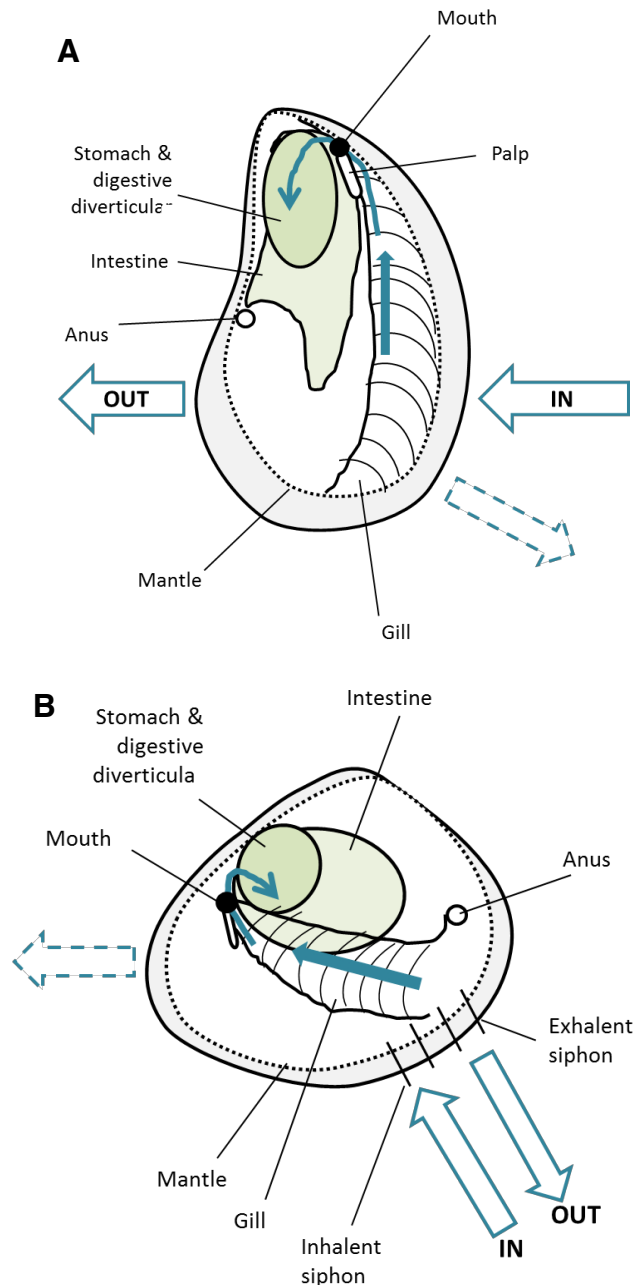


Figure 4: Anatomy of **A:** *Crassostrea virginica* and **B:** *Mercenaria mercenaria* showing the process of feeding. Blue arrows indicate the movement of water and algal cells into the bivalve, over the gills, to the labial palps where cells are either rejected (dotted blue line) or, ingested through the mouth into the digestive gland (where digestion occurs), followed by ejection of produced faeces out of the bivalve.

2.4 Life cycle

Life cycles of both *C. virginica* and *M. mercenaria* are characterized by a planktonic larval stage and a sessile (oyster) or sedentary (clam) benthic adult phase (Figure 5). The planktonic stage begins with the release of gametes into the water column, external fertilization, embryogenesis and development of free-swimming larvae (Figure 5). These planktonic stages are vulnerable to predation and environmental stressors (Carriker, 2001; Gosselin and Qian, 1997; Hedgecock, 1986; Pechenik 1987; Thompson et al., 1996; Thorson, 1950). Upon attaining a large enough size (~ 2 weeks after fertilization), and for oysters, finding a suitable substrate for settlement, larvae settle, undergo metamorphosis and resemble their adult form (Figure 5, Bachelet et al., 1992; Galtsoff, 1964; Kennedy, 1996; Rodríguez et al., 1993; Thompson et al., 1996). Oysters attach to substrate such as oyster shell and clams settle on fine sediment, in which they can burrow (Bachelet et al., 1992; Kennedy, 1996; Rodríguez et al., 1993). Although more protected than the larval stages, juveniles and adults cannot move away from predators and environmental stressors, so are also vulnerable to changes in the environment. The maturation of juveniles to reproductively active adults typically takes between 1- 3 years, although oysters in the Gulf of Mexico may be reproductively active before this time (Hayes and Menzel, 1981).

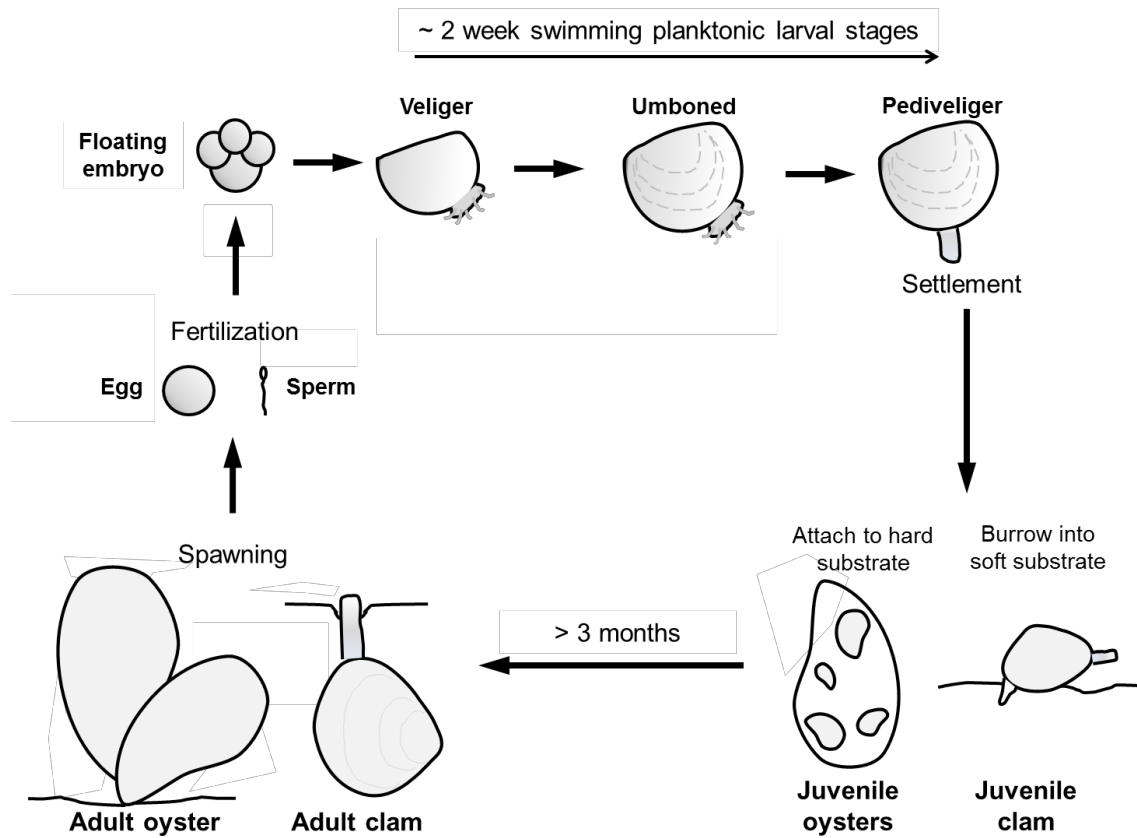


Figure 5: the life cycle of typical bivalve species such as *Crassostrea virginica* and *Mercenaria mercenaria* in south west Florida. Images modified from Karen Swanson (COSEE SE/NSF).

2.5 Reproduction

Eastern oysters are alternate hermaphrodites, usually starting their reproductive life as males and maturing to females; however, they may change sex (male- female- male- etc) between and even within a spawning season based on many factors including the proportion of males and females in a population and environmental stress (Arias-De León et al., 2013; Galtsoff, 1964; Thompson et al., 1996). Hard clams on the other hand are consecutive hermaphrodites, changing sex once in their life, from male to female (Dalton and Menzel, 1983; Eversole, 2001).

To spawn, adult bivalves and clams undergo gametogenesis, a period of gamete development and ripening. This is suspected to be a period of intensive physiological change in the adult because most of the energy acquired by the bivalve is used for the production of gametes (Soletchnik et al., 1997; Royer et al., 2008). Both species exhibit a seasonal cycle in the synthesis, storage and utilization of biochemical energy reserves. In the summer and autumn, when food is plentiful, energy storage reserves are built up. These reserves are used to maintain metabolism (during periods of low food supply) and start gametogenesis during the winter or spring (Thompson et al., 1996). Once initiated (by a mix of endogenous and exogenous cues), germinal cells develop and mature into either spermatozoa or oocytes over a period of weeks to months. Maximum ripening of gametes occurs just prior to spawning. Following cues to spawn (usually a sharp rise or drop in temperature), *C. virginica* and *M. mercenaria* eject mature gametes into the water column where external fertilization occurs. When spawning is complete, bivalves may re-ripen (by the ripening of remaining germinal follicles) or residual gametes may be resorbed by hemocytes, the gonad shrinks and individuals enter a 'resting' period.

In SW FL, oysters are found in a spawning state in all months, although the main spawning season is from May through October and peak spawning events are typically at the start and end of this period (Volety, 2008; Volety et al., 2009). Spawning of hard clams (including *M. mercenaria*, *M. campachensis* and their hybrids) is also essentially continuous with peaks in the spring and autumn (Hesselman et al., 1989).

2.6 Encounters with *Karenia brevis*

Blooms of *K. brevis* occur mainly in the late summer and autumn and last weeks to years; thus, these economically and environmentally important bivalve species may encounter blooms of *K. brevis* throughout their lifecycle. Indeed, as inhabitants of shallow estuaries/ near-shore areas, eastern oysters and hard clams are likely exposed to *K. brevis* and its associated toxins more frequently than other more mobile animals. Although a salinity barrier in estuaries was thought to protect benthic inhabitants from contact with *K. brevis* (Ray and Aldrich, 1967); studies showing the tolerance of *K. brevis* to lower salinities (Maier Brown et al., 2006) and detection of *K. brevis* blooms in near-fresh water in estuaries (Dortch et al., 1998; FWC, monitoring data) have proved that these organisms are exposed. Eastern oysters and hard clams may be exposed during a period of intense physiological change (gametogenesis) followed by a weakened state (post-spawning), which may affect not only the adult but also subsequently produced offspring. Furthermore, once spawning has occurred, it is highly likely the vulnerable early planktonic lifecycle stages of these species will be exposed to *K. brevis* blooms, either for part of or for the entire duration of the pelagic larval stage.

Bloom concentrations vary from low (1- 10 cells mL⁻¹) to extremely high (> 10⁵ cells mL⁻¹) and, between bloom events, *K. brevis* is often present at low concentrations in the environment (<1 cells mL⁻¹, Finucane, 1964; Heil and Steidinger, 2009). In addition, *K. brevis* has also been documented in marine sediments where *C. virginica* and *M. mercenaria* are found (Mendoza et al., 2008). Thus, these bivalve species may be exposed to chronic, low levels of *K. brevis* and associated toxins for periods extending beyond bloom events. The effects of chronic bloom exposure of *K. brevis* and its associated toxins on bivalves are poorly understood.

2.7 Brevetoxin accumulation

Filter-feeding bivalves such as *C. virginica* and *M. mercenaria* accumulate PbTx very quickly as they filter large quantities of algae, including *K. brevis*, from the water (Hemmert, 1975; Bricelj et al., 2012). Brevetoxin levels up to hundreds of µg g⁻¹ may be accumulated, primarily in the digestive gland and fatty tissues (Baden et al., 1982; Hemmert, 1975; Landsberg et al., 2009; McFarren et al., 1965; Roberts et al., 1979;

Steidinger et al., 1973, 1993, 1998a, 2008; Tester and Fowler, 1990). The amount of PbTx accumulated differs between species and between individuals within a population and is thought to be determined by the sensitivity of the individual to the toxin (Bricelj et al., 2005; Twarog et al., 1972). However, the major toxins found in cultures of *K. brevis* do not accumulate in bivalves (or in fish and mammals). Rather, accumulated brevetoxin is metabolized into other polar and non-polar compounds through largely unknown but presumed enzymatic (i.e. by the cytochrome P450 system) and chemical conversions (Fleming et al., 2011; Mello et al., 2012; Nicolas et al., 2004; Snyder, 2000; Washburn et al., 1994). Following an outbreak of NSP in New Zealand in 1992-1993, several PbTx metabolites (named BTX-B1, BTX-B2, BTX-B3, BTX-B4, BTX-B5, referring to specific taurine, cysteine and fatty-acid conjugates and an oxidation product of PbTx-2) were recorded in Greenshell mussel (*Perna canaliculus*) and cockle (*Austrovenus stutchburyi*) tissues (Ishida et al., 1995, 2004a; Morohashi et al., 1995, 1999; Murata et al., 1998). The abundance and composition of these accumulated metabolites vary between species (Abraham et al., 2012; Morohashi et al., 1999; Plakas et al., 2004). For example, the taurine conjugate of PbTx-1 has only been reported in the brevetoxin profile of *M. mercenaria* (Abraham et al., 2012). In bivalve species such as *C. virginica*, PbTx-2 is readily metabolized into PbTx-3 and a suite of metabolites, dominant in cysteine conjugates derived from PbTx-2 (Abraham et al., 2006, 2008 Dickey et al., 1999; Plakas et al., 2002, 2004; Plakas and Dickey, 2010; Radwan and Ramsdell, 2006). The effects of these metabolites on other organisms are complex and create management problems. Although they are not thought to be toxic to fish (Tester et al., 2000; Flewelling et al., 2005; Naar et al., 2007 Landsberg 2002), they are at least in part responsible for causing NSP in humans (Ishida et al, 2004b; Nozawa et al 2003; Pierce et al., 2004; Plakas et al 2004; Poli et al., 2000). In order to avoid possible cases of NSP, shellfish harvest areas are closed when *K. brevis* cell counts reach $> 5 \text{ cells mL}^{-1}$ and/or PbTx values of 20 mouse units (MU) 100 g^{-1} shellfish meat [equivalent to $0.8 \text{ mg PbTx-2 equivalents kg}^{-1}$] (NSSP, 2011).

Once accumulated, the elimination of brevetoxins from bivalve tissues may take weeks to months during which many more unknown metabolites with varying toxicities may be produced (Baden et al., 2005; Plakas et al., 2004, 2008; Steidinger et al., 2008a). Depuration times depend on several factors including the bivalve

species, the amount of toxin accumulated, availability of non-toxic food and continuing exposure to toxic algal cells (Bricelj and Shumway, 1998). As *K. brevis* may be present at low levels throughout non-bloom periods, it is possible that some chronically exposed species never fully depurate.

2.8 Sensitivity

The negative effect of PbTx exposure and accumulation are determined by the sensitivity of the individual to the effects of the toxin (Bricelj et al., 2005; Twarog et al., 1972). Voltage sensitive channels (including Na⁺) are involved in spike generating mechanisms in bivalves (Anderson et al., 2005; Devlin, 1993) thus, it is possible that the nervous system of bivalves may be affected by PbTx. Sodium channel blocking biotoxins, tetrodotoxin (TTX) and saxitoxin (STX) were shown to affect *in vitro* nervous transmission in bivalves (Twarog et al., 1972). In that study, the cerebral visceral nerves of *M. mercenaria* were more resistant to TTX and STX than those of *C. virginica*, as shown by the blocking of action potentials on nerves isolated from cerebral- visceral connective tissue (Twarog et al., 1972). Twarog et al. (1972) also noted a difference in the sensitivity of nerves isolated from different populations of blue mussels, *Mytilus edulis*, exposed to TTX. Bricelj et al. (2005) reported that different populations of the softshell clam, *Mya arenaria*, had differing sensitivity to STX. Clams from areas frequently exposed to PST producing algae accumulated 5-fold higher concentrations of PST (STX) compared to clams from areas with no history of exposure to PST-producing algae (Bricelj et al., 2005). Clams from PST un-affected areas also showed increased mortalities and severe physiological effects such as the inability to re-burrow, compared to clams from PST affected populations. Resistance in these previously exposed populations to PST was due to a natural mutation on an amino acid residue on the VSSC, which led to a one-thousand fold decrease in the binding affinity of STX to the VSSC (Bricelj et al., 2005). Such mutations can be small and occur regularly within even the same population, allowing species regularly exposed to certain toxins, a degree of insensitivity (Anderson et al., 2005). As a biotoxin which also acts on binding with the VSSC, it is possible that such small mutations and differences in the structure of the VSSC between individuals/ populations/ species, may also alter the affinity of PbTx binding to the VSSC.

2.9 Lethal and sublethal effects

As previously mentioned, exposure of bivalves to bloom concentrations of *K. brevis* has caused lethal and sub-lethal effects. Field exposures have led to observations of mortalities in several species. For example, a *K. brevis* bloom lasting from November 1946 to the latter half of August 1947 covering the SW FL coast, caused mortalities of *C. virginica* and clams (unknown species) (Gunter et al., 1947,1948). Simon and Dauer (1972) reported mortalities of benthic invertebrates including the clam (*Mulinia lateralis*) following a bloom of *K. brevis* in SW FL from July- August 1971 that reached nearly 1.8×10^4 cells mL⁻¹. Similarly, Tiffany and Heyl, (1978) also recorded mortalities of the Atlantic surf clam (*Spisula solidissima similis*) and Coquina clam (*Donax variabilis*) exposed for ~ a month to a bloom in SW FL up to 5×10^3 cells mL⁻¹. Short- term (< 2 days) laboratory exposures of adult bivalve species, shown to be affected by *K. brevis* in the field, have however shown no mortalities or effects on the behaviour of these species (i.e. Ray and Aldrich, 1967; Sievers, 1969). Yet, a more recent study by Leverone et al. (2007) in which *C. virginica*, *M. mercenaria* and *A. irradians* were exposed to bloom concentrations of whole cells of *K. brevis* in either static or flow through systems, resulted in a significant reduction in algal clearance rates, which differed between bivalve species, suggesting a cytotoxic effect. Behaviours such as shell or valve closure, reduced filtration, burrowing and pseudofaeces production have been demonstrated in bivalves exposed to toxic algae to avoid contact with cells (Basti et al., 2009; Bricelj et al., 2005; Hégaret et al., 2007; Shumway et al., 1985). Furthermore, physiological effects (such as tissue inflammation and alterations in hemocyte responses) have also been observed in bivalve species exposed to different toxic algae, indicating cytotoxic effects (i.e. Basti et al., 2009, 2011a; da Silva et al., 2008; Galimany et al., 2008a,b,c; Haberkorn et al., 2010a, b; Hégaret et al., 2009, 2010). Possible physiological effects were also reported by Leverone (2007) following exposure of adult *A. irradians* to whole and lysed preparations of *K. brevis* at 500 cells mL⁻¹ for 2 weeks. Although significant effects could not be identified due to the additional effect of poor nutrition in all *K. brevis* exposed and control scallops, results suggested there were some inflammatory responses in the digestive gland of the scallops (Leverone, 2007).

As detailed above, physiological changes may also be linked with changes in reproductive parameters and *visa versa*. A study by Haberkorn et al. (2010b) in which adult pacific oysters (*C. gigas*) were continually exposed to the PST producing dinoflagellate, *Alexandrium minutum*, for 4 days, revealed not only significant inflammatory responses within oyster tissues, but also morphological and functional alterations in the spermatozoa from exposed adults compared to sperm from non-exposed adults. These changes in gamete quality could lead to reduced sperm fertility and reproduction success. The potential physiological and reproductive effects of longer-term *K. brevis* exposure on adult *C. virginica* and *M. mercenaria* are unknown.

Exposure of the early life cycle stages of bivalves have also been shown to be susceptible to toxic algal exposure with susceptibility differing between species, dependent on developmental stage of the bivalve and, the toxin content and toxicological mechanisms of the algae (Basti et al., 2011b; Glibert et al., 2007; Matsuyama et al., 2001; Stoecker et al., 2008; Wang et al., 2006; Yan et al., 2001; 2003; Wazniak and Glibert, 2004). The susceptibility of early bivalve life stages exposed to *K. brevis* both in the field and under controlled conditions has previously been demonstrated (Leverone et al., 2006; Summerson and Peterson, 1990). In 1987, a bloom of *K. brevis* transported along the south east coast of FL, up to North Carolina by the Gulf Stream, caused almost total recruitment failure of newly settled bay scallops, *A. irradians* (Summerson and Peterson, 1990; Tester et al., 1989). The effect of this bloom was long lasting as recruitment remained low for the subsequent year (Summerson and Peterson, 1990). Leverone et al. (2006) exposed 3 and 7 day old larvae of *C. virginica* and *M. mercenaria* to concentrations of up to 5000 cells mL⁻¹ *K. brevis* for 3 and 7 days respectively. Although exposure only lasted a few days the *K. brevis* treatments had dose-dependent lethal and sub-lethal effects on larvae (Leverone et al., 2006). However, the effects of exposing early life stages of *C. virginica* and *M. mercenaria* to *K. brevis* are not fully understood. Furthermore, earlier life stages than those exposed by Leverone et al. (2006) (i.e. gametes, embryos and < 3 day-old larvae) would also be exposed in the field and possibly would be more susceptible to *K. brevis* exposure than at 3 or 7 days. Other bioactive compounds associated with *K. brevis* (in addition to PbTx) may also be involved in the toxic effects on these early life stages.

As demonstrated by Summerson and Peterson (1990), exposure to *K. brevis* may also have significant ramifications for recruitment and therefore possibly population structure.

2.10 Summary

Acute bloom concentrations of *K. brevis* have been shown to be lethal to economically and environmentally important bivalve species; however, the sublethal effects of chronic exposure on these species are relatively unknown. Blooms of *K. brevis* and the reproductive periods of *C. virginica* and *M. mercenaria* overlap, thus these species may be exposed to *K. brevis* and its associated toxins for prolonged periods, during times of reproductive conditioning and spawning. The effect of these longer term exposures of *K. brevis* on the reproductive capacities of these species has yet to be determined. The early development of bivalves is a particularly important and sensitive period in the life cycle, which if affected can reduce recruitment and possibly population stability (i.e. Yan et al., 2003, Glibert et al., 2007). It is thus also important to determine the effects of *K. brevis* during the early development of these species.

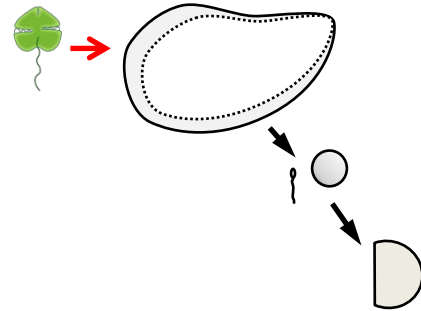
Furthermore, an apparent increase in the abundance of *K. brevis* blooms in recent years, identification of new PbTx producing algal species and, production of brevetoxin by algal species previously thought not to be brevetoxic (Bourdelais et al., 2002; Brand and Compton, 2007; Bridgers et al., 2004); have highlighted the need for research into this area.

Part 3: Objectives and Rationale

3.1 Objectives

The objectives of this project were to determine:

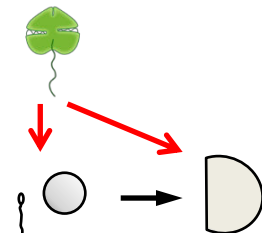
1. The effects of field and laboratory exposure of *K. brevis* on the reproductive and related physiological processes of adult *M. mercenaria* and *C. virginica* and the quality of subsequently produced offspring from exposed adults (Figure 6).



Hypotheses:

- Exposure to *K. brevis* has no effect on the survival, or on the reproductive and related physiological processes of adult *M. mercenaria* and *C. virginica*.
- Brevetoxin accumulated within the adult is not present within the gametes.
- Adult exposure to *K. brevis* has no effect on the gametes and development of subsequently produced offspring.

2. The effects of direct *K. brevis* exposure on gamete, embryo and larval development of *M. mercenaria* and *C. virginica* (Figure 6).



Hypotheses:

- Brevetoxin is the cause of toxic effects on early life stages during *K. brevis* exposure.
- Sensitivity to *K. brevis* exposure is not life cycle stage dependent.
- Sperm and oocytes do not differ in their sensitivity to *K. brevis* exposure.
- Deleterious effects on early life stages do not continue after an exposure has finished.

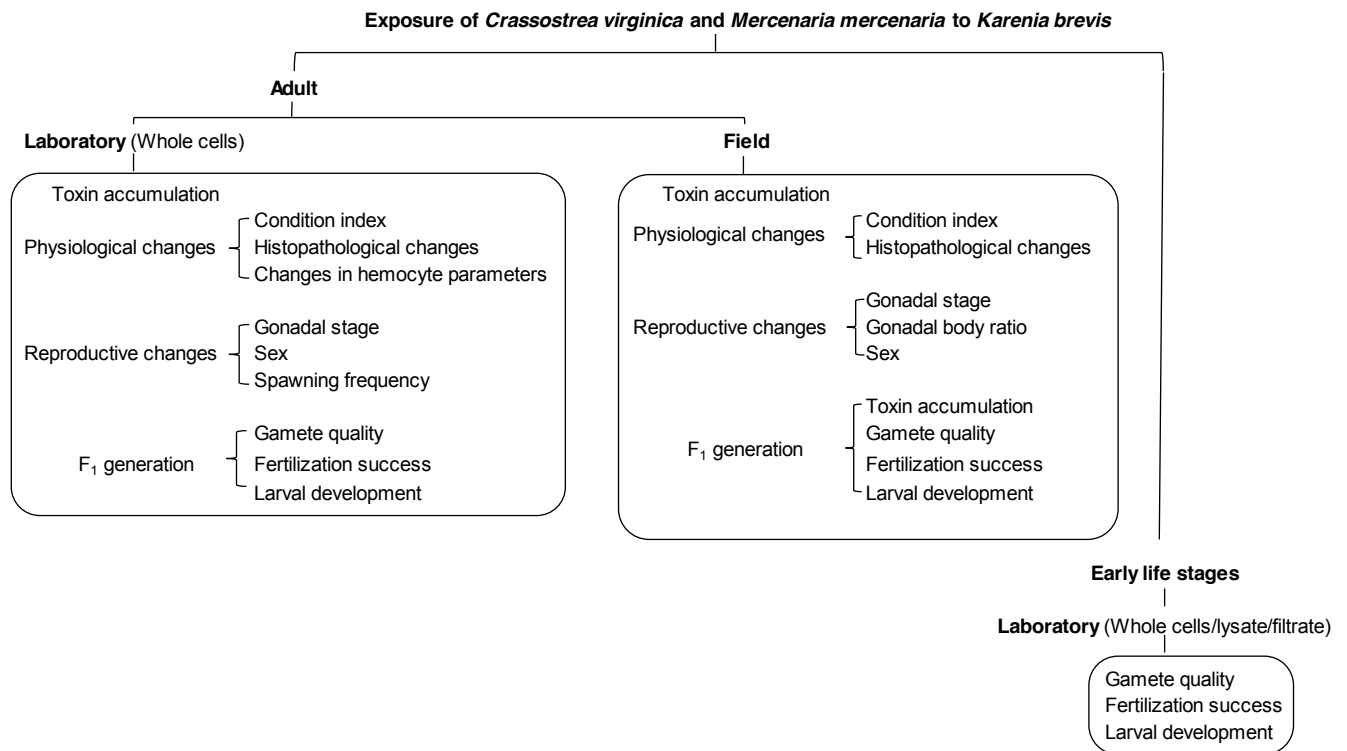


Figure 6: Structure of the project, showing the different life stages studied, both in the laboratory and the field and, the physiological and reproductive parameters measured.

3.2 Rationale

Results will improve our understanding of the effects of *K. brevis* on the reproduction and early life histories of commercially and ecologically important species and will have practical and applied uses. Results will help elucidate the relative contribution and significance of *K. brevis* bloom exposure on adult bivalves and resulting offspring against other environmental factors. Data from this project may aid in bloom modeling, allowing predictions of both acute and chronic impacts of *K. brevis* blooms on the physiology, fecundity, recruitment and therefore structure of populations of clams and oysters in SW FL and other affected areas of the GoM. This will enable informed decisions on suitable sites and other cost effective practices for fisheries/ aquaculture and informed decisions on restoration and enhancement of wild stock to be made.

As other brevetoxin producing algal species and brevetoxin-like compounds are found throughout the world (Bourdelaïs et al., 2002; Chang et al., 2006), data may also be of significance to other regions.

References

- Abbott, B. C., Siger, A., Spigelstein, M. 1975. Toxins from the blooms of *Gymnodinium breve*. In: Proceedings of the First International Conference on Toxic Dinoflagellate Blooms. Massachusetts Science and Technology Foundation. No. 8. p. 355.
- Abbott R. T. 1974. American seashells; the marine Mollusca of the Atlantic and Pacific coasts of North America. 2d ed. Van Nostrand Reinhold, N.Y. pp. 663.
- Abraham, A., Wang, Y., El Said, K. R., Plakas, S. M. 2012. Characterization of brevetoxin metabolism in *Karenia brevis* bloom-exposed clams (*Mercenaria* sp.) by LC-MS/MS. *Toxicon*, 60(6), 1030-1040.
- Abraham, A., Plakas, S. M., Flewelling, L. J., El Said, K. R., Jester, E. L. E., Granade, H. R., White, K.D., Dickey, R. W. 2008. Biomarkers of neurotoxic shellfish poisoning. *Toxicon*. 52(2): 237–45.
- Abraham, A., Plakas, S. M., Wang, Z., Jester, E.L.E., El Said, K. R., Granade, H. R., Henry, M. S. Blum, P.C., Pierce, R.H., Dickey R. W. 2006. Characterization of polar brevetoxin derivatives isolated from *Karenia brevis* cultures and natural blooms. *Toxicon* 48(1): 104-115.
- Abraham, W. M., Bourdelais, A. J., Ahmed, A., Serebriakov, I., Baden, D. G. 2005. Effects of inhaled brevetoxins in allergic airways: toxin-allergen interactions and pharmacologic intervention. *Environ. Health Perspect.* 113(5): 632-637.
- Alcock, F. 2007. An assessment of Florida red tide: Causes, consequences and management strategies, p. 40. Technical Report #1190. Sarasota, FL: Mote Marine Laboratory.
- Anderson, D. M., Glibert, P. M., Burkholder, J. M. 2002. Harmful algal blooms and eutrophication nutrient sources, composition, and consequences. *Estuaries*. 25(4): 704-726.
- Anderson, P. A., Roberts-Misterly, J., Greenberg, R. M. 2005. The evolution of voltage-gated sodium channels: were algal toxins involved?. *Harmful Algae*. 4(1): 95-107.
- Andrews, J. 1971. Sea shells of the Texas coast. University of Texas Press. Austin, TX. pp.285.
- Arzul, G., Turki, S., Namza, A., Daniel, P., Merceron, M., 1995. Fish kills induced by phycotoxins. *Toxicon* 33: 11- 19.
- Bachelet, G., Butman, C. A., Webb, C. M., Starczak, V. R., Snelgrove, P. V. 1992. Non-selective settlement of *Mercenaria mercenaria* (L.) larvae in short-term, still-water, laboratory experiments. *J. Exp. Mar. Biol. Ecol.* 161(2): 241-280.
- Baden, D. G., Bourdelais, A. J., Jacocks, H., Michelliza, S., Naar, J. 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ. Health Perspect.* 113(5): 621-625.
- Baden, D. G., Tomas, C. R. 1988. Variations in major toxin composition for six clones of *Ptychodiscus brevis*. *Toxicon*. 26(10): 961-963.
- Baden, D. G., Mende, T. J. 1982. Toxicity of two toxins from the Florida red tide dinoflagellate (*Ptychodiscus brevis*). *Toxicon*. 20: 457–461.
- Baden, D. G., Mende, T. J., Bikhazi, G., Leung, I. 1982. Bronchoconstriction caused by Florida red tide toxins. *Toxicon*. 20: 929–932.

- Basti, L., Endo, M., Segawa, S. 2011a. Physiological, pathological, and defense alterations in Manila clams (short-neck clams), *Ruditapes philippinarum*, induced by *Heterocapsa circularisquama*. J. Shellfish Res. 30(3): 829-844.
- Basti, L., Go, J., Higuchi, K., Nagai, K., Segawa, S. 2011b. Effects of the toxic dinoflagellate *Heterocapsa circularisquama* on larvae of the pearl oyster *Pinctada fucata martensii* (Dunker, 1873). J. Shellfish Res. 30(1): 177-186.
- Basti, L., Nagai, K., Shimasaki, Y., Oshima, Y., Honjo, T., Segawa, S. 2009. Effects of the toxic dinoflagellate *Heterocapsa circularisquama* on the valve movement behaviour of the Manila clam *Ruditapes philippinarum*. Aquaculture. 291(1): 41-47.
- Bialojan, C., Takai, A. 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem. J. 256: 283-290.
- Bossart, G. D., Baden, D. G., Ewing, R. Y., Roberts, B., Wright, S. D. 1998. Brevetoxicosis in manatees (*Trichechus manatus latirostris*) from the 1996 epizootic: gross, histologic and immunohistochemical features. Toxicol. Pathol. 26: 276-282.
- Bourdelais, A. J., Jacocks, H. M., Wright, J. L., Bigwarfe, P. M., Baden, D. G. 2005. A New Polyether Ladder Compound Produced by the Dinoflagellate *Karenia brevis*. J. Nat. Prod. 68(1): 2-6.
- Bourdelais, A. J., Campbell, S., Jacocks, H., Naar, J., Wright, J. L. C., Carsi, J., Baden, D. G. 2004. Brevenal is a natural inhibitor of brevetoxin action in sodium channel receptor binding assays. Cell. Mol. Neurobiol. 24(4): 553-63.
- Bourdelais, A. J., Tomas, C. R., Naar, J., Kubanek, J., Baden, D. G. 2002. New fish-killing alga in coastal Delaware produces neurotoxins. Environ. Health Perspect. 110(5): 465-470.
- Brand, L., Campbell, L., Bresnan, E. 2012. *Karenia*: The biology and ecology of a toxic genus. Harmful Algae. 14: 156-178.
- Brand, L.E., Compton, A. 2007. Long-term increase in *Karenia brevis* abundance along the Southwest Florida Coast. Harmful Algae. 6(2): 232-252.
- Bricelj, V. M., Haubois, A. G., Sengco, M. R., Pierce, R. H., Culter, J. K., Anderson, D. M. 2012. Trophic transfer of brevetoxins to the benthic macrofaunal community during a bloom of the harmful dinoflagellate *Karenia brevis* in Sarasota Bay, Florida. Harmful Algae. 16: 27-34.
- Bricelj, V. M., Connell, L., Konoki, K., MacQuarrie, S. P., Scheuer, T., Catterall, W. A., Trainer, V. L. 2005. Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP. Nature. 434(7034): 763-767.
- Bricelj, V. M., Shumway, S. E. 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics, and biotransformation. Rev. Fish. Sci. 6(4): 315-383.
- Bricelj, V.M., Wards, J.E., Cembella, A., MacDonald, B. A. 1998. Application of video-endoscopy to the study of bivalve feeding on toxic dinoflagellates. In: Reguera, B., Blanco, J., Fernandez, M., Wyatt, T. Harmful Microalgae. Xunta de Galicia: IOC de UNESCO. pp. 453- 456.
- Bridgers, A., McConnel, E., Naar, J., Weidner, A., Tomas, L., Tomas, C. R. 2004. Comparison of regional clones of the genera *Chattonella* and *Fibrocapsa* for growth characteristics and potential toxin production. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds). Harmful Algae 2002. St. Petersburg, Florida: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 405-407.

- Bronk, D.A., Sanderson, M.P., Mulholland, M.R., Heil, C.A., O'Neil, J.M. 2004. Organic and inorganic nitrogen uptake kinetics in field populations dominated by *Karenia brevis*. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds). Harmful Algae 2002. St. Petersburg, Florida: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 80–82.
- Burkholder, J.M., Shumway, S.E. 2012. Bivalve shellfish aquaculture and eutrophication. In: Shumway, S. E. (Ed.), Shellfish Aquaculture and the Environment. Wiley and Sons Inc., Hoboken, NJ. pp. 155–215.
- Carlton, J. T., Mann, R. 1996. Transfers and world-wide introductions. The eastern oyster *Crassostrea virginica*. Maryland Sea Grant, College Park, Maryland. pp. 691-706.
- Carriker, M.R., 2001. Embryogenesis and organogenesis of veligers and early juveniles. In: Kraeuter, J. N., Castagna, M. (Eds). Biology of the hard clam. Vol. 31. Gulf Professional Publishing. pp 77–115.
- Chang, F. H., Bourdelais, A. J., Baden, D. G., Gall, M., Hulston, D., Webb, V. 2006. *Karenia concordia* (Dinophyceae) as a brevetoxin-producer and comparison with two closely related species *K. brevisulcata* and *K. mikimotoi*. In: 12th International Conference on Harmful Algae, Program and Abstracts, Copenhagen, Denmark. pp. 4-8.
- Costanza, R., D'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., Solomons, M.D. 1997. The value of the world's ecosystem services and natural capital. *Nature*. 387: 253- 260.
- Daily, G.C., Alexander, S., Ehrlich, P.R., Goulder, L., Lubchenco, J., Matson, P.A., Mooney, H.A., Postel, S.L., Schneider, S.H., Tilman, D., Woodwell, G.M. 1997. Ecosystem services: benefits supplied to human societies by natural ecosystems. *Issues Ecol.* 2: 1–16.
- Dame, R. F. 1993. The role of bivalve filter feeder material fluxes in estuarine ecosystems. NATO ASI Series G 33, Bivalve Filter Feeders; In: Dame R. F. (Ed.), Estuarine and coastal ecosystem processes, Heidelberg Springer-Verlag. pp. 246-68.
- Deeds, J. R., Schwartz, M. D., 2010. Human risk associated with palytoxin exposure. *Toxicon*. 56: 150–162.
- Devlin, C. L. 1993. An analysis of control of the ventricle of the mollusc *Mercenaria mercenaria*. *J. Experimental Biol.* 179(1): 47-61.
- Dickey, R.E., Jester, R.G., Mowdy, D., Moncreiff, C., Rebarchik, D., Robl, M., Musser, S., Poli, M. 1999. Monitoring brevetoxins during a *Gymnodinium breve* red tide: comparison of sodium channel specific cytotoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. *Natural Toxins*. 7(4): 157-165.
- Dortch, Q., Moncreiff, C.A., Mendenhall, W., Parsons, M.L., Franks, J.S., Hemphill, K.W., 1998. Spread of *Gymnodinium breve* into the NGOMEX. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, GRAFISANT, Santiago de Compostela, Spain. pp. 143–144.
- Draisci, R., Lucentini, L., Giannetti, L., Boria, P., Poletti, R. 1996. First report of pectenotoxin-2 (PTX-2) in algae (*Dinophysis fortii*) related to seafood poisoning in Europe. *Toxicon*. 34(8): 923-935.
- Errera, R. M., Campbell, L. 2011. Osmotic stress triggers toxin production by the dinoflagellate *Karenia brevis*. *Proceedings of the National Academy of Sciences*. 108(26): 10597-10601.

- Errera, R. M., Bourdelais, A., Drennan, M. A., Dodd, E. B., Henrichs, D. W., Campbell, L. 2010. Variation in brevetoxin and brevenal content among clonal cultures of *Karenia brevis* may influence bloom toxicity. *Toxicon*, 55(2): 195-203.
- Eversole, A. G. 2001. Reproduction in *Mercenaria mercenaria*. In: Kraeuter, J.N., Castagna, M. (Eds.), *Biology of the Hard Clam. Developments in Aquaculture and Fisheries Science*, 31. Elsevier, Amsterdam. pp 221-260.
- Finucane, J. H., 1964. Distribution and seasonal occurrence on *Gymnodinium breve* on the West Coast of Florida, 1954–57. United States Fish and Wildlife Service Special Scientific Report. Fisheries No. 487.
- Fire, S. E., Fauquier, D., Flewelling, L. J., Henry, M., Naar, J., Pierce, R., Wells, R. S. 2007. Brevetoxin exposure in bottlenose dolphins (*Tursiops truncatus*) associated with *Karenia brevis* blooms in Sarasota Bay, Florida. *Mar. Biol.* 152(4): 827-834.
- Flaherty, K. E., Landsberg, J. H. 2011. Effects of a persistent red tide (*Karenia brevis*) bloom on community structure and species-specific relative abundance of nekton in a Gulf of Mexico estuary. *Estuar. Coast.* 34(2): 417-439.
- Fleming, L. E., Kirkpatrick, B., Backer, L. C., Walsh, C. J., Nierenberg, K., Clark, J., Reich, A., Hollenbeck, J., Benson, J., Cheng, Y.S., Naar, J., Pierce, R., Bourdelais, A.J., Abraham, W.M., Kirkpatrick, G., Zaias, J., Wanner, A., Mendes, E., Shalat, S., Hoagland, P., Stephan, W., Bean, J., Watkins, S., Clarke, T., Byrne, M., Baden, D. G. 2011. Review of Florida Red Tide and Human Health Effects. *Harmful Algae*. 10(2): 224–233.
- Flewelling, L. J., Adams, D. H., Naar, J. P., Atwood, K. E., Granholm, A. A., O’Dea, S. N., Landsberg, J. H. 2010. Brevetoxins in sharks and rays (*Chondrichthyes*, *Elasmobranchii*) from Florida coastal waters. *Mar. Biol.* 157(9): 1937-1953.
- Flewelling, L. J., Naar, J. P., Abbott, J. P., Baden, D. G., Barros, N. B., Bossart, G. D., Bottein, M. Y., Hammond, D. G., Haubold, E. M., Heil, C. A., Henry, M. S., Jacocks, H. M., Leighfield, T. A., Pierce, R. H., Pitchford, T. D., Rommel, S. A., Scott, P. S., Steidinger, K. A., Truby, E. W., Van Dolah, F. M., Landsberg, J. H. 2005. Brevetoxicosis: red tides and marine mammal mortalities. *Nature*. 435: 755-756.
- Forrester, D. J., Gaskin, J. M., White, F. H., Thompson, N. P., Quick, J. A., Henderson, G., Woodard, J. C., Robertson, W. D. 1977. An epizootic of waterfowl associated with a red tide episode in Florida. *J. Wildl. Dis.* 13(2): 160–167.
- Franchini, A., Malagoli, D., Ottaviani, E. 2010. Targets and effects of yessotoxin, okadaic acid and palytoxin: A differential review. *Mar. Drugs*. 8(3): 658-677.
- Franchini, A., Marchesini, E., Poletti, R., Ottaviani, E. 2004a. Acute toxic effect of the algal yessotoxin on Purkinje cells from the cerebellum of Swiss CD1 mice. *Toxicon*. 43(3): 347-352.
- Franchini, A., Marchesini, E., Poletti, R., Ottaviani, E. 2004b. Lethal and sub-lethal yessotoxin dose-induced morpho-functional alterations in intraperitoneal injected Swiss CD1 mice. *Toxicon*. 44(1): 83-90.
- Galimany, E., Sunila, I., Hégaret, H., Ramon, M., Wikfors, G. H. 2008a. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: Histopathology, immune responses, and recovery. *Harmful Algae*. 7(5): 702-711.

- Galimany, E., Place, A. R., Ramón, M., Jutson, M., Pipe, R. K. 2008b. The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*. *Harmful Algae*. 7(1): 91–98.
- Galimany, E., Sunila, I., Hégaret, H., Ramón, M., Wikfors, G. H. 2008c. Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. *Harmful Algae*. 7(5): 630–638.
- Galtsoff, P. S. 1964. The american oyster *Crassostrea virginica* Gmelin. *Fish. Bull.* 64: 1- 480.
- Gawley, R. E., Rein, K. S., Jeglitsch, G., Adams, D. J., Theodorakis, E. A., Tiebes, J., Nicolaou, K. C., Baden, D. G. 1995. The relationship of brevetoxin 'length' and A-ring functionality to binding and activity in neuronal sodium channels. *Chem. Biol.* 2(8): 533-541.
- Geesey, M., Tester, P. A. 1993. *Gymnodinium breve*: Ubiquitous in Gulf of Mexico waters? In: Smayda, T. J., Shimizu, Y. (Eds.), *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam. pp. 251–255.
- Gentien, P., Arzul, G. 1990. Exotoxin production by *Gyrodinium cf. aureolum* (Dinophyceae). *J. Mar. Biol. Assoc. U.K.* 70(3): 571-581.
- Geraci, J.R. 1989 Clinical investigations of the 1987–1988 mass mortality of bottlenose dolphins along the U.S. central and south Atlantic coast. Final report to the National Marine Fisheries Service, U.S. Navy Office of Naval Research and Marine Mammal Commission. Ontario Veterinary College, Guelph, Ontario. pp 63.
- Glibert, P., Alexander, J., Meritt, D., North, E., Stoecker, D. 2007. Harmful algae pose additional challenges for oyster restoration: Impacts of the harmful algae *Karlodinium veneficum* and *Prorocentrum minimum* on early life stages of the oysters *Crassostrea virginica* and *Crassostrea ariakensis*. *J. Shellfish Res.* 26(4): 919-925.
- Glasgow, H. B., Burkholder, J. M., Morton, S. L., Springer, J. 2001. A second species of ichthyotoxic *Pfiesteria* (Dinamoebales, Dinophyceae). *Phycologia*. 40(3): 234-245.
- Gosselin, L. A., Qian, P. Y. 1997. Juvenile mortality in benthic marine invertebrates. *Oceanogr. Lit. Rev.* 44(7): 730-731.
- Gunter, G., Williams, R. H., Davis, C. C., Smith, F. W. 1948. Catastrophic mass mortality of marine animals and coincident phytoplankton bloom on the west coast of Florida, November 1946 to August 1947. *Ecol. Monogr.* 18: 310-324.
- Gunter, G., Walton Smith, F. G., Williams, R. H. 1947. Mass Mortality of Marine Animals on the Lower West Coast of Florida, November 1946-January 1947. *Science*. 105(2723): 256-257.
- Haberkorn, H., Lambert, C., Le Goïc, N., Guéguen, M., Moal, J., Palacios, E., Lassus, P., Soudant, P. 2010a. Effects of *Alexandrium minutum* exposure upon physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. *Aquat. Toxicol.* 97(2): 96-108.
- Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Gueguen, M., Sunila, I., Soudant, P. 2010b. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. *Harmful Algae*. 9(5): 427-439.
- Hallegraeff, G. M. 1993. A review of harmful algal blooms and their apparent global increase*. *Phycologia*. 32(2): 79-99.

Hanna, G.D. 1966. Introduced mollusks of western North America. Calif. Acad. Sci. Occasional Papers 48:1-108.

Hayes, P. F., Menzel, R. W. 1981. The reproductive cycle of early setting *Crassostrea virginica* (Gmelin) in the northern Gulf of Mexico, and its implications for population recruitment. Biol. Bull. 160: 80-88.

Haywood, A. J., Steidinger, K. A., Truby, E. W., Bergquist, P. R., Bergquist, P. L., Adamson, J. Mackenzie, L. 2004. Comparative morphology and molecular phylogenetic analysis of three new species of the genus *Karenia* (Dinophyceae) from New Zealand. J. Phycol. 40: 165–79.

Heck, K. L., Nadeau, D. A., Thomas, R. 1997. The nursery role of seagrass beds. Gulf of Mexico Sci. 15: 50-54.

Hedgecock, D. 1986. Is gene flow from pelagic larval dispersal important in the adaptation and evolution of marine invertebrates?. Bul. Mar. Sci. 39(2): 550-564.

Hégaret, H., Smolowitz, R. M., Sunila, I., Shumway, S. E., Alix, J., Dixon, M., Wikfors, G. H. 2010. Combined effects of a parasite, QPX, and the harmful-alga, *Prorocentrum minimum* on northern quahogs, *Mercenaria mercenaria*. Mar. Environ. Res. 69(5): 337-344.

Hégaret, H., Da Silva, P. M., Sunila, I., Shumway, S. E., Dixon, M. S., Alix, J., Wikfors, G. H., Soudant, P. 2009. Perkinsosis in the Manila clam *Ruditapes philippinarum* affects responses to the harmful-alga, *Prorocentrum minimum*. J. Exp. Mar. Biol. Ecol. 371(2): 112-120.

Hégaret, H., Wikfors, G. H., Shumway, S. E. 2007. Diverse feeding responses of five species of bivalve mollusc when exposed to three species of harmful algae. J. Shellfish Res. 26(2): 549-559.

Hemmert, W. H. 1975. The public health implications of *Gymnodinium breve* red tides, a review of literature and recent events. In: LoCicero, V. R. (Ed.). Proceedings of the First International Conference on Toxic Dinoflagellate Blooms, Wakefield, Massachusetts Science and Technology Foundation. pp. 489–497.

Heil, C. A., Bronk, D.A., Dixon, L.K., Hitchcock, G. L., Kirkpatrick, G. J., Mulholland, M. R., O'Neil, J. M., Walsh, J. J., Weisberg, R., Garrett, M. 2014. The Gulf of Mexico ECOHAB: *Karenia* Program 2006–2012. Harmful Algae. 38: 3-7.

Heil, C. A., Steidinger, K. A. 2009. Monitoring, management, and mitigation of *Karenia* blooms in the eastern Gulf of Mexico. Harmful Algae. 8(4): 611-617.

Heil, C. A., Mulholland, M. R., Bronk, D. A., Bernhardt, P., O'Neil, J. M. 2004. Bacterial and size fractionated primary production within a large *Karenia brevis* bloom on the west Florida shelf. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds). Harmful Algae 2002. St. Petersburg, Florida: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 38-40.

Hellyer, S. D., Selwood, A. I., Rhodes, L., Kerr, D. S. 2011. Marine algal pinnatoxins E and F cause neuromuscular block in an *in vitro* hemidiaphragm preparation. Toxicon. 58(8): 693-699.

Hesselman, D.M., Barber, B.J., Blake, N.J., 1989. The reproductive cycle of adult hard clams, *Mercenaria* spp. in the Indian River Lagoon, Florida. J. Shellfish Res. 8:43–49.

Hoagland, P. Scatasta, S. 2006. The economic effects of harmful algal blooms. In: Granéli, E., Turner, J. T. (Eds). Ecology of Harmful Algae. Springer-Verlag: Berlin, 2006; pp 391-401.

Hu, C., Muller-Karger, F. E., Swarzenski, P. W. 2006. Hurricanes, submarine groundwater discharge, and Florida's red tides. *Geophys. Res. Lett.* 33: L11601.

Ishida, H., Nozawa, A., Hamano, H., Naoki, H., Fujita, T., Kaspar, H. F., Tsuji, K. 2004a. Brevetoxin B5, a new brevetoxin analog isolated from cockle *Austrovenus stutchburyi* in New Zealand, the marker for monitoring shellfish neurotoxicity. *Tetrahedron Lett.* 45(1): 29-33.

Ishida, H., Nozawa, A., Nukaya, H., Tsuji, K. 2004b. Comparative concentrations of brevetoxins PbTx-2, PbTx-3, BTX-B1 and BTX-B5 in cockle, *Austrovenus stutchburyi*, greenshell mussel, *Perna canaliculus*, and Pacific oyster, *Crassostrea gigas*, involved neurotoxic shellfish poisoning in New Zealand. *Toxicon.* 43(7): 779-789.

Ishida, H., Nozawa, A., Totoribe, K., Muramatsu, N., Nukaya, H., Tsuji, K., Kentaro Yamaguchi, K., Yasumoto, T., Kaspar, H., Berkett, N., Kosuge, T. 1995. Brevetoxin B 1, a new polyether marine toxin from the New Zealand shellfish, *Austrovenus stutchburyi*. *Tetrahedron Lett.* 36(5): 725-728.

Jefferson, J. P., Porter, J. Y., Moore, T. 1879. On the destruction of fish in the vicinity of the Tortugas during the months of September and October, 1878. *Proc. U.S. Nat. Mus.* 2: 244-246.

Jensen, A.C. 1975. The economic halo of a red tide. In: LoCicero, V. R. (Ed.), *Proceedings of the First International Conference on Toxic Dinoflagellate Blooms*. Wakefield, MA. MIT Science and Technology Foundation, pp. 507–516.

Kennedy, V. S. 1996. Biology of larvae and spat. In: Kennedy, V. S. (Ed.), *The Eastern Oyster Crassostrea virginica*. Maryland Sea Grant College, University of Maryland, College Park, Maryland. pp 371-421.

Kirkpatrick, B., Fleming, L. E., Squicciarini, D., Backer, L. C., Clark, R., Abraham, W., Benson, J., Cheng, Y.S., Johnson, D., Pierce, R., Zaias, J., Bossart, G. D., Baden, D. G. 2004. Literature review of Florida red tide: implications for human health effects. *Harmful Algae.* 3(2): 99-115.

Kubanek, J., Snell, T.W., Pirkle, C. 2007. Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing. *Limnol. Oceanogr.* 52(3): 1026–1035.

Kubanek, J., Hicks, M. K., Naar, J., Villareal, T. A. 2005. Does the red tide dinoflagellate *Karenia brevis* use allelopathy to outcompete other phytoplankton? *Limnol. Oceanogr.* 50(3): 883–895.

Lackey, J. B., 1956. Known geographic range of *Gymnodinium breve* Davis. *Q. J. Fl. Acad. Sci.* 19: 71.

Landsberg, J. H., Flewelling L. J., Naar, J. 2009. *Karenia brevis* red tides, brevetoxins in the food web, and impacts on natural resources: Decadal advancements. *Harmful Algae.* 8: 598–607.

Landsberg, J. H. 2002. The Effects of Harmful Algal Blooms on Aquatic Organisms. *Rev. Fish. Sci.* 10(2): 113–390.

Landsberg, J. H., Steidinger, K. A. 1998. A historical review of *Gymnodinium breve* red tides implicated in mass mortalities of the manatee (*Trichechus manatus latirostris*) in Florida, USA. In: Reguera, B., Blanco, J., Fernandez, M. L., Wyatt, T. (Eds), *Harmful Algae*. Xunta de Galicia and Intergovernmental Oceanographic Commission, UNESCO, pp. 97–100.

Larkin, S.L., Adams, C.M., Ballyram, D., Mulkey, A., Hodges, A. 2003. Red Tides and Coastal Business: Measuring Economic Consequences in Florida. Working paper, Food and Resource Economics Department, University of Florida, Gainesville, FL. pp 21.

- Lefebvre, K. A., Robertson, A. 2010. Domoic acid and human exposure risks: a review. *Toxicon*. 56(2): 218-230.
- Lekan, D. K., Tomas, C. R. 2010. The brevetoxin and brevenal composition of three *Karenia brevis* clones at different salinities and nutrient conditions. *Harmful Algae*. 9(1): 39-47.
- Lelong, A., Hégaret, H., Soudant, P., Bates, S. S. 2012. *Pseudo-nitzschia* (*Bacillariophyceae*) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. *Phycologia*. 51(2): 168-216.
- Leverone, J. R. 2007. Comparative effects of the toxic dinoflagellate, *Karenia brevis*, on bivalve molluscs from Florida. University of South Florida. Thesis. pp 144.
- Leverone, J. R., Shumway, S. E., Blake, N. J. 2007. Comparative effects of the toxic dinoflagellate *Karenia brevis* on clearance rates in juveniles of four bivalve molluscs from Florida, USA. *Toxicon*. 49(5): 634-645.
- Leverone, J. R., Blake, N. J., Pierce, R. H., Shumway, S. E. 2006. Effects of the dinoflagellate *Karenia brevis* on larval development in three species of bivalve mollusc from Florida. *Toxicon*. 48: 75-84.
- Loret, P., Tengs, T., Villareal, T. A., Singler, H., Richardson, B., McGuire, P., Morton, S., Busman, M., Campbell, L. 2002. No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. *J. Plankton Res.* 24(7): 735-739.
- Magaña, H. A., Villareal, T. A. 2006. The effect of environmental factors on the growth rate of *Karenia brevis* (Davis) G. Hansen and Moestrup. *Harmful Algae*. 5(2): 192-198.
- Maier Brown, A. F., Dortch, Q., Van Dolah, F.M., Leighfield, T. A., Morrison, W., Thessen, A.E., Steidinger, K. E., Richardson, B., Moncreiff, C. A., Pennock, J. R. 2006. Effect of salinity on the distribution, growth, and toxicity of *Karenia* spp. *Harmful Algae*. 5(2): 199-212.
- Marshall, J., Ross, T., Pyecroft, S., Hallegraeff, G., 2005. Superoxide production by marine microalgae 2: Towards understanding ecological consequences and possible functions. *Mar. Biol.* 147: 541-549.
- Matsuyama, Y., Shumway, S.E. 2009. Impacts of harmful algal blooms on shellfisheries aquaculture. In: Burnell, G., Allan, G. (Eds.), *New technologies in aquaculture. Improving production efficiency, quality and environmental management*. Cambridge, Woodhead. pp 580-609.
- Matsuyama, Y., Usuki, H., Uchida, T., Kotani, Y. 2001. Effects of harmful algae on the early planktonic larvae of the oyster, *Crassostrea gigas*. In: Hallegraeff, G., Blackburn, S., Bolch, C., Lewis, R. (Eds.), *Harmful Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO. pp 411-414.
- McFarren, E. F., Tanabe, H., Silva, F. J., Wilson, W. B., Campbell, J. E., Lewis, K. H. 1965. The occurrence of a ciguatera-like poison in oysters, clams and *Gymnodinium breve* cultures. *Toxicon*. 3:111-123.
- Mello, D. F., De Oliveira, E. S., Vieira, R. C., Simoes, E., Trevisan, R., Dafre, A. L., Barracco, M. A. 2012. Cellular and transcriptional responses of *Crassostrea gigas* hemocytes exposed in vitro to brevetoxin (PbTx-2). *Mar. Drugs*. 10(3): 583-597.
- Mendoza, W. G, Mead, R. N. Brand, L. E. Shea, D. 2008. Determination of brevetoxin in recent marine sediments. *Chemosphere*. 73(8): 1373-1377.

Meyer, K.A., O'Neil, J.M., Hitchcock, G.L., Heil, C.A. 2014. Microbial production along the West Florida Shelf: Responses of bacteria and viruses to the presence and phase of *Karenia brevis* blooms. *Harmful Algae*. 38: 110–118.

Micheli, F., Peterson, C. H. 1999. Estuarine vegetated habitats as corridors for predator movements. *Conservation Biol.* 13(4): 869-881.

Morohashi, A., Satake, M., Naoki, H., Kaspar, H. F., Oshima, Y., Yasumoto, T. 1999. Brevetoxin B4 isolated from greenshell mussels *Perna canaliculus*, the major toxin involved in neurotoxic shellfish poisoning in New Zealand. *Nat. Toxins*. 7(2): 45-48.

Morohashi, A., Satake, M., Murata, K., Naoki, H., Kaspar, H. F., Yasumoto, T. 1995. Brevetoxin B3, a new brevetoxin analog isolated from the greenshell mussel *Perna canaliculus* involved in neurotoxic shellfish poisoning in New Zealand. *Tetrahedron Lett.* 36(49): 8995-8998.

Morton, R. A., Burklew, M. A. 1969. Florida shellfish toxicity following blooms of the dinoflagellate, *Gymnodinium breve*. Marine Research Laboratory, Florida Department of Natural Resources. pp 26.

Mulholland, M. R., Bernhardt, P. W., Heil, C. A., Bronk, D. A., O'Neil, J. M. 2006. Nitrogen fixation and release of fixed nitrogen by *Trichodesmium* spp. in the Gulf of Mexico. *Limnol. Oceanogr.* 51(4): 1762-1776.

Mulholland, M. R., Heil, C. A., Bronk, D. A., O'Neil, J. M., Bernhardt, P. W. 2004. Does nitrogen regeneration from the N₂ fixing cyanobacteria *Trichodesmium* spp. fuel *Karenia* blooms in the Gulf of Mexico? In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds). *Harmful Algae 2002*. St. Petersburg, Florida: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 47–49.

Munday, R., Towers, N. R., Mackenzie, L., Beuzenberg, V., Holland, P. T., Miles, C. O. 2004. Acute toxicity of gymnodimine to mice. *Toxicon*. 44(2): 173-178.

Murata, K., Satake, M., Naoki, H., Kaspar, H. F., Yasumoto, T. 1998. Isolation and structure of a new brevetoxin analog, brevetoxin B2, from greenshell mussels from New Zealand. *Tetrahedron*. 54(5): 735-742.

Music, S.I., Howell, J.T., Brumback, L.C. 1973. Red tide: its public health implications. *Florida Med. J.* 60(11): 27–29.

Naar, J. P., Flewelling, L.J., Lenzi, A., Abbott, J.P., Granholm, A., Jacocks, H.M., Gannon, D., Henry, M., Pierce, R., Baden, D.G., Wolny, J., Landsberg, J.H. 2007. Brevetoxins, like ciguatoxins, are potent ichthyotoxic neurotoxins that accumulate in fish. *Toxicon*. 50(5): 707-723.

National Marine Fisheries Service (NMFS). 2012. Annual commercial landing statistics, Fisheries statistics. Available at: http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html. Accessed 29th October 2014.

National oceanic and Atmospheric Administration (NOAA). 2004. Action Plan for harmful Algal Blooms and the Gulf of Mexico Coastal ocean observing System: Results from a Regional Workshop. NOAA Coastal Services Center. NoAA/CSC/20516-PUB 27.

Neely, T., Campbell, L. 2006. A modified assay to determine hemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae*. 5: 592–598.

Newell, C. R., Shumway, S. E., Cucci, T. L., Selvin, R. 1989. The effects of natural seston particle size and type on feeding rates, feeding selectivity and food resource availability for the mussel *Mytilus edulis* Linnaeus, 1758 at bottom culture sites in Maine. *J. Shellfish Res.* 8(1): 187-196.

- Newell, R. I. E., 2004. Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. *J. Shellfish Res.* 23(1): 51–62.
- Newell, R. I. E., Jordan, S. J. 1983. Preferential ingestion of organic material by the American oyster *Crassostrea virginica*. *Mar. Ecol. Prog. Ser.* 13(1): 47-53.
- Nicolas, J., Hendriksen, P. J., Gerssen, A., Bovee, T. F., Rietjens, I. M. 2014. Marine neurotoxins: State of the art, bottlenecks, and perspectives for mode of action based methods of detection in seafood. *Mol. Nutr. Food Res.* 58(1): 87-100.
- Nozawa, A., Tsuji, K., Ishida, H. 2003. Implication of brevetoxin B1 and PbTx-3 in neurotoxic shellfish poisoning in New Zealand by isolation and quantitative determination with liquid chromatography-tandem mass spectrometry. *Toxicon.* 42(1): 91-103.
- Otero, P., Pérez, S., Alfonso, A., Vale, C., Rodríguez, P., Gouveia, N. N., Gouveia, N., Delgado, J., Vale, M., Hirama, M., Ishihara, Y., Molgo, J., Botana, L. M. 2010. First toxin profile of ciguateric fish in Madeira Arquipelago (Europe). *Anal. Chem.* 82(14): 6032-6039.
- Palmer, K. V. W. 1927. The Veneridae of eastern America. *Cenozoic and Recent. Palaeontogr. Americana.* 1(5): 209-522.
- Pechenik, J.A., 1987. Environmental influences on larval survival and development. In: Giese, A.C., Pearse, J.S., Pearse, V.B. (Eds.), *Reproduction of Marine invertebrates*. Blackwell Scientific Publications, Palo Alto, CA. pp. 551–608.
- Pérez-Gómez, A., Ferrero-Gutierrez, A., Novelli, A., Franco, J. M., Paz, B., Fernández-Sánchez, M. T. 2006. Potent neurotoxic action of the shellfish biotoxin yessotoxin on cultured cerebellar neurons. *Toxicol. Sci.* 90(1): 168-177.
- Persson, A., Smith, B. C., Morton, S., Shuler, A., Wikfors, G. H. 2013. Sexual life stages and temperature dependent morphological changes allow cryptic occurrence of the Florida red tide dinoflagellate *Karenia brevis*. *Harmful Algae.* 30: 1-9.
- Pshenichkin, S. P., Wise, B. C. 1995. Okadaic acid increases nerve growth factor secretion, mRNA stability, and gene transcription in primary cultures of cortical astrocytes. *J. Biol. Chem.* 270(11): 5994-5999.
- Pierce, R. H., Henry, M. 2008. Harmful algal toxins of the Florida red tide (*Karenia brevis*): natural chemical stressors in South Florida coastal ecosystems. *Ecotoxicol.* 17(7): 623-631.
- Pierce, R., Henry, M., Blum, P. 2008. Brevetoxin abundance and composition during ECOHAB-Florida field monitoring cruises in the Gulf of Mexico. *Continental Shelf Res.* 28(1): 45-58.
- Pierce, R. H., Henry, R.S., Dickey, R., Plakas, S. 2004. NSP Toxins and metabolites in oysters, clams and whelks. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.) *Harmful Algae 2002*. St. Petersburg, Florida: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 294-296.
- Pierce, R.H., M. Henry, P. Blum, Payne, S. 2001. *Gymnodinium breve* toxins without cells: intra-cellular and extra-cellular toxins. In: Hallegraeff, G., Blackburn, S., Bolch, C., Lewis, R. (Eds.), *Harmful Algal Blooms*, Intergovernmental Oceanographic Commission of UNESCO. pp. 421–424.
- Pierce, R. H., Henry, M.S., Proffitt, L.S., Hasbrouck, P.A. 1990. Red tide toxin (brevetoxin) enrichment in marine aerosol. In: Graneli, E., Sundstrom, B., Elder, L., Anderson, D.M. (Eds), *Toxic Marine Phytoplankton*. Elsevier; Amsterdam. pp. 397-402.

- Pierce, R. H. 1986. Red tide (*Ptychodiscus brevis*) toxin aerosols: A review. *Toxicon*. 24(10): 955-965.
- Plakas, S., Dickey, R. 2010. Advances in monitoring and toxicity assessment of brevetoxins in molluscan shellfish. *Toxicon*. 56: 137-149.
- Plakas, S.M., Wang, Z.H., El Said, K.R., Jester, E.L.E., Granade, H.R., Flewelling, L., Scott, P., Dickey, R.W. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. *Toxicon*. 44: 677–685.
- Plakas, S.M., El Said, K.R., Jester, E.L.E., Granade, H.R., Musser, S.M., Dickey, R.W. 2002. Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. *Toxicon*. 40: 721–729.
- Poirier, L., Quiniou, F., Ruiz, N., Montagu, M., Amiard, J. C., Pouchus, Y. F. 2007. Toxicity assessment of peptaibols and contaminated sediments on *Crassostrea gigas* embryos. *Aquat. Toxicol.* 83(4): 254-262.
- Poli, M. A., Musser, S. M., Dickey, R. W., Eilers, P. P., Hall, S. 2000. Neurotoxic shellfish poisoning and brevetoxin metabolites: a case study from Florida. *Toxicon*. 38(7): 981-993.
- Poli, M. A., Mende, T. J., Baden, D. G. 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol. Pharmacol.* 30(2): 129-135.
- Prince, E.K., Poulson, K.L., Myers, T.L., Sieg, R.D., Kubanek, J. 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. *Harmful Algae*. 10: 39–48.
- Quick, J. A., Henderson, G. E. 1974. Effects of *Gymnodinium breve* red tide on fishes and birds: a preliminary report on behavior, anatomy, hematology and histopathology. In *Proceedings of the Gulf Coast regional symposium on diseases of aquatic animals*. Louisiana State University: Louisiana Sea Grant, Louisiana, USA. pp 85- 113.
- Radwan, F.F.Y., Ramsdell, J. S. 2006. Characterization of in vitro oxidative and conjugative metabolic pathways for brevetoxin (PbTx-2). *Toxicol. Sci.* 89: 57–65.
- Ray, S. M., Aldrich, D. V. 1967. Ecological interactions of toxic dinoflagellates and molluscs in the Gulf of Mexico. In: Russell, F., Saunders, P. (Eds.), *Animal Toxins*. Oxford: Pergamon. pp. 75–83.
- Roberts, B. S., Henderson, G. E., Medlyn, R.A. 1979. The effect of *Gymnodinium breve* toxin(s) on selected mollusks and crustaceans. In: Taylor, D. L., Seliger, H. H. (Eds.), *Toxic Dinoflagellate Blooms*, New York: Elsevier. pp. 419–424.
- Rodríguez, S. R., Ojeda, F. P., Inestrosa, N. C. 1993. Settlement of benthic marine invertebrates. *Mar. Ecol. Prog. Ser.* 97(2): 193-207.
- Rossini, G.P., and Hess, P. 2010. Phycotoxins: chemistry, mechanisms of action and shellfish poisoning. In: Luch, A. (Ed.), *Molecular, Clinical and Environmental Toxicology*. Birkhäuser-Verlag AG, Basel. pp 65-122.
- Roszell, L E, Schulman, L S, Baden D G. 1990. Toxin profiles are dependent on growth stages in cultures *Phytodiscus brevis*. In: Graneli, E., Sundstrom, B., Elder, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton*. Elsevier. pp 403-406.
- Roth, P. B., Twiner, M. J., Wang, Z., Bottein Dechraoui, M. Y., Doucette, G. J. 2007. Fate and distribution of brevetoxin (PbTx) following lysis of *Karenia brevis* by algicidal bacteria, including analysis of open A-ring derivatives. *Toxicon*. 50(8): 1175-1191.

- Rounsefell, G. A., Nelson, W. R., 1966. Red-tide research summarized to 1964 including an annotated bibliography. In: United States Department of the interior Fish and Wildlife Service. Contribution number 20. Bureau of Commercial Fisheries Special Scientific Report. Fisheries number 535. Washington, D.C. pp. 1–85.
- Royer, J., Segueineau, C., Park, K. I., Pouvreau, S., Choi, K. S., Costil, K. 2008. Gametogenetic cycle and reproductive effort assessed by two methods in 3 age classes of Pacific oysters, *Crassostrea gigas*, reared in Normandy. *Aquaculture*. 277(3): 313-320.
- Ruesink, J.L., Lenihan, H.S., Trimble, A.C., Heiman, K.W., Micheli, F., Byers, J.E., Kay, M. C. 2005. Introduction of non-native oysters: ecosystem effects and restoration implications. *Annu. Rev. Ecol. Evol. Syst.* 36: 643–89.
- Seki, T., Satake, M., Mackenzie, L., Kaspar, H. F., Yasumoto, T. 1995. Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. *Tetrahedron Lett.* 36(39): 7093-7096.
- Shen, M., Xu, J., Tsang, T. Y., Au, D. W. 2010. Toxicity comparison between *Chattonella marina* and *Karenia brevis* using marine medaka (*Oryzias melastigma*): Evidence against the suspected ichthyotoxins of *Chattonella marina*. *Chemosphere*. 80(5): 585-591.
- Sheridan, P. F. 1992. Comparative habitat utilization by estuarine macrofauna within the mangrove ecosystem of Rookery Bay, Florida. *Bull. Mar. Sci.* 50(1): 21-39.
- Shi, F., McNabb, P., Rhodes, L., Holland, P., Webb, S., Adamson, J., Immers, A., Gooneratne, R., Holland, J. 2012. The toxic effects of three dinoflagellate species from the genus *Karenia* on invertebrate larvae and finfish. *New Zealand J. Mar. and Freshwater Res.* 46(2): 149-165.
- Shimizu, Y., Chou, H. N., Bando, H., Van Duyne, G., Clardy, J. 1986. Structure of brevetoxin A (GB-1 toxin), the most potent toxin in the Florida red tide organism *Gymnodinium breve* (*Ptychodiscus brevis*). *J. Am. Chem. Soc.* 108(3): 514-515.
- Shumway, S. E. 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquac. Soc.* 21(2): 65-104.
- Shumway, S. E., Cucci, T. L. 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. *Aquat. Toxicol.* 10(1): 9-27.
- Shumway, S. E., Cucci, T. L., Gainey, L., Yentsch, C. M. 1985. A preliminary study of the behavioral and physiological effects of *Gonyaulax tamarensis* on bivalve molluscs. *Toxic Dinoflagellates*. 389-394.
- Sievers, A. M. 1969. Comparative toxicity of *Gonyaulax monilata* and *Gymnodinium breve* to annelids, crustaceans, molluscs and a fish. *J. Protozool.* 16(3): 401-404.
- Simon, J. L., Dauer, D. M. 1972. A quantitative evaluation of red-tide induced mass mortalities of benthic invertebrates in Tampa Bay, Florida. *Environ. Lett.* 3(4): 229-234.
- Sipler, R. E., McGuinness, L. R., Kirkpatrick, G. J., Kerkhof, L. J., Schofield, O. M. 2014. Bacteriocidal effects of brevetoxin on natural microbial communities. *Harmful Algae*. 38: 101-109.
- Smayda, T. J. 1997. Harmful algal blooms: their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnol. Oceanogr.* 42(5): 1137-1153.
- Snyder, M.J. 2000. Cytochrome P450 enzymes in aquatic invertebrates: Recent advances and future directions. *Aquat. Toxicol.* 48: 529–547.

Soletchnik, P., Razet, D., Geairon, P., Faury, N., Gouletquer, P. 1997. Ecophysiology of maturation and spawning in oyster *Crassostrea gigas*: metabolic (respiration) and feeding (clearance and absorption rates) responses at different maturation stages. *Aquatic Living Res.* 10: 177-185.

Starr, T. J. 1958. Notes on a toxin from *Gymnodinium breve*. *Tex. Rep. Biol. Med.* 16: 500-507.

Steidinger, K. A. 2009. Historical perspective on *Karenia brevis* red tide research in the Gulf of Mexico. *Harmful Algae.* 8(4): 549-561.

Steidinger, Karen A., J. H. Landsberg, Flewelling, L.J., Kirkpatrick, B.A. 2008a. Toxic dinoflagellates. In: Walsh, P.J., Smith, S.L., Fleming, L.E., Solo-Gabriele, H.M., Gerwick, W.H. (Eds.) *Oceans and Human Health; Risks and Remedies From the Seas.* Elsevier, Burlington, Ma, U.S.A. pp 239-56.

Steidinger, K. A., Wolny, J. L., Haywood, A. J. 2008b. Identification of *Kareniaceae* (Dinophyceae) in the Gulf of Mexico (With 9 figures and 1 table). *Nova Hedwigia Beihefte.* 133: 269–84.

Steidinger, K. A., Carlson, P., Baden, D., Rodriguez, C., Seagle, J. 1998. Neurotoxic shellfish poisoning due to toxin retention in the clam *Chione cancellata*. In: Reguera, B., Blanco, J., Fernandez M. L., Wyatt, T. (Eds.) *Harmful Algae.* Paris: Xunta de Galicia, IOC pp. 457–458.

Steidinger, K. A. 1993. Some taxonomic and biological aspects of toxic dinoflagellates. In: Falconer, I. R. (Ed.). *Algal Toxins in Seafood and Drinking Water,* London: Academic Press pp. 1–28.

Steidinger, K. A. 1975. Implications of dinoflagellate life cycles on initiation of *Gymnodinium breve* red tides. *Environ. Lett.* 9(2): 129-139.

Steidinger, K. A., Burklew, M. Ingle, R. M. 1973. The effects of *Gymnodinium breve* toxin on estuarine animals. In: Martin D.F., Padilla, G. M. (Eds.), *Marine Pharmacognosy: Action of Marine Toxins at the Cellular Level,* New York: Academic Press pp. 179–202.

Steidinger, K. A., Ingle, R. M. 1972. Observations on the 1971 summer red tide in Tampa Bay, Florida. *Environ. Lett.* 3(4): 271-278.

Stoecker, D., Adolf, J., Place, A., Glibert, P., Meritt, D. 2008. Effects of the toxic dinoflagellate *Karlodinium veneficum* and *Prorocentrum minimum* on early life history stages of the eastern oyster (*Crassostrea virginica*). *Mar. Biol.* 154: 81-90.

Stumpf, R. P., Litaker, R. W., Lanerolle, L., Tester, P. 2008. Hydrodynamic accumulation of *Karenia* off the west coast of Florida. *Continental Shelf Res.* 28(1): 189–213.

Stumpf, R. P., Ransibrahmanakal, V., Steidinger, K. A., Tester, P. A. 1998. Observations of sea surface temperature and winds in association with Florida USA red tides (*Gymnodinium breve* blooms). *Harmful Algae. Proc. VIII International Conference on Harmful Algae* (June 1997, Vigo, Spain). Santiago de Compostela/Paris, Xunta de Galicia/Intergovernmental Oceanographic Commission of UNESCO. pp. 145- 148.

Sudaranam, S., Duke-Virca, G., March, D.J., Srinivasan, S. 1992. An approach to computer aided inhibitor design: application to cathepsin I. *J Comput Aided Mol Des.* 6: 223–233.

Summerson, H., Peterson, C. 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Ptychodiscus brevis*, outbreak recorded in North Carolina. *Estuaries.* 13(3): 322-331.

Tammes, P.M.L., Dral, A.D.G., 1955. Observations of the straining of suspensions by mussels. *Arch. Meerl. Zool.* 11: 87–112.

- Tatters, A.O, Muhlstein, H.I., Tomas, C.R. 2010. The hemolytic activity of *Karenia selliformis* and two clones of *Karenia brevis* throughout a growth cycle. *J. Appl. Phycol.* 22: 435–442.
- Taylor, H.F., 1917. Mortality of fishes on the west coast of Florida, Rep. U.S.A. Commun. Fish. Doc. No. 848, pp 24.
- Tester, P. A., Turner, J. T., Shea, D. 2000. Vectorial transport of toxins from the dinoflagellate *Gymnodinium breve* through copepods to fish. *J. Plankton Res.* 22(1): 47-62.
- Tester, P. A., Steidinger, K. A. 1997. *Gymnodinium breve* red tide blooms: initiation, transport, and consequences of surface circulation. *Limnol. Oceanogr.* 45: 1039–1051.
- Tester, P. A. and P. K. Fowler. 1990. Brevetoxin contamination of *Mercenaria mercenaria* and *Crassostrea virginica*: a mangagement issue. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.). *Toxic Marine Phytoplankton*. New York: Academic Press. pp. 499–503.
- Tester, P. A., Fowler, P. K., Turner, J. T. 1989. Gulf Stream transport of the toxic red tide dinoflagellate *Ptychodiscus brevis* from Florida to North Carolina. In: Cosper, E.M., Bricelj, V.M., Carpenter, E.J. (Eds.), *Novel Phytoplankton Blooms*. Springer, Berlin. pp. 349-358.
- Tiffany III, W. J., Heyl, M. G. 1978. Invertebrate mass mortality induced by a *Gymnodinium breve* red tide in Gulf of Mexico waters at Sarasota, Florida. *J. Environ. Sci. Heal. A.* 13(9): 653-662.
- Thompson, R. J., Newell, R. I. E., Kennedy, V. S., Mann, R. 1996. Reproductive processes and early development. In: Kennedy, V.S. (Ed.), *The Eastern Oyster Crassostrea virginica*. Maryland Sea Grant College, University of Maryland, College Park, Maryland. pp 335-370.
- Thorson, G. 1946. Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the South (øresund). *Meddr. Kommn. Danm. Fisk.-og Havunders. Ser. Plankton* 4: 1-523.
- Tubaro, A., Dell'Ovo, V., Sosa, S., Florio, C. 2010. Yessotoxins: A toxicological overview. *Toxicon.* 56(2): 163-172.
- Turner, J. T., Tester, P. A. 1997. Toxic marine phytoplankton, zooplankton grazers, and pelagic food webs. *Limnol. Oceanogr.* 42(5): 1203-1214.
- Twarog, B. M., Hidaka, T., Yamaguchi, H. 1972. Resistance to tetrodotoxin and saxitoxin in nerves of bivalve molluscs: a possible correlation with paralytic shellfish poisoning. *Toxicon.* 10(3): 273-278.
- Twiner, M. J., Doucette, G. J., Rasky, A., Huang, X. P., Roth, B. L., Sanguinetti, M. C. 2012. Marine algal toxin azaspiracid is an open-state blocker of HERG potassium channels. *Chem. Res. Toxicol.* 25(9): 1975-1984.
- Twiner, M. J., Hess, P., Dechraoui, M. B., McMahan, T., Samons, M. S., Satake, M., Yasumoto, T., Ramsdell, J. S., Doucette, G. J. 2005. Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines. *Toxicon.* 45(7): 891-900.
- United States Department of Agriculture (2002). *Census of Aquaculture 2005: United States Summary and State Data. Geographic Area Series. Part 51 Volume 3: special studies part 2. AC-02-SP-2. Vol 1.* United States Department of Agriculture. Washington D.C. pp 59-65.
- Vale, P. 2006. Differential dynamics of dinophysistoxins and pectenotoxins, part II: offshore bivalve species. *Toxicon.*, 47(2): 163-173.

- Vargo, G. A. 2009. A brief summary of the physiology and ecology of *Karenia brevis* Davis (G. Hansen and Moestrup comb. nov.) red tides on the West Florida Shelf and of hypotheses posed for their initiation, growth, maintenance, and termination. *Harmful Algae*. 8(4): 573-584.
- Van Deventer, M., Atwood, K., Vargo, G. A., Flewelling, L. J., Landsberg, J. H., Naar, J. P., Stanek, D. 2012. *Karenia brevis* red tides and brevetoxin-contaminated fish: a high risk factor for Florida's scavenging shorebirds?. *Botanica Marina*. 55(1): 31-37.
- Van Dolah, F. M., Lidie, K. B., Monroe, E. A., Bhattacharya, D., Campbell, L., Doucette, G. J., Kamykowski, D. 2009. The Florida red tide dinoflagellate *Karenia brevis*: New insights into cellular and molecular processes underlying bloom dynamics. *Harmful Algae*. 8(4): 562-572.
- Vinagre, T.M., Alciati, J.C., Regoli, F., Bocchetti, R., Yunes, J.S., Bianchini, A., Monserrat, J.M. 2003. Effect of microcystin on ion regulation and antioxidant system in gills of the estuarine crab *Chasmagnathus granulatus* (Decapoda, Grapsidae). *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* 135(1): 67-75.
- Volety, A.K., Savarese, M., Tolley, S.G., Arnold, W.S., Sime, P., Goodman, P., Cham-berlain, R.H., Doering, P.H., 2009. Eastern oysters (*Crassostrea virginica*) as an indicator for restoration of everglades ecosystems. *Ecol. Indic.* 9(6):120–136.
- Volety, A. K. 2008. Effects of salinity, heavy metals and pesticides on health and physiology of oysters in the Caloosahatchee Estuary, Florida. *Ecotoxicol.* 17: 579–590.
- Walker, L. M. 1982. Evidence for a sexual cycle in the Florida red tide dinoflagellate, *Ptychodiscus brevis* (= *Gymnodinium breve*). *Trans. Am. Microbiol. Soc.* 101: 287–293.
- Wang, L., Yan, T., Zhou, M. 2006. Impacts of HAB species *Heterosigma akashiwo* on early development of the scallop *Argopecten irradians* Lamarck. *Aquaculture*. 255: 374-383.
- Ward, J. E., Shumway, S. E. 2004. Separating the grain from the chaff: particle selection in suspension-and deposit-feeding bivalves. *J. Exp. Mar. Biol. Ecol.* 300(1): 83-130.
- Ward, J. E., Levinton, J. S., Shumway, S. E., Cucci, T. 1997. Site of particle selection in a bivalve mollusc. *Nature*. 390(6656): 131-132.
- Ward, J. E., Newell, R. I., Thompson, R. J., MacDonald, B. A. 1994. In vivo studies of suspension-feeding processes in the eastern oyster, *Crassostrea virginica* (Gmelin). *Biol. Bull.* 186(2): 221-240.
- Washburn, B.S.; Baden, D.G.; Gassman, N.J.; Walsh, P.J. 1994. Brevetoxin: Tissue distribution and effect on cytochrome P450 enzymes in fish. *Toxicon*. 32: 799–805.
- Watkins, S. M., Reich, A., Fleming, L. E., Hammond, R. 2008. Neurotoxic shellfish poisoning. *Mar. Drugs*. 6(3): 431–455.
- Wazniak, C., Glibert, P. 2004. Potential impacts of brown tide, *Aureococcus anophagefferens*, on juvenile hard clams, *Mercenaria mercenaria*, in the Coastal Bays of Maryland, USA. *Harmful Algae*. 3: 321-329.
- Weisberg, R. H., Barth, A., Alvera-Azcárate, A., Zheng, L. 2009. A coordinated coastal ocean observing and modeling system for the West Florida Continental Shelf. *Harmful Algae*. 8(4): 585-597.
- Wells, H. W. 1961. The fauna of oyster beds, with special reference to the salinity factor. *Ecol. Monogr.* 31: 239-266.

Introduction

Wilson, W. B. 1967. Forms of the dinoflagellate *Gymnodinium breve* Davis in cultures. Contr. Mar. Sci. Univ. Texas. 13: 120-134.

Yan, T., Zhou, M., Fu, M., Wang, Y., Yu, R., Li, J. 2001. Inhibition of egg hatching success and larvae survival of the scallop, *Chlamys farreri*, associated with exposure to cells and cell fragments of the dinoflagellate *Alexandrium tamarense*. Toxicon. 39: 1239-1244.

Yan, T., Zhou, M., Fu, M., Yu, R., Wang, Y., Li, J. 2003. Effects of the dinoflagellate *Alexandrium tamarense* on early development of the scallop *Argopecten irradians concentricus*. Aquaculture. 217: 167- 178.

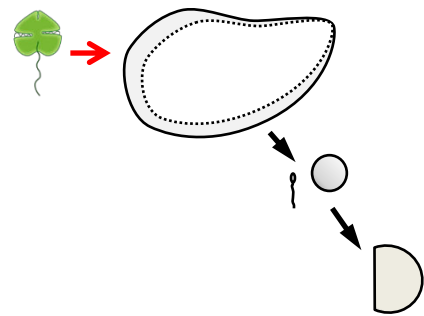
Yonge, C. M. 1960. Oysters, Collins London. pp 209.

Zhang, T., Yang, H. S., Liu, B. Z., Liu, Y., Wen, H. X., Liu, S. L., Zhang, F. S. 2003. Primary study on modes of seed hard clam *Mercenaria mercenaria* (Linnaeus, 1758) culture in field. Oceanologia Et Limnologia Sinica. 34(6): 648-655.

Chapter 1

Exposure of adult *Crassostrea virginica* to *Karenia brevis*: Toxin accumulation, histopathological and cellular responses in adults and, effects on gametogenesis, gamete and larval quality.

The reproductive and physiological effects of exposing adult *C. virginica* to *K. brevis* were studied. Three laboratory experiments were performed; the first two experiments considered these effects independently on separate individuals and the 3rd considered both the reproductive and physiological effects of exposure on the same individuals.



The hypotheses tested were:

- Exposure to *K. brevis* has no effect on the survival or on the reproductive and related physiological processes of adult oysters.
- Adult exposure to *K. brevis* has no effect on the gametes and development of subsequently produced offspring.

Exposure of adult *Crassostrea virginica* to *Karenia brevis*: Toxin accumulation, histopathological and cellular responses in adults and, effects on gametogenesis, gamete and larval quality.

Anne Rolton^{1*,2}, Julien Vignier^{1,2}, Ludovic Donaghy², Sandra Shumway³, Monica Bricelj⁴, Richard Pierce⁵, Michael Henry⁵, Patricia Blum⁵, Aswani Volety^{2,6}, Philippe Soudant¹.

^{*1}Université de Bretagne Occidentale—IUEM, LEMAR CNRS UMR 6539, Place Nicolas Copernic, Technopôle Brest Iroise, 29280 Plouzané, France;

²Florida Gulf Coast University, College of Arts and Sciences, 10501 FGCU Blvd South, Fort Myers, Florida 33965;

³Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340;

⁴Department of Marine and Coastal Sciences and Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349;

⁵The Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, Florida 34236;

⁶Present address: University of North Carolina Wilmington, 601 S. College Rd, Wilmington, NC 28403

*Email: annerolton@gmail.com Phone: +33 298 498 623

Abstract

Blooms of the brevetoxin producing dinoflagellate species, *Karenia brevis*, are a recurrent and sometimes devastating phenomenon in the Gulf of Mexico. The eastern oyster, *Crassostrea virginica*, is exposed regularly during blooms yet, little is known about the effect of *K. brevis* upon this species. In 3 separate experiments, the effects of bloom-type scenarios on reproductive and related physiological parameters of *C. virginica* were studied. The first two relatively short-term (10 days) experiments exposed unripe and ripe adult oysters daily to whole cells of *K. brevis* at high bloom concentrations (1000 and 5000 cells mL⁻¹) and recorded either physiological or reproductive responses. In a 3rd exposure, recently spawned adult oysters were exposed long-term (10 weeks) to a lower bloom concentration (500 cells mL⁻¹), which was designed to induce reproductive conditioning and both physiological and reproductive responses were recorded. High concentrations of brevetoxin were recorded in oyster tissues from all exposures, reaching up to $\sim 22 \times 10^3$ ng g⁻¹ PbTx-3 equivalents in the short-term exposures and $\sim 11 \times 10^3$ ng g⁻¹ in the long-term exposure. Significant histopathological changes were recorded in tissues of exposed oysters in both the short-term and long-term exposures compared to controls. Tissues significantly affected were those in direct contact with *K. brevis* cells and/ or the associated toxins. In the short-term experiment, significant inflammatory responses were recorded in the mantle, gills, connective tissue and stomach/intestine. A corresponding increase in the total number of circulating hemocytes was also recorded suggesting that i) new hemocytes may be moving to sites of tissue inflammation or, ii) are released in the circulatory system from inflamed tissues where they may be produced. Exposed oysters also had less food/debris present in the stomach/intestine but, oysters were not starving. This leads us to consider that toxic algal exposure had modified their feeding; however, this trend was not seen in the long-term exposure. Similarly, to the short-term exposure, increased hemocyte infiltration was recorded in the stomach/intestine of oysters exposed to *K. brevis* for 10 weeks.

In the short-term exposure of ripe oysters, significantly fewer ripe gametes were obtained from oysters (37% and 34% for the 1000 and 5000 cells mL⁻¹ *K. brevis* respectively) compared to the control (64%, p=0.048).

Results demonstrate that eastern oysters are irritated by relatively acute and chronic exposures to *K. brevis*. The reproductive fitness of eastern oysters may be affected by high bloom concentrations of *K. brevis* throughout the gametogenic ripening period. As blooms will likely continue into periods of spawning, the stress of direct exposure of gametes and early stages to *K. brevis* should also be considered in addition to any effects following adult exposure.

Key words: *Crassostrea virginica*, *Karenia brevis*, brevetoxin, inflammation, physiology, reproduction.

1. Introduction

The effect of harmful algal blooms (HAB) contaminating bivalve molluscs destined for human consumption by the accumulation of toxins within their tissues is well documented (e.g. McFarren et al., 1965; Steidinger et al., 1973, 1993, 1998; Shumway 1990; Shumway, 1995; Tester and Fowler, 1990). The effect of HAB of toxic algae directly upon bivalves however, is relatively poorly understood (Landsberg, 2002) yet, filter-feeding shellfish that ingest high concentrations of phytoplankton are at high risk of exposure to toxic algae and their associated toxins (Hégaret and Wikfors, 2005a). The effects of toxic algal exposure on bivalve survival, behavior, metabolism, physiology and reproduction have increasingly been recognized (i.e. Basti et al., 2011; Galimany et al., 2008a,b,c; Haberkorn et al., 2010a,b; Hégaret et al., 2007a; Hégaret and Wikfors, 2005b; Leverone et al., 2006, 2007; Rolton et al., 2014, 2015; Shumway and Cucci, 1987; Shumway and Gainey, 1992; Wikfors and Smolowitz, 1995).

The HAB forming algae, *Karenia brevis*, is a producer of lipophilic brevetoxins (PbTx) and other hemolytic, ichthyotoxic, allelopathic and chemically uncharacterized harmful compounds, found primarily within the Gulf of Mexico (GoM) (Baden et al., 2005; Kubanek et al., 2005, 2007; Neely and Campbell, 2006; Steidinger, 2009). The near-annual occurrence of *K. brevis* blooms, which are most likely to form in autumn and may last weeks to many months (Brand and Compton, 2007; Florida Fish and Wildlife Conservation Commission, FWC, monitoring data), is interspersed with occasional catastrophic bloom events, which have resulted in mass mortalities of marine mammals, birds, fish, crustaceans, bivalve molluscs and other marine invertebrates (Gunter et al., 1948; Finucane, 1964; Flewelling et al., 2005; Tester and Fowler, 1990; Hu et al., 2006; Simon and Dauer, 1972; Stumpf et al., 2003; Summerson and Peterson, 1990; Vargo et al., 2004). In addition, the suggestion that the frequency of *K. brevis* blooms increasing (Brand and Compton, 2007) has highlighted the need for further research into the effects of *K. brevis* exposure on these species. Although mass mortality events caused by severe bloom concentrations of *K. brevis* are well reported, the effects of more typical bloom concentrations of *K. brevis* cells ($\leq 5 \times 10^3$ cells mL⁻¹: FWC, monitoring data; Steidinger, 2009) and their associated toxins on the reproductive and related

physiological processes of important bivalve species in the GoM region are not (Landsberg, 2002).

Filter-feeding bivalves, such as the ecologically and economically important eastern oyster (*Crassostrea virginica*), are found in estuaries and near-shore areas along the south west Florida (SW FL) coast and are regularly exposed to blooms of *K. brevis* (Brand and Compton, 2007; FWC, monitoring data). Eastern oysters accumulate high levels of PbTx as they ingest *K. brevis* cells from the water and accumulated PbTx may be very persistent in the tissues, lasting weeks to months (Baden, 1983; Plakas et al., 2004, 2008; Steidinger et al., 1998). Eliminating PbTx depends on several factors including any continuing exposure to the toxic algae, the concentration of the toxic algae and the bivalves' rates of feeding and elimination (Baden, 1983; Shumway et al., 1990). Reports into the effects of *K. brevis* exposure on *C. virginica* are mixed; Gunter et al. (1948), reported mortalities of eastern oysters exposed in the field to a severe bloom of *K. brevis*; whereas, Taylor et al. (1917) and Steidinger and Ingle (1972) reported that *C. virginica* was unaffected following exposure to *K. brevis* blooms which killed other marine mammal, fish and invertebrate species. Results of short-term laboratory exposures of *C. virginica* to *K. brevis* are also mixed; Sievers (1969) and Ray and Aldrich (1967) reported no effects on the behaviour and pseudofaeces production of adults; whereas, Leverone et al. (2007) exposed juveniles to a mixed diet of *Isochrysis galbana* and *K. brevis* cells at 1000 cells mL⁻¹ for 1 hour and found a 38% reduction in the clearance rate compared with control oysters. However, following a 2 day exposure to 1000 cells mL⁻¹ *K. brevis*, the clearance rate of oysters was no longer significantly affected (Leverone et al., 2007).

Other non-brevetoxin producing species of *Karenia* have been shown to affect physiological processes in bivalves. Smolowitz and Shumway (1997) recorded inflammatory responses in several juvenile bivalve species, including *C. virginica*, exposed daily for 1 week to 100 cells mL⁻¹ of *Karenia mikimotoi* (the toxic mechanism of which has yet to be elucidated). Responses differed between species as exposed oysters showed increased lesions in tissues, which were not observed in other exposed bivalve species (Smolowitz and Shumway, 1997). da Silva et al. (2008) continuously exposed the Manila clam (*Ruditapes philippinarum*) to the gymnodimine producing species, *Karenia selliformis*, at 100 and 1000 cells mL⁻¹ for

6 weeks and recorded reduced hemocyte size compared to control clams. In a shorter experiment, Hégaret et al. (2007b) showed a reduction in hemocyte size and complexity and an increase in the number of circulating hemocytes of *R. philippinarum* exposed to whole cells of *K. selliformis* and *K. mikimotoi* for 6 days. Effects on the hemocytes were attributable to the toxins produced by these algal species (Hégaret et al., 2007b).

Ripe eastern oysters are found in all months in SW FL; however in the Caloosahatchee estuary area, the main spawning period is from March to November, with peak spawning in the spring and autumn (Volety, 2008; Volety et al., 2009). Oysters may be exposed to blooms of *K. brevis* for long periods during reproductive conditioning and spawning. A previous report of near- total recruitment failure of the bay scallop (*Argopecten irradians*) exposed to a *K. brevis* bloom, highlights that exposure to *K. brevis* could cause significant negative effects on the reproductive processes of *C. virginica* (Summerson and Peterson, 1990). Effects of toxic algal exposure on the reproductive and related physiological parameters of bivalves have been demonstrated. Haberkorn et al. (2010 b), showed that a continuous 4- day exposure of ripe adult Pacific oysters, *Crassostrea gigas*, to the paralytic shellfish toxin producing dinoflagellate, *Alexandrium minutum*, at 5×10^3 cells mL⁻¹, significantly reduced the motility and ATP content of obtained sperm. Inflammation in oyster tissues and an increase in circulating hemocytes, were also recorded in the same study, and suggest that physiological changes may also indicate modified reproductive responses (Haberkorn et al., 2010 b). Gametogenesis is suspected to be a period of intensive physiological change, because most of the energy acquired by the bivalve is used for the production of gametes (Soletchnik et al., 1997; Royer et al., 2008). The effect of longer term exposure to bloom concentrations of *K. brevis*, on the reproductive parameters of bivalves have yet to be determined.

This study was designed to identify changes in the reproductive and related physiological parameters of adult *C. virginica* exposed to 2 *K. brevis* bloom scenarios over a short- term (10 day) and also a long- term (10 week) period. Brevetoxin accumulation, condition index, gonadal stage, sex distribution, spawning frequency, histopathological features in oyster tissues and changes in the cellular parameters of spermatozoa obtained from adults were examined. In addition, adult oysters

exposed to *K. brevis* over a 10 day period were examined for changes in hemocyte responses and, the fertilization success and subsequent development of larvae obtained from adult oysters were monitored.

2. Methods

2.1 *Karenia brevis* cultures

The ‘Manasota Key’ strain of the toxic dinoflagellate, *Karenia brevis*, was obtained from the Mote Marine Laboratory, Sarasota, FL. One-litre cultures of *K. brevis* stock were grown according to Rolton et al. (2014). Cultures used for all exposures were grown in 9 L (8 quart) Camwear® Round Containers (Cambro Manufacturing) filled with 3 L of 0.1 µm-carbon filtered and UV-treated seawater (FSW) from Estero Bay, Bonita Springs, FL (26.35, -81.84), which had been autoclaved, allowed to cool, before L1 nutrients (NCMA) and approximately 1 L of 14-day-old *K. brevis* stock culture were added aseptically. Up-scaled 9 L cultures were kept at a salinity of 29 (±2) and temperature of 22°C (±1) on a 12:12 light: dark cycle. The growth and purity of cultures were monitored microscopically. Once stationary phase in the 9 L cultures had been reached and typically at a density of 1×10^4 – 2×10^4 cells mL⁻¹, cultures were used for experiments.

Representative early stationary phase, fourteen-day-old 1 L stock cultures of *K. brevis* were sampled throughout a 2 year period (2011- 2013) for toxin profile analyses by LC-MS/MS (Plakas et al., 2008). Cell counts were between 1.3×10^4 - 3.1×10^4 cells mL⁻¹, cultures were confirmed as toxic and were comparable to other studies (Table 1, Rolton et al., 2014, 2015; Pierce and Henry, 2008; Leverone et al., 2006).

Table 1: Brevetoxin concentrations (pg cell⁻¹) of representative 14- day –old 1 L stock cultures of *Karenia brevis* (Manasota Key strain), harvested over a 2 year period. Nine- litre *K. brevis* cultures used for experiments were grown from these stock cultures. Data are presented as mean ± standard error, $n = 8$.

PbTx conjugate	PbTx- 1	PbTx- 2	PbTx- 3	PbTx- carboxylic acid	Total	Brevetoxin
Concentration (pg cell ⁻¹)	0.80 ± 0.10	9.54 ± 1.13	0.38 ± 0.06	0.13 ± 0.05	10.86 ± 1.19	2.57 ± 0.64

2.2 Experimental design and sampling

Three separate exposures, studying the reproductive and physiological effects of *K. brevis* exposure, were performed.

2.2.1 Effects of short-term exposure on physiological responses

Adult *C. virginica* (mean total oyster fresh weight \pm standard error (SE), 49.1 ± 7.1 g and mean shell length 42.5 ± 2.3 mm), were collected in October 2012 from naturally occurring native populations in Iona Cove, Fort Myers, FL (26.52, -81.98). Low *K. brevis* concentrations (1- 100 cells mL⁻¹) were recorded in this area from the start of October 2012 and cell counts were ~ 600 cells mL⁻¹ at the time of collection (FWC, monitoring data). Oysters were kept in 400 L tanks at the Vester Marine Field Station (VMFS), Florida Gulf Coast University (FGCU) for 7 days, at a salinity of 25, temperature of 23 ± 1 °C. During this time, oysters were fed a mixed diet of shellfish diet 1800® (Reed Mariculture), *Tisochrysis lutea* sp. nov [Bendif and Probert, 2013] (T-Iso, CCMP 1324), *Tetraselmis chui* [Butcher, 1959] (CCMP 882) and *Chaetoceros muelleri* [Lemmermann, 1898] (CCMP 1318) *ad libitum*. Algal species were obtained from the Provasoli-Guillard National Center for Marine Microalgae and Microbiota (NCMA), Bigelow Laboratory, Maine (previously the CCMP). Flagellates T-Iso and *T. chui* were grown at a salinity of 30 in CELL-HI F2P medium based on Guillard F/2 medium (Guillard, 1975; Varicon Aqua) and the diatom *C. muelleri* was grown at a salinity of 24 also in CELL-HI F2P medium with added Si. At the end of the 7 days, oysters contained 638 ± 124 ng g⁻¹ of brevetoxin (PbTx-3 equivalents, mean \pm SE, $n= 3$). Twenty individuals were randomly sampled for CI ($n= 10$) and, GS and histological features ($n= 10$) prior to exposure.

Three replicate tanks were prepared per treatment. Whole cell treatments of stationary-phase *K. brevis* were prepared to reach 1000 cells mL⁻¹ and 5000 cells mL⁻¹ concentration in experimental aquaria tanks. Triplicate cell counts of 9 L cultures were performed using a nanoplankton chamber (PhycoTech Inc.) and aliquots added to the 20 L aquaria tanks to reach the desired final concentration in 10 L total volume. Controls were prepared with no *K. brevis*.

Eleven oysters were distributed per tank in a plastic basket. Temperature was maintained at $24 \pm 1^\circ\text{C}$, salinity at 27, light aeration was supplied in tanks to maintain the dissolved oxygen $>7 \text{ mg L}^{-1}$ and pH levels were maintained at 8 ± 0.5 . Water quality parameters (temperature, dissolved oxygen, pH and salinity) were monitored daily using a Pro ODO optic probe (YSI), 'Pinpoint' pH monitor (American Marine Inc.) and a salinity refractometer (Aquatic ecosystems). Each day, baskets containing oysters were carefully removed from aquaria tanks, and the water and *K. brevis* treatments were changed before replacing the basket containing the oysters. At the start of exposure and each day after the water change, all tanks were fed with the same ration of shellfish diet 1800® (Reed Mariculture) [in addition to any *K. brevis* already present in exposure tanks] at 5% dry weight of algae per dry weight of oyster meat. Dry weight of oyster soft tissue was calculated from total oyster wet weight to tissue dry weight measurements using a linear regression function relating these two variables generated from previously sacrificed individuals ($n= 30$) of the same batch of oysters (data not shown), according to Hégaret et al. (2004). This daily food ration was split into 2 feedings, 4 hours apart to ensure the concentration of algae in the tank did not exceed the 10 mg L^{-1} pseudofeces threshold (Hégaret et al., 2004; May et al., 2010; Morton, 1983). Exposure continued for 10 days.

Following the 10 day exposure, 5 oysters were sampled from each tank for condition index ($n= 5$ per tank). From those sampled for CI, five individuals were randomly selected per treatment to assess total PbTx content ($n= 5$ per treatment). Of the 6 remaining oysters per tank, five were sampled firstly for hemocyte analyses ($n= 5$ per tank) before all were examined for gonadal stage (GS) and histopathological features ($n= 6$ per tank).

2.2.2 Effects of short- term exposure on reproductive responses

Ripe adult *C. virginica* (mean total oyster fresh weight $40.7 \pm 6.2 \text{ g}$ and mean shell length $38.4 \pm 2.2 \text{ mm}$), were collected from naturally occurring native populations in Estero Bay, in September 2012. Oysters were acclimatized for 2 weeks as detailed above before 5 oysters were selected randomly and sacrificed to verify ripeness. These oysters contained $382 \pm 43 \text{ ng g}^{-1}$ PbTx (mean \pm SE, $n= 3$). Prior to exposing oysters to *K. brevis* treatments, 20 individuals were also sampled to assess total condition index (CI, $n= 10$) and gonadal stage (GS, $n= 10$).

Aliquots of whole- cell stationary-phase *K. brevis* were prepared in experimental tanks at the same concentrations as detailed above (section 2.2.1) in a 10 L total volume. Control tanks were also prepared with no *K. brevis* as above and 3 replicate tanks were prepared per treatment. Twelve oysters were distributed per 20 L aquaria tank in a plastic basket and were exposed, fed and maintained as detailed above except, the temperature was maintained at $21 \pm 1^\circ\text{C}$.

Following the 10 day exposure, 3 oysters from each tank were sampled for CI ($n= 3$ per tank). From those sampled for CI, 5 were selected randomly from each treatment to assess total PbTx content ($n= 5$ per treatment). The 9 remaining oysters per tank were strip-spawned to determine the prevalence of fertile gametes and, changes in sperm cell parameters, fertilization success and subsequent larval development for 4 days post-fertilization (PF).

Sex of adult oysters was determined microscopically prior to strip spawning. Keeping individuals separate, the gonads were scored using a scalpel and $0.1 \mu\text{m}$ FSW and both male and female gametes filtered through nitex mesh ($150 \mu\text{m}$). Oocytes were retained on $20 \mu\text{m}$ mesh, sperm were further filtered through $55 \mu\text{m}$ mesh, and subsamples of both were checked for purity. Gamete quality was assessed under the microscope, and individuals with fertile gametes were used for further studies on gamete and larval quality. Oocytes were considered fertile if they were full with a round shape, $\sim 60 \mu\text{m}$ diameter and there was high density of oocytes. Sperm was considered fertile if the sample was dense and contained $> 90\%$ motile sperm. The prevalence of fertile gametes (%) was expressed as the number of individuals in a tank with fertile gametes divided by the total number of individuals in that tank. Sperm were sampled for flow cytometry analysis (described in section 2.3.6).

Fertile gametes of the same sex were pooled per replicate. Washed oocytes were pooled in a sterile 1 L beaker to give a range of 500-2500 oocytes mL^{-1} . Oocytes were allowed to round-up and were fertilized with sperm from the same treatment. Six- thousand oocytes were stocked per glass beaker to give a final concentration of 20 oocytes mL^{-1} in a final volume of 300 mL. Sperm was added to each beaker to reach an oocyte: sperm ratio of 1:300 (Alliegro and Wright, 1983; Song et al., 2009) and mixed gently. Three replicate beakers were prepared per treatment. Glass beakers (600 mL) contained $0.1\mu\text{m}$ FSW at a salinity 27. Beakers were exposed to

natural daylight, but kept away from direct sunlight. Water quality parameters (temperature, dissolved oxygen, pH and salinity) were measured as detailed above and temperature was maintained at $24 \pm 1^\circ\text{C}$. Gentle aeration and daily 'swirling' of the uncovered beakers helped maintain DO levels. One hour after fertilization, at Day 1 and Day 4, 10 mL sub-samples were taken from each beaker and fixed in 0.1% buffered formalin. After sampling at Day 1 and at Day 3, larvae were fed T-Iso and *C. muelleri* according to Rolton et al. (2014). Samples taken 1 hour after fertilization, were assessed for numbers of fertilized (polar body up to 4 cell stage) and unfertilized (no polar body) oocytes. At least 30 oocytes/ embryos were counted per replicate using a light microscope and a Sedgewick Rafter Cell and data are expressed as fertilization success in percent (%). Percent of normal, abnormal (live) and dead embryos/larvae and the mean shell length of 20 live larvae from each replicate, were assessed at Day 1 and 4 according to Rolton et al. (2014).

2.2.3 Effects of long- term exposure on physiological and reproductive responses

In April 2013, broodstock *C. virginica* (mean total oyster fresh weight: 79.7 ± 8.1 g and mean shell length: 104.4 ± 3.9 mm) were collected from naturally occurring native populations in Iona Cove, FL (26.52, -81.98), during a bloom of *K. brevis*. Cell counts were ~ 100 cells mL^{-1} at the time of collection (FWC, monitoring data). Oysters were acclimatized for 1 week prior to experimental exposure as described above (section 2.2.1) and spawned via standard thermal cycling to ensure 'synchronization' of the reproductive stage. Prior to the 'synchronizing' spawn and experimental exposure, oysters contained 668 ± 73 ng g^{-1} brevetoxin (PbTx- 3 equivalents, mean \pm SE, $n = 3$). Thirty oysters were also sampled to assess pathological features within tissues ($n = 30$). Based on the gametes obtained from spawning, sexes were kept separate.

Whole cell treatments of stationary phase *K. brevis* were prepared at 500 cells mL^{-1} concentration in 20 L aquaria tanks. Controls were also prepared with no *K. brevis*. As detailed in section 2.2.1, triplicate cell counts of 9 L cultures were performed and aliquots of culture were added to aquaria tanks to reach the desired final concentration in 17 L total volume.

Seventeen spawned oysters (7 female and 10 male) were distributed per tank in a plastic basket. Temperature at the start of exposure was maintained at $21.5 \pm 0.5^\circ\text{C}$.

To induce ripening of the oysters, after 2 weeks of exposure, the temperature was slowly raised to $26 \pm 0.5^\circ\text{C}$ by week 10. Other water quality parameters were maintained and measured as detailed above. For 10 weeks, daily water (and *K. brevis* treatment) changes and feeding were performed as for the 10 day exposures. The 5% feeding ration of shellfish diet was maintained at the initial amount throughout exposure, regardless of removal of oysters and any mortalities incurred. After 2 and 8 weeks of exposure, 1 oyster per tank (of unknown sex) was randomly sampled for PbTx content ($n= 3$ per treatment). At 10 weeks, 5 oysters were removed per tank for CI ($n= 5$ per tank) and from these, 5 were randomly selected per treatment for PbTx analysis ($n= 5$ per treatment).

After sampling for CI and PbTx at 10 weeks, plastic baskets containing remaining oysters were placed in 400 L black tanks filled with approximately 100 L of $0.1 \mu\text{m}$ FSW at a salinity of 27 and at 18°C and were induced to spawn via standard thermal cycling from 18 to 30°C . Obtained gametes were filtered through nitex mesh as detailed above (section 2.2.2) and kept separate. Fertile sperm were sampled for flow cytometry analyses (section 2.3.6). The spawning frequency (%) was determined as the number of individuals that spawned divided by the total number of individuals put to spawn in that treatment. No females spawned so subsequent changes in fertilization success and larval development could not be determined.

Histopathological features, sex distribution and GS were determined for all of the oysters that spawned and those that did not, combined. As mortalities were incurred during exposure, the resulting number of oysters put-to-spawn and sampled for histopathological, sex and GS parameters were different between replicates. Control replicates 1, 2 and 3 contained 6, 6 and 3 oysters respectively, and the 500cells mL^{-1} treatment replicates 1, 2 and 3 contained 9, 6 and 7 oysters respectively.

2.3 Measurements

2.3.1 Condition index

Whole soft tissue wet weight (g) / shell dry weight (g) x 100 was used to determine the condition index of individuals according to Delaporte et al. (2005) and Lassudrie et al. (2014).

2.3.2 *Brevetoxin content of Crassostrea virginica*

Whole tissues of shucked oysters were frozen at -80°C until extraction. Individual tissues were homogenized under liquid nitrogen using a Laboratory Mixer Mill MM400 (Retsch). One gram aliquots were taken from homogenized whole tissue, mixed with 80% methanol and incubated at 60°C for 20 minutes before transfer on ice for 10 min (Griffith et al., 2013). Samples were then centrifuged for 10 min at 3000 g and the supernatant extract saved. The process was repeated and extracts were combined for each sample before being washed in hexane and stored at -20°C (Griffith et al., 2013). Extracted samples were analyzed within 7 days for brevetoxin content using a competitive ELISA (Naar et al., 2002) and following the method of Griffith et al. (2013). The antibody used in the ELISA is directed against the PbTx-2 backbone and measures any conjugates with this backbone structure (Naar et al., 2002). Brevetoxin concentration is expressed as ng g⁻¹ of PbTx-3 equivalents.

2.3.3 *Characterisation of hemolymph*

A 3 mL syringe equipped with a 25-G needle was used to withdraw ~1.5 mL hemolymph from each oyster. Purity of collected hemolymph was examined microscopically and then filtered through a 41 µm mesh and held on ice to prevent clumping of cells. The total number of circulating hemocytes, size, complexity, mortality, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) production, lysosomal content and the phagocytic capacity of hemocytes were assessed using protocols adapted from Delaporte et al. (2003) Donaghy et al. (2012); Donaghy and Volety (2011); Hégaret et al. (2003a, b) and Soudant et al. (2004). Analyses were performed on individuals using a Cytomics FC500 flow cytometer (Beckman Coulter), equipped with a 488 nm Argon laser and data were analysed using WinMDI 2.9 software.

2.3.4 *Histopathological features*

Soft tissues were carefully removed from shucked oysters and two ~ 4mm cross-sections of tissue which included gills, mantle, stomach/ intestine, digestive diverticula, connective tissue and gonad were cut. Dissected tissues were placed in individual cassettes and fixed in Davidson's fixative (Shaw and Battle, 1957) at 4°C.

After 7 days, the cassettes were moved to 70% ethanol for at least 24 hours, following which, tissues were embedded in paraffin. Six μm tissue sections were cut, mounted on glass slides, and stained with Harris' Hematoxylin and Eosin (Martoja and Martoja-Pierson, 1967; Humason, 1979; Howard et al., 2004). Slides were examined under a light microscope to determine pathological and non-pathological features in tissues.

Pathological features observed in tissues such as hemocyte infiltration, occlusion of hemolymph vessels and edema and a non-pathological feature (the presence of food/ debris in the stomach/ intestine), were recorded as '0' for absent or '1' when present. Presence or absence of features were converted to percent presence of feature (%) by counting the number of individuals within each replicate showing a particular feature and dividing by the number of individuals inspected for that feature within that replicate x 100.

The average sum of pathological features per tissue (i.e. mantle, gills, stomach/ intestine, connective tissue) per oyster was also assessed by adding the number of pathological features in each tissue for all individuals per replicate and dividing by the number of individuals within that replicate. The presence of food/ debris in the stomach/ intestine was excluded from these calculations

2.3.5 Gonadal stage (GS)

The sex and gonadal stage (GS) of adult oysters was determined from prepared histological sections. The GS was determined according to Fisher et al (1996, adapted from the International Mussel Watch Program, 1980) and data are presented as the mean GS per tank.

2.3.6 Flow cytometric analysis of sperm

Fertile sperm from individuals [from stripped oysters in the short-term exposure and thermally spawned oysters in the long- term exposure] were kept separate and the concentration was adjusted to have a final concentration of approximately 300-500 sperm μL^{-1} per individual. Sperm morphology, viability, relative DNA content, MMP and ROS production were determined using protocols modified from Delaporte et al.

(2003) and Hégaret et al. (2003a,b) (using SYBR-14 instead of SYBR-green at a final concentration of 100 nM) and Donaghy and Volety (2011). Analyses were performed on a Cytomics FC500 flow cytometer (Beckman Coulter), equipped with a 488 nm Argon laser. Collected data were analysed using WinMDI 2.9 software.

2.4 Statistical analysis

Analyses were performed keeping tank or beaker replication ($n= 3$) where possible. Adult oyster PbTx content and spermatozoa data obtained on the flow cytometer, were combined from the 3 tank replicates. Results for percent presence of pathological or non-pathological feature (%), prevalence of fertile gametes (%), spawning frequency (%), sex of individuals (%), adult mortalities incurred (%), percent data recorded by the cytometer, fertilization success (%) and percent of larval normality, abnormality and mortality, were arcsine square root transformed to meet normality assumptions. A one-way ANOVA with either Tukeys multiple comparison post hoc test (Levene's test, $p > 0.05$), or Dunnetts T3 post-hoc test (Levene's test, $p < 0.05$), were performed on transformed percent data and, for the average sum of pathological features per tissue per oyster, CI, GS, hemocyte data and mean larval size using treatment as an independent variable. A Pearson chi-square test was used to compare the presence of pathological and non-pathological features between the start and end of exposure for controls. Independent t- tests were used to compare CI and GS at day 10 to day 0 and to compare the PbTx content between short- term experiments at the same concentration.

The statistical software program SPSS 22 was used to compare differences between treatments. Results were considered significant when p-value was < 0.05 .

3. Results

3.1 Effects of short- term exposure on physiological responses

Oysters contained $691 \pm 130 \text{ ng g}^{-1}$ (mean \pm SE) after 10 days in the controls (Figure 1 A). Following 10 days of exposure to $1000 \text{ cells mL}^{-1}$ *K. brevis*, oysters

accumulated $5.5 \times 10^3 \pm 512 \text{ ng g}^{-1}$ and those exposed to the highest *K. brevis* concentration contained $22.0 \times 10^3 \pm 2.3 \times 10^3 \text{ ng g}^{-1}$ (Figure 1 A).

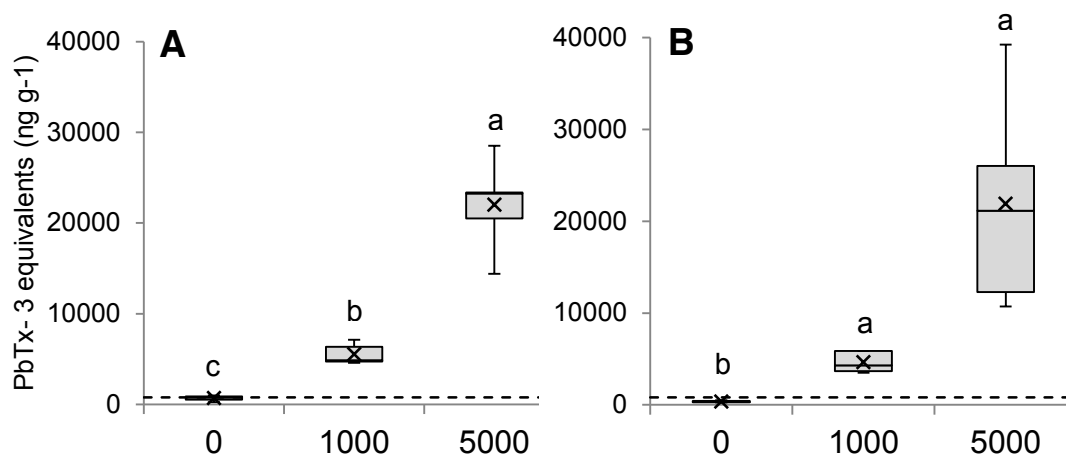


Figure 1: PbTx (as measured by brevetoxin ELISA) in whole homogenized tissue of *Crassostrea virginica* after a 10 day exposure to whole cells of *Karenia brevis* at 0, 1000 and 5000 cells mL⁻¹ studying; **A**: physiological responses and **B**: reproductive responses. The dotted line represents the threshold of 800 ng g⁻¹ brevetoxin at which shellfish beds are closed. The top and bottom of boxes represent the interquartile range, with the median line within. Bars are the maximum and minimum values and 'x' represents the mean, $n = 5$. Treatments with the same letter were not significantly different ($p > 0.05$).

There was no significant difference in the CI of oysters prior to exposure (18.0 ± 1.2 , mean \pm SE) compared to controls 10 days later (15.3 ± 1.2) or after a 10 day exposure to *K. brevis* treatments (16.7 ± 2.1 for 1000 and 17.1 ± 0.9 for 5000 cells mL⁻¹ concentrations respectively). Similarly, the GS of oysters remained unchanged from day 0 (1.8 ± 0.3) in any of the treatments after 10 days (between 1.7- 1.8).

Prior to exposure to *K. brevis*, oysters had a low percent presence of pathological features (0 -30% in the mantle, gill, stomach/ intestine and connective tissue), which remained low in the control after 10 days (Pearson Chi Square $p > 0.05$). As shown in Figures 2 and 3 and Table 2, *K. brevis* exposed oysters showed significant Pathological and non-pathological changes in the mantle, gills, stomach/ intestine and connective tissue. Significant pathological features observed were; edema in the mantle (Figures 2 A and 3 D), the presence of hemocytes in the water tubules of the gill and mucus and hemocytes between the gills (Figures 2 B and 3 A), and occlusion of hemolymph vessels in the connective tissue (Figure 2 D). Hemocyte infiltration in

the mantle ($p= 0.069$) and stomach/ intestine ($p= 0.051$) were also observed (Figures 2 A, C and 3 E). The average sum of pathological features per oyster recorded in the mantle, gill, stomach/ intestine and connective tissue, were significantly higher in oysters exposed to *K. brevis* than in controls (Table 2). All of the oysters observed in the controls, showed the presence of food/ debris within the stomach/ intestine, compared to 61% in the treatment exposed to the lower *K. brevis* concentration and 39% in the higher *K. brevis* treatment (Figure 2 C, $p < 0.001$).

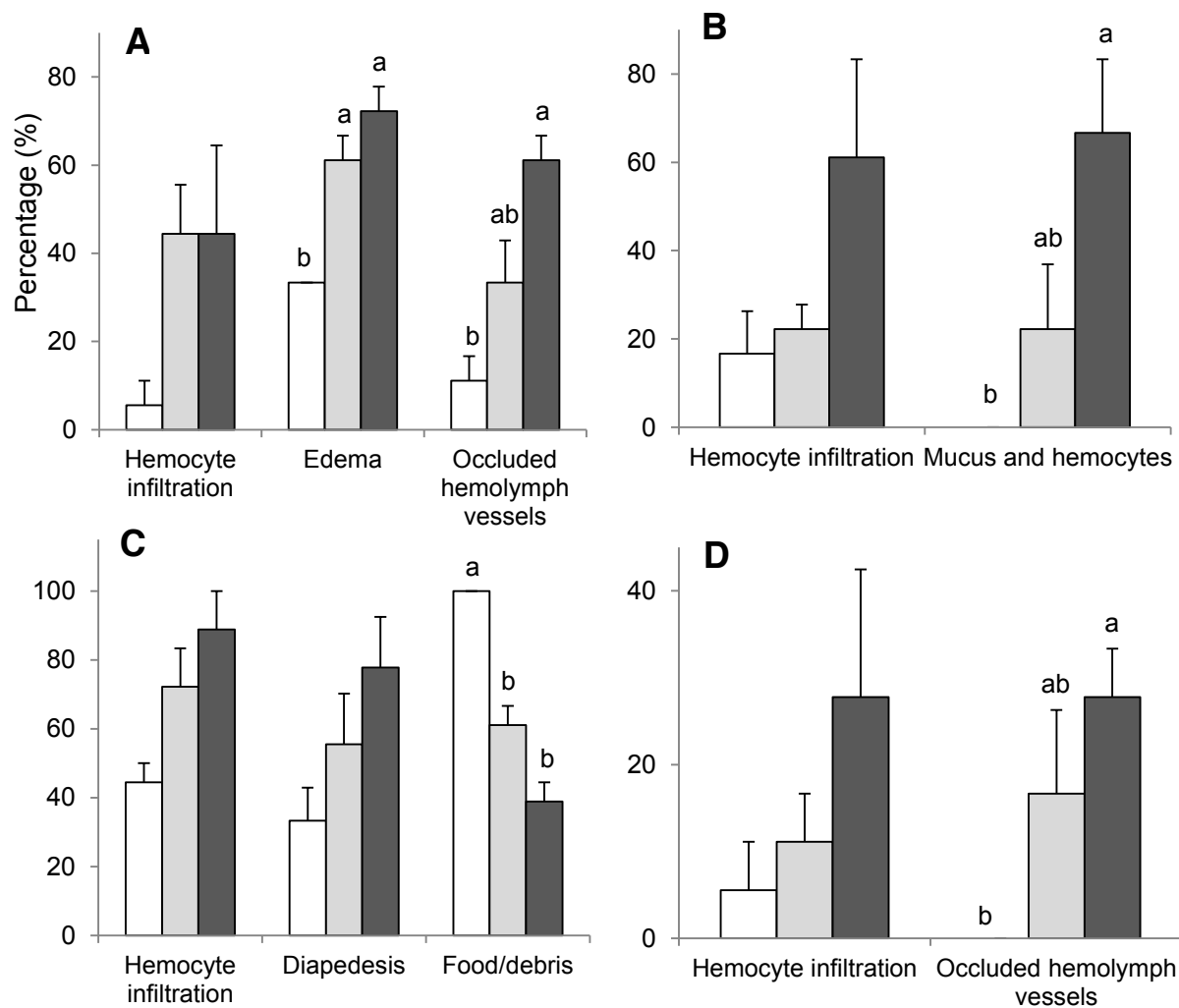


Figure 2: Percent of pathological and non-pathological features (%) in tissues of *Crassostrea virginica* exposed daily to whole cells of *Karenia brevis* at 1000 \square and 5000 \blacksquare cells mL^{-1} and a control \square with no *K. brevis* for 10 days. Features were observed in the **A**: mantle; **B**: gill; **C**: stomach/ intestine; **D**: connective tissue. Treatments with the same letter were not significantly different ($p > 0.05$). Data are presented as mean \pm standard error, $n = 3$.

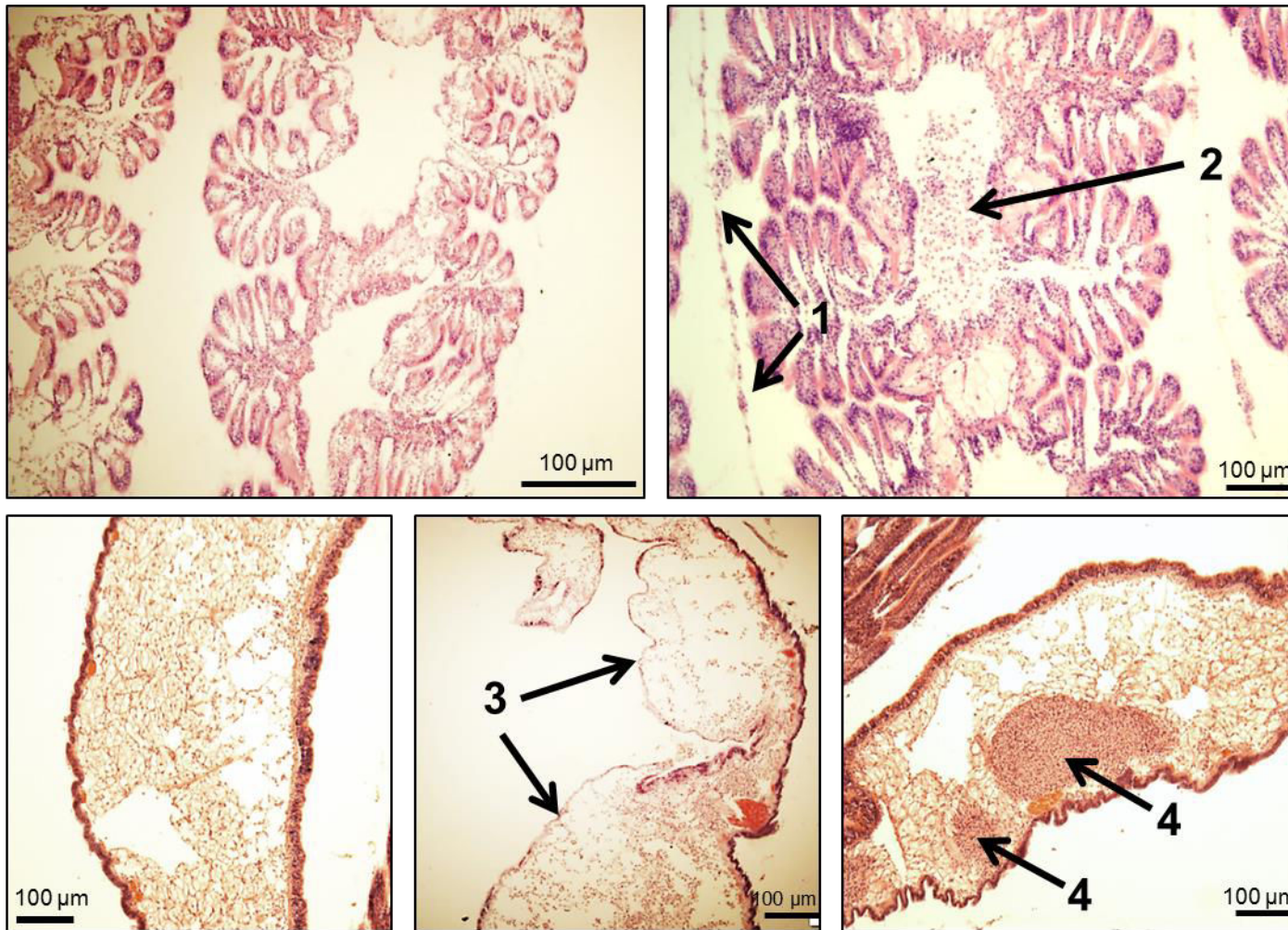


Figure 3: Examples of histopathological features recorded in *Crassostrea virginica* exposed to *Karenia brevis*. **A**: Normal gill, **B**: gill of exposed oyster showing hemocytes within the water tubules (1) and mucus and hemocytes between the gill (2), **C**: normal mantle, **D**: edema of the mantle (3) and **E**: occluded hemolymph vessels (4) in the mantle of an exposed oyster.

Table 2: The average sum of pathological features per individual in different tissues of *Crassostrea virginica* exposed for 10 days to 0, 1000 and 5000 cells mL⁻¹ *Karenia brevis*. For each tissue, treatments with the same letter were not significantly different ($p > 0.05$). Significance levels: ** $p < 0.01$, * $p < 0.05$. Data are presented as mean \pm standard error, $n = 3$.

	0	1000	5000	Significance
Mantle	0.50 \pm 0.10 ^b	1.39 \pm 0.20 ^a	1.78 \pm 0.20 ^a	**
Gill	0.17 \pm 0.10 ^b	0.44 \pm 0.20 ^b	1.28 \pm 0.20 ^a	**
Stomach/ intestine	0.78 \pm 0.11 ^b	1.28 \pm 0.06 ^{ab}	1.67 \pm 0.25 ^a	*
Connective Tissue	0.06 \pm 0.06 ^b	0.28 \pm 0.06 ^{ab}	0.56 \pm 0.15 ^a	*

The total number of circulating hemocytes significantly increased in the oysters exposed to the 1000 and 5000 cells mL⁻¹ *K. brevis* treatments compared to controls ($p = 0.023$, Table 3). Two hemocyte populations were identified on the flow cytometer: hyalinocytes and granulocytes. Granulocytes of oysters exposed to the 1000 cells mL⁻¹ *K. brevis* had a significantly lower lysosomal content than oysters exposed to the 5000 cells mL⁻¹ treatment ($p = 0.041$, Table 3).

Table 3: Hemocyte responses of *Crassostrea virginica* exposed to whole cells of *Karenia brevis* (at 1000 and 5000 cells mL⁻¹) for 10 days. Significance levels: NS= not- significant, * p < 0.05. Treatments with the same letter were not significantly different (p > 0.05). Data are presented as mean ± standard error, n = 3.

		0	1000	5000	Significance
Total circulating hemocytes (cells mL ⁻¹)		1.7 x 10 ⁵ ± 1.9 x 10 ⁴ ^b	3.5 x 10 ⁵ ± 3.8 x 10 ⁴ ^a	3.5 x 10 ⁵ ± 5.3 x 10 ⁴ ^a	*
Mortality %		7.0 ± 1.0	9.1 ± 1.4	9.5 ± 1.2	NS
Phagocytosis %		19.1 ± 2.8	17.9 ± 0.5	18.1 ± 0.8	NS
Hyalinocytes	Size	157.0 ± 0.4	156.6 ± 0.7	157.6 ± 1.6	NS
	Complexity	28.2 ± 1.5	25.8 ± 0.6	24.6 ± 0.6	NS
	ROS production	73.8 ± 4.9	69.5 ± 4.5	68.6 ± 4.2	NS
	Mitochondrial membrane potential	0.4 ± 0.03	0.5 ± 0.1	0.3 ± 0.01	NS
	Lysosomal content	96.0 ± 11.1	86.5 ± 5.5	95.7 ± 2.6	NS
Granulocytes	Size	128.0 ± 2.2	133.3 ± 5.2	133.4 ± 2.0	NS
	Complexity	432.1 ± 12.1	442.4 ± 17.6	430.1 ± 11.3	NS
	ROS production	355.6 ± 22.0	375.0 ± 11.5	394.5 ± 38.1	NS
	Mitochondrial membrane potential	0.4 ± 0.03	0.3 ± 0.04	0.6 ± 0.1	NS
	Lysosomal content	428.9 ± 10.4 ^{ab}	402.6 ± 20.6 ^b	490.4 ± 23.0 ^a	*
	% of total hemocytes	65.6 ± 4.8	68.1 ± 4.2	71.9 ± 2.9	NS

3.2 Effects of short- term exposure on reproductive responses

Ripe oysters contained $354 \pm 46 \text{ ng g}^{-1}$ after 10 days in the controls and accumulated high levels of PbTx following a 10 day exposure to *K. brevis* (Figure 1 B). Following 10 days of exposure to $1000 \text{ cells mL}^{-1}$ *K. brevis*, oysters contained $4.65 \times 10^3 \pm 516 \text{ ng g}^{-1}$ and those exposed to the highest concentration contained $21.9 \times 10^3 \pm 5.2 \times 10^3 \text{ ng g}^{-1}$ (Figure 1 B). Oysters from both short- term exposures accumulated similar amounts of PbTx ($p > 0.05$) at 1000 and 5000 cells mL^{-1} concentrations. There were no mortalities during exposure.

The CI was 16.0 ± 1.3 (mean \pm SE) at day 0, and did not change significantly in the control at day 10 (16.9 ± 0.5) or between any of the treatments (13.8 ± 0.8 in the 1000 cells mL^{-1} treatment and 15.2 ± 0.7 in the 5000 cells mL^{-1} treatment). Oysters were considered ripe at the start of exposure (GS was 4.4 ± 0.4) and ripe gametes were obtained at the end of the 10 days. Oysters exposed to the 5000 cells mL^{-1} treatment had a significantly lower ripeness frequency than in the control ($p = 0.048$, $n = 3$, Table 4). Sixty-three percent of oysters stripped in the control treatment had ripe gametes, compared to 37% of oysters in the lower *K. brevis* treatment and 34% in the higher 5000 cells mL^{-1} *K. brevis* treatment (Table 4). Flow cytometric analyses of obtained sperm from individuals however, revealed no significant differences in the morphology, viability, DNA content, mitochondrial membrane potential or ROS production and, no significant differences were recorded in fertilization success and subsequent larval development (up to 4 days post- fertilization, PF) between oysters exposed to *K. brevis* treatments and those in the controls (Table 4).

Table 4: The ripeness frequency (%) of adult *Crassostrea virginica*, differences in fertilization success (%), mean larval size (μm) and normality, abnormality and mortality (%) of obtained gametes and larvae from adult *C. virginica* exposed to 0, 1000 and 5000 cells mL^{-1} *K. brevis* for 10 days. Significance levels: NS= not- significant, * $p < 0.05$. Data are presented as mean \pm standard error, $n=3$.

		0	1000	5000	Significance
Ripeness frequency (%)		63.0 \pm 9.8	37.0 \pm 3.7	34.2 \pm 5.6	*
Fertilization success (%)		94.0 \pm 1.2	95.2 \pm 0.9	94.7 \pm 0.4	NS
Size (μm)	Day 1	69.5 \pm 0.3	69.3 \pm 0.4	69.8 \pm 0.2	NS
	Day 4	80.0 \pm 0.1	76.6 \pm 1.0	79.8 \pm 0.4	NS
Day 1	normality (%)	93.7 \pm 2.3	82.0 \pm 1.3	96.7 \pm 0.6	NS
	abnormality (%)	4.6 \pm 2.1	14.1 \pm 1.8	2.1 \pm 0.5	NS
	mortality (%)	1.7 \pm 0.2	3.9 \pm 0.9	1.2 \pm 0.3	NS
Day 4	normality (%)	94.8 \pm 1.1	90.7 \pm 3.5	97.1 \pm 1.1	NS
	abnormality (%)	1.0 \pm 0.2	5.0 \pm 1.5	1.3 \pm 0.7	NS
	mortality (%)	4.2 \pm 1.0	4.3 \pm 2.1	1.6 \pm 0.5	NS

3.3 Effects of long- term exposure on physiological and reproductive responses

Oysters accumulated high levels of brevetoxin within 2 weeks of exposure to 500 cells mL^{-1} *K. brevis* (mean $3.7 \times 10^3 \pm 832 \text{ ng g}^{-1}$). Brevetoxin concentrations continued to rise until the end of exposure reaching $7.6 \times 10^3 \pm 476 \text{ ng ng}^{-1}$ at week 8 and $10.8 \times 10^3 \pm 236 \text{ ng g}^{-1}$ by week 10 (significantly more than at day 0, $p < 0.05$, Figure 4). Oysters in the non-exposed treatment maintained similar levels of brevetoxin throughout the 10 week exposure (805- 1012 ng g^{-1} PbTx, $p > 0.05$, Figure 4).

At week 10, there was no difference in the CI and GS between controls (CI: 14.1 ± 0.8 and GS: 3.6 ± 0.6) and exposed (CI: 13.6 ± 0.6 and GS: 2.9 ± 0.1) treatments. A total of 15 oysters in the controls and 8 in the treatments exposed to 500 cells mL^{-1} *K. brevis* died between week 3 and week 9 of exposure; however, differences in mortalities between the exposed and non-exposed treatments were not significant ($p= 0.157$, Table 5).

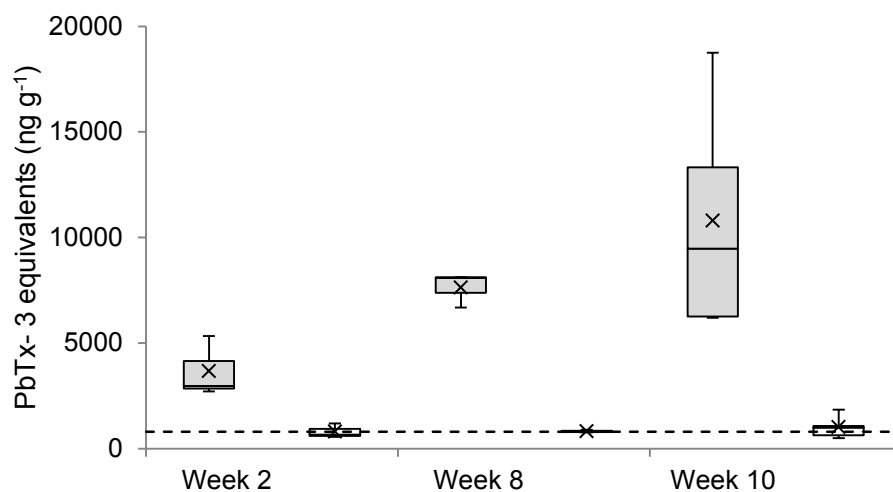


Figure 4: PbTx in whole *Crassostrea virginica* tissue after 2, 8 and 10 weeks of exposure to whole cells of *Karenia brevis* at 500 cells mL⁻¹ (grey boxes) and those that remained unexposed throughout the same time (white boxes). The dotted line represents the threshold of 8000 ng g⁻¹ PbTx at which shellfish beds are closed. The top and bottom of boxes represent the interquartile range, with the median line within. Bars are the maximum and minimum values and 'x' represents the mean. For weeks 2 and 8, n= 3; for week 10, n= 5.

Table 5: The mortality (%), sex (%) and spawning frequency (%) of adult *Crassostrea virginica* exposed to 0 and 500 cells mL⁻¹ *K. brevis* for 10 weeks. Data are presented as mean ± standard error, n= 3. NS= not- significant.

		0	500	Significance
Mortality (%)		33.3 ± 6.7	17.8 ± 5.9	NS
Sex	Female	44.4 ± 5.6	25.1 ± 12.5	NS
	Male	50.0 ± 9.6	62.7 ± 6.5	
	Hermaphrodite	0 ± 0	3.7 ± 3.7	
	Undetermined	5.5 ± 5.5	8.5 ± 4.3	
Spawning frequency (%)		44.4 ± 14.7	23.3 ± 5.5	NS

Prior to *K. brevis* exposure, there was 37% hemocyte infiltration and nearly 100% prevalence of food in the stomach/ intestine and; a 33% presence of edema and 50% presence of occluded hemolymph vessels in the mantle. The presence of all other recorded pathological features was low (< 20%). After 10 weeks of exposure, oysters in control treatments showed a significant increase in the presence of diapodesis and hemocyte infiltration in the stomach/ intestine and a significant

decrease in presence of food/ debris in the stomach/ intestine, and in the presence of edema and occluded hemolymph vessels in the mantle compared to oysters prior to exposure (Pearson chi square, $p < 0.05$). Oysters exposed to $500 \text{ cells mL}^{-1}$ *K. brevis* for 10 weeks showed a significantly higher percent presence of hemocyte infiltration in the stomach/ intestine (94%) than in control oysters (56%), ($p = 0.031$, Figure 5).

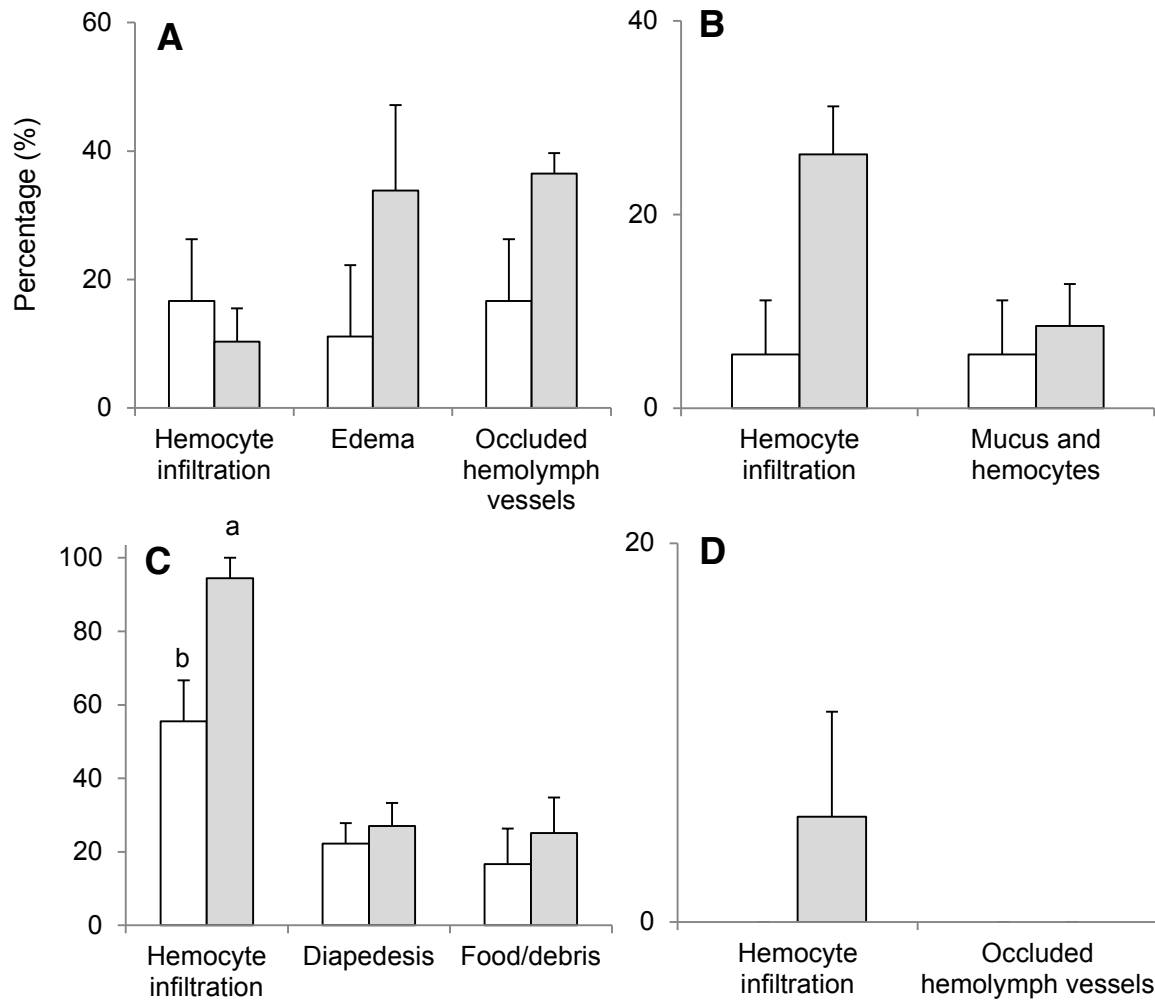


Figure 5: Presence of pathological and non-pathological features (%) in tissues of *Crassostrea virginica* exposed daily to whole cells of *Karenia brevis* at 500 cells mL⁻¹ (■) and a control with no *K. brevis* (□) for 10 weeks. **A:** Mantle; **B:** gill; **C:** stomach/ intestine; **D:** connective tissue. Letters indicate a significant difference between treatments ($p < 0.05$). Data are presented as mean \pm standard error, $n = 3$.

At the start of exposure, 10 male and 7 female oysters were stocked per tank, equating to 59% and 41% male and female respectively. After 10 weeks, remaining oysters (after mortalities and removal of $n=2$ oysters of unknown sex per tank) in the control treatment contained 50.0% male, 44.4% female, 5.5% undetermined sex and 0% hermaphrodite. Oysters exposed to 500 cells mL⁻¹ *K. brevis* contained 62.7% male, 25.1% female, 8.5% undetermined sex and 3.7% hermaphrodite (Table 5, $p > 0.05$). Forty-four percent of oysters in the control treatments spawned after 10 weeks exposure, compared with 23% in the exposed (Table 5, $p = 0.263$). As only male oysters spawned in both treatments, further analysis on fertilization and larval development were not possible. Flow cytometric analyses of obtained sperm from individuals revealed no significant differences in the morphology, viability, DNA content, mitochondrial membrane potential or ROS production between treatments.

4. Discussion

4.1 Toxin accumulation

Following short- and long- term exposure to whole- cells of *K. brevis*, oysters accumulated high levels of brevetoxin comparable with other laboratory exposures (Griffith et al, 2013; Plakas et al, 2002, 2004). Following 10 days of exposure of both ripe and unripe *C. virginica* to 5000 cells mL⁻¹ *K. brevis*, the PbTx concentration in oyster tissues was $\sim 22.0 \times 10^3$ ng g⁻¹, nearly 30 times the regulatory limit at which shellfish beds are closed for harvesting (800 ng g⁻¹, NSSP, 2011). This may reflect oysters having low sensitivity to PbTx, resulting in the potential for high toxin accumulation. In an *in vitro* assay, bivalve species which showed low nerve sensitivity to saxitoxin (STX), accumulated more STX due to low binding characteristics of this toxin on the voltage sensitive sodium channel (Twarog et al., 1972). Ripe and unripe oysters in the short- term exposure accumulated similar levels of PbTx in both 1000 and 5000 cells mL⁻¹ treatments. These oysters may have had similar feeding activities, and accumulated the same amount of PbTx. Brevetoxin concentrations were also highly variable between individuals both after 10 days exposure to the higher 5000 cells mL⁻¹ *K. brevis* and after 10 weeks exposure to the lower 500 cells mL⁻¹ concentration. This may reflect individual variation in filtration and feeding activities. Indeed, the amount of marine biotoxin

accumulated by bivalves is depends on several factors including, the concentration of toxic algae, cell toxicity, the duration of exposure, bivalve filtration, condition, population differences and even variation between individual bivalves from the same population (Bricelj et al., 2005, 2012; Bricelj and Shumway, 1998; Haberkorn et al., 2010a, 2011).

Control oysters in the short- term exposures contained low levels of PbTx. Similarly to the short-term exposure recording reproductive responses, Griffith et al. (2013) found that PbTx was present in oysters collected from the field in SW FL when no *K. brevis* was recorded in the area. Cells of *K. brevis* are ubiquitous in the GoM region below background levels of 1 cell mL⁻¹ (Finucane, 1964), so oysters may be frequently exposed to some level of toxin and never fully depurate. Brevetoxin depuration times in bivalves are variable and may be very persistent (Dickey et al., 1999; Griffith et al., 2013; Plakas et al., 2004, 2008; Steidinger and Ingle, 1972; Volety et al, unpublished results). Brevetoxin was detected in oyster tissues up to 8 months after the dissipation of a *K. brevis* bloom (Plakas et al., 2008). Indeed, the PbTx concentration did not appear to decline in the control oysters over the 10 day or during the 10 week exposures and is likely due to the persistence of PbTx within tissues. The apparent lack of depuration as measured using a competitive ELISA (which detects brevetoxins with the PbTx-2 backbone structure) does not account for any metabolic conversions likely taking place. Shellfish depurate PbTx through different metabolic pathways, producing metabolites that have variable levels of toxicity (Baden et al., 2005; Echevarria et al., 2012; Plakas et al., 2004, 2002; Poli et al., 2000; Wang et al., 2004). Previous studies indicate that parent toxins PbTx-1 and -2 are rapidly metabolised into PbTx-3 and a suite of cysteine, peptide and fatty acid-amino acid conjugates and other hydrolysis products (Plakas et al., 2002, 2004, 2008; Wang et al., 2004). Although the peptide conjugates are thought to be eliminated from the oyster relatively quickly (within days to a couple of weeks), other products, notably the cysteine conjugates, are very persistent in the oyster (Plakas et al., 2004, 2008). The persistent PbTx- 2 cysteine conjugate (m/z 1018) and PbTx-2 cysteine sulphate conjugate (m/z 1034) are the principal PbTx metabolites found in *C. virginica* tissue (Plakas et al., 2004, 2008) and would be detected with the ELISA assay. Toxin depuration is influenced by several factors, including food availability (Medhioub et al., 2012). The presence of food and debris in the digestive gland

decreased significantly in the 10 week exposure, from 97% at day 0 to 17% and 25% in control and oysters exposed to 500 cells mL⁻¹ *K. brevis* respectively. This apparent reduction in food intake may have slowed the elimination of PbTx.

4.2 Pathological impacts

For both short- and long- term exposures, significant differences in pathological and non-pathological features were recorded in the tissues of *K. brevis*-exposed oysters, which were overall dose- dependent. Oysters exposed to 5000 *K. brevis* cells mL⁻¹ for 10 days showed histological changes in several tissues, whereas, those exposed to 1000 cells mL⁻¹ for the same period showed comparatively fewer changes and the mantle was the only tissue significantly impacted. Histopathology results from the long- term exposure, in which oysters were exposed to 500 cells mL⁻¹ are more difficult to interpret because significant pathological increases were also recorded in the control at week 10 compared to the start of exposure; notably in the stomach/ intestine. Long-term laboratory exposure studies are often compromised by the difficulty in keeping experimental animals under optimal nutritional conditions (Barber, 2004). Indeed, mortalities recorded in both control and exposed treatments between 3 and 9 weeks exposure indicate oysters were additionally stressed by the experimental conditions.

Effects may have been caused by contact with the *K. brevis* cell / associated toxins (including PbTx) and/ or PbTx (and other toxins?) accumulated within tissues. Both *K. brevis* and PbTx have been shown to cause negative effects *in vivo* and *in vitro* and it is possible that one or both are involved in the recorded negative effects herein. Indeed, in addition to intracellular PbTx, cells of *K. brevis* produce ichthyotoxic, allelopathic and hemolytic compounds and may also produce other as-yet uncharacterized bioactive compounds which could elicit deleterious effects in oyster tissues (Marshall et al., 2005; Kubanek et al., 2005, 2007; Prince et al., 2010; Tatters et al., 2010; Neeley and Campbell, 2006). Although PbTx- 2 was shown not to be cytotoxic following *in vitro* exposure of *Crassostrea gigas* hemocytes (Mello et al., 2012), PbTx-1 was ichthyotoxic for Medaka fish (*Oryzias latipes*) embryos (Kimm- Brinson and Ramsdell, 2001), demonstrating that brevetoxin alone may cause deleterious impacts. Following a 2 week exposure of the bay scallop (*Argopecten irradians*) to whole and lysed- cell preparations of *K. brevis* at 500 cells

mL⁻¹, Leverone (2007) recorded a higher incidence of hemocytic infiltrations in the digestive diverticula in individuals either starved or exposed to lysed culture of *K. brevis*. This suggested a cytotoxic response rather than a reaction to the *K. brevis* cells.

The tissues in which most histopathological changes were recorded in the present study, were those that came into direct contact with *K. brevis* cells; i.e. external tissues (mantle and gills) and internal tissues (stomach/ intestine). This suggests that direct contact with *K. brevis* cells and/or its associated toxins caused the observed effects. Brevetoxin is intracellular, however as cultures containing whole-cells of *K. brevis* were added to treatment tanks, external oyster tissues may have been in contact with the free PbTx in the culture supernatant (from cells naturally lysing during culture growth, Pierce et al., 2001, 2008). Other bioactive compounds produced by *K. brevis* either in culture medium or associated with the outside of the algal cell, may also have caused impacts in these external tissues. The increased production of mucus (along with hemocytes) in the gill recorded in the 10 day exposure may be a reaction to contact with toxins in the culture supernatant and/ or those associated with the outside of the algal cell. Mucus has lytic enzymes that can act against dissolved organic compounds and/or particles suspended in seawater and play an important role in defense (Galimany et al., 2008 b; Shumway and Cucci, 1987; Fisher, 1992; Brun et al., 2000). Rotifer (*Brachionus plicatilis*) mortalities were recorded following contact with cells of the similar *Karenia* species, *K. mikimotoi* (Zou et al., 2010). Harmful compounds located within the surrounding fatty acid layer outside of the *K. mikimotoi* cell were responsible for the ichthyotoxic effects (Parrish et al., 1998). Once ingested, the *K. brevis* cells lyse during digestion and the stomach/ intestine may therefore be exposed to additional PbTx.

Inflammatory responses accounted for most of the histopathological changes recorded in the present study and suggest *K. brevis* cells and/or associated toxins are causing hemocyte-related responses. Toxic algae are known to cause inflammatory responses in many bivalve species (e.g. Estrada et al., 2007; Galimany et al., 2008a,b,c; Hégaret et al., 2009; Lassudrie et al., 2014; Smolowitz and Shumway, 1997). Increased diapedesis of hemocytes into the digestive tract has been suggested as a mechanism to eliminate toxins through the lumen of the intestine (Franchini et al., 2003; Haberkorn et al., 2010 b; Hégaret et al., 2009; Lassudrie et al., 2014).

Corresponding with the inflammatory responses observed in tissues, a significant increase in the total number of circulating hemocytes was recorded. Similarly, Hégaret et al. (2007b) recorded an increase in the number of circulating hemocytes and a reduction in hemocyte size and complexity in Manila clams, *R. philippinarum*, exposed to whole cells of *K. selliformis* and *K. mikimotoi* for 6 days. Effects on the hemocytes were attributable to toxins produced by those algae (Hégaret et al., 2007b). Although the site(s) of hematopoiesis in bivalves are not known (Bachère et al., 2004), more new hemocytes may be produced and move to sites of inflammation within oyster tissues. It cannot be ruled out that hemocytes may also be produced near the site of inflammation and subsequently pass into circulation.

4.3 Feeding processes

A lack of food/ debris was recorded in the stomach/ intestine of exposed oysters in the short- term exposure but, the digestive tubules in these oysters appeared 'plump', indicating that oysters were not starving. The reduction of food presence in the digestive tract suggests that the feeding process may have been affected by the presence of *K. brevis* cells. Oysters are capable of regulating their ingestion of toxic dinoflagellates by behavior such as shell closure, reduction of filtration rate and increasing pseudofaeces production (Hégaret et al., 2007a; May et al., 2010; Shumway et al., 1985; Shumway and Cucci, 1987). However, the high toxin content of animals implies that a lot of *K. brevis* cells were digested and not rejected as pseudofaeces (which was not quantitatively measured). This leads us to consider that oysters exposed to *K. brevis* may have fed more slowly on algae than non-exposed oysters. The significant increase in the presence of mucus and hemocytes in the gills indicates a reaction to exposure which may have impaired the feeding ability of the oyster.

No difference in the presence of food in the stomach/ intestine was recorded for the long- term exposure. This suggests that oysters may have acclimated to the daily exposure to lower concentrations of *K. brevis* cells over a 10 week period.

4.4 Reproduction

The significant reduction in the prevalence of fertile gametes in the short-term exposure suggests there were subtle effects on reproductive parameters. Oysters used in the present short-term exposure were already ripe which suggests there was likely little gamete ripening during exposure to *K. brevis*. Exposure may not have had as much of an impact on the reproductive success had the oysters been exposed whilst still ripening. However, no significant effects were recorded on the reproductive parameters measured for the long-term exposure, an experiment designed to mimic an exposure of *C. virginica* to a bloom of *K. brevis* throughout gametogenesis and gamete ripening. Responses recorded for this long-term exposure are complex however, as non-significant changes in the spawning frequency and sex ratio of *K. brevis*-exposed oysters allude to some impact on reproductive success. Based on the availability of individuals of known sex, oysters were stocked at a ratio of 1:1.4, female to male at the start of exposure. At the end of the long-term exposure, this ratio had changed in both control and exposed treatments. Oysters in the control had a 1:1.2 female to male ratio and exposed oysters had a ratio of 1 female to 4 males. In addition, hermaphrodites and more oysters of undetermined sex were recorded in the 500 cells mL⁻¹ treatment. In SW FL, hermaphrodites are more common than at temperate latitudes (Volety et al., unpublished data). Eastern oysters are also sequential hermaphrodites and individuals may change sex between and within a reproductive season (Arias-De León et al., 2013; Galtsoff, 1964; Needler, 1932). Although populations may differ in their sex ratio (Burkenroad, 1931; Harding et al., 2013; Haley, 1977), most populations are thought to have a sex ratio close to 1:1 (Kennedy, 1983). Poor environmental conditions (such as toxic algal exposure or lack of food) may change this ratio, resulting in an excess of males over females (Tranter, 1958, Thompson et al., 1996). Alternatively, the majority of oysters which died in exposed treatments may have been female. Care must be taken interpreting the data, because oysters were removed from the tanks throughout exposure (due to mortalities or for sampling for PbTx content) and some randomly occurring artefact cannot be ruled out.

Although the feeding ration and the increase in temperature used to induce gamete conditioning and spawning, are well established for bivalves (Hayes and Menzel, 1981; Helm and Bourne, 2004; Loosenhoff and Davis, 1963; Thompson et al., 1996;

Thorson 1946; Utting and Millican, 1997), it is possible that conditions may not have been optimal. Poor nutritional conditions may cause less extensive development of the gonad (Thompson et al., 1996), which may explain why no females spawned in either exposed or control treatments after 10 weeks gametogenic conditioning.

The main spawning period of *C. virginica* in the Iona Cove area (from where the oysters used in the long-term exposure were collected), is from March through November, with two main peaks at the start and end of this season (Volety et al., 2008). Future studies that expose oysters to *K. brevis* during gametogenesis and gamete ripening at different times during the reproductive season may indicate more effects on reproductive parameters.

4.5 Conclusion

HAB effects upon bivalves are more common and widespread than generally recognized. Contrary to the belief that oysters are an unaffected vector of brevetoxins, results demonstrate that eastern oysters are affected by bloom concentrations of *K. brevis*. The ripeness frequency of gametes obtained from oysters exposed to high bloom concentrations of *K. brevis* for 10 days was significantly reduced; however, no other significant differences were recorded for other reproductive parameters measured following exposure to two *K. brevis* bloom scenarios. The ability to maintain optimal environmental and nutritional conditions during long term exposures should be considered when assessing impacts of this toxic algal species on bivalves. The responses of oysters shown herein, suggest that certain high-concentration bloom scenarios, timed during peak ripening and spawning periods may significantly affect reproductive (and physiological) parameters in individuals. Effects of *K. brevis* exposure directly on the gametes and early life stages (released into the bloom during spawning) should also be considered in addition to effects caused directly on the adults.

Acknowledgements

We are grateful to Dr Inke Sunila, Shellfish pathologist at the Bureau of Aquaculture for the State of Connecticut, for her invaluable histopathology training, expertise and time. We thank Nelly Le Goïc, from Université de Bretagne Occidentale (UBO) for her assistance in flow cytometry techniques and data analysis. Thanks also to Audrey Barbe, Marine Remize, Lindsay Castret, Lesli Haynes, and staff at the Marine Laboratory, FGCU for help collecting and processing oysters and; to Diane Blackwood at the Florida Fish and Wildlife Conservation Commission (FWC) for providing *K. brevis* field count data. Funding support for this research was provided in part by UBO, and by “Laboratoire d’Excellence” LabexMER (ANR-10-LABX-19); Outgoing Ph.D. Fellowship, co-funded by a grant from the French government under the program “Investissements d’Avenir”.

References

- Alliegro, M. C., Wright, D. A. 1983. Polyspermy inhibition in the oyster, *Crassostrea virginica*. J. Exper. Zool. 227(1): 127-137.
- Arias-De León, C., Lango-Reynoso, F., Chávez-Villalba, J., Castañeda-Chávez, M. R., Ramírez-Gutiérrez, S. C. 2013. Oocyte cohort analysis: reproductive patterns of *Crassostrea virginica* (Bivalvia) in tropical lagoons of the Gulf of Mexico. Invertebr. Reprod. Dev. 57(2): 85-94.
- Bachère, E., Gueguen, Y., Gonzalez, M., De Lorgeril, J., Garnier, J., Romestand, B. 2004. Insights into the anti-microbial defence of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. Immunol. Rev. 198(1): 149-168.
- Baden, D. G., Bourdelais, A. J., Jacocks, H., Michelliza, S., Naar, J. 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. Environ. Health Persp. 113(5): 621-625.
- Baden, D. G. 1983. Marine food-borne dinoflagellate toxins. Int. Rev. Cytol. 82: 99-150.
- Barber, B. J. 2004. Neoplastic diseases of commercially important marine bivalves. Aquat. Living Resour. 17(4): 449-466.
- Basti, L., Endo, M., Segawa, S. 2011. Physiological, Pathological, and Defense Alterations in Manila Clams (Short-Neck Clams), *Ruditapes philippinarum*, Induced by *Heterocapsa circularisquama*. J. Shellfish Res. 30(3): 829–844.
- Brand, L.E. Compton, A. 2007. Long-term increase in *Karenia brevis* abundance along the Southwest Florida Coast. Harmful Algae. 6(2): 232-252.
- Bricelj, V. M., Haubois, a. G., Sengco, M. R., Pierce, R. H., Culter, J. K., Anderson, D. M. 2012. Trophic transfer of brevetoxins to the benthic macrofaunal community during a bloom of the harmful dinoflagellate *Karenia brevis* in Sarasota Bay, Florida. Harmful Algae. 16: 27–34.
- Bricelj, V. M., Connell, L., Konoki, K., MacQuarrie, S.P., Scheuer, T., Catterall, W.A., Trainer, V.L. 2005. Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP. Nature. 434(7034): 763-767.
- Bricelj, V. M., Shumway, S. E. 1998. Paralytic Shellfish Toxins in Bivalve Molluscs: Occurrence, Transfer Kinetics, and Biotransformation. Rev. Fish. Sci. 6(4): 315–383.
- Brun, N. T., Ross, N.W., Bogen, A.D. 2000. Changes in the electrophoretic profiles of gill mucus proteases of the eastern Oyster *Crassostrea virginica* in response to infection by the turbellarian *Urastoma cyprinae*. J. Invertebr. Pathol. 75(2): 163-170.
- Burkenroad, M. D. 1931. Sex in the Louisiana oyster, *Ostrea virginica*. Science. 74(1907): 71-72.
- Carriker, M.R., 2001. Embryogenesis and organogenesis of veligers and early juveniles. In: Kraeuter, J. N., Castagna, M. (Eds), Biology of the hard clam. Vol. 31. Gulf Professional Publishing. pp. 77–115.
- da Silva, P. M., Hégaret, H., Lambert, C., Wikfors, G. H., Le Goïc, N., Shumway, S. E., Soudant, P. 2008. Immunological responses of the Manila clam (*Ruditapes philippinarum*) with varying parasite (*Perkinsus olseni*) burden, during a long-term exposure to the harmful alga, *Karenia selliformis*, and possible interactions. Toxicon. 51(4): 563–73.
- Delaporte, M., Soudant, P., Moal, J., Kraffe, E., Marty, Y., Samain, J.-F. 2005. Incorporation and modification of dietary fatty acids in gill polar lipids by two bivalve species *Crassostrea gigas* and *Ruditapes philippinarum*. Comp. Biochem. Phys. A. 140(4): 460–70.

- Delaporte, M., Soudant, P., Moal, J., Lambert, C., Quéré, C., Miner, P., Choquet, G., Paillard, C., Samain, J.-F. 2003. Effect of a mono-specific algal diet on immune functions in two bivalve species *Crassostrea gigas* and *Ruditapes philippinarum*. *J. Exp. Biol.* 206(17): 3053–3064.
- Dickey, R.E., Jester, R.G., Mowdy, D., Moncreiff, C., Rebarchik, D., Robl, M., Musser, S., Poli, M. 1999. Monitoring brevetoxins during a *Gymnodinium breve* red tide: comparison of sodium channel specific cytotoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. *Nat. Toxins.* 7(4): 157-165.
- Donaghy, L., Jauzein, C., Volety, A. K. 2012. First report of severe hemocytopenia and immunodepression in the sunray venus clam, *Macrocallista nimbosa*, a potential new aquaculture species in Florida. *Aquaculture.* 364-365: 247–251.
- Donaghy, L., Volety, A. K. 2011. Functional and metabolic characterization of hemocytes of the green mussel, *Perna viridis*: In vitro impacts of temperature. *Fish Shellfish Immun.* 31(6): 808–814.
- Echevarria, M., Naar, J.P., Tomas, C., Pawlik, J.R. 2012. Effects of *Karenia brevis* on clearance rates and bioaccumulation of brevetoxins in benthic suspension feeding invertebrates. *Aquat. Toxicol.* 106: 85-94.
- Estrada, N., de Jesús Romero, M., Campa-Córdova, A., Luna, A., Ascencio, F. 2007. Effects of the toxic dinoflagellate, *Gymnodinium catenatum* on hydrolytic and antioxidant enzymes, in tissues of the giant lions-paw scallop *Nodipecten subnodosus*. *Comp. Biochem. Phys. C.* 146(4): 502–510.
- Finucane, J.H. 1964. Distribution and seasonal occurrence of *Gymnodinium breve* on the west coast of Florida 1954–1957. US Department of Interior Fish and Wildlife Service Special Scientific Reports, Fisheries no. 487. Washington, D.C. pp 14.
- Fisher, W. S., Winstead, J. T., Oliver, L. M., Edmiston, H. L., Bailey, G. O. 1996. Physiologic variability of eastern oysters from Apalachicola Bay, Florida. *J. Shellfish Res.* 15(3): 543-553.
- Fisher, W. S. 1992. Occurrence of agglutinins in the pallial cavity mucus of oysters. *J. Exp. Mar. Biol. Ecol.* 162(1): 1–13.
- Flewelling, L.J., Naar, J.P., Abbott, J.P., Baden, D.G., Barros, N.B., Bossart, G.D., Bottein, M.Y., Hammond, D.G., Haubold, E.M., Heil, C.A., Henry, M.S., Jacocks, H.M., Leighfield, T.A., Pierce, R.H., Pitchford, T.D., Rommel, S.A., Scott, P.S., Steidinger, K.A., Truby, E.W., Van Dolah, F.M., Landsberg, J.H. 2005. Brevetoxicosis: red tides and marine mammal mortalities. *Nature.* 435: 755-756
- Franchini, A., Milandri, A., Poletti, R., Ottaviani, E. 2003. Immunolocalization of yessotoxins in the mussel *Mytilus galloprovincialis*. *Toxicon.* 41: 967–970.
- Galimany, E., Place, A. R., Ramón, M., Jutson, M., Pipe, R. K. 2008a. The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*. *Harmful Algae.* 7(1): 91–98.
- Galimany, E., Sunila, I., Hégarret, H., Ramón, M., Wikfors, G. H. 2008b. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: Histopathology, immune responses, and recovery. *Harmful Algae.* 7(5): 702–711.
- Galimany, E., Sunila, I., Hégarret, H., Ramón, M., Wikfors, G. H. 2008c. Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. *Harmful Algae.* 7(5): 630–638.
- Galtsoff, P. S. 1964: The American oyster. *Fishery bulletin (US.)* 64: 1-480.

Griffith, A. W., Shumway, S. E., Volety, A. K. 2013. Bioaccumulation and depuration of brevetoxins in the eastern oyster (*Crassostrea virginica*) and the northern quahog (= hard clam, *Mercenaria mercenaria*). *Toxicon*. 66 : 75–81.

Guillard, R. R. 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W. L. Chanley, M. H. (Ed.), *Culture of marine invertebrate animals*, Springer US. pp. 29-60.

Gunter, G., Williams, R. H., Davis, C. C., Smith, F. W. 1948. Catastrophic mass mortality of marine animals and coincident phytoplankton bloom on the west coast of Florida, November 1946 to August 1947. *Ecol. Monogr.* 18: 310-324.

Haberkorn, H., Tran, D., Massabuau, J. C., Ciret, P., Savar, V., Soudant, P. 2011. Relationship between valve activity, microalgae concentration in the water and toxin accumulation in the digestive gland of the Pacific oyster *Crassostrea gigas* exposed to *Alexandrium minutum*. *Mar. Pollut. Bull.* 62(6): 1191-1197.

Haberkorn, H., Lambert, C., Le Goïc, N., Guéguen, M., Moal, J., Palacios, E., Lassus, P., Soudant, P. 2010a. Effects of *Alexandrium minutum* exposure upon physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. *Aquat. Toxicol.* 97(2): 96–108.

Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P. 2010b. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. *Harmful Algae.* 9(5): 427–439.

Haley, L. E. 1977. Sex determination in the American oyster. *J. Hered.* 68(2): 114-116.

Harding, J. M., Powell, E. N., Mann, R., Southworth, M. J. 2013. Variations in eastern oyster (*Crassostrea virginica*) sex-ratios from three Virginia estuaries: protandry, growth and demographics. *J. Mar. Biol. Assoc. U.K.* 93(2): 519-531.

Hayes, P. F., Menzel, R. W. 1981. The reproductive cycle of early setting *Crassostrea virginica* (Gmelin) in the northern Gulf of Mexico, and its implications for population recruitment. *Biol. Bull.* 160(1): 80-88.

Hégaret, H., da Silva, P. M., Sunila, I., Shumway, S. E., Dixon, M. S., Alix, J., Wickfors, G.H., Soudant, P. 2009. Perkinsosis in the Manila clam *Ruditapes philippinarum* affects responses to the harmful-alga, *Prorocentrum minimum*. *J. Exp. Mar. Biol. Ecol.* 371(2): 112–120.

Hégaret, H., Wikfors, G. H., Shumway, S. E. 2007a. Diverse feeding responses of five species of bivalve mollusc when exposed to three species of harmful algae. *J. Shellfish Res.* 26(2): 549-559.

Hégaret, H., da Silva, P. M., Wikfors, G. H., Lambert, C., De Bettignies, T., Shumway, S. E., Soudant, P. 2007b. Hemocyte responses of Manila clams, *Ruditapes philippinarum*, with varying parasite, *Perkinsus olseni*, severity to toxic-algal exposures. *Aquat. Toxicol.* 84(4): 469–479

Hégaret, H., Wikfors, G. H. 2005a. Time-dependent changes in hemocytes of eastern oysters, *Crassostrea virginica*, and northern bay scallops, *Argopecten irradians irradians*, exposed to a cultured strain of *Prorocentrum minimum*. *Harmful Algae.* 4(2): 187–199.

Hégaret, H., Wikfors, G. H. 2005b. Effects of natural and field-simulated blooms of the dinoflagellate *Prorocentrum minimum* upon hemocytes of eastern oysters, *Crassostrea virginica*, from two different populations. *Harmful Algae.* 4(2): 201–209.

Hégaret, H., Wikfors, G.H., Soudant, P., Delaporte, M., Alix, J.H., Smith, B. C., Dixon, M.S., Quéré, C., Le Coz, J.R., Paillard, C., Moal, J., Samain, J.-F. 2004. Immunological competence of eastern

oysters, *Crassostrea virginica*, fed different microalgal diets and challenged with a temperature elevation. *Aquaculture*, 234: 541–560.

Hégaret, H., Wikfors, G. H., Soudant, P. 2003a. Flow-cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation I. Haemocyte types and morphology. *J. Exp. Mar. Biol. Ecol.* 293(2): 237–248.

Hégaret, H., Wikfors, G. H., Soudant, P. 2003b. Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation II. Haemocyte functions: Aggregation, viability, phagocytosis, and respiratory burst. *J. Exp. Mar. Biol. Ecol.* 293(2): 249–265.

Helm, M. M., Bourne, N. 2004. Hatchery culture of bivalves: A practical manual (Vol. 471). Lovatelli, A. (Ed.). Food and agriculture organization of the United Nations. Rome. pp. 59-80.

Howard, D.W., Lewis, E.J., Keller, B.J., Smith, C.S., 2004. Histological Techniques for Marine Bivalve Mollusks and Crustaceans. NOAA Technical Memorandum. NOSNCCOS, Oxford, MD, USA. pp. 218.

Hu, C., Muller-Karger, F. E., Swarzenski, P. W. 2006. Hurricanes, submarine groundwater discharge, and Florida's red tides. *Geophys. Res. Lett.* 33: L11601.

Humason, G. L. *Animal Tissue Techniques*. 1979. W.N. Freeman and Company. San Francisco, CA, U.S.

International Mussel Watch Program (IMW). 1980. National Academy of Sciences, Washington D.C. pp. 248.

Kennedy, V. S. 1983. Sex- ratios in oysters, emphasizing *Crassostrea virginica* from Chesapeake Bay, Maryland. *Veliger*. 25(4): 329- 338.

Kimm-Brinson, K. L., Ramsdell, J. S. 2001. The red tide toxin, brevetoxin, induces embryo toxicity and developmental abnormalities. *Environ. Health Persp.* 109(4): 377- 381.

Kubanek, J., Snell, T.W., Pirkle, C. 2007. Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing. *Limnol. Oceanogr.* 52(3): 1026–1035.

Kubanek, J., Hicks, M.K., Naar, J., Villareal, T.A. 2005. Does the red tide dinoflagellate *Karenia brevis* use allelopathy to outcompete other phytoplankton? *Limnol. Oceanogr.* 50(3): 883–895.

Landsberg, J.H. 2002. The Effects of Harmful Algal Blooms on Aquatic Organisms. *Rev. Fish. Sci.* 10(2): 113–390.

Lassudrie, M., Soudant, P., Richard, G., Henry, N., Medhioub, W., da Silva, P. M., Donval, A., Bunel, M., Le Goïc, N., Lambert, C., de Montaudouin, X., Fabioux, C., Hégaret, H. 2014. Physiological responses of Manila clams *Venerupis (=Ruditapes) philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure. *Aquat. Toxicol.* 154: 27–38.

Leverone, J. R. 2007. Comparative effects of the toxic dinoflagellate, *Karenia brevis*, on bivalve molluscs from Florida (Doctoral dissertation, University of South Florida).

Leverone, J. R., Shumway, S. E., Blake, N. J. 2007. Comparative effects of the toxic dinoflagellate *Karenia brevis* on clearance rates in juveniles of four bivalve molluscs from Florida, U.S. *Toxicon.* 49(5): 634–645.

Leverone, J.R., Blake, N.J., Pierce, R.H., Shumway, S.E. 2006. Effects of the dinoflagellate *Karenia brevis* on larval development in three species of bivalve mollusc from Florida. *Toxicon.* 48: 75–84.

- Marshall, J., Ross, T., Pyecroft, S., Hallegraeff, G., 2005. Superoxide production by marine microalgae 2: Towards understanding ecological consequences and possible functions. *Mar. Biol.* 147: 541–549.
- Martoja, R., Martoja-Pierson, M. 1967. *Initiation aux techniques de l'histologie animale*, Paris. pp. 1232.
- May, S. P., Burkholder, J. M., Shumway, S. E., Hégaret, H., Wikfors, G. H., Frank, D. 2010. Effects of the toxic dinoflagellate *Alexandrium monilatum* on survival, grazing and behavioral response of three ecologically important bivalve molluscs. *Harmful Algae*. 9(3): 281-293.
- McFarren, E. F., Tanabe, H., Silva, F. J., Wilson, W. B., Campbell, J. E., Lewis, K. H. 1965. The occurrence of a ciguatera-like poison in oysters, clams and *Gymnodinium breve* cultures. *Toxicon*. 3:111–123.
- Medhioub, W., Lassus, P., Truquet, P., Bardouil, M., Amzil, Z., Sechet, V., Sibat, M., Soudant, P. 2012. Spirolide uptake and detoxification by *Crassostrea gigas* exposed to the toxic dinoflagellate *Alexandrium ostenfeldii*. *Aquaculture*. 358: 108-115.
- Mello, D.F., de Oliveira, E.S., Vieira, R.C., Simoes, E., Trevisan, R., Dafre, A.L., Barracco, M.A. 2012. Cellular and Transcriptional Responses of *Crassostrea gigas* Hemocytes Exposed *in Vitro* to Brevetoxin (PbTx-2). *Mar. Drugs*. 10: 583-597.
- Morton, B., 1983. Feeding and digestion in Bivalvia. In: Saleuddin, A.S.M., Wilbur, K.M. (Eds.). *The Mollusca. Physiology, Part 2*, vol. 5, Academic Press, New York. pp. 65 – 340.
- Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P. L., Flewelling, L., Steidinger, K., Lancaster, J., Baden, D. G. 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. *Environ. Health Perspect.* 110(2): 179–185.
- Needler, A. B. 1932. Sex reversal in *Ostrea virginica*. *Contrib. Can. Biol. Fish.* 7(1): 283-294.
- Neely, T., Campbell, L. 2006. A modified assay to determine hemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae*. 5: 592–598.
- NSSP. 2011. National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish. 2011 Revision U. S. Department of Health and Human Services Public Health Service Food and Drug Administration Interstate Shellfish Sanitation Conference.
- Parrish, C. C., Bodennec, G., Gentien, P. 1998. Haemolytic glycolipids from *Gymnodinium* species. *Phytochemistry*. 47(5): 783–787.
- Pierce, R.H., Henry, M. 2008. Harmful algal toxins of the Florida red tide (*Karenia brevis*): natural chemical stressors in South Florida coastal ecosystems. *Ecotoxicol.* 17(7): 623-631.
- Pierce, R., Henry, M., Blum, P. 2008. Brevetoxin abundance and composition during ECOHAB-Florida field monitoring cruises in the Gulf of Mexico. *Continental Shelf Res.* 28: 45–58.
- Pierce, R., Henry, M., Blum, P., Payne, S., 2001. *Gymnodinium breve* toxins without cells: intra-cellular and extra-cellular toxins. In: Hallegraeff, G., Blackburn, S., Bolch, C., Lewis, R. (Eds.), *Harmful Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO, pp. 421–424.
- Plakas, S. M., Jester, E.L.E., El Said, K.R., Granade, H.R., Abraham, A., Dickey, R.W., Scott, P.S., Flewelling, L.J., Henry, M., Blum, P., Pierce, R. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (*Crassostrea virginica*). *Toxicon*. 52(1): 32-38.

- Plakas, S.M., Wang, Z.H., El Said, K.R., Jester, E.L.E., Granade, H.R., Flewelling, L., Scott, P., Dickey, R.W. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. *Toxicon*. 44: 677–685.
- Plakas, S.M., El Said, K.R., Jester, E.L.E., Granade, H.R., Musser, S.M., Dickey, R.W. 2002. Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. *Toxicon*. 40: 721–729.
- Poli, M. A., Musser, S. M., Dickey, R. W., Eilers, P. P., Hall, S. 2000. Neurotoxic shellfish poisoning and brevetoxin metabolites: A case study from Florida. *Toxicon*. 38(7): 981–993.
- Prince, E.K., Poulson, K.L., Myers, T.L., Sieg, R.D., Kubanek, J. 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. *Harmful Algae*. 10: 39–48.
- Ray, S. M., Aldrich, D.V. 1967. Ecological interactions of toxic dinoflagellates and molluscs in the Gulf of Mexico. In: Russell, F., Saunders, P. (Eds.). *Animal Toxins*. Oxford: Pergamon. pp. 75–83.
- Rolton, A., Soudant, P., Vignier, J., Pierce, R., Henry, M., Shumway, S. E., Bricelj, V. M., Volety, A. K. 2015. Susceptibility of gametes and embryos of the eastern oyster, *Crassostrea virginica*, to *Karenia brevis* and its toxins. *Toxicon*. 99: 6-15.
- Rolton, A., Vignier, J., Soudant, P., Shumway, S. E., Bricelj, V. M., Volety, A. K. 2014. Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*. *Aquat. Toxicol.* 155: 199–206.
- Royer, J., Segueineau, C., Park, K. I., Pouvreau, S., Choi, K. S., Costil, K. 2008. Gametogenetic cycle and reproductive effort assessed by two methods in 3 age classes of Pacific oysters, *Crassostrea gigas*, reared in Normandy. *Aquaculture*. 277(3): 313-320.
- Sengco, M. R. 2009. Review Prevention and control of *Karenia brevis* blooms. *Harmful Algae*. 8: 623–628.
- Shaw, B. ., Battle, H. I. 1957. The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Can. J. Zool.* 35: 325– 347.
- Shumway, S. E. 1995. Phycotoxin-related shellfish poisoning: Bivalve molluscs are not the only vectors. *Rev. Fish. Sci.* 3(1): 1-31.
- Shumway, S. E., Gainey, L. F. 1992. A review of physiological effects of toxic dinoflagellates on bivalve molluscs. In: *Proceedings of Ninth International Malac. Congress*. pp. 357-362.
- Shumway, S.E. 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquac. Soc.* 21 (2): 65–104.
- Shumway, S. E., Cucci, T. L. 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. *Aquat. Toxicol.* 10(1): 9-27.
- Shumway, S.E., Cucci, T.L., Gainey, L., Yentsch, C.M., 1985. A preliminary study of the behavioral and physiological effects of *Gonyaulax tamarensis* on bivalve mollusks. In: Anderson, D.M., White, A.W., Baden, D.G. (Eds.), *Toxic dinoflagellates*. Elsevier, New York, pp. 389–394.
- Sievers, A. M. 1969. Comparative toxicity of *Gonyaulax monilata* and *Gymnodinium breve* to annelids, crustaceans, molluscs and a fish. *J. Protozool.* 16(3): 401-404.
- Simon, J. L., Dauer, D. M. 1972. A quantitative evaluation of red-tide induced mass mortalities of benthic invertebrates in Tampa Bay, Florida. *Environ. Lett.* 3(4): 229-234.

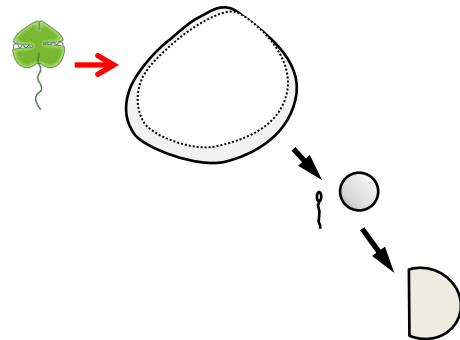
- Smolowitz, R., Shumway, S. E. 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve molluscs. *Aquacult. Int.* 5(4): 291–300.
- Soletchnik, P., Razet, D., Geairon, P., Faury, N., Gouletquer, P. 1997. Ecophysiology of maturation and spawning in oyster *Crassostrea gigas*: metabolic (respiration) and feeding (clearance and absorption rates) responses at different maturation stages. *Aquat. Living Res.* 10: 177- 185
- Song YP, Suquet M, Quéau I, Lebrun L. 2009. Setting of a procedure for experimental fertilisation of Pacific oyster (*Crassostrea gigas*) oocytes. *Aquac.* 287:311-314.
- Soudant, P., Paillard, C., Choquet, G., Lambert, C., Reid, H. I., Marhic, A., Donaghy, L., Birkbeck, T. H. 2004. Impact of season and rearing site on the physiological and immunological parameters of the Manila clam *Venerupis* (= *Tapes*, = *Ruditapes*) *philippinarum*. *Aquaculture.* 229(1-4): 401–418.
- Steidinger, K.A. 2009. Historical perspective on *Karenia brevis* red tide research in the Gulf of Mexico. *Harmful Algae.* 8(4): 549–561.
- Steidinger, K. A., Carlson, P., Baden, D., Rodriguez, C., Seagle J. 1998. Neurotoxic shellfish poisoning due to toxin retention in the clam *Chione cancellata*. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.). *Harmful Algae.* Xunta de Galicia, Paris. pp. 457–458.
- Steidinger, K. A. 1993. Some taxonomic and biological aspects of toxic dinoflagellates. In: Falconer, I. R., (Ed). *Algal Toxins in Seafood and Drinking Water.* Academic Press, London. pp. 1–28.
- Steidinger, K. A., Burklew, M., Ingle, R. M. 1973. The effects of *Gymnodinium breve* toxin on estuarine animals. In: Martin D.F., Padilla, G. M. (Eds.). *Marine Pharmacognosy: Action of Marine Toxins at the Cellular Level.* New York: Academic Press. pp. 179–202.
- Steidinger, K. A., Ingle, R. M. 1972. Observations on the 1971 summer red tide in Tampa Bay, Florida. *Environ. Lett.* 3(4): 271-278.
- Stumpf, R.P., Culver, M.E. Tester, P.A., Tomlinson, M., Kirkpatrick, G.J., Pederson, B.A., Truby, E., Ransibrahmanakul, V., Soracco, M. 2003. Monitoring *Karenia brevis* blooms in the Gulf of Mexico using satellite ocean color imagery and other data. *Harmful Algae.* 2: 147–160.
- Summerson, H., Peterson, C., 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Ptychodiscus brevis*, outbreak recorded in North Carolina. *Estuaries.* 13(3): 322–331.
- Tatters, A.O, Muhlstein, H.I., Tomas, C.R. 2010. The hemolytic activity of *Karenia selliformis* and two clones of *Karenia brevis* throughout a growth cycle. *J. Appl. Phycol.* 22: 435–442.
- Taylor, H.F. 1917. Mortality of fishes on the west coast of Florida, Rep. U.S.. *Commun. Fish. Doc. No.* 848, pp. 24.
- Tester, P. A., Fowler, P. K. 1990. Brevetoxin contamination of *Mercenaria mercenaria* and *Crassostrea virginica*: A management issue. In: Granéli, E., Sundström, B., Edler, L., Anderson, D.M. (Eds.). *Toxic marine phytoplankton.* New York: Academic Press. pp. 499-503.
- Thompson, R. J., Newell, R. I. E., Kennedy, V. S., Mann, R. 1996. Reproductive processes and early development. In: Kennedy, V.S. (Ed.) *The Eastern Oyster Crassostrea virginica.* Maryland Sea Grant College, University of Maryland, College Park, Maryland, 335-370.
- Thorson, G. 1946. Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the South (øresund). *Meddr. Kommn. Danm. Fisk.-og Havunders. Ser. Plankton* 4: 1-523.

- Tranter, D. J. 1958. Reproduction in Australian pearl oysters (*Lamelli branchia*). III. *Pinctada albina* (Lamarck): Breeding season and sexuality. *Mar. and Freshwater Res.* 9(2): 191-216.
- Twarog, B. M., Hidaka, T., Yamaguchi, H. 1972. Resistance to tetrodotoxin and saxitoxin in nerves of bivalve molluscs: a possible correlation with paralytic shellfish poisoning. *Toxicon.* 10(3): 273-278.
- Utting, S. D., Millican, P. F. 1997. Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability. *Aquaculture.* 155(1): 45-54.
- Vargo, G. A., Heil, C. A., Ault, D. N., Neely, M. B., Murasko, S., Havens, J., Lester, K.M., Dixon, L.K., Merkt, R., Walsh, J., Weisberg, R., Steidinger, K. A. 2004. Four *Karenia brevis* blooms: A comparative analysis. In: Steidinger K.A., Landsberg J.H., Tomas C.R. and Vargo, G.A. (Eds.), *Harmful Algae 2002; Proceedings of the Xth International Conference on Harmful Algae*, Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and the Intergovernmental Oceanographic Commission of UNESCO, St Petersburg, FL; 2004. pp. 14–16.
- Volety, A.K., Savarese, M., Tolley, S.G., Arnold, W.S., Sime, P., Goodman, P., Chamberlain, R.H., Doering, P.H., 2009. Eastern oysters (*Crassostrea virginica*) as an indicator for restoration of everglades ecosystems. *Ecol. Indic.* 9(6):120–136.
- Volety, A.K. 2008. Effects of salinity, heavy metals and pesticides on health and physiology of oysters in the Caloosahatchee Estuary, Florida. *Ecotoxicology.* 17: 579–590.
- Wang, Z., Plakas, S. M., El Said, K. R., Jester, E. L. E., Granade, H. R., Dickey, R. W. 2004. LC/MS analysis of brevetoxin metabolites in the Eastern oyster (*Crassostrea virginica*). *Toxicon.* 43(4): 455–65.
- Wikfors, G.H., Smolowitz, R.M., 1995. Experimental and histological studies of four life-history stages of the eastern oyster, *Crassostrea virginica*, exposed to a cultured strain of the dinoflagellate *Prorocentrum minimum*. *Biol. Bull.* 188, 313–328.
- Zou, Y., Yamasaki, Y., Matsuyama, Y., Yamaguchi, K., Honjo, T., Oda, T. 2010. Possible involvement of hemolytic activity in the contact-dependent lethal effects of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. *Harmful Algae.* 9: 367–373.

Chapter 2

Effects of *Karenia brevis* exposure on adult *Mercenaria mercenaria*: Histopathological responses in adults and effects on gamete and larval quality.

In this chapter, the reproductive and related physiological effects of exposing adult clams, *M. mercenaria*, were studied. In addition, the response of clams and oysters were compared and, possible effects of using clams from different geographical areas, with different exposure histories to *K. brevis* were studied.



The hypotheses tested were:

- Exposure to *K. brevis* has no effect on the survival or on the reproductive and related physiological processes of adult clams.
- Adult exposure to *K. brevis* has no effect on the gametes and development of subsequently produced offspring.
- Previous exposure to *K. brevis* has no effect on the response of clams.

Effects of *Karenia brevis* exposure on adult *Mercenaria mercenaria*: Histopathological responses in adults and effects on gamete and larval quality.

Anne Rolton^{1*,2}, Julien Vignier^{1,2}, Sandra E. Shumway³, V. Monica Bricelj⁴, Aswani K. Volety^{2,5}, Philippe Soudant¹.

¹LEMAR CNRS UMR 6539, Institut Universitaire Européen de la Mer - Université de Bretagne Occidentale, Place Nicolas Copernic, Technopôle Brest Iroise, 29280 Plouzané, France;

²Florida Gulf Coast University, Vester Marine Field Station, 5164 Bonita Beach Road, Bonita Springs, FL 34134;

³Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340;

⁴Department of Marine and Coastal Sciences and Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349;

⁵Present address: University of North Carolina Wilmington, 601 S. College Rd, Wilmington, NC 28403.

*email: annerolton@gmail.com Phone: +33 298 498 623

Abstract

The effects of exposing two populations of *Mercenaria mercenaria*, with differing exposure histories, to bloom concentrations of the toxic dinoflagellate, *Karenia brevis*, were investigated. In separate experiments, ripe adult *M. mercenaria* from a site with a history of exposure to bloom concentrations of *K. brevis* ('previously exposed') and from a site approximately 200 miles north, which had no previous exposure to *K. brevis* blooms ('non-exposed') were exposed to whole cells of cultured *K. brevis* at 1000 and 5000 cells mL⁻¹ for 10 days. Brevetoxin (PbTx) accumulation, condition index (CI), gonadal stage (GS) and histopathological features in the tissues of adults; and, effects on the spawning frequency, spermatozoa characteristics, fertilization success and larval development (up to 4 days post fertilization, PF) were recorded. Clams from both sites accumulated ~1000 ng g⁻¹ PbTx in the 1000 cells mL⁻¹ treatment and ~5000 ng g⁻¹ PbTx in the 5000 cells mL⁻¹ treatment and showed no changes in CI and GS following exposure. Following exposure to *K. brevis*, clams from the 'previously exposed' site showed a significant increase in hemocytes diapedesis in the stomach/ intestine. Clams from the 'previously exposed' site did not spawn following thermal stimulation and further investigations on the gametes and offspring were performed on clams from the 'non-exposed' site only. Significantly reduced fertilization success ($p < 0.001$) was recorded from exposed clams compared to control. No significant changes were recorded in any of the spermatozoa cellular parameters measured when analyzing sperm used for subsequent fertilization; however, a significant reduction in sperm ROS production was recorded when including sperm ejected from exposed clams after a delayed period in analyses. Larval development was significantly affected from 1 day PF. Four days PF, percent mortality and abnormality were significantly higher in larvae obtained from clams exposed to the 5000 cells mL⁻¹ treatment (4.4% and 5.3 % respectively) compared to the lower treatment (2.1% and 1.9% respectively) and control (2.5% and 2.4% respectively). Exposure to *K. brevis* and/ or associated toxins negatively affects clam tissues and, the uptake of toxins into the adult may affect the gametes and subsequent offspring.

Key words: *Mercenaria mercenaria*, *Karenia brevis*, brevetoxin, histopathology, larval development.

1. Introduction

Hard clam (= northern quahog, *Mercenaria mercenaria*) aquaculture is the largest and most valuable of the shellfish aquaculture industries on the east coast of the U.S. (Whetstone et al., 2005). The native range of this economically important species is from the Gulf of St. Lawrence in Canada to the Gulf of Mexico (GoM) in the U.S. (Palmer, 1927; Andrews, 1971); however, it has been introduced both intentionally and accidentally to the west coast of the U.S., northern Europe and, recently to areas of China for aquaculture purposes (Hanna, 1966; Heppell, 1961; Tebble, 1966; Kaas, 1937; Zhang et al., 2003). The U.S. produced nearly 40,000 tonnes of food-size clams in 2005, primarily in Virginia, Florida and Connecticut; worth over \$60 million (United States Department of Agriculture (US DOA), 2005). A frequently re-occurring event in the productive south west Florida (SW FL) region of hard clam culture is the harmful algal bloom (HAB) forming species, *Karenia brevis* (Brand and Compton, 2007; Heil et al., 2014; Sengco, 2009).

Near-annual blooms of *K. brevis* ranging from ≥ 1 cell mL^{-1} to $> 10^5$ cells mL^{-1} have been reported along the Gulf Coast of FL (Finucane, 1964; Sengco, 2009; Steidinger 2009). Cells of *K. brevis* produce brevetoxins (PbTx) and other harmful compounds (Baden et al., 2005; Kubanek et al., 2007; Neely and Campbell, 2006; Poli et al., 1986; Prince et al., 2010) which accumulate in bivalve mollusc filter feeders such as *M. mercenaria* (McFarren et al., 1965; Landsberg, 2002; Naar et al., 2004) and can lead to neurotoxic shellfish poisoning (NSP) in humans, due to consumption of contaminated shellfish (Steidinger et al., 1998, Poli et al., 2000). To prevent NSP, states which border the GoM in the U.S. close shellfish beds when concentrations of >5 cells of *K. brevis* mL^{-1} are reached and/ or shellfish reach 20 mouse units (MU) 100 g^{-1} shellfish meat [equivalent to 0.8 mg PbTx-2 equivalents kg^{-1}] (NSSP, 2011).

Although bivalves are generally thought to tolerate exposure to *K. brevis* (i.e. Sievers, 1969; Cummins et al., 1971), negative effects directly on bivalves have been reported. Simon and Dauer (1972) and Tiffany and Heyl (1978) recorded mortalities of the dwarf surf clam (*Mulinia lateralis*), pod razor clam (*Ensis minor*), Atlantic surf clam (*Spisula solidissima siliis*) and coquina (*Donax variabilis*) which had been exposed to field concentrations of between 9.5×10^2 - 1.7×10^4 cells mL^{-1} *K. brevis* during red tide blooms in SW FL in 1971 and 1978, respectively. Leverone

et al. (2006) and Rolton et al. (2014) reported increased mortalities, abnormalities and a reduction in larval size of *M. mercenaria* embryos and larvae exposed to bloom concentrations of *K. brevis* for only a few days.

Based on their history of exposure, bivalves of the same species may also differ in their susceptibility following exposure to toxic algae such as *K. brevis* (Shumway and Cucci, 1987). Summerson and Peterson (1990), highlighted the vulnerability of scallops, *Argopecten irradians*, exposed in 1987- 1988 to a bloom of *K. brevis* which had been transported by the Gulf Stream from areas of frequent exposure in the GoM, past the tip of FL and along the coast to North Carolina (Tester et al., 1989). Possible mortalities of adult scallops and mass recruitment failure were recorded in these 'naïve' populations. Differential susceptibility has been shown in between populations of bivalves exposed to the paralytic shellfish toxin (PST) producing dinoflagellate, *Alexandrium tamarense*. Shumway et al. (1985) exposed adult mussels *Mytilus edulis* from 'naïve' and 'previously exposed' sites to 500 cells mL⁻¹ of *A. tamarense* for 1 hour. Mussels from the local, previously exposed site, showed no alteration in shell valve closure after addition of the algae, whereas, mussels from the 'naïve' site closed their valves. Differential sensitivity to toxic algal exposure between the same species may be due to genetic differences. Although populations of the softshell clam (*Mya arenaria*) were thought to be genetically similar, Bricelj et al. (2005) proved that juvenile *M. arenaria* from areas exposed to red tides of *A. tamarense* were more resistant to PSTs than naïve clams due to a mutation of a single amino acid residue on the voltage dependent sodium channel. These mutations can be small and occur regularly even within the same population (Anderson et al., 2005).

The present study identified if populations of clams with different *K. brevis* bloom exposure histories, respond similarly to *K. brevis* exposure. Physiological effects on tissues and possible impacts on the reproductive fitness of clams from frequently exposed areas ('previously exposed') and those from relatively near-by areas with no previous exposure to blooms ('non-exposed') were studied. The accumulation of PbTx within whole adult clam tissues and pathological and non-pathological features in tissues were assessed after 10 days of exposure to two bloom concentrations of whole cells of *K. brevis*. Clams were spawned to determine any effects on

spermatozoa cellular parameters, fertilization success and subsequent larval development.

2. Methods

2.1 *Karenia brevis* cultures

The toxic dinoflagellate, *Karenia brevis* (Manasota Key strain) was obtained from the Mote Marine Laboratory, Sarasota, Florida (MML). Cultures were grown in 9 L (8 quart) Camwear® Round Containers (Cambro Manufacturing) filled with 3 L of 0.1 µm carbon- filtered and UV-treated seawater (FSW) from Estero Bay which had been autoclaved, allowed to cool, L1 nutrients (NCMA;) and approximately 1 L of 14-day-old *K. brevis* stock culture added aseptically. Cultures were kept at a salinity of 29 ± 2 and temperature of $22 \pm 1^\circ\text{C}$ on a 12:12 light: dark cycle. The growth and purity of cultures were monitored microscopically. Once stationary phase had been reached (~ 14 days) and typically at a density of 2×10^4 – 2.5×10^4 cells mL⁻¹, cultures were used for experiments. The brevetoxin profiles of representative *K. brevis* stock cultures (1 L), from which 9 L cultures used for experimental clam exposures were grown, were determined by liquid chromatography, tandem mass spectrometry (LC-MS/MS) at the MML according to the method of Plakas et al. (2008). Cultures were toxic and contained 0.8 and 9.54 pg cell⁻¹ of the parent toxins PbTx-1 and PbTx-2 respectively. Metabolites PbTx-3, PbTx- CA and the brevetoxin antagonist brevenal were also present at 0.38, 0.13 and 2.57 pg cell⁻¹ respectively (Rolton et al., in prep, a/ Chapter 1).

2.2 Broodstock

Clams from Pine Island, FL (Lat. 26.60, Long. -82.15) and Cedar Key, FL (Lat 29.14, long -83.01), were obtained from commercial harvest areas and were estimated to be 2- 4 years old. Harvested adults originated from hatchery produced stock in Cedar Key, FL. Broodstock used in Cedar Key hatcheries originated from *M. mercenaria* imported from the east coast of FL in the 1990's for aquaculture purposes.

In April 2012, adult *M. mercenaria* were obtained from an area previously exposed to blooms of *K. brevis* (Pine Island, FL) and are referred to as 'previously exposed'.

Bloom concentrations of *K. brevis* had been recorded in the Pine Island area from September 2011- January 2012 (Florida Fish and Wildlife Conservation Commission, FWC, monitoring data). Mean total clam fresh weight was 49.6 ± 3.0 g (mean \pm standard error, SE) and mean shell length (greatest anterior-posterior length) was 52.9 ± 1.4 mm.

In March 2013, adult *M. mercenaria* were obtained from Dog Island, Cedar Key, FL. Clams supplied from this area had no previous exposure to bloom concentrations of *K. brevis* and are referred to as 'non-exposed'. Mean total clam fresh weight was 64.5 ± 1.3 g (\pm SE) and mean shell length was 60.2 ± 0.4 mm.

Clams were acclimated in 400 L tanks at the Vester Marine Field Station, Florida Gulf Coast University, at a salinity of 28, temperature of $23 \pm 1^\circ\text{C}$ and were fed a mixed diet of shellfish diet 1800® (Reed Mariculture), *Tisochrysis lutea* sp. nov (T-Iso; CCMP 1324), *Chaetoceros muelleri* (CCMP 1318) and *Tetraselmis chui* (CCMP 882) *ad libitum*. Algal stock cultures were obtained from the Provasoli-Guillard National Center for Marine Microalgae and Microbiota (NCMA), Bigelow Laboratory, Maine (previously the CCMP). The flagellates T-Iso and *T. chui* were grown at a salinity of 30 in CELL-HI F2P based on Guillard F/2 medium (Guillard, 1975; Varicon Aqua). The diatom, *C. muelleri* was grown in CELL-HI F2P based on Guillard F/2 medium and Si at a salinity of 24.

Following 5 days of acclimatization, 3 clams were sampled for total PbTx content, 10 clams were sampled for condition index (CI) and another 10 were sampled to determine histopathological status, gonadal stage (GS) and sex distribution.

2.3 Experimental design

Both exposures had the same experimental design. Three replicate whole-cell treatments of *K. brevis* were prepared in aquaria tanks at 1000 and 5000 cells mL⁻¹ concentrations and controls were prepared with no *K. brevis*. Triplicate cell counts of 9L *K. brevis* cultures were performed using a nanoplankton chamber (PhycoTech Inc.) and aliquots added to the 20 L aquaria tanks to reach the desired final concentration in 10 L total volume. Eleven individuals were distributed per tank in a plastic basket. Temperature was maintained at $23 \pm 1^\circ\text{C}$, salinity at 28, light aeration in tanks maintained the dissolved oxygen (DO) $> 7\text{mg L}^{-1}$ and pH levels were

maintained at 8 ± 0.5 . Temperature, DO, pH and salinity were monitored daily using a Pro ODO optic probe (YSI), 'Pinpoint' pH monitor (American Marine Inc.) and a salinity refractometer (Aquatic ecosystems). Each day, baskets containing clams were removed from the tanks and the water and *K. brevis* treatment were changed before replacing the basket containing the clams. After the water change and addition of fresh *K. brevis* to exposure treatment tanks, all tanks were fed with shellfish diet 1800® (Reed Mariculture) at a ration of 3% dry weight of algae per dry weight of clam meat. Dry weight of clam soft tissue was calculated according to Hégaret et al. (2004). Total wet weight to tissue dry weight measurements using a linear regression function relating these two variables was generated from previously sacrificed individuals of the same batch of clams ($n= 30$, data not shown). The daily shellfish diet ration was split into 2 feedings, 4 hours apart to ensure the concentration of algae in the tank did not exceed the 10 mg L^{-1} pseudofeces threshold (Hégaret et al., 2004). Exposure continued for 10 days.

2.4 Clam sampling

After 10 days of exposing 'previously exposed' (Pine Island) clams to *K. brevis* treatments, 5 individuals were selected at random from each tank for condition index (CI). After exposure of 'non-exposed' (Cedar Key) clams to *K. brevis* treatments for 10 days, 3 individuals were randomly sampled from each tank for CI. For both exposures, of those selected for CI, 5 individuals were randomly selected per treatment for PbTx analysis. Thermal stimulation was used to spawn remaining clams from both sites ($n= 18$ for 'previously exposed' and $n= 24$ for 'non-exposed' per treatment); however, clams from the 'previously-exposed' site did not spawn and were all sampled to determine pathological and non-pathological features in tissues and GS ($n= 18$). Only clams from the 'non-exposed' site were used to determine the spawning frequency, changes in spermatozoa cell parameters, fertilization success and subsequent larval development for 4 days post fertilization (PF). Clams which did not spawn were also sampled to determine pathological and non-pathological features in tissues and GS ($n= 6, 13$ and 11 in the control, 1000 and 5000 cells mL^{-1} treatments respectively).

2.5 Measurements

2.5.1 Condition index

Bivalve CI was assessed by shucking clams, removing whole soft tissues from shells and weighing them in a pre-weighed aluminum crucible. The remaining shells were dried for 48h at 60°C and weighed in the same way. The CI of each individual was calculated as tissue wet meat weight (g) / shell dry weight (g) x 100 according to Delaporte et al. (2005) and Lassudrie et al. (2014).

2.5.2 Brevetoxin content of adults

Whole tissues of shucked individuals were frozen at -80°C until extraction. Tissues from individuals were homogenized under liquid nitrogen using a Laboratory Mixer Mill MM400 (Retsch). One gram aliquots were taken from homogenized whole tissue and extracted from shellfish tissues according to Griffith et al. (2013). Briefly, 80% methanol was mixed with the homogenized tissue and incubated at 60°C for 20 minutes before transfer on ice for 10 min. Samples were then centrifuged for 10 min at 3000 *g* and the supernatant extract saved. The process was then repeated; extracts were combined for each sample before being washed in hexane and stored at -20°C. Extracted samples were analyzed within 7 days for brevetoxin using a competitive ELISA and following the method of Griffith et al. (2013) and Naar et al. (2002). Brevetoxin concentration was expressed as ng g⁻¹ of PbTx-3 equivalents.

2.5.3 Histopathological features

Individuals were carefully shucked and two ~ 4mm thick cross-sections of tissue which included gills, digestive gland, connective tissue, foot and gonad were cut. Dissected tissues were placed in individual cassettes and fixed in Davidson's fixative (Shaw and Battle, 1957) at 4°C for 7 days, after which they were transferred to 70% ethanol for at least 24 hours. Following this, dissected tissues were dehydrated and embedded in paraffin. Six µm sections were cut using a microtome, mounted on glass slides, and stained with Harris' Hematoxylin and Eosin according to Martoja and Martoja-Pierson (1967), Humason (1979) and Howard et al. (2004). Slides were examined under a light microscope to determine pathological and non-pathological features in tissues. Pathological features observed such as hemocyte infiltration, occlusion of hemolymph vessels and edema in various tissues and a non-pathological feature; presence of food/ debris in the stomach/ intestine, were recorded as either 0 for absent or, 1 when present. For presentation in Figure 2, results were converted into the percent presence of feature (%), by adding the

number of features observed for all individuals in a treatment and dividing by the number of individuals observed per treatment x 100.

The sum of pathological features per tissue (gill and stomach/ intestine) for each individual was also assessed. For presentation in Table 1, results were converted to a mean with standard error by dividing the sum of pathologies in each tissue by the number of individuals per treatment. The presence of food/ debris in the stomach/ intestine was excluded from these calculations.

2.5.4 Gonadal stage (GS)

Clam gonadal stage (GS) was determined according to Eversole (1997) and expressed as a mean \pm standard error per treatment.

2.6 Bivalve spawning

Clams were placed in a tank of 0.1 μm FSW at a salinity of 30 and temperature of 18°C for 30 min, before slowly raising the temperature to 30°C and maintaining it for 30 min. Spawning individuals were removed from the tank, rinsed with FSW and transferred to individual 1 L containers containing a small amount of 0.1 μm FSW to complete spawning and ensure gametes were not contaminated or fertilized. Spawning was considered finished 30 min after the first individual had begun to spawn. Gamete quality was assessed under the microscope and all gametes obtained within the 30 minute spawning period were used for fertilization. A total of 13 individuals in the control, 6 in the 1000 and 7 in the 5000 cells mL^{-1} treatments spawned within this time. Motile sperm from males were individually filtered through a 55 μm mesh and kept separate. Oocytes from females were individually filtered first through a 150 μm mesh to remove debris, washed and retained on a 35 μm mesh, re-suspended in 0.1 μm FSW and kept separate.

2.6.1 Flow cytometric assessment of spermatozoa

Filtered sperm from individuals was kept separate and the concentration was adjusted to have a final concentration of approximately 300-500 sperm μL^{-1} per individual. Analyses were performed using a Cytomics FC500 flow cytometer (Beckman Coulter), equipped with a 488 nm Argon laser. Collected data were analysed using WinMDI 2.9 software. Methods for assessing the morphology,

viability, relative DNA content and ROS production of the spermatozoa were determined according to Le Goïc et al. (2013) and Rolton et al. (submitted).

2.6.2 Larval development

Fertile gametes of the same sex, obtained from adult clams from the 3 replicate aquaria tanks, were pooled per treatment. Washed oocytes were pooled in a sterile 1 L beaker to give a range of 500-2500 oocytes mL⁻¹ and could round-up before being stocked in 600 mL beakers to give a final concentration of 20 oocytes mL⁻¹ in a final volume of 500 mL of 0.1 µm FSW at a salinity of 27. Four replicate beakers were prepared per treatment. For each of the 4 replicate beakers containing pooled oocytes from adults exposed to each treatment, sperm from the same treatment as oocytes was added to have a range of 300- 700 sperm oocyte⁻¹ and mixed gently. The concentration of pooled sperm from each treatment was determined by photometric absorbance at 640 nm according to Bricelj (1979) and was between 2.9-4.7 x 10⁷ sperm mL⁻¹. Beakers were exposed to natural daylight, but kept away from direct sunlight. Water quality parameters were measured as above and temperature was maintained at 25± 1°C. Gentle aeration and daily 'swirling' of the uncovered beakers helped maintain DO levels. One hour after fertilization, at Day 1 and Day 4, 10 mL sub-samples were taken from each beaker and fixed in 0.1% buffered formalin. After sampling at Day 1, newly hatched larvae were fed with T-Iso and *C. muelleri* at 5 x 10⁴ cells mL⁻¹. At Day 3, T-Iso was again added at 5 x 10⁴ cells mL⁻¹ and *C. muelleri* at 3.5 x 10⁴ cells mL⁻¹ (total cell density = 10⁵ cells mL⁻¹ [T-Iso cell volume-equivalent]).

2.6.3 Fertilization success and larval measurements

Samples taken ~1 hour after fertilization, were assessed for numbers of fertilized (polar body - 4 cell stage) and unfertilized (no polar body) oocytes. At least 30 oocytes were counted for each of the 4 replicate beakers using a light microscope and a sedgewick rafter and data were expressed as fertilization success in percent (%). Percent of normal, abnormal (live) and dead embryos/larvae were calculated at Day 1 and 4 using a light microscope and a sedgewick rafter according to Carriker (2001) and Rolton et al. (2014). The mean shell length (greatest antero-posterior dimension) of twenty live larvae from each of the 4 replicate beakers per treatment were measured at Day 1 and 4 with a micrometer on a compound light microscope.

2.7 Statistical analysis

Where the same parameters were recorded for both Pine Island and Cedar Key clams, the same statistical tests were performed. The CI was compared between treatments keeping tank replication ($n= 5$ (Pine Island) or $n= 3$ (Cedar Key) per tank) and clam PbTx content and spermatozoa data obtained on the flow cytometer were combined from the 3 tank replicates. Percent data recorded on the flow cytometer, results for fertilization success (%) and, percent larval normality, abnormality and mortality data were arcsine square root transformed to normalize distributions. CI, PbTx, spermatozoa and mean larval size data and, transformed percentage data were compared using a one-way ANOVA with either Tukeys multiple comparison post- hoc test (Levene's test, $p > 0.05$), or Dunnetts T3 post-hoc test (Levene's test, $p < 0.05$) using treatment as an independent variable. Differences in the spawning frequency of clams from each treatment were compared using Pearson Chi square. Independent t-tests were used to compare the PbTx content of both batches of clams at the same concentration.

As a low number of Cedar Key clams were sampled for histopathology in control tanks; GS, the sum of pathological features in each tissue for each clam and the presence of each pathological and non-pathological feature were combined from the 3 replicate tanks and analysed on all individuals per treatment. [For Pine Island clams, $n= 18$ for each treatment. For Cedar Key clams, $n= 6$ in the control; $n= 13$ at $1000 \text{ cells mL}^{-1}$ and $n= 11$ at $5000 \text{ cells mL}^{-1}$]. The GS and sum of pathological features per tissue for each clam were compared per treatment using a Mann-Whitney test and the presence of each pathological and non-pathological feature were compared per treatment using Pearson Chi square.

Statistics were run using SPSS 22 statistical software and results were considered significant when p-value was < 0.05 .

3. Results

Prior to exposure, clams from the 'previously exposed' (Pine Island) and 'non-exposed' (Cedar Key) sites contained no detectable PbTx ($< 5 \text{ ng g}^{-1}$). Clams from both sites accumulated similar levels of PbTx following a 10 day exposure to whole cell *K. brevis* treatments ($p > 0.05$). 'Previously exposed' clams accumulated $1228 \pm 236 \text{ ng g}^{-1}$ (mean \pm SE) in the $1000 \text{ cells mL}^{-1}$ treatment and $5224 \pm 750 \text{ ng g}^{-1}$ in the $5000 \text{ cells mL}^{-1}$ treatment (Figure 1). Clams from the 'non-exposed' site accumulated $1444 \pm 296 \text{ ng g}^{-1}$ and $5229 \pm 1282 \text{ ng g}^{-1}$ in the 1000 and $5000 \text{ cells mL}^{-1}$ treatments respectively (Figure 1, $p > 0.05$).

The CI of 'previously exposed' clams prior to *K. brevis* exposure was 25.6 ± 1.4 and GS was 2.6 ± 0.2 . The CI and GS for 'non-exposed' clams prior to *K. brevis* exposure were 28.5 ± 1.0 and 3.5 ± 0.3 respectively. 'Previously exposed' clams contained 20% male clams, 60% female clams and the sex of 20% of the clams were undetermined. 'Non-exposed' clams contained 50: 50 males: females. After 10 days exposure to *K. brevis* treatments, the CI and GS were not significantly different to the start of exposure, nor were there significant differences between clams exposed to different *K. brevis* treatments for both populations of clams.

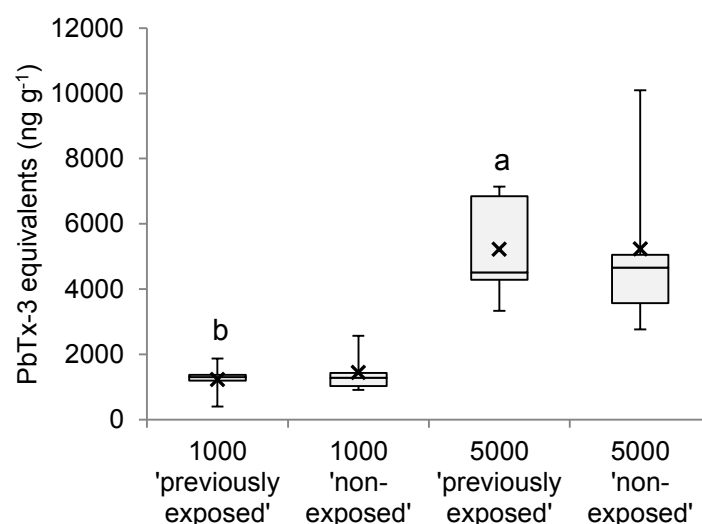


Figure 1: A comparison of PbTx content in individual whole tissue samples of *Mercenaria mercenaria* from a 'previously exposed' site (Pine Island) and a 'non-exposed' site (Cedar Key) after a 10 day exposure to whole cells of *Karenia brevis* at 1000 and $5000 \text{ cells mL}^{-1}$. The top and bottom of boxes represent the interquartile range with the median line within. Bars are the minimum and maximum values and 'x' represents the mean. Day 0 and control day 10 samples were below the minimum detectable concentration (5 ng g^{-1}) and have not been reported in the figure. Letters indicate a significant difference between treatments ($n=5$).

Prior to exposure, clams from both 'previously exposed' and 'non-exposed' sites had a low presence of pathological features within tissues (10- 20% hemocyte infiltration in the stomach/ intestine).

Following 10 days exposure of 'previously exposed' clams to *K. brevis* treatments, a significant increase in diapedesis was recorded in the stomach/ intestine compared to controls ($p= 0.018$, Figures 2 A and 3). Clams from the 'previously exposed' site exposed to the highest *K. brevis* concentration showed a significant increase in the mean sum of pathological features per clam in the gill (0.3) and stomach/ intestine (1.7) as compared to the lower *K. brevis* treatment (0.2 and 0.9 respectively) and control (0 and 0.7 respectively, Table 1).

Exposed clams from Cedar Key (non-exposed site) showed a significant increase in the mean sum of pathological features per clam in the stomach/ intestine of 'non-exposed' clams exposed to 5000 cells mL⁻¹ *K. brevis* (1.0) compared to clams exposed to 1000 cells mL⁻¹ *K. brevis* was recorded (0.3, $p= 0.021$, Table 1).

No pathological features were observed in the gonad of clams from either site exposed to *K. brevis* treatments compared to control.

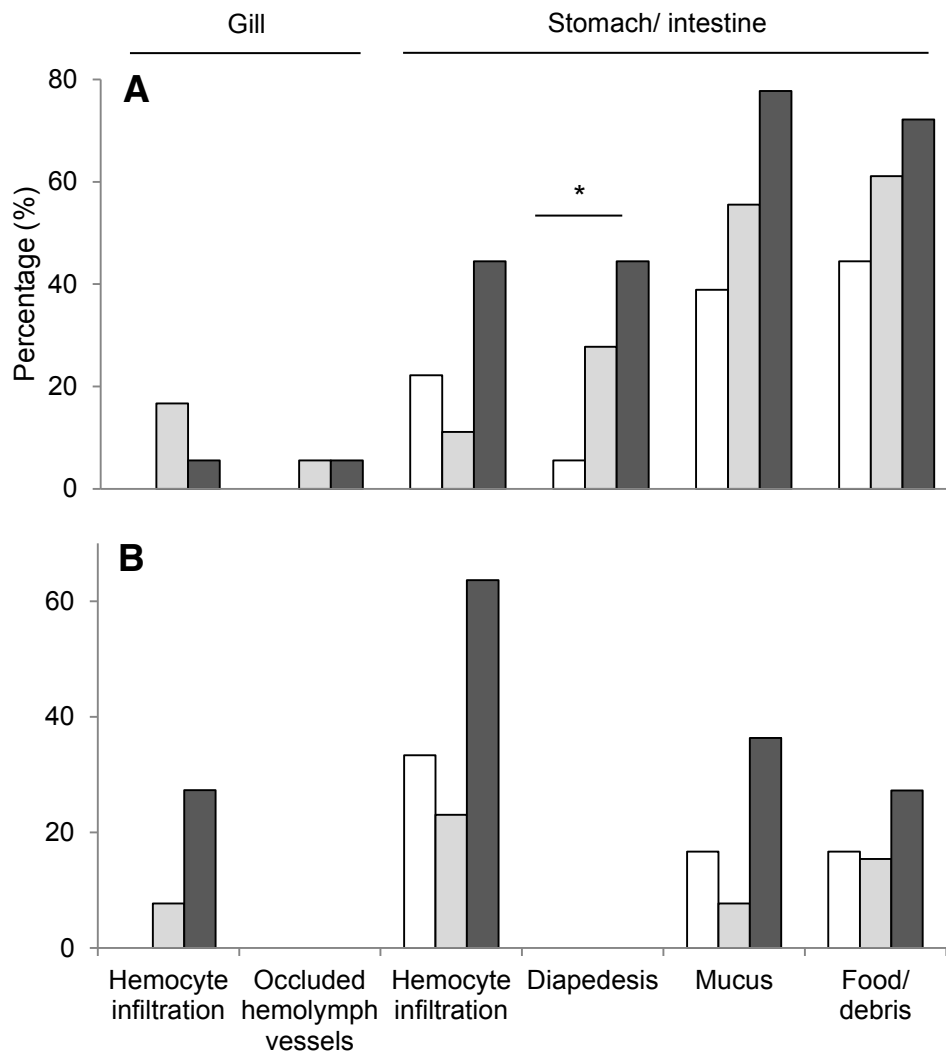


Figure 2: Percent of pathological and non-pathological features (%) in the stomach/ intestine and gill of *Mercenaria mercenaria* from **A**: Pine Island ('previously exposed') and **B**: Cedar Key ('non-exposed') sites exposed daily to whole cells of *Karenia brevis* at 0 \square , 1000 \square and 5000 \blacksquare cells mL⁻¹ and a control with no *K. brevis* for 10 days. For Pine Island clams, $n= 18$ for each treatment. For Cedar Key clams, $n= 6$ in the control (0 cells mL⁻¹); $n= 13$ at 1000 cells mL⁻¹ and $n= 11$ at 5000 cells mL⁻¹. * denotes significant difference ($p < 0.05$) between treatments.

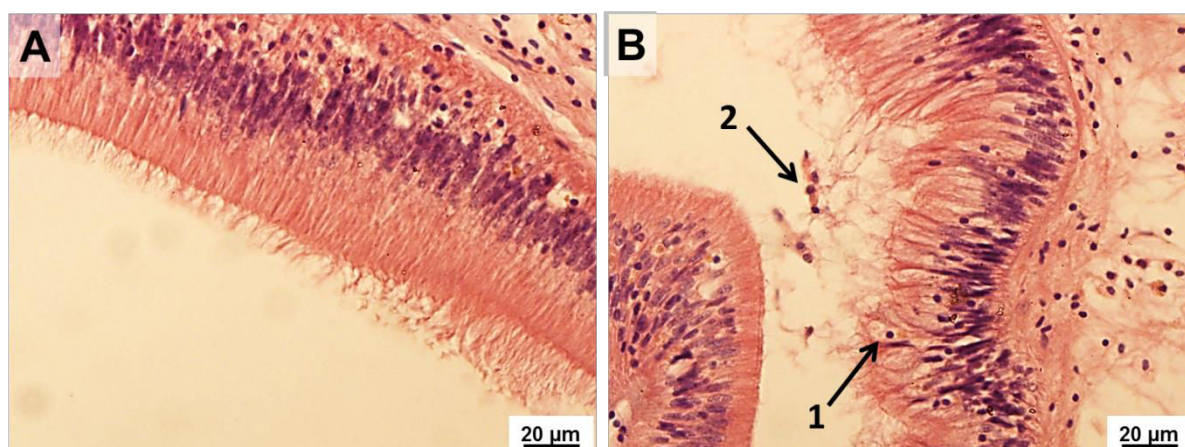


Figure 3: An example of a histopathological feature recorded in *Mercenaria mercenaria* exposed to *Karenia brevis*. **A**: Normal intestine (digestive gland), **B**: intestine of clam exposed to *K. brevis* showing diapedesis of hemocytes into the lumen of the intestine (1) and hemocytes in the lumen of the intestine (2).

Table 1: Mean number of pathological features per individual in the gill and stomach/ intestine of *Mercenaria mercenaria* from; Pine Island ('previously exposed') and; Cedar Key ('non-exposed'), exposed for 10 days to whole-cell treatments of *Karenia brevis* at 0, 1000 and 5000 cells mL⁻¹ concentrations. Data are presented as mean \pm standard error, SE. For Pine Island clams, $n= 18$ for each treatment. For Cedar Key clams, $n= 6$ in the control (0 cells mL⁻¹); $n= 13$ at 1000 cells mL⁻¹ and $n= 11$ at 5000 cells mL⁻¹. Treatments with the same letter were not significantly different ($p > 0.05$).

	'previously exposed' (Pine Island) <i>M. mercenaria</i>			'non-exposed' (Cedar Key) <i>M. mercenaria</i>		
	0	1000	5000	0	1000	5000
Gill	0.0 \pm 0.0 ^b	0.2 \pm 0.1 ^b	0.3 \pm 0.1 ^a	0.0 \pm 0.0	0.1 \pm 0.1	0.3 \pm 0.1
Stomach/ intestine	0.7 \pm 0.2 ^b	0.9 \pm 0.2 ^b	1.7 \pm 0.2 ^a	0.5 \pm 0.3 ^{ab}	0.3 \pm 0.1 ^b	1.0 \pm 0.2 ^a

Clams from the 'previously exposed' (Pine Island) site did not spawn following thermal stimulation. All female clams from the 'non-exposed' site (Cedar Key) that had spawned within the 30 minute period after the start of spawning were used for subsequent larval development assays; oocytes from 2 females and 3 females were used in the 1000 and 5000 cells mL⁻¹ treatment respectively compared to oocytes from 8 females in the control. Flow cytometric analysis of the sperm obtained from the spawning showed no significant differences in the parameters measured. However, the fertilization success of gametes obtained from Cedar Key clams

exposed to *K. brevis* for 10 days was significantly reduced (46% for gametes from clams exposed to 1000 cells mL⁻¹ *K. brevis* and 61% for those exposed to 5000 cells mL⁻¹ *K. brevis*) compared to the control (86%; $p < 0.001$, Figure 4).

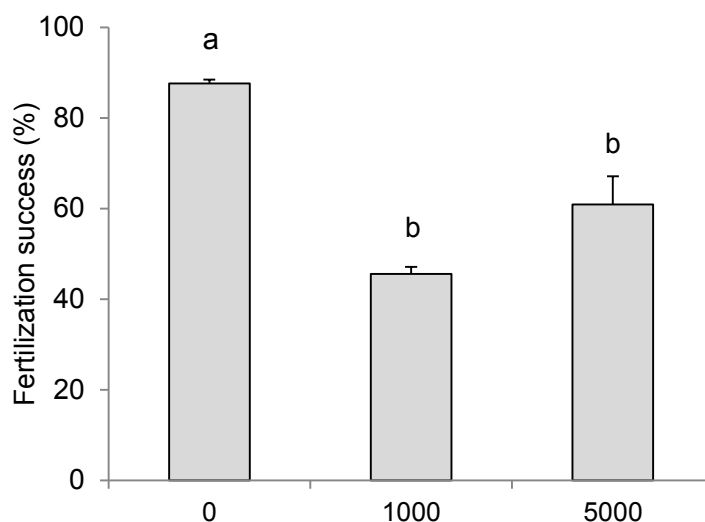


Figure 4: Fertilization success (%) of gametes from previously 'non-exposed' clams (Cedar Key) (*Mercenaria mercenaria*) exposed for 10 days to whole cells of *Karenia brevis* at 0, 1000 and 5000 cells mL⁻¹ *K. brevis*. Data presented are means \pm standard error, $n = 4$. Treatments with the same letter were not significantly different ($p > 0.05$).

Although the mean size of larvae at Day 1 and 4 was not significantly different between treatments, significant differences were seen in percent abnormality and normality at Day 1 and in mortality, abnormality and normality at Day 4 (Figure 5). At Day 1 PF, percent abnormality of larvae obtained from clams exposed to 5000 cells mL⁻¹ of *K. brevis* for 10 days was significantly higher than from control clams (7.9% vs 3.1%, $p = 0.023$) and percent normality of larvae was significantly lower (88.9% vs 95.3%, $p = 0.009$). By Day 4 PF, larvae from clams exposed to the 5000 cells mL⁻¹ *K. brevis* treatment showed significantly more mortalities (4.4%, $p = 0.001$), abnormalities (5.3%, $p = 0.008$) and significantly fewer normal larvae (90.3%, $p = 0.001$) than larvae from clams exposed to the 1000 cells mL⁻¹ treatment (2.1%, 1.9%, 95.9% respectively) and control (2.5%, 2.4%, 95.1% respectively) (Figure 5).

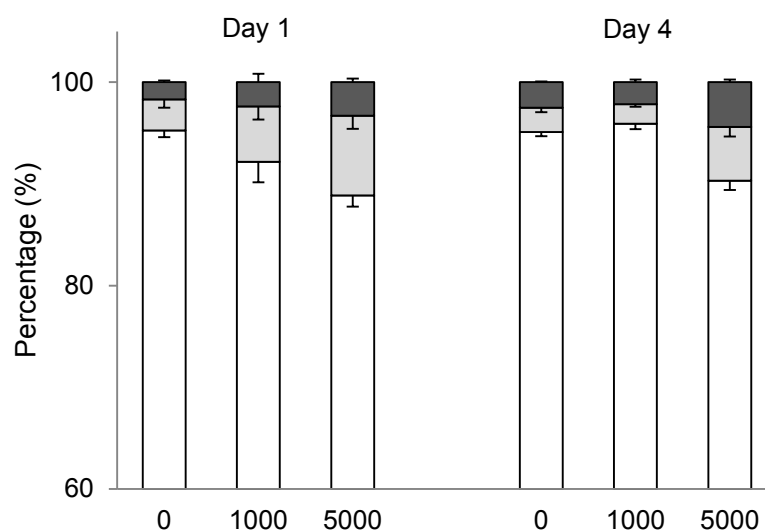


Figure 5: Larvae obtained from Cedar Key clams (non-exposed) at Day 1 (left) and Day 4 (right) post-fertilization. □ represents percent of normal larvae; ■, percent of abnormal larvae and ■, percent mortality of larvae. Data are presented as mean \pm standard error, $n = 4$.

4. Discussion

4.1 Toxin accumulation

Within 10 days, clams from ‘previously exposed’ and ‘non-exposed’ sites exposed to *K. brevis* accumulated similar levels of PbTx (\sim 1000 and \sim 5000 ng g⁻¹ at 1000 and 5000 cells mL⁻¹ respectively) above the 800 ng g⁻¹ regulatory limit at which shellfish beds are closed for harvesting (Naar et al., 2002). A similar study by Griffith et al. (2013) showed that upon daily exposure to 500 cells mL⁻¹ of *K. brevis* for 5 days, the PbTx content of clams also from Pine Island (the ‘previously exposed’ site) reached 1000 ng g⁻¹. Monitoring the toxicity of juvenile hard clams exposed to field blooms of between 100-1200 *K. brevis* cells mL⁻¹, Bricelj et al. (2012) found clams became toxic quickly, accumulating around 1500 ng g⁻¹ PbTx after 3 days.

Biotoxin accumulation in bivalves differs between species (Bricelj and Shumway, 1998). Indeed, clams in the present study accumulated 4 times less PbTx than did *C. virginica* exposed under the same conditions (Rolton et al., in prep. a/ Chapter 1). The filtration rate of bivalves exposed to toxic algae also differs between species (Bricelj et al., 1990; Hégaret et al., 2007a; Lesser and Shumway, 1993; Leverone et al., 2007; Shumway, 1990; Shumway and Cucci, 1987; Wikfors and Smolowitz, 1993). Monitoring the clearance rate of juvenile *M. mercenaria* and *C. virginica*,

exposed to 1000 cells mL⁻¹ of *K. brevis* for 1 hour, Leverone et al. (2007) observed that as well clearing less T-Iso than oysters, clams also cleared fewer *K. brevis* cells. Oysters cleared 54% of *K. brevis* cells compared to just 9% for clams (Leverone et al., 2007). Adult clams in the present study may also have filtered less *K. brevis* than did oysters. Although not quantitatively measured, pseudofaeces production was noted in the tanks of clams exposed to *K. brevis*, suggesting clams may have eliminated toxic material prior to ingestion. Leverone et al. (2007) did not however, record any pseudofaeces production in juvenile clams exposed to *K. brevis*. This may be due to the different exposure duration and/ or the life stage exposed to that of the present study.

4.2 Effects on adults

A significant increase of hemocyte diapedesis into the stomach/ intestine was observed in exposed clams from the 'previously exposed' site (Pine Island). Toxic algae are known to cause inflammatory responses in many bivalve species, due to contact with the cell and/ or associated toxin (Basti et al., 2011; Galimany et al., 2008a,b,c; Haberkorn et al., 2010; Lassudrie et al., 2014; Smolowitz and Shumway, 1997). The significantly affected tissues in the present study (gills and stomach/ intestine) were those that came into direct contact with *K. brevis* cells/ associated toxins. Brevetoxin is intracellular, yet during culture growth, cells of *K. brevis* may lyse and release PbTx, resulting in extracellular PbTx, free in the culture supernatant (Pierce et al., 2001, 2008). In addition, cells of *K. brevis* also produce other harmful bioactives which can be allelopathic, hemolytic and ichthyotoxic and possibly other uncharacterized harmful compounds (Kubanek et al., 2005, 2007; Marshall et al., 2005; Neely and Campbell, 2006; Prince et al., 2010; Tatters et al., 2010). These compounds may be free in the culture supernatant and also possibly associated with the *K. brevis* cell as with *K. mikimotoi* (Parrish et al., 1998; Zou et al., 2010). The observed responses, may be in part due to contact with these PbTx/ bioactives.

However, once *K. brevis* is in the stomach/ intestine, cells lyse during digestion, releasing previously intracellular PbTx. We speculate there is additional exposure to PbTx in the stomach/ intestine. Leverone (2007) observed hemocyte aggregations and infiltrations in the digestive diverticula of the bay scallop, *Argopecten irradians*, exposed to whole and lysed preparations of *K. brevis* up to 500 cells mL⁻¹ for 2

weeks. The cause of the pathology (i.e. exposure to *K. brevis* or PbTx) could not be definitively proved; however, results suggested a cytotoxic response rather than a response to the dinoflagellate cell (Leverone, 2007). Diapedesis, as observed in the present study and hemocyte infiltration as recorded by Leverone (2007), have been suggested as a mechanism to eliminate toxins through the lumen of the intestine (Franchini et al., 2003; Galimany et al., 2008b; Haberkorn et al., 2010; Hégaret et al., 2009; Lassudrie et al., 2014).

The present response may therefore be caused by contact with *K. brevis* cells and associated toxins (in the gill and stomach/ intestine), followed by additional exposure in the stomach/ intestine to previously intracellular PbTx when cells lyse during digestion. Once accumulated in the bivalve, PbTx (and other toxins?) may be removed via hemocyte diapedesis. This is in agreement with a previous study by Rolton et al. (in prep. a/ Chapter 1), in which *C. virginica* were exposed to cells of *K. brevis* under the same conditions as the present study and inflammatory responses were recorded in the tissues also in direct contact with *K. brevis* cells/ associated toxins.

4.3 Effects on offspring

Exposure of adult clams from Cedar Key ('non-exposed') to *K. brevis* cells/ toxic compounds may have also caused the significant differences observed in fertilization success and subsequent larval development. Results from Rolton et al. (in prep. b/ Chapter 3), also suggest that fertilization and larval development of hard clams are negatively affected following exposure to a bloom of *K. brevis* in the field, which may be due in part to PbTx presence in the gametes. The significant negative effects on larval development suggest that the uptake of toxins/harmful compounds into the adult may affect gametes and offspring indirectly. A possible effect of using oocytes from a small numbers of females in exposed treatments however, cannot be ruled out. During hatchery spawning procedures, it is desirable to use viable eggs from several females to ensure successful fertilization and larval development (Eversole, 2001; Utting and Spencer, 1991). Therefore, fertilization assays using oocytes from exposed females ($n= 2$ and $n= 3$ for 1000 and 5000 cells mL⁻¹ treatments respectively) were not as optimal as they could have been with more females. Although cellular parameters of spermatozoa used for fertilization and larval studies

was not significantly affected by exposure, after spawning was considered finished (30 minutes after the first individual began spawning), some clams from exposed treatments continued to spawn. Flow cytometric analysis of these additional sperm and those used for fertilization, revealed a significant reduction in sperm ROS production ($p= 0.007$, data not shown) from individuals exposed to *K. brevis* treatments. This leads us to consider that exposure of clams may cause delayed ejection of 'lesser quality' sperm that has been more affected by adult exposure to *K. brevis*. Haberkorn et al. (2010), recorded morphological and functional changes in *Crassostrea gigas* sperm maternally exposed to the PST producing dinoflagellate, *A. minutum* for 4 days. The motility, ATP content and DNA content of spermatozoa were significantly lower and the sperm complexity was significantly higher than in controls. These alterations in spermatozoa cell parameters, as observed in the present study and by Haberkorn et al. (2010) could cause an additional decrease in fertilization and larval development.

As blooms may continue during times of bivalve spawning and larval development, it is likely that early stages may be directly exposed to *K. brevis* cells in addition to this 'maternal' exposure. A previous study by Summerson and Peterson (1990) demonstrated a significant reduction in the recruitment of *A. irradians* following direct exposure of early life stages to a bloom *K. brevis* in North Carolina. This study also highlighted that these scallops may have been vulnerable to *K. brevis* exposure due to the rarity of *K. brevis* blooms in that area (Summerson and Peterson, 1990; Tester et al., 1989).

4.4 Stock differences

Clams from both sites used in the present study had differing exposure histories to *K. brevis* and, although subtle, differences were recorded between these 2 stocks following controlled exposure to *K. brevis*. Overall, clams from the 'previously exposed' site showed a dose-dependent histological response to *K. brevis* exposure, while clams from the 'non-exposed' site, did not. The pathological responses of clams from both sites may be related to food presence in the stomach/ intestine. Clams which had a higher presence of food in the stomach/ intestine may have fed more (on shellfish diet and *K. brevis*) which resulted in increased contact with *K. brevis*/ toxins and a higher prevalence pathologies in the stomach/ intestine and gill. Clams with a lower presence of food in the stomach/ intestine ('non-exposed' Cedar

Key clams) may have fed less (and so showed less pathologies within tissues) possibly indicating that Cedar Key clams were more sensitive to *K. brevis* exposure, agreeing with previous findings that 'naïve' populations may be more susceptible to HAB exposure (Shumway et al., 1985; Bricelj et al., 2005). However, only the remaining un-spawned clams from Cedar Key (non-exposed) were sampled for histology and more effects might have been observed in clams that spawned, although no histological features were observed in the gonad of clams from both populations. Brevetoxin accumulation appears disconnected from food/ debris presence in the stomach/ intestine, as both stocks of clams accumulated the same PbTx. Different stocks of clams may not accumulate or depurate PbTx with the same kinetics.

Clams from Pine Island (previously exposed) did not spawn at the end of the exposure period, and could not be investigated for subsequent effects on sperm, fertilization and larval development. This may be an adaptive response to previous *K. brevis* exposure; however, clams in the controls also did not spawn, leading us to consider that clams from Pine Island were not yet fully ripe. The GS of Cedar Key (non-exposed) clams was higher.

Differing responses between clams from both sites following laboratory exposure to *K. brevis* could be due to genetic differences between these stocks. Although *M. mercenaria* from both sites used in the present study originated from hatchery produced spat in Cedar Key, FL and are genetically homogeneous, future studies must determine if there are any genetic differences between these stocks.

Differing responses between clams from both sites may also be due to environmental factors. Clams previously exposed to bloom concentrations of *K. brevis* may show adaptive responses and tolerate exposure to *K. brevis* more than previously non-exposed clams. Although not previously exposed to bloom concentrations of *K. brevis*, the Cedar Key clams may have been exposed to below background levels ($< 1 \text{ cell mL}^{-1}$) as cells are ubiquitous in the GoM (Finucane, 1964) and hence cannot be considered truly 'naïve' to *K. brevis*/ PbTx exposure. In their first year of exposure, 'naïve' *Mytilus edulis* transplanted to areas exposed to *Alexandrium* sp. blooms, showed 50% less accumulation of paralytic shellfish toxins (PST) compared to frequently exposed *M. edulis* from bloom areas (Chebib et al.,

1993). During exposure to a bloom the next year however, these previously 'naïve' mussels accumulated the same toxin as chronically exposed mussels (Chebib et al., 1993). A combination of abiotic and biotic environmental differences between sites may also have resulted in interactive effects with *K. brevis* exposure. Interactive effects between parasite/bivalve host/HAB exposures which may cause synergistic or antagonistic effects of HAB exposure have been demonstrated (da Silva et al., 2008; Hégaret et al., 2007b, 2009; Lassudrie et al., 2014). Finally, observed responses could be related to the environmental history of these stocks and a combination of adaptive and interactive responses.

4.5 Conclusion

Upon exposure to high bloom concentrations of *K. brevis*, adult *M. mercenaria* accumulated high levels of brevetoxin and inflammatory responses were recorded in the stomach/ intestine. Clams from different stocks may respond differently to *K. brevis* exposure. Significant negative effects on the fertilization success and subsequent larval development of clams from Cedar Key (previously not exposed to *K. brevis*) were recorded. This suggests that exposure of adult hard clams to high bloom concentrations of *K. brevis* may significantly affect gamete and larval development. If blooms continue during spawning events, this may cause cumulative negative effects, as gametes and larvae will continue to be directly exposed to *K. brevis*.

Acknowledgements

We thank Dr. Inke Sunila, for histopathology training and Dr. Richard Pierce, Michael Henry, Patricia Blum and the team at the Mote Marine Laboratory, FL, for supplying cultures of *K. brevis* and for LC-MS/MS toxin analysis of *K. brevis* cultures. Thanks also to Leslie Sturmer and Toney Heebe ('Cut-throat clams') for supply of adult *M. mercenaria* and to Dr. Hélène Hégaret and Caroline Fabioux for critical review of an earlier version of the manuscript. This collaborative work was partially supported by Université de Bretagne Occidentale, and by "Laboratoire d'Excellence" LabexMER (ANR-10-LABX-19), co-funded by a grant from the French government under the program "Investissements d'Avenir".

References

- Anderson, P. A., Roberts-Misterly, J., Greenberg, R. M. 2005. The evolution of voltage-gated sodium channels: were algal toxins involved?. *Harmful Algae*. 4(1): 95-107.
- Andrews, J. 1971. *Sea shells of the Texas coast*. University of Texas Press. Austin, TX. pp. 285.
- Baden, D. G., Bourdelais, A. J., Jacocks, H., Michelliza, S., Naar, J. 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ. Health Perspect.* 113(5): 621-625.
- Basti, L., Endo, M., Segawa, S. 2011. Physiological, pathological, and defense alterations in Manila clams (short-neck clams), *Ruditapes philippinarum*, induced by *Heterocapsa circularisquama*. *J. Shellfish Res.* 30(3): 829-844.
- Brand, L. E., Compton, A. 2007. Long-term increase in *Karenia brevis* abundance along the Southwest Florida Coast. *Harmful Algae*. 6(2): 232-252.
- Bricelj, V. M., Haubois, A. G., Sengco, M. R., Pierce, R. H., Culter, J. K., Anderson, D. M. 2012. Trophic transfer of brevetoxins to the benthic macrofaunal community during a bloom of the harmful dinoflagellate *Karenia brevis* in Sarasota Bay, Florida. *Harmful Algae*. 16: 27–34.
- Bricelj, V. M., Connell, L., Konoki, K., Macquarrie, S. P., Scheuer, T., Catterall, W., Trainer, V. L. 2005. Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP. *Nature*. 434(7034): 763–767.
- Bricelj, V. M., Shumway, S. E. 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics, and biotransformation. *Rev. Fish. Sci.* 6(4): 315-383.
- Bricelj, V. M., Lee, J. H., Cembella, A., Anderson, D. M. 1990. Uptake of *Alexandrium fundyense* by *Mytilus edulis* and *Mercenaria mercenaria* under controlled conditions. In: Granéli, E., Sundström, B., Edler, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton*, Elsevier, New York. pp. 269-274.
- Bricelj V.M. 1979. Fecundity and related aspects of the hard clam (*Mercenaria mercenaria*) reproduction in Great South Bay, NY. MSc Thesis. State University of New York at Stony Brook.
- Carriker, M.R., 2001. Embryogenesis and organogenesis of veligers and early juveniles. *Dev. Aquac. Fish. Sci.* 31: 77–115.
- Chebib, H. A., Cembella, A. D., Anderson, P. 1993. Differential paralytic shellfish toxin accumulation and detoxification kinetics in transplanted populations of *Mytilus edulis* exposed to natural blooms of *Alexandrium excavatum*. In: Smayda, T. J. and Shimizu, Y. (Eds.), *Toxic Phytoplankton Blooms in the Sea*. New York, Elsevier Science Publishers. pp 383–388.
- Cummins, J. M., Jones, A. C., Stevens, A. A. 1971. Occurrence of toxic bivalve molluscs during a *Gymnodinium breve* "red tide". *T. Am. Fish. Soc.* 100(1): 112-116.
- da Silva, P. M., Hégaret, H., Lambert, C., Wikfors, G. H., Le Goïc, N., Shumway, S. E., Soudant, P. 2008. Immunological responses of the Manila clam (*Ruditapes philippinarum*) with varying parasite (*Perkinsus olseni*) burden, during a long-term exposure to the harmful alga, *Karenia selliformis*, and possible interactions. *Toxicon*. 51(4): 563–73.
- Delaporte, M., Soudant, P., Moal, J., Kraffe, E., Marty, Y., Samain, J.-F. 2005. Incorporation and modification of dietary fatty acids in gill polar lipids by two bivalve species *Crassostrea gigas* and *Ruditapes philippinarum*. *Comp. Biochem. Phys. A*. 140(4): 460–70.

- Eversole, A. G. 1997. Gametogenesis of *Mercenaria mercenaria*, *M. campechiensis* and their hybrids. *Nautilus*. 110(4): 107-110.
- Eversole, A. G. 2001. Reproduction in *Mercenaria mercenaria*. In: Kraeuter, J. N., Castagna, M. (Eds.). *Biology of the hard clam* (Vol. 31). Elsevier Science, Amsterdam. pp 221-260.
- Finucane, J.H., 1964. Distribution and seasonal occurrence on *Gymnodinium breve* on the West Coast of Florida, 1954–57. In: United States Fish and Wildlife Service Special Scientific Report. Fisheries No. 487.
- Franchini, A., Milandri, A., Poletti, R., Ottaviani, E. 2003. Immunolocalization of yessotoxins in the mussel *Mytilus galloprovincialis*. *Toxicon*. 41: 967–970.
- Galimany, E., Place, A. R., Ramón, M., Jutson, M., Pipe, R. K. 2008a. The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*. *Harmful Algae*. 7(1): 91–98.
- Galimany, E., Sunila, I., Hégarret, H., Ramón, M., Wikfors, G. H. 2008b. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: Histopathology, immune responses, and recovery. *Harmful Algae*. 7(5): 702–711.
- Galimany, E., Sunila, I., Hégarret, H., Ramón, M., Wikfors, G. H. 2008c. Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. *Harmful Algae*. 7(5): 630–638.
- Griffith, A. W., Shumway, S. E., Volety, A. K. 2013. Bioaccumulation and depuration of brevetoxins in the eastern oyster (*Crassostrea virginica*) and the northern quahog (= hard clam, *Mercenaria mercenaria*). *Toxicon*. 66: 75–81.
- Guillard, R. R. 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W. L. Chanley, M. H. (Ed.), *Culture of marine invertebrate animals*, Springer US. pp. 29-60.
- Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P. 2010. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. *Harmful Algae*. 9(5): 427–439.
- Hanna, G.D. 1966. Introduced mollusks of western North America. *Calif. Acad. Sci. Occasional Papers* 48:1-108.
- Hégarret, H., da Silva, P. M., Sunila, I., Shumway, S. E., Dixon, M. S., Alix, J., Wikfors, G.H., Soudant, P. 2009. Perkinsosis in the Manila clam *Ruditapes philippinarum* affects responses to the harmful alga, *Prorocentrum minimum*. *J. Exp. Mar. Biol. Ecol.* 371(2): 112–120.
- Hégarret, H., Wikfors, G. H., Shumway, S. E. 2007a. Diverse feeding responses of five species of bivalve mollusc when exposed to three species of harmful algae. *J. Shellfish Res.* 26(2): 549-559.
- Hégarret, H., da Silva, P. M., Wikfors, G. H., Lambert, C., De Bettignies, T., Shumway, S. E., Soudant, P. 2007b. Hemocyte responses of Manila clams, *Ruditapes philippinarum*, with varying parasite, *Perkinsus olseni*, severity to toxic-algal exposures. *Aquat. Toxicol.* 84(4): 469–479
- Hégarret, H., G. H. Wikfors, P. Soudant, M. Delaporte, J. H. Alix, B. C. Smith, M. S. Dixon C. Que´re, J.R. Le Coz, C. Paillard, J. Moal, J.-F. Samainet. 2004. Immunological competence of eastern oysters, *Crassostrea virginica*, fed different microalgal diets and challenged with a temperature elevation. *Aquaculture*. 234: 541-560.

- Heil, C. A., Bronk, D.A., Dixon, L.K., Hitchcock, G.L., Kirkpatrick, G.J., Mulholland, M.R., O'Neil, J.M., Walsh, J.J., Weisberg, R., Garrett, M. 2014. The Gulf of Mexico ECOHAB: *Karenia* Program 2006–2012. *Harmful Algae*. 38: 3-7.
- Hepell, D. 1961. The naturalization in Europe of the quahog, *Mercenaria mercenaria* (L.). *J. Conchol.* 25: 21-34.
- Howard, D.W., Lewis, E.J., Keller, B.J., Smith, C.S. 2004. *Histological Techniques for Marine Bivalve Mollusks and Crustaceans*. NOAA Technical Memorandum. NOSNCCOS, Oxford, MD, USA, pp. 218.
- Humason, G. L. *Animal Tissue Techniques*, 1979. San Francisco, CA USA: WN Freeman and Company.
- Kaas, P. 1937. *Venus mercenaria* L., een nieuwe mollusk voor de Nederlandse fauna. *Basteria*. 2(4): 58-60.
- Kubanek, J., Snell, T. W., Pirkle, C. 2007. Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing. *Limnol. Oceanogr.* 52(3): 1026–1035.
- Kubanek, J., Hicks, M.K., Naar, J., Villareal, T.A. 2005. Does the red tide dinoflagellate *Karenia brevis* use allelopathy to outcompete other phytoplankton? *Limnol. Oceanogr.* 50(3): 883–895.
- Landsberg, J. H. 2002. The effects of harmful algal blooms on aquatic organisms. *Rev. Fish. Sci.* 10(2): 113-390.
- Lassudrie, M., Soudant, P., Richard, G., Henry, N., Medhioub, W., da Silva, P. M., Donval, A., Bunel, M., Le Goïc, N., Lambert, C., de Montaudouin, X., Fabioux, C., Hégaret, H. 2014. Physiological responses of Manila clams *Venerupis (=Ruditapes) philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure. *Aquat. Toxicol.* 154: 27–38.
- Le Goïc, N., Hégaret, H., Fabioux, C., Miner, P., Suquet, M., Lambert, C., Soudant, P. 2013. Impact of the toxic dinoflagellate *Alexandrium catenella* on Pacific oyster reproductive output: application of flow cytometry assays on spermatozoa. *Aquat. Living Resour.* 26(3): 221-228.
- Lesser, M.P., Shumway, S.E. 1993. Effects of toxic dinoflagellates on clearance rates and survival in juvenile bivalve molluscs. *J. Shellfish Res.* 12(2): 377-381.
- Leverone, J. R., Shumway, S. E., Blake, N. J. 2007. Comparative effects of the toxic dinoflagellate *Karenia brevis* on clearance rates in juveniles of four bivalve molluscs from Florida, USA. *Toxicon.* 49(5): 634–645.
- Leverone, J.R. 2007. Comparative effects of the toxic dinoflagellate, *Karenia brevis*, on bivalve molluscs from Florida. *Theses and Dissertations*. Paper 2260.
- Leverone, J.R., Blake, N.J., Pierce, R.H., Shumway, S.E., 2006. Effects of the dinoflag-ellate *Karenia brevis* on larval development in three species of bivalve mollusk from Florida. *Toxicon.* 48: 75–84.
- Marshall, J., Ross, T., Pyecroft, S., Hallegraeff, G., 2005. Superoxide production by marine microalgae 2: Towards understanding ecological consequences and possible functions. *Mar. Biol.* 147: 541–549.
- Martoja, R., Martoja-Pierson, M. 1967. *Initiation aux techniques de l'histologie animale*, Paris, 1232 pp.

- McFarren, E. F., Tanabe, H., Silva, F. J., Wilson, W. B., Campbell, J. E., Lewis, K. H. 1965. The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. *Toxicon*. 3: 111-123.
- Naar, J., Kubanek, J., Weidner, A., Flewelling, L., Bourdelais, A., Steidinger, K., Baden, D. G. 2004. Brevetoxin Depuration in Shellfish via Production of Non-toxic Metabolites: Consequences for Seafood Safety and the Environmental Fate of Biotoxins, In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A., (Eds). *Harmful Algae*. 2002. Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and the Intergovernmental Oceanographic Commission of UNESCO. St. Petersburg, FL, USA. pp. 488- 490.
- Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P. L., Flewelling, L., Steidinger, K., Lancaster, J., Baden, D. G. 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. *Environ. Health Perspect.* 110(2): 179–185.
- Neely, T., Campbell, L. 2006. A modified assay to determine hemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae*. 5(5): 592–598.
- Nice, H. E. 2005. Sperm motility in the Pacific oyster (*Crassostrea gigas*) is affected by nonylphenol. *Mar. Pollut. Bull.* 50(12): 1668–74.
- NSSP, 2011. National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish 2011 Revision U. S. Department of Health and Human Services Public Health Service Food and Drug Administration Interstate Shellfish Sanitation Conference.
- Palmer, K.V.W. 1927. The Veneridae of Eastern America: Cenozoic and Recent. *Palaeontogr. Americana* 1(5): 209-522.
- Parrish, C.C., Bodennec, G., Gentien, P. 1998. Haemolytic glycolipids from *Gymnodinium* species. *Phytochemistry*. 47(5): 783-787.
- Pierce, R. H., Henry, M. S., Dickey, R., Plakas, S. 2002. NSP (*Karenia brevis*) toxins and metabolites in oysters, clams, and whelks. *Harmful Algae*. 2004, 294-296.
- Plakas, S. M., Jester, E.L.E., El Said, K.R., Granade, H.R., Abraham, A., Dickey, R.W., Scott, P.S., Flewelling, L.J., Henry, M., Blum, P., Pierce, R. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (*Crassostrea virginica*). *Toxicon*. 52(1): 32-38.
- Poli, M. A., Mende, T. J., Baden, D. G. 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol. Pharmacol.* 30(2): 129-135.
- Poli, M. A., Musser, S. M., Dickey, R. W., Eilers, P. P., Hall, S. 2000. Neurotoxic shellfish poisoning and brevetoxin metabolites: A case study from Florida. *Toxicon*. 38(7): 981–993.
- Prince, E. K., Poulson, K. L., Myers, T. L., Sieg, R. D., Kubanek, J. 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. *Harmful Algae*. 10(1): 39–48.
- Rolton, A., Vignier, J., Soudant, P., Shumway, S. E., Bricelj, V. M., Volety, A. K. 2014. Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*. *Aquat. Toxicol.* 15: 199–206.
- Rolton, A., Soudant, P., Vignier, J., Pierce, R., Henry, M., Shumway, S. E., Bricelj, V. M., Volety, A. K. submitted. Susceptibility of gametes and embryos of the eastern oyster, *Crassostrea virginica*, to *Karenia brevis* and its toxins. *Toxicon*.

- Rolton, A., Vignier, J., Soudant, P., Donaghy, L., Shumway, S., Bricelj, V.M., Volety, A.K. In prep a. Short and long-term effects of *Karenia brevis* exposure on *Crassostrea virginica*: Toxin accumulation and histopathological responses in adults.
- Rolton, A., Vignier, J., Soudant, P., Barbe, A., Shumway, S. E., Bricelj, V. M., Volety, A. K. in prep b. Transfer of brevetoxin to the gametes of eastern oysters (*Crassostrea virginica*) and northern quahogs (= hard clam, *Mercenaria mercenaria*) following field exposure to *Karenia brevis*.
- Sengco, M. R. 2009. Prevention and control of *Karenia brevis* blooms. *Harmful Algae*. 8(4): 623–628.
- Sievers, A. M. 1969. Comparative toxicity of *Gonyaulax monilata* and *Gymnodinium breve* to annelids, crustaceans, molluscs and a fish. *J. Protozool.* 16(3): 401-404.
- Simon, J. L., Dauer, D. M. 1972. A quantitative evaluation of red-tide induced mass mortalities of benthic invertebrates in Tampa Bay, Florida. *Environ. Lett.* 3(4): 229-234.
- Shaw, B. ., Battle, H. I. 1957. The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Can. J. Zoolog.* 325– 347.
- Shumway, S. E., Cucci, T. L. 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. *Aquat. Toxicol.* 10(1): 9-27.
- Shumway, S. E., Cucci, T. L., Gainey, L., Yentsch, C. M. 1985. A preliminary study of the behavioral and physiological effects of *Gonyaulax tamarensis* on bivalve molluscs. *Toxic Dinoflagellates*. 389-394.
- Smolowitz, R., Shumway, S. E. 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve molluscs. *Aquacult. Int.* 5(4): 291–300.
- Soudant, P., Paillard, C., Choquet, G., Lambert, C., Reid, H. I., Marhic, A., Donaghy, L., Birkbeck, T. H. 2004. Impact of season and rearing site on the physiological and immunological parameters of the Manila clam *Venerupis* (= *Tapes*, = *Ruditapes*) *philippinarum*. *Aquaculture*. 229(1-4): 401–418.
- Steidinger, K.A. 2009. Historical perspective on *Karenia brevis* red tide research in the Gulf of Mexico. *Harmful Algae*. 8(4): 549–561.
- Steidinger, K. A., Vargo, G. A., Tester, P. A., Tomas, C. R. 1998. Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico. *NATO Adv. Sci. I. G-Eco.* 41: 133-154.
- Summerson, H., Peterson, C., 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Ptychodiscus brevis*, outbreak recorded in North Carolina. *Estuaries*. 13(3): 322–331.
- Tatters, A.O, Muhlstein, H.I., Tomas, C.R. 2010. The hemolytic activity of *Karenia selliformis* and two clones of *Karenia brevis* throughout a growth cycle. *J. Appl. Phycol.* 22: 435–442.
- Tebble, N. 1966. *British bivalve seashells*. Alden Press, London, 212pp.
- Tester, P. A., Fowler, P. K., Turner, J. T. 1989. Gulf Stream transport of the toxic red tide dinoflagellate *Ptychodiscus brevis* from Florida to North Carolina. In: Cosper, E.M., Bricelj, V.M., Carpenter, E.J. (Eds), *Novel Phytoplankton Blooms*. Springer, Berlin. pp. 349-358.
- Tiffany III, W. J., Heyl, M. G. 1978. Invertebrate mass mortality induced by a *Gymnodinium breve* red tide in Gulf of Mexico waters at Sarasota, Florida. *J. Environ. Sci. Heal. A.* 13(9): 653-662.

United States Department of Agriculture (2002). Census of Aquaculture 2005: United States Summary and State Data. Geographic Area Series. Part 51 Volume 3: special studies part 2. AC-02-SP-2. Vol 1. United States Department of Agriculture. Washington D.C., pp 59-65.

Utting, S. D., Spencer, B. E. 1991. The hatchery culture of bivalve mollusc larvae and juveniles. Laboratory leaflet no. 68. Ministry of Agriculture, fisheries and food. Directorate of fisheries research. 31pp.

Whetstone, J. M., Sturmer, L. N., Oesterling, M. J. 2005. Biology and culture of the Hard Clam. Southern Regional Aquaculture Center. (433): 1-6.

Wikfors, G. H., Smolowitz, R. M. 1993. Detrimental effects of a *Prorocentrum* isolate upon hard clams and bay scallops in laboratory feeding studies. Dev. Mar. Biol. 1993.

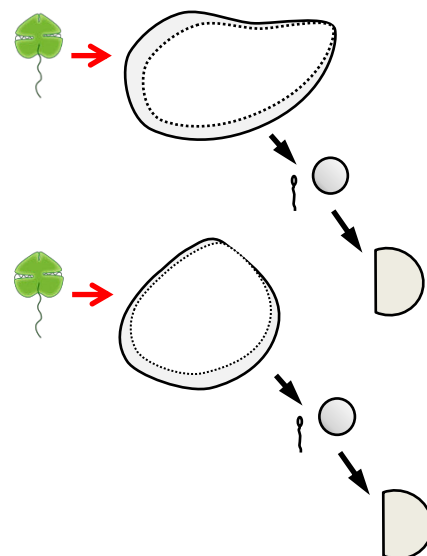
Zhang, T., Yang, H. S., Liu, B. Z., Liu, Y., Wen, H. X., Liu, S. L., Zhang, F. S. 2003. Primary study on modes of seed hard clam *Mercenaria mercenaria* (Linnaeus, 1758) culture in field. Oceanologia Et Limnologia Sinica. 34(6): 648-655.

Zou, Y., Yamasaki, Y., Matsuyama, Y., Yamaguchi, K., Honjo, T., Oda, T. 2010. Possible involvement of hemolytic activity in the contact-dependent lethal effects of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. Harmful Algae. 9: 367–373.

Chapter 3

Transfer of brevetoxin to the gametes of eastern oysters (*Crassostrea virginica*) and northern quahog (=hard clam, *Mercenaria mercenaria*) following field exposure to *Karenia brevis*.

Chapters 1 and 2 demonstrated that adult *C. virginica* and *M. mercenaria* are affected by exposure to *K. brevis* and, different responses were recorded between these 2 bivalve species (i.e. differences in PbTx accumulation, possible differences in the feeding process, histopathological effects and impacts on offspring). The present chapter relates these previous findings to the field. By analyzing clams and oysters which were exposed throughout gametogenesis to *K. brevis*, we looked for consistencies with previous findings.



The hypotheses tested were:

- Exposure to natural blooms of *K. brevis* has no effect on the reproductive and related physiological processes of adult clams and oysters.
- Brevetoxin accumulated in the adult is not present within the gametes.
- Adult exposure to *K. brevis* has no effect on the gametes and development of subsequently produced offspring.

Transfer of brevetoxin to the gametes of eastern oysters (*Crassostrea virginica*) and northern quahogs (= hard clam, *Mercenaria mercenaria*) following field exposure to *Karenia brevis*.

Anne Rolton^{1*,2}, Julien Vignier^{1,2}, Audrey Barbe², Sandra Shumway³, Monica Bricelj⁴, Aswani Volety^{2,5}, Philippe Soudant¹.

¹Université de Bretagne Occidentale—IUEM, LEMAR CNRS UMR 6539, Place Nicolas Copernic, Technopôle Brest Iroise, 29280 Plouzané, France;

²Florida Gulf Coast University, College of Arts and Sciences, 10501 FGCU Blvd South, Fort Myers, Florida 33965;

³Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340;

⁴Department of Marine and Coastal Sciences and Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349;

⁵Present address: University of North Carolina Wilmington, 601 S. College Rd, Wilmington, NC 28403.

*email: annerolton@gmail.com Phone: +33 298 498 623

Abstract

The 2012-2013 bloom of the red-tide dinoflagellate, *Karenia brevis*, was present in Lee county, FL, from September 2012 through May 2013. The eastern oyster (*Crassostrea virginica*) and hard clam (*Mercenaria mercenaria*), which contribute markedly to the economy and ecology of the region, were exposed to this bloom during a period of gametogenesis and gamete ripening. In the present study, ripe adult clams and oysters were collected from a site exposed to *K. brevis*, and a site 200 miles north which was not exposed to the bloom. Bivalves from both sites were examined for histopathological features, condition index and gonadal stage and area. The brevetoxin (PbTx) content of whole adult tissue and of stripped, filtered and concentrated gametes were assessed. Morphological and physiological characteristics of spermatozoa from spawned adults were determined and, differences in fertilization success and subsequent larval quality were assessed for 11 days post-fertilization (PF). Significant pathological and non-pathological differences were recorded in the mantle, gill and stomach/ intestine of adult bivalves of both species between exposed and non-exposed sites. Significantly more instances of mucus in the stomach/ intestine were observed for clams (23.3%) and oysters (20%) from exposed sites than those from non-exposed sites (0%). Significantly more clams from the exposed site showed edema in the gill (30%) than clams from the non-exposed site (3%). Oysters from the exposed site showed significantly higher instances of edema (33%) and occluded hemolymph vessels (50%) in the mantle than from the non-exposed site (3% and 17% respectively). There was accumulation of PbTx within the spermatozoa and oocytes of both species and was estimated to be 16- 30% of the adult PbTx load. Gametes and larvae obtained from exposed clams showed significantly lower fertilization success and larval size at Day 1 PF. A significant increase in mortalities and a smaller larval size were noted for the first 6 days PF for larvae obtained from exposed adult oysters. Negative effects may be due to presence of PbTx in the oocytes, which is mobilized by the larvae during embryonic and lecithotrophic larval development. Results highlight the need to consider not just effects of *K. brevis* blooms on adults, but also the effects on any offspring produced during blooms of toxic algae. Ecotoxicological studies and bivalve restoration projects should also consider wider

ranging environmental factors, such as the presence of toxic algae, when making assessments in the field.

Key words: *Karenia brevis*, brevetoxin, accumulation, *Mercenaria mercenaria*, *Crassostrea virginica*, gametes.

1. Introduction

Harmful algal blooms (HAB) of the brevetoxin (PbTx), producing algae, *Karenia brevis*, are common in the Gulf of Mexico (Sengo, 2009; Steidinger, 2009). Brevetoxin is an intracellular, lipid-soluble, ladder-frame polyether biotoxin, which binds to site 5 on the voltage sensitive sodium channel of nerve cells, causing permanent excitation of the cell and disrupting nervous transmission (Baden et al., 2005; Gawley et al., 1995; Poli et al., 1986). Blooms of *K. brevis* (classed as ≥ 1 cell mL^{-1}) cause economic problems due to negative impacts on recreation and tourism and, closure of shellfish harvest beds (when cell counts reach above 5 cells mL^{-1}) (NSSP, 2011). Human health problems, caused by inhalation of aerosolized toxins or ingestion of toxic shellfish leading to neurotoxic shellfish poisoning (NSP), may also occur (Pierce et al., 2003; Steidinger et al., 1998). Finally, environmental problems caused by blooms, such as increased mortalities and sublethal negative effects on wildlife, including marine bivalve molluscs may be severe (Landsberg, 2002; Shumway, 1990; Summerson and Peterson, 1990). As *K. brevis* blooms are most likely to form in late summer and autumn months and may last weeks to many months (Hu et al., 2006; Rounsefell and Nelson, 1966), bivalves, such as the economically and environmentally important species, *Mercenaria mercenaria* (= hard clam), and *Crassostrea virginica* (= eastern oyster), are likely to be exposed during a time of gametogenesis, gamete ripening, spawning and larval recruitment (Volety et al., 2009; Hesselman et al., 1989). The 2012- 2013 *K. brevis* bloom lasted from September 2012 through May 2013 and extended from Pinellas, FL, to the Florida Keys (Florida Fish and Wildlife Conservation Commission (FWC), monitoring data). This is not a rare phenomenon; bloom concentrations of *K. brevis* have been present along the south west coast of Florida (SW FL) in every one of the past 10 years, although fluctuations in the cell concentrations and duration of blooms vary considerably between years (National Oceanic and Atmospheric Administration (NOAA), available online at: <http://service.ncddc.noaa.gov/website/AGSViewers/HABSOS/maps.htm>; FWC, monitoring data). Bivalves, including the eastern oyster and hard clam are therefore frequently exposed throughout periods of gametogenesis.

Shellfish, such as *M. mercenaria* and *C. virginica*, exposed to *K. brevis* concentrations as low as 5 cells mL^{-1} , accumulate sufficient amounts of brevetoxin to

cause NSP in humans (Baden et al., 1995; Pierce et al., 2008). However, it is unknown if PbTx accumulates within the gametes of these species and are transferred directly to the offspring. Miller (1993) showed that organochlorine compounds (PCBs and *p,p'*-DDE) were transferred to the eggs of chinook salmon (*Oncorhynchus tshawytscha*) and lake trout (*Salvelinus namaycush*) and the amount passed on from the adult was found to be related to egg lipid content. It is speculated that other lipophilic compounds, including the marine biotoxin ciguatoxin (a similar lipophilic toxin to brevetoxin), could pose a substantial risk to populations of fin fish through transfer from maternal stores to the ova during oogenesis (Edmunds et al., 1999). Although confirming the presence of PbTx in the embryos of mice and sharks, previous studies have not considered the consequences of this transfer (Benson et al., 2006 and Flewelling et al., 2010). Kimm-Brinson and Ramsdell (2001) found concentrations as low as 0.1 ng PbTx-1 egg⁻¹ microinjected into 6 h embryos of Medaka fish (*Oryzias latipes*) caused adverse effects, such as convulsions, on 4 day old embryos. Parent toxins PbTx-1 and PbTx-2 do not accumulate in bivalves however, rather it is the polar metabolites to which developing embryos may be exposed (Naar et al., 2004, 2007; Plakas et al., 2004; Plakas and Dickey, 2010; Poli et al., 1990a).

Previous laboratory exposures by Rolton et al. (in prep a, b/ Chapters 1 and 2) have showed increased histopathological features in the tissues of adult *M. mercenaria* and *C. virginica* exposed to cultured *K. brevis* at high bloom concentrations (1000 and 5000 cells mL⁻¹) for 10 days and for *C. virginica*, additionally to a lower bloom concentration (500 cells mL⁻¹) for 10 weeks. Inflammatory responses were noted in the tissues in direct contact with *K. brevis* cells and/ or its associated toxins. Results obtained following spawning and stripping of adult bivalves also suggested that *K. brevis* exposure may affect gametogenesis and the reproductive fitness of these species (Rolton et al., in prep a,b/ Chapters 1 and 2). Clams showed a significant reduction in fertilization success and increased mortalities in larvae obtained from adults exposed to whole cells of *K. brevis* for 10 days (up to 4 days post-fertilization, (PF); Rolton et al, in prep b/ Chapter 2). Following a 10 week exposure of *C. virginica* to much lower concentrations of *K. brevis* (500 cells mL⁻¹), only male oysters spawned (Rolton et al, in prep a/ Chapter 1). Relating these findings to the field is important. A previous field study by Summerson and Peterson (1990) showed near-total recruitment failure of the scallop (*Argopecten irradians*) exposed to a

bloom of *K. brevis* in North Carolina. Many factors besides toxic algal exposure affect bivalves in the field and these may increase or diminish any apparent effects from *K. brevis*. Blooms of *K. brevis* may also contain other biotoxin producing *Karenia* species (Heil and Steidinger, 2009; Steidinger et al., 2008) and in each bloom, cells of *K. brevis* and the bloom as a whole varies in toxicity (Baden and Tomas, 1988; Pierce et al., 2003, 2008; Hu et al., 1996; Roszell et al., 1990). The patchy nature of blooms also means that bivalves may not be exposed to *K. brevis* cells at all times (Riley et al., 1989).

In the present study, ripe adult *M. mercenaria* and *C. virginica* were collected from a site exposed to *K. brevis* and a site with no recent exposure to *K. brevis*. The condition index, gonadal stage and area, sex distribution, and histopathological features were determined for clams and oysters from exposed and non-exposed sites. Brevetoxin content was assessed in adults and in stripped, filtered and concentrated gametes. Bivalves were then spawned and differences in the spermatozoa, fertilization success and larval development were assessed on obtained offspring up to 11 days PF.

The aim of this study was to increase the understanding of the toxinokinetics and tissue transfer of brevetoxin and help allude to the mode of action of this toxic alga in these commercially and environmentally important bivalve species.

2. Methods

2.1 Collection of bivalves

In March 2013, approximately 200 ripe adult *M. mercenaria* were collected from a site exposed to a bloom of *K. brevis* (Shellfish Harvesting Area (SHA) 6212, Lat. 26.6, Long. -82.2, Figure 1A, D) and a site ~200 miles north which was not exposed to the bloom (SHA 3012, Lat. 29.1, Long. -83.0, Figure 1A, B). For the 2 weeks prior to collection of 'exposed' clams, the mean cell concentration of *K. brevis* in a ~10 mile area surrounding the sites from where clams were collected ranged from 0.7-1.6x10³ cells mL⁻¹ (Figure 2, FWC, monitoring data), temperature was 19.9 ± 0.8 °C and salinity was 35.9 ± 0.9 (mean ± standard error, SE; FWC and Charlotte Harbour National Estuary Program (CHNEP), monitoring data). For the area from where 'non-exposed' clams were collected, temperature and salinity were 16.2 ± 0.1°C and 25.2

± 0.1 respectively (<http://shellfish.ifas.ufl.edu/>). Mean total clam fresh weight of exposed clams was 69.5 ± 9.3 g per individual (mean \pm SE) and shell length was 56.3 ± 2.5 mm ($n= 20$). Unexposed clam mean total fresh weight was 65.4 ± 2.6 g and shell length was 59.8 ± 0.8 mm ($n= 20$).

In April 2013, approximately 200 ripe adult *C. virginica* were collected from a site exposed to *K. brevis* (Lat. 26.5, Long. -82.0, Figure 1A, D) and a site ~200 miles north which was not exposed to the bloom of *K. brevis* (SHA 3402, Lat. 29.0, Long. -82.8, Figure 1A, C). For the 2 weeks prior to collection of oysters, the mean cell concentration of *K. brevis* in an 10 mile area surrounding the sites from where exposed oysters were collected ranged from 0- 9.0×10^2 cells mL⁻¹ (Figure 2), temperature was $19.0 \pm 0.9^\circ\text{C}$ and salinity was 32.8 ± 0.6 (FWC, monitoring data). For the area from where 'non-exposed' oysters were collected, temperature was $17.6 \pm 0.3^\circ\text{C}$ and salinity was 22.7 ± 0.1 (<http://shellfish.ifas.ufl.edu/>).

Total oyster fresh weight of exposed oysters was 79.7 ± 5.2 g (mean \pm SE) and shell length was 102.8 ± 2.6 mm ($n= 20$). For unexposed oysters, total fresh weight was 85.5 ± 5.1 g and shell length was 88.4 ± 2.7 mm ($n= 20$).

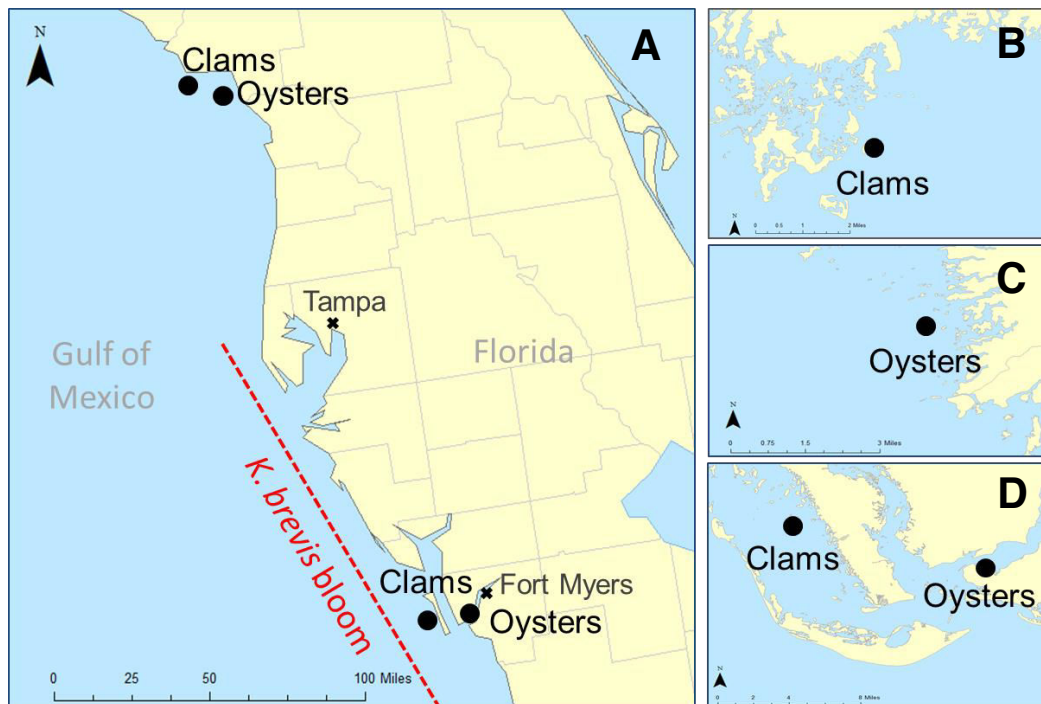


Figure 1: Sites where clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*) were collected along the south west Florida coast. **A**: Sites in the south were exposed to a bloom of *Karenia brevis* (the range of the bloom extending along the coast is represented by the dotted line) and sites in the north were not exposed to the *K. brevis* bloom. More detailed view from where non-exposed clams, **B**; and oysters, **C** and 'exposed' clams and oysters, **D**, were collected.

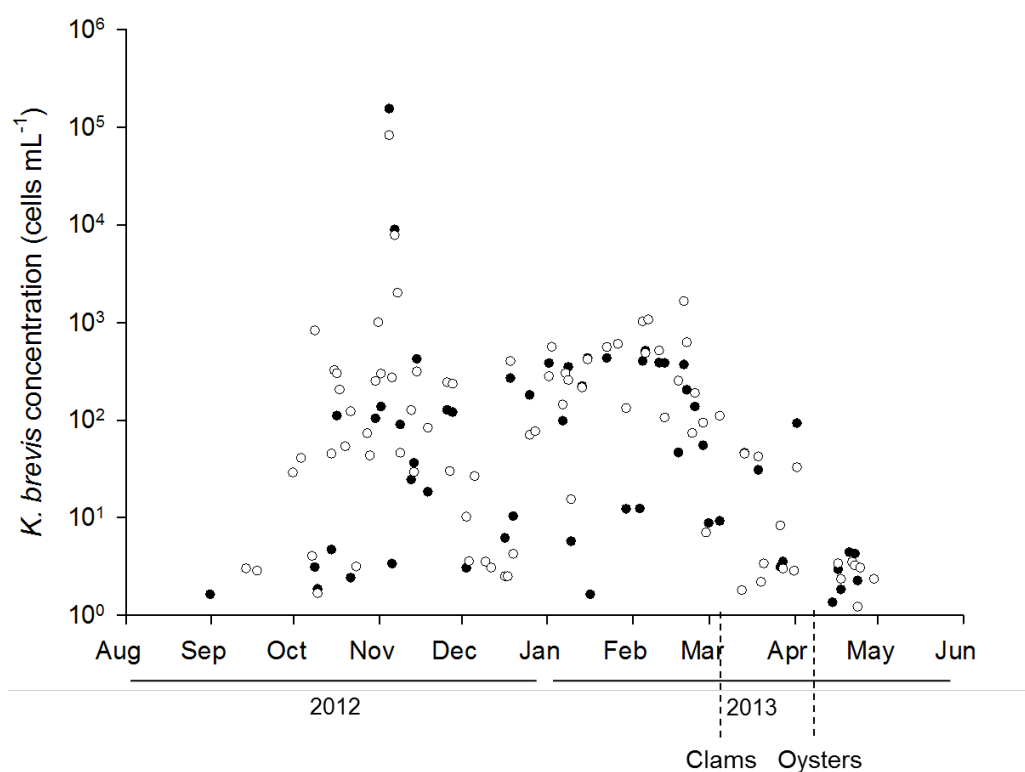


Figure 2: Mean *K. brevis* cell concentrations measured within a ~10 mile radius of where exposed clams (*Mercenaria mercenaria*), ○ ; and oysters (*Crassostrea virginica*), ● ; were collected during a bloom (> 1 cells mL⁻¹) along the south west Florida coast. Clams were collected in March 2013, and oysters were collected in April 2013 (indicated by dotted line). Count data provided courtesy of FWC.

2.2 Sampling of adults

2.2.1 Condition index

Bivalve condition index (CI) was assessed by shucking 20 animals per site, removing whole soft tissues from shells and weighing them individually in a pre-weighed aluminum cup. Remaining shells from individuals were dried for 48h at 60°C and then weighed in the same way. The CI of each individual was calculated as tissue wet meat weight (g) / shell dry weight (g) x 100 according to Delaporte et al. (2005) and Lassudrie et al. (2014).

2.2.2 Brevetoxin content of adults

Five adults per site were shucked and whole tissues were frozen at -80°C until extraction. Tissues from individuals were homogenized under liquid nitrogen using a Laboratory Mixer Mill MM400 (Retsch). One gram aliquots were taken from homogenized whole shellfish tissue and extracted according to Griffith et al. (2013). Briefly, 80% methanol was mixed with the homogenized tissue and incubated at 60°C for 20 minutes before transfer on ice for 10 min. Samples were then centrifuged for 10 min at 3000 *g* and the supernatant extract was saved. The process was repeated, extracts for each sample were combined, washed in hexane, stored at -20°C and used within 7 days of extraction. Extracts were analyzed using a competitive Enzyme- Linked Immunosorbent Assay (ELISA), which detects all PbTx type- 2 brevetoxins (Naar et al., 2002, 2004; Dickey et al., 2004; Plakas et al., 2004). Results are expressed as ng g⁻¹ PbTx-3 equivalents, and reflect the overall concentration of brevetoxins and brevetoxin-like compounds present in the sample.

2.2.3 Histological processing

Thirty individuals per site were carefully shucked and two ~ 4mm thick cross-sections of tissue which included gills, mantle, intestine, connective tissue, kidney, foot, gonad and heart were cut. Dissected tissues were placed in individual cassettes and fixed in Davidson's fixative (Shaw and Battle, 1957) at 4°C for 7 days, after which they were transferred to 70% ethanol for at least 24 hours. Cassettes were then dehydrated and embedded in paraffin and 6 µm tissue sections were cut, mounted on glass slides and stained with Harris' Hematoxylin and Eosin (Martoja and Martoja-Pierson, 1967; Humason, 1979; Howard et al., 2004). From slides, the gonadal stage (GS), gonadal area and pathological and non-pathological features in tissues were assessed.

2.2.4 Gonadal stage (GS)

Clam GS was determined according to Eversole (1997) and, for oysters according to Fisher et al. (1996) (adapted from the International Mussel Watch Program, 1980). GS is expressed as a mean of all individuals sampled per site ± standard error.

2.2.5 Gonadal area

Each histology slide was scanned into the computer. Each electronic image of the whole tissue section was traced and converted into pixel numbers using 'Image J' software. The area of the whole tissue section taken up by the gonad was also traced and converted into pixel numbers. From these pixel numbers, the percent (%) of gonadal area was calculated by dividing the total pixel number of the gonadal area by the pixel number of the whole tissue section and multiplying by 100 (Kang et al., 2003; Morales-Alamo and Mann, 1989). This value represents the reproductive effort (corresponding to the proportion of energy allocated to reproduction) (Todd and Havenhand, 1983; Normand et al., 2009).

2.2.6 Histopathological features

The presence of pathological and non-pathological features were assessed under a light microscope and categorized as '0' when absent and '1' when present. The presence of parasites (protists, digenic trematodes, cestodes, ciliates and coccidians) was recorded as present or absent in the individual as a whole. The presence of other features were recorded per tissue. Hemocyte infiltration, diapedesis, mucus and food/ debris were recorded in the stomach/ intestine. Presence of hemocyte infiltration, mucus containing hemocytes, mucus, edema, and occluded hemolymph vessels were recorded in the gill. The presence of hemocyte infiltration, edema and occluded hemolymph vessels in the mantle, the presence of hemocyte infiltration in the connective tissue and kidney stones were also recorded. For presentation in Figure 3, presence and absence data were converted into percent presence of feature in all individuals from each site.

The sum of all pathological features per tissue (i.e. mantle, gill, stomach/ intestine) for each individual was also assessed. The presence of food/ debris in the stomach/ intestine was excluded from these calculations. For presentation in table 3, results were converted into the average number of pathologies per tissue per individual [by adding the number of pathologies in each tissue for all individuals per site and dividing by the number of individuals sampled at that site] ($n= 30$).

2.2.7 Brevetoxin content of gametes

Sex of adult bivalves was determined microscopically. Keeping sexes separate, bivalves were strip spawned by scoring the gonad with a scalpel and collecting male and female gametes filtered through a Nitex mesh (150 μm). Twenty female and 20 male clams were stripped from both exposed and non-exposed sites. Eighteen female and 16 male oysters were stripped from the exposed site and 20 female and 20 male oysters were stripped from the non-exposed site. Pooled sperm was further filtered through 55 μm mesh and pooled oocytes were retained on 35 μm mesh (clams) or 20 μm mesh (oysters). To further concentrate gametes, they were centrifuged at 1500 g for 10 mins, allowed to settle, and frozen at -80°C until analysis. For analysis, samples were allowed to thaw at room temperature and were centrifuged again at 1500 g to re-concentrate gametes. One gram aliquots taken from this solid gamete tissue mass (< 5 g total) were homogenized using a tissue homogenizer before following the same tissue extraction and ELISA procedure as above. Three, one-gram tissue samples were analyzed from each pool of gametes.

2.3 Analysis of offspring

2.3.1 Algal cultures and preparation

Algal stock cultures were obtained from the Provasoli-Guillard National Center for Marine Microalgae and Microbiota (NCMA), Bigelow Laboratory, Maine (previously the CCMP). The flagellate *Tisochrysis lutea* sp. nov (T-Iso) was grown at a salinity of 30 in CELL-HI F2P based on Guillard F/2 medium (Varicon Aqua). The diatom, *Chaetoceros muelleri* was grown in CELL-HI F2P based on Guillard F/2 medium and Si (Varicon Aqua) at a salinity of 24.

2.3.2 Bivalve spawning

Following the above sampling of adults, spawning of remaining adult bivalves was induced by thermal stimulation. Keeping bivalves from different sites separate, ~ 60 individuals were placed in a tank of 0.1 μm carbon filtered and UV sterilized sea water (FSW) at 18°C and a salinity of 30 for clams and 25 for oysters. After 30 min the temperature was slowly raised to 30°C and maintained for 30 min. Spawning individuals were removed from the tank, rinsed in 0.1 μm FSW and individually

transferred to separate 1 L containers with 0.1 μm FSW to complete spawning and prevent gamete contamination or fertilization. Spawning was considered finished 30 min after the first individual had begun to spawn. Gamete quality was assessed under the microscope.

2.3.3 Flow cytometric assessment of sperm

Dense, motile sperm from individuals was filtered through a 55 μm mesh and kept separate. Sperm from 10 non-exposed and 7 exposed clams and 8 non-exposed and 8 exposed oysters were analysed. For flow cytometric analysis, sperm concentration was adjusted to have a final concentration of approximately 300-500 sperm μL^{-1} per individual. Analyses were performed using a Cytomics FC500 flow cytometer (Beckman Coulter), equipped with a 488 nm Argon laser. Collected data were analysed using WinMDI 2.9 software.

i) Morphology, viability and relative DNA content

The shape and size of spermatozoa was determined using side scatter and forward scatter light (Delaporte et al., 2003). Spermatozoa viability was assessed using a double-staining procedure to determine sperm membrane integrity (Le Goïc et al., 2013a). Sperm treatments and dyes were incubated at room temperature in the dark for 10 min with SYBR-14 (used at a final concentration of 100 nM, Invitrogen) and propidium iodide (PI, used at a final concentration of 12 μM , Sigma). SYBR-14 enters live spermatozoa and binds to DNA, emitting a green fluorescence. PI enters spermatozoa which have lost membrane integrity (i.e. dying and dead cells) emitting an orange/red fluorescence. Proportions of viable (live) sperm were estimated from this double staining. Relative DNA content of sperm could also be estimated from the intensity of SYBR-14 staining (in arbitrary units (AU) of green fluorescence).

ii) Mitochondrial membrane potential (MMP)

The fluorescent dye JC-10 (Enzo Life Sciences, used at a final concentration of 5 μM) was used to determine the mitochondrial membrane potential of oyster sperm (Le Goïc et al., 2013a). JC-10 selectively enters into mitochondria, emitting an orange fluorescence when the membrane potential increases. When the membrane potential of sperm decreases, it results in a shift to green fluorescence. The sperm treatments and dye were incubated at room temperature in the dark for 5 minutes.

Proportions of more and less active sperm were estimated from the amount of green and orange light detected. A protocol could not be established for clam sperm.

iii) *ROS production*

ROS production was measured using 2'7'- dichlorofluorescein diacetate (DCFH-DA, Molecular probes, Invitrogen) at a final concentration of 10 μM (Le Goïc et al., 2013b). DCFH-DA measures intracellular oxidative metabolism by diffusing into the cells, where it is hydrolysed into DCFH. Trapped within the cell, DCFH is oxidized by reactive oxygen species (ROS) into DCF which emits a green fluorescence. Green fluorescence is thus proportional to the ROS production of the sperm cell. Sperm treatments and dye were incubated at room temperature in the dark for 30 min prior to flow cytometric analysis

2.3.4 *Larval development*

The best gametes [dense, >90% motile sperm and, round full, $\sim 75\mu\text{m}$ (clam) or $\sim 60\mu\text{m}$ (oyster) diameter oocytes] were selected for fertilization. Four to five males and females were selected for clams and oysters from the exposed and non-exposed sites. Motile sperm from males were filtered through a 55 μm mesh and pooled per site in a sterile 1 L beaker. The concentration of pooled *M. mercenaria* sperm was determined by photometric absorbance at 640 nm according to Bricelj (1979) [and was $\sim 3.3 \times 10^7$ sperm mL^{-1} for exposed clams and $\sim 1.6 \times 10^7$ sperm mL^{-1} for non-exposed clams]. The concentration of pooled *C. virginica* sperm was determined by photometric absorbance at 581 nm according to Dong et al. (2005) [and was $\sim 1.4 \times 10^7$ sperm mL^{-1} for exposed oysters and $\sim 2.9 \times 10^7$ sperm mL^{-1} for non-exposed oysters]. Pooled oocytes from females were filtered first through a 150 μm Nitex mesh to remove debris and were then retained on either a 35 μm mesh (clam) or a 20 μm mesh (oyster). Oocytes were washed and pooled per site in a sterile 2 L beaker to give a range of 500-2500 oocytes mL^{-1} and to allow them to round- up. For both species, oocytes were then diluted in glass beakers containing 500 mL of 0.1 μm FSW at a salinity of 30 for *M. mercenaria* and 27 for *C. virginica*, to give a final concentration of 15 oocytes mL^{-1} . Five replicate beakers were prepared from pooled gametes from each site. Pooled sperm from adults from the same site were added to the oocyte solution to have a range of 500- 800 sperm oocyte $^{-1}$ and mixed gently. Larval development was recorded for 11 days post-fertilization (PF).

Beakers were exposed to natural daylight, but kept away from direct sunlight. For both species, the temperature was maintained at $24 \pm 1^\circ\text{C}$ and water quality parameters (temperature, dissolved oxygen (DO), pH and salinity) were monitored daily using a Pro ODO optic Probe (YSI), 'Pinpoint' pH monitor (American Marine Inc.) and a refractometer (Aquatic Ecosystems). Gentle aeration (~ 5 bubbles second⁻¹) and daily 'swirling' of the uncovered beakers helped maintain DO levels $>7\text{mg L}^{-1}$.

One-hour after fertilization, at Day 1, 4, 6 and 8 PF, 10 mL sub-samples were taken from each beaker and fixed in 0.1% buffered formalin. At Day 11 PF, larvae were filtered through a 20 μm mesh, ensuring all non-degraded individuals were saved, re-suspended in clean water and preserved in 0.1% buffered formalin.

After sampling at Day 1, newly hatched larvae were fed with T-Iso and *C. muelleri* at 5×10^4 cells mL^{-1} T-Iso equivalents. At Day 3, 4, 6 and 8, T-Iso was again added at 5×10^4 cells mL^{-1} and *C. muelleri* at 3.5×10^4 cells mL^{-1} (total cell density = 10^5 cells mL^{-1} [T-Iso cell volume-equivalent]). For both species, water changes were done on Day 4, 6 and 8. Larvae were filtered through a 20 μm Nitex mesh to remove decaying larvae/algae, beakers were rinsed and wiped clean, and new 0.1 μm FSW was added. Larvae were re-stocked without grading. After the water change at Day 4, 6 and 8, larvae were fed as detailed above.

2.3.5 Fertilization success and larval measurements

The fertilization success (%) was assessed on samples taken 1 h after fertilization. Numbers of fertilized (polar body- 4-cell stage) and un-fertilized (no polar body) oocytes were counted using a light microscope and Sedgewick- Rafter counting chamber. At least 30 oocytes were counted per replicate and data were expressed as mean fertilization success (%). Percent mortality, abnormality and normality of larvae were recorded and calculated at Day 1, 4, 6, 8 and 11 using a light microscope and a Sedgewick- Rafter counting chamber according to Carriker (2001) and Rolton et al. (2014). At Day 8 and 11, abnormal larvae were considered those which had not reached veliger or early umboned stage and those with shell deformities and/or mantle abnormalities. The mean shell length (greatest antero-posterior dimension) at Day 1, 4, 6, 8, and 11 was assessed from twenty live larvae from each replicate according to Rolton et al. (2014).

2.4 Statistical analysis

Gonadal area (%), percent data recorded for spermatozoa, fertilization success (%), percent larval normality, abnormality and mortality were arcsine squareroot transformed to normalize distributions. Transformed gonadal area, spermatozoa, fertilization and larval percent data and, adult CI and mean larval length data were compared between exposed and non-exposed sites for each bivalve species using independent t-tests. Sex distribution and the presence of each pathological and non-pathological feature were compared between exposed and non-exposed sites using Pearsons Chi square. A Mann-Whitney test was used to assess differences attributable to exposed and non-exposed sites for the GS and for the sum of pathological features per tissue per individual.

Statistical tests were run using SPSS 22 statistical software and results were considered significant when $p < 0.05$.

3. Results

Clams and oysters from exposed sites accumulated brevetoxin (clams: 1508 ± 508 ng g⁻¹; oysters: 922 ± 55 ng g⁻¹, mean \pm SE). Oocytes and sperm obtained from stripped adult clams contained 244 ± 50 ng g⁻¹ and 470 ± 82 ng g⁻¹ PbTx respectively ($n= 3$). Conversely, oocytes obtained from stripped adult oysters contained more PbTx (237 ± 10 ng g⁻¹) than in spermatozoa (163 ± 20 ng g⁻¹). Whole tissues of adult bivalves from non-exposed sites and subsequent gametes collected from non-exposed adults, showed no detectible concentrations of brevetoxin (< 5 ng g⁻¹).

The condition index (CI) of exposed clams was significantly lower (24.8 ± 1.0 , mean \pm SE) than for non-exposed clams (27.5 ± 0.8 , $p= 0.044$); however, the CI of exposed oysters was significantly higher (21.1 ± 1.3) than for non-exposed oysters (16.2 ± 1.1 $p= 0.007$, Table 1). The sex distribution of clams and oysters were also significantly different between exposed and non-exposed sites ($p= 0.008$ and $p= 0.017$ respectively, Table 1). Clams from the exposed sites contained 47% male, 33% female and 20% undetermined sex. The non-exposed clams were 47% male and 53% female. Exposed oysters were 30% male, 43% female, 20% undetermined sex and 7% hermaphrodite. Non-exposed oysters were 20% male, 77% female and 3% undetermined. The area of whole tissues occupied by the gonad was significantly higher in bivalves from non-exposed sites ($p< 0.001$, Table 1). The gonadal area for clams from non-exposed and exposed sites was 43.4 ± 1.5 and 23.1 ± 2.9 respectively and, for oysters was 22.5 ± 1.5 and 12.1 ± 2.2 from non-exposed and exposed sites respectively. The gonadal stage (GS) of both bivalve species was however not significantly different between sites ($p= 0.112$ for clams and $p= 0.070$ for oysters, Table 1).

Table 1: The sex distribution (%), gonadal stage, gonadal area (%) and, condition index of adult *Mercenaria mercenaria* and *Crassostrea virginica* obtained from sites exposed and non-exposed to a bloom of *Karenia brevis*. Statistical comparisons: Sex (%), $n=30$, Pearson Chi square; Gonadal stage, $n=30$, Mann-Whitney test; Gonadal area (%), $n=29$ and $n=23$ for non-exposed and exposed clams respectively and $n=27$ for oysters and condition index, $n=20$: independent sample t-tests. NS= non-significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean \pm standard error where applicable.

	<i>Mercenaria mercenaria</i>			<i>Crassostrea virginica</i>			
	Non- exposed	Exposed	Significance	Non-exposed	Exposed	Significance	
Sex (%)	Male	46.7	46.7	20.0	30.0		
	Female	53.3	33.3	**	76.7	43.3	*
	Hermaphrodite	0	0		0	6.7	
	Undetermined	0	20.0		3.3	20.0	
Gonadal stage	3.3 \pm 0.1	2.7 \pm 0.2	NS	4.4 \pm 0.2	3.6 \pm 0.3	NS	
Gonadal area (%)	43.3 \pm 1.5	23.1 \pm 2.9	***	22.5 \pm 1.5	12.1 \pm 2.2	***	
Condition index	27.5 \pm 0.8	24.8 \pm 1.0	*	16.2 \pm 1.1	21.1 \pm 1.3	**	

Significant pathological and non-pathological differences were observed in the mantle, gill and stomach/ intestine of bivalves collected from exposed and non-exposed sites (Table 2 and 3, Figure 3).

The presence of the cestode *Tylocephalum* sp. was significantly (~6x) higher in clams from the exposed site compared to clams from the non-exposed site (Table 2). Exposed clams showed increased instances of mucus in the stomach/ intestine (23% in exposed to 0% in non-exposed, Figure 3 A) and edema in the gills (30% in exposed to 3% in non-exposed, Figure 3 A). The presence of food/ debris in the stomach/ intestine and also the stomach contents were different between exposed and non-exposed clams (Figures 3 A and 4 A, B). Significantly more clams from the exposed site showed the presence of food/ debris in the stomach/ intestine (97% in exposed and 50% in non-exposed) and also a diet rich in dinoflagellates which were all similar in appearance (Figures 3 A and 4 B). These dinoflagellate cells share morphological characteristics of agarose-enrobed *K. brevis* cells, which had been fixed and processed in the same way as clam tissue sections (Figure 4 C, D). The average number of pathologies recorded per individual in each tissue showed that there were significantly more pathological features in the gill of clams from the exposed site (Table 3).

The presence of edema (30%) and occluded hemolymph vessels (50%) in the mantle and mucus in the stomach/ intestine (20%), were significantly higher in oysters from the exposed site compared to the non-exposed oysters (which were 3.3%, 16.7% and 0%, respectively, Figure 3 B). Significantly more oysters from the non-exposed site showed the presence of the protist *Nematopsis* sp. (70%) compared to oysters from the exposed site (30%, Table 2). Overall, oysters showed significantly more histological features in the mantle than non-exposed oysters (Table 3).

Table 2: The presence of parasites observed in tissues of *Mercenaria mercenaria* and *Crassostrea virginica* collected from a site exposed to a bloom of *Karenia brevis* and a non-exposed site. Data is presented as percent presence of feature of all individuals from each site (%). Features were classed as present or absent and Pearson Chi square tests were performed, $n= 30$. NS= non-significant ($p > 0.05$), ** $p < 0.01$.

	<i>Mercenaria mercenaria</i>			<i>Crassostrea virginica</i>		
	Non-exposed	Exposed	Significance	Non-exposed	Exposed	Significance
<i>Nematopsis</i> sp.	0.0	3.3	NS	70.0	30.0	**
<i>Bucephalus</i> sp.	0.0	0.0	NS	10.0	3.3	NS
<i>Tylocephalum</i> sp.	6.7	40.0	**	3.3	3.3	NS
Ciliates	0.0	3.3	NS	13.3	16.7	NS
<i>Pseudoclossia</i> sp.	6.7	0.0	NS	0.0	0.0	NS

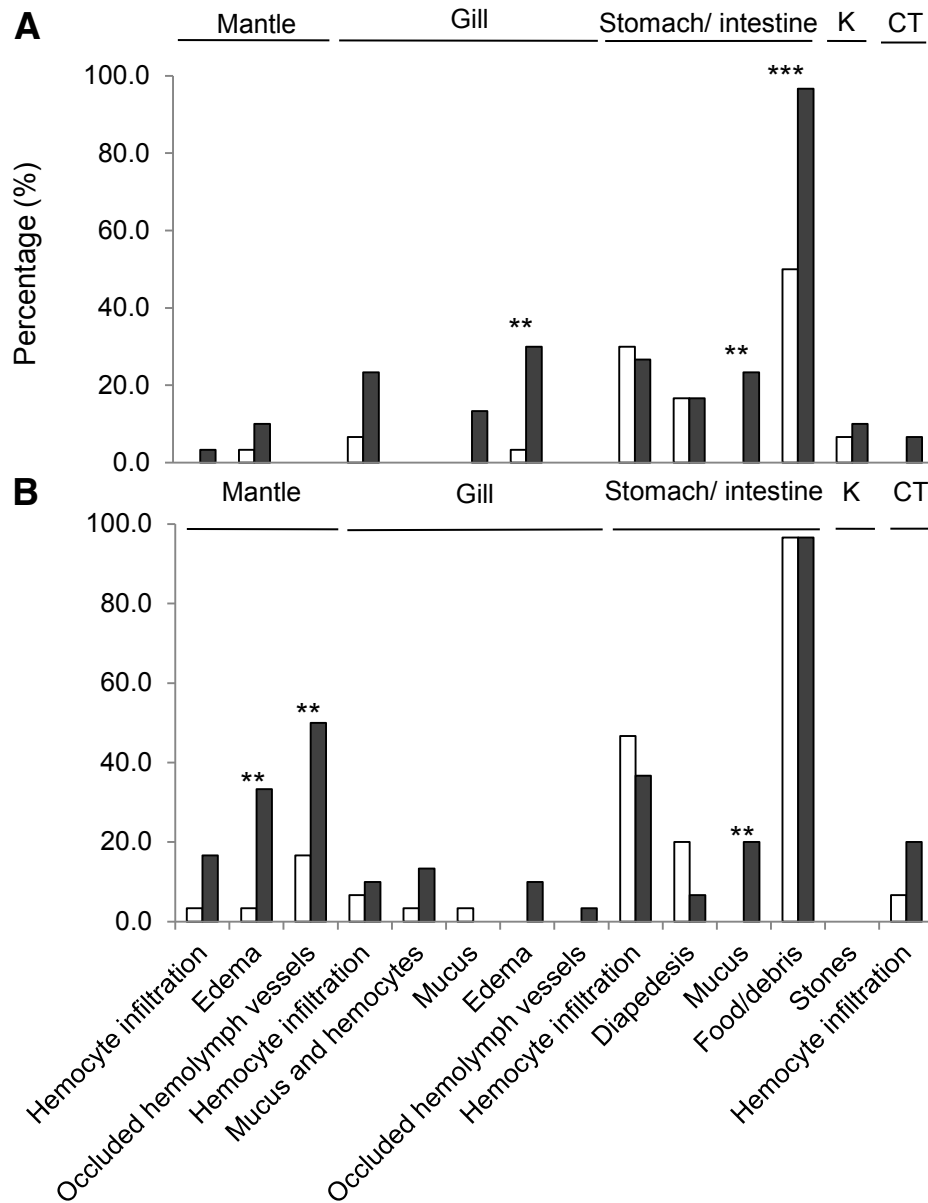


Figure 3: Pathological and non-pathological features observed in tissues of **A:** *Mercenaria mercenaria* and **B:** *Crassostrea virginica* collected from a site exposed to a bloom of *Karenia brevis* (■) and a site not exposed to the *K. brevis* bloom (□). Data is presented as percent presence of pathological or non-pathological feature of all individuals from each site (%). Features were classed as present or absent and Pearson Chi square tests were performed, $n= 30$. ** $p < 0.01$, *** $p < 0.001$. K= Kidney; CT= Connective Tissue.

Table 3: The average number of pathological features observed per individual in each tissue of *Mercenaria mercenaria* and *Crassostrea virginica* collected from a site exposed to *Karenia brevis* and a site not exposed to the *K. brevis* bloom. The sum of the pathological features (classed as present or absent) in each tissue for each individual were compared using a Mann-Whitney test. The presence of parasites is excluded for this table. NS= non-significant ($p > 0.05$), *** $p < 0.001$. Data are presented as mean \pm standard error, $n = 30$.

	<i>Mercenaria mercenaria</i>			<i>Crassostrea virginica</i>		
	Non-exposed	Exposed	Significance	Non-exposed	Exposed	Significance
Mantle	0.0 \pm 0.03	0.1 \pm 0.1	NS	0.2 \pm 0.1	1.0 \pm 0.2	***
Gills	0.1 \pm 0.1	0.7 \pm 0.1	***	0.1 \pm 0.1	0.4 \pm 0.1	NS
Stomach/ intestine	0.5 \pm 0.1	0.7 \pm 0.1	NS	0.7 \pm 0.1	0.6 \pm 0.1	NS
Connective tissue	0.0 \pm 0.0	0.1 \pm 0.05	NS	0.1 \pm 0.05	0.2 \pm 0.1	NS
Kidney	0.1 \pm 0.04	0.1 \pm 0.1	NS	0.0 \pm 0.0	0.0 \pm 0.0	NS

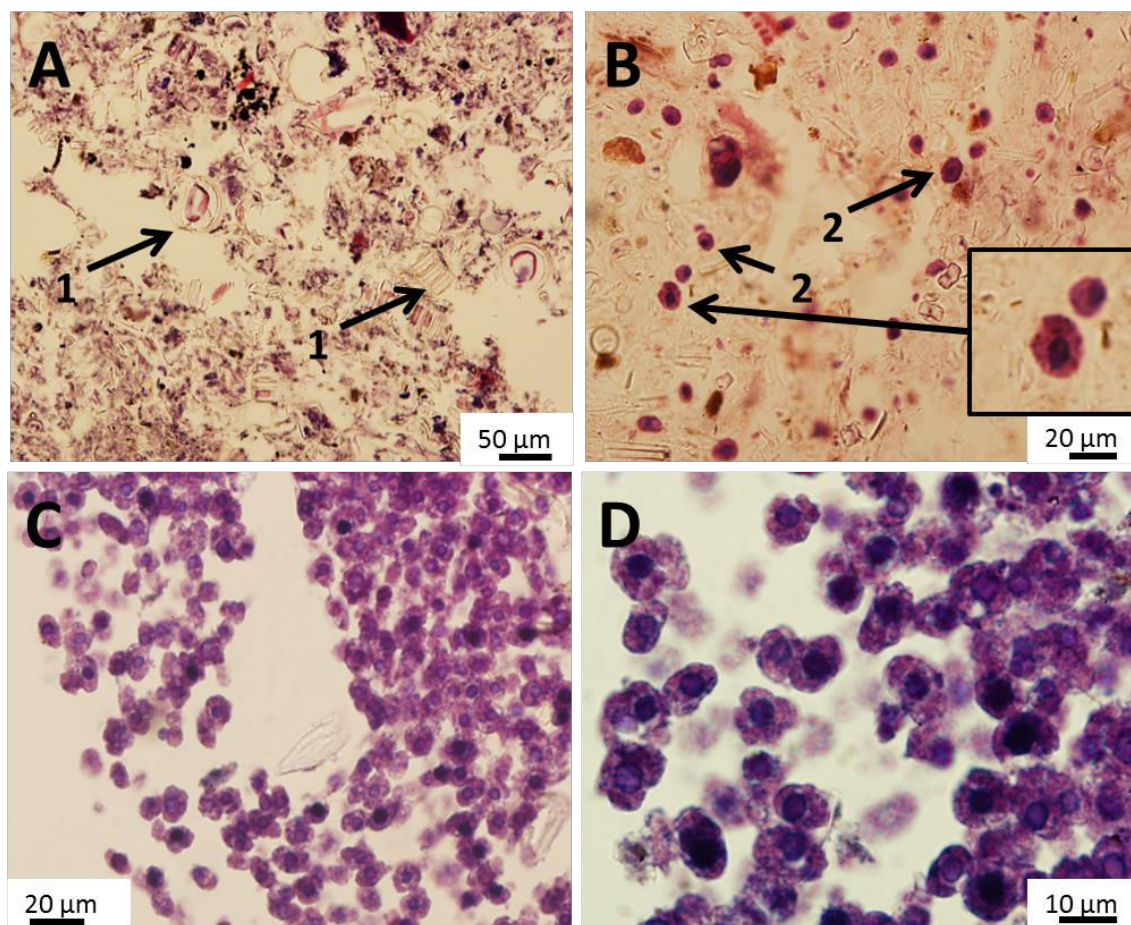


Figure 4: Stomach/ intestine contents of clams from **A**: the non-exposed site, showing a diet predominantly consisting of diatoms, as shown by diatom skeletons (1); **B**: the exposed site, showing a mixed diet of diatoms and dinoflagellates and high numbers of similar dinoflagellate cells (2), which share morphological characteristics with cells of *Karenia brevis* (**C** and **D**), which were agarose-embedded according to the method of Inke Sunila (personal communication) and processed via histology as above (section 2.2.3).

Significantly lower ROS production was recorded in the spermatozoa of clams and oysters from the exposed sites compared to non-exposed sites ($p < 0.01$, Table 4). The complexity of clam spermatozoa and the size of oyster spermatozoa obtained from the exposed sites were also significantly lower than clam and oyster spermatozoa from the non-exposed sites ($p = 0.016$ and $p = 0.043$ respectively, Table 4).

Table 4: Spermatozoa parameters of *Mercenaria mercenaria* and *Crassostrea virginica* adults collected from a *K. brevis*- bloom exposed and non-exposed site. Data are presented as mean \pm standard error, $n= 8$ for clams, $n= 10$ for non-exposed oysters and $n= 7$ for exposed oysters. ROS= reactive oxygen species production. MMP = mitochondrial membrane potential. NA= non-applicable: a protocol could not be established for measuring MMP for clam sperm. NS= non-significant ($p > 0.05$), ** $p < 0.01$, * $p < 0.05$.

	<i>Mercenaria mercenaria</i>			<i>Crassostrea virginica</i>		
	Non-exposed	Exposed	Significance	Non-exposed	Exposed	Significance
ROS	100.9 \pm 5.6	69.8 \pm 4.8	**	94.2 \pm 18.3	31.0 \pm 3.1	**
Alive %	89.4 \pm 1.2	88.5 \pm 1.5	NS	99.0 \pm 0.3	99.0 \pm 0.3	NS
Size	157.2 \pm 1.2	156.2 \pm 1.3	NS	105.8 \pm 1.5	102.0 \pm 0.9	*
Complexity	232.7 \pm 6.2	211.7 \pm 2.6	*	95.9 \pm 5.5	105.9 \pm 4.5	NS
DNA content	126.9 \pm 16.0	90.0 \pm 16.4	NS	167.2 \pm 22.8	167.4 \pm 29.0	NS
MMP	NA	NA	NA	1.9 \pm 0.3	1.6 \pm 0.2	NS

Significantly lower fertilization success was obtained using gametes from exposed clams ($89 \pm 2.0\%$, mean \pm SE), than gametes from non-exposed clams ($96 \pm 0.7\%$, $p = 0.007$). At Day 1 PF, clam larvae from exposed clams were smaller than those from non-exposed clams (Figure 5 C). By Day 8, however, larvae from exposed clams showed significantly lower mortalities and higher percent of normality compared to maternally non-exposed larvae and at Day 11, exposed clam larvae were also significantly bigger than non-exposed clam larvae (Figure 5 A, C).

Whilst oysters showed no differences in fertilization success ($99 \pm 0.7\%$ in non-exposed and $94 \pm 2.6\%$ in exposed, $p = 0.224$), significant differences were observed in larval development up to 6 days after fertilization. Larvae from exposed oysters were significantly smaller than those from non-exposed oysters up to 6 days PF (Figure 5 C). Significantly more abnormalities and mortalities were also recorded in larvae from maternally exposed oysters at Day 1, 4 (and 6 for abnormalities) (Figure 5 A, B). Less normal larvae were recorded at Days 1, 4 and 6 in exposed oysters. By Day 11, there were no significant differences between larvae from exposed and non-exposed oysters.

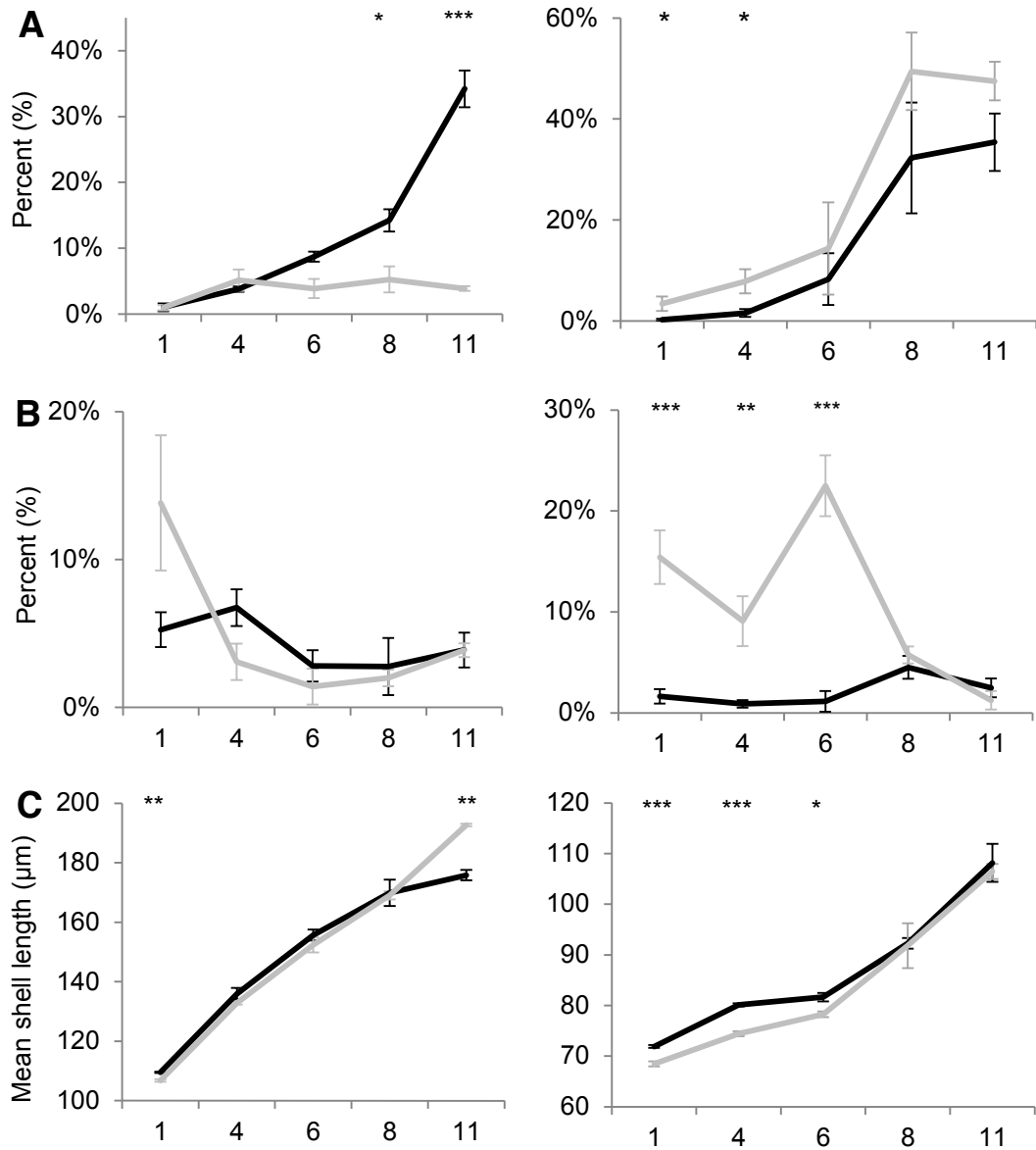


Figure 5: **A:** percent mortality of *Mercenaria mercenaria* (left) and *Crassostrea virginica* (right) larvae, **B:** percent abnormality of clam (left) and oyster (right) larvae and. **C:** mean shell length of clam (left) and oyster (right) larvae from maternally exposed- (grey line) and non-exposed (black line) individuals up to 11 days post-fertilization. Significance levels: : *** p < 0.001, ** p < 0.01, * p < 0.05. data are presented as mean ± standard error, n= 5.

4. Discussion

4.1 Toxin accumulation

Adult bivalves exposed to field blooms of *K. brevis* accumulated brevetoxin above the 800 ng g⁻¹ threshold at which shellfish harvest beds are closed. The accumulation of PbTx in bivalves above threshold levels in the field is well documented (i.e. Bricelj et al., 2012; Plakas et al., 2008; Tester and Fowler, 1990). Biotoxins such as PbTx accumulate in the gut and hepatopancreas of bivalves (Landsberg, 2002, McFarren et al., 1965; Steidinger et al., 1973, 1993; Roberts et al., 1979; Baden et al., 1982; Tester and Fowler, 1990; Shumway and Cembella, 1993; Steidinger et al., 1998); however, to our knowledge, no previous studies have shown there is transfer of PbTx from adult bivalves to the gametes. Herein, we have shown there was accumulation of PbTx within the gametes of adult clams and oysters exposed to *K. brevis* in the field. Clams and oysters accumulated between 16- 31% of the adult body burden of PbTx in the spermatozoa or oocytes. Flewelling et al. (2010) recorded PbTx in utero embryos of 3 species of shark. The total PbTx content of near-term embryos obtained from viviparous sharks (which obtain nutrients from the mother throughout development) was 0.2- 16% of the total PbTx content of the mother. Washburn et al. (1994) recorded little accumulation (~1% of the adult body burden) of orally administered, carbon labelled PbTx-3 in the gonad of the gulf toadfish (*Opsanus beta*). However, as the study did not use sexually mature fish and only the ¹⁴C PbTx-3 metabolite was searched for, accumulation of PbTx in the gametes cannot be inferred from their study. The accumulation of organic lipophilic contaminants such as PCBs in the eggs of *C. virginica*, has been demonstrated by Chu et al. (2000). Roughly 11% of the total adult PCB content was found in oocytes following a 30 day exposure of adults to 1 µg g⁻¹ PCBs.

It is unknown how PbTx accumulates within the oocytes. Following bivalve digestion, it is speculated there is a flux of lipids from the digestive gland to the gonad (Barber and Blake, 1985), which PbTx may follow. Edmunds et al. (1999) speculated that the similar marine biotoxin, ciguatoxin, may be transferred into the natural lipid droplets of oocytes. As a lipid soluble, ladder frame polyether, PbTx, can move across cell membranes by active transport (Poli et al., 1990a, b) and therefore may also be present within the lipid droplets of oocytes. Oysters accumulated more PbTx in the oocytes than in the same weight of sperm, whereas clams accumulated similar

levels of PbTx in sperm and oocytes. Brevetoxin accumulation, metabolism and elimination is thought to differ between species (Pierce et al., 2004, Griffith et al., 2013). Indeed, differences in PbTx accumulation in the gametes may be due to differing lipid composition of oocytes between species. Oocytes of *C. virginica* have a lipid content of 21% of the dry weight of the oocyte, of which a high proportion is triglyceride (76%, Lee and Heffernan, 1991). Clam oocytes by contrast contain a lower portion of lipid (14%) of which only 30% is triglycerides (Lee and Heffernan, 1991). The transfer of PbTx from the digestive gland to sperm or oocytes may also happen at different rates between species. However, differences in accumulation due to environmental differences cannot be ruled out. Oysters were collected a month later than clams, when *K. brevis* concentrations were lower (Figure 2), and may have eliminated PbTx from specific tissues.

4.2 Effects on Adults

Various pathological and non-pathological tissue differences were recorded between bivalves from exposed and non-exposed sites. The protist, *Nematopsis* sp., digenic trematode, *Bucephalus* sp., cestode, *Tylocephalum* sp and ciliates are commonly reported in SW FL for these bivalve species and except for heavy infections, cause little effect on the host (Cake and Menzel, 1980; Lauckner, 1983; Overstreet, 1978; Winstead et al., 2004). Indeed, *Nematopsis* sp. is considered a symbiont in *C. virginica* (Winstead et al., 2004). The coccidian, *Pseudoclossia* sp. is a kidney parasite of mollusc species (Desser et al., 1998; Friedman et al., 1995; Morado et al., 1984); however, we have found no previous reports of its presence in *M. mercenaria*. As only a few of these coccidians were present in clam kidney tissue, we considered this infection light. For these reasons, the presence of parasites were not included in Table 3 (showing the average number of pathologies per tissue per individual). Differences in the presence of *Tylocephalum* sp. and *Nematopsis* sp. in clams and oysters between sites may be due to environmental differences such as, temperature, salinity, substrate, sediment load, etc; however, *K. brevis* may affect the parasites. On one hand the toxic algae may weaken the host and make it more susceptible to the parasite (i.e. increasing the load of *Tylocephalum* sp. in clams), but on the other; the presence of the toxic algae may weaken the parasite. Interactive effects between parasites and harmful algae were previously shown by

da Silva et al. (2008) who showed that *Perkinsus olseni* burdens on Manila clams (*Ruditapes philippinarum*) exposed to the gymnodimine producing *Karenia* species, *K. selliformis* were reduced. The apicomplexan, *Nematopsis* sp. is phylogenetically related to *P. olseni* and may also be sensitive to harmful compounds from *K. brevis*. However, *Nematopsis* sp. burdens in the Caloosahatchee estuary are highly variable between closely occurring oyster populations (Winstead et al., 2004). Future studies using bivalves collected from several close proximity sites within *K. brevis* bloom areas may help distinguish if parasite burdens are due to the site from where oysters were collected, or toxic algal presence.

Cedar Key was the closest non-exposed area along the SW FL coast to the exposed study area from where clams and oysters could be obtained. Physiological differences (i.e. in the CI, gonadal area, sex ratio, and for oysters, shell length) of clams and oysters between the different sites may be due to underlying environmental differences besides *K. brevis* exposure. Temperature and salinity data for the 2 weeks prior to collection of bivalves in either March (for clams) or April (for oysters), indicate that bivalves were subject to significantly different conditions between exposed and non-exposed sites. Clams and oysters from the non-exposed sites were subject to lower temperatures and salinities than those from exposed areas (<http://shellfish.ifas.ufl.edu/>; CHNEP and FWC, monitoring data). Oysters from the exposed site were also collected near the mouth of the Caloosahatchee river; an area subject to occasional high sediment loads coinciding with freshwater releases from Lake Okeechobee/ inland canals (Volety et al., 2009). Future studies linking physiological parameters to toxin content in the same individual and studies which use bivalves from several exposed areas may help elude if physiological changes recorded are due to *K. brevis* exposure and/ or other underlying environmental differences.

The presence of mucus in the stomach/ intestine of clams and oysters recorded herein can be considered a normal physiological process. However, more mucus was recorded in the stomach/ intestine of exposed clams and oysters and may be related to increased digestion in bivalves from these sites. Clams from exposed sites contained significantly more food/ debris in the stomach/ intestine than non-exposed. The increased presence of mucus recorded may also be related to the difference in

stomach/ intestine contents. Although we could not distinguish the stomach/ intestine contents of the oyster, a clear difference in the diet of clams from different sites was noted. The stomach of exposed clams contained a diet rich in dinoflagellates which shared morphological similarities with cells of *K. brevis*, present in the area at the time of collection (Figure 2). Dinoflagellates may be a less desirable food source for bivalves than diatoms (Wikfors, 2005), which were recorded in abundance at the non-exposed site. Mucus contains lytic enzymes that act against a variety of stressors such as dissolved organic compounds and particles suspended in seawater and play an important role in defense (Galimany et al., 2008a, b; Shumway and Cucci, 1987; Fisher, 1992; Brun et al., 2000). Therefore, these dinoflagellate cells may cause mucus production. Other dinoflagellate HAB species have also been shown to affect nutrition related processes in bivalves (Haberkorn et al., 2010; Galimany et al., 2008a, b, c; Hégaret et al., 2009).

Clams from exposed sites showed increased edema and increased pathologies in the gill. Exposed oysters showed increased edema and occluded hemolymph vessels in the mantle and an overall increase in the average number of pathologies in the mantle. These pathologies could be due to contact with *K. brevis* and/or associated toxins including PbTx. Edema is a commonly described observation in physiologically compromised bivalves and may be caused by other biotic and abiotic factors (Jones et al., 2010; Humphrey et al., 1998). Infecting agents such as bacteria, viruses and parasites may also lead to increased edema (Jones et al., 2010), although high parasite loads were not recorded in the present study. In the absence of external forces such as large variations in salinity, edema requires an active mechanism affecting sodium and chloride pumping (Wehner et al., 2003; Jones et al., 2010). Occluded hemolymph vessels as recorded in the mantle of oysters may be caused by many environmental (i.e. high sediment loads) or pathogenic agents (Apeti et al., 2014). Increased instances of occluded hemolymph vessels in the mantle of oysters were also recorded by Rolton et al. (in prep a/ Chapter 1) exposing *C. virginica* to whole cell concentrations of 5000 cells mL⁻¹ *K. brevis* for 10 days. Indeed, Rolton et al. (in prep a, b/ Chapters 1 and 2) also recorded increased pathologies in the same tissues of these bivalve species following a 10 day laboratory exposure of clams and oysters to 1000 and 5000 cells mL⁻¹ *K. brevis*. In those studies however, responses recorded in clam and oyster

tissues were mainly hemocytic in nature and were possibly caused by direct contact with an irritating agent. i.e. *K. brevis* cells/ associated toxins and/ or accumulated PbTx (and other toxins?) within the bivalve tissues. Toxic algae are known to cause hemocyte responses in the tissues of several bivalve species (i.e. Estrada et al., 2007; Hégaret et al., 2009; Smolowitz and Shumway, 1997). Although differences recorded in clam and oyster tissues herein differ between species, they may also be due to the different concentration of toxic algae/ duration of exposure to which each species was exposed in the field.

4.3 Effects on offspring

Decreased ROS production was recorded in the sperm from *K. brevis* exposed clams and oysters. ROS is produced following mitochondrial respiration or as a stress response during normal cellular function (Halliwell, 2006). A decrease in ROS may indicate a reduction of cellular respiration which over time could reduce the energy production of the sperm. Such a reduction of energy associated with a decrease of respiration did not result from compromised mitochondria however, as the mitochondrial membrane potential remained unchanged.

Fertilization success of gametes from exposed clams was significantly lower than for non-exposed clams. This could be related to the presence of PbTx within the eggs leading to poorer egg quality. Using oocyte dry weight values from the literature (51 ng oocyte⁻¹ for *M. mercenaria* and 12 ng oocyte⁻¹ for *C. virginica*; Lee and Heffernan, 1991) and tissue wet weight to dry weight ratios [calculated from drying pre-weighed whole wet tissue of 20 adult individuals from each population of bivalves at 60°C for 2 days before re-weighing the dried tissue]; we could estimate the toxin content per egg. The PbTx content for clams was 6.3×10^{-5} ng oocyte⁻¹ and for oysters was 1.8×10^{-5} ng oocyte⁻¹. This difference may be due to the larger size of clam oocytes but may be due to the higher concentration of PbTx in the adults as compared to oysters. Embryos may be at greater risk to biotoxins than adults due to the reduced ratio of body mass to toxin load and the bioavailability of the toxin during sensitive stages of development (Edmunds et al., 1999). Rolton et al. (in prep b/ Chapter 2) also found reduced fertilization success in clams exposed in laboratory to high bloom concentrations (5000 cells mL⁻¹) of *K. brevis* for 10 days.

No effect was observed on the fertilization success of gametes from exposed oysters, despite the presence of PbTx within the oocytes. Although there was no significant difference in the fertilization success in oysters, there was a clear effect on larvae up to 6 days PF. Up to pediveliger stage (120-300 μm for oysters; Carriker, 1996; Thompson et al., 1996), *C. virginica* larvae are significantly affected by reserves of lipid laid down in the egg (Gallager and Mann, 1986), lipids which may have contained PbTx. We hypothesize that as larvae stop living off these maternal lipid reserves (lipid droplets from the egg are resorbed within ~ 4 days of the initiation of feeding on nutritious algae, Gallager et al., 1986), the negative effects disappear. The study by Rolton et al. (in prep a/ Chapter 1) recorded no significant negative effects on fertilization success and larval development (up to 4 days PF) however; in that study, oysters were already ripe at the time of the 10 day experimental exposure, thus we speculated that negligible ripening and PbTx accumulation was taking place in the oocytes, unlike in the present study where oysters were fully conditioned in the field with *K. brevis*.

Larvae from exposed clams obtained a smaller size at Day 1 PF, which similarly to oysters may be due to PbTx in the oocyte. However, unlike for oysters, negative effects on the larvae were not as long-lasting. Clams have proportionally less lipid reserves as compared to oysters (Lee and Heffernan, 1991) and may also have a shorter lecithotrophic phase (Gallager et al., 1986) therefore, the duration of PbTx effect may be shorter. Results from the study by Rolton et al. (in prep b/ Chapter 2) showed significant increases in larval mortalities and abnormalities up to 4 days PF. Differences between these 2 studies are perhaps due to higher *K. brevis* concentrations to which clams were exposed (5000 cells mL^{-1}) or the amount of PbTx accumulated (~ 5000 ng g^{-1} PbTx) than in this field study (variable but over-all lower cell counts and ~ 1500 ng g^{-1} PbTx).

The higher larval mortalities at Day 8 and 11 and lower larval size recorded at Day 11 PF in the non-exposed clam treatment in the present study may be due to the presence of bacteria. Although oocytes were filtered and placed in 0.1 μm FSW; during spawning there may be vertical transmission of bacteria from the parent to the gametes (Sharp et al., 2007). Populations of bacteria can quickly multiply and cause significant mortalities (Figure 5).

4.4 Conclusion

The presence of PbTx within spermatozoa and oocytes of clams and oysters has not been shown before and may have repercussions for gamete quality and larval survival. The early development of both oyster and clam larvae appears to be less robust if parents were exposed to *K. brevis*; however, provision of a non-contaminated food source to larvae appears to mitigate the early negative effects of neonatal exposure. Differential effects between species may be related to the way energy is stored and/ or used during development. As blooms may continue into spawning times, larvae may have the additional stressor of direct exposure to cells and/or associated toxic compounds. Differences in the pathological and non-pathological features recorded in tissues between bivalves from exposed and non-exposed sites are difficult to interpret due to the number of unknown influences in the field; however, consistencies between histopathological features recorded herein and in previous laboratory exposures suggest that *K. brevis* may influence the physiology of bivalves. This field study highlights the importance of considering wider ranging environmental factors, such as toxic algal presence, when performing ecotoxicological studies and in restoration programs.

Acknowledgements

The authors would like to thank Dr Inke Sunila from the Bureau of Aquaculture for the State of Connecticut for histopathology training and expertise; Diane Blackwood from FWC for providing *Karenia brevis* count data and bloom history information; FWC and CHNEP for water quality data; and Katie McFarland, Lesli Haynes, Lindsay Castret and staff of the marine laboratory FGCU for their help collecting and processing oysters. We also thank Tony Heebe (Cut throat clams), Schooner Bay Seafood/ Cookes Seafood and Leslie Sturmer (University of Florida) for the supply of clam and oyster broodstock and water quality data. This work was part funded by Université de Bretagne Occidentale, and by “Labo-ratoire d’Excellence” LabexMER (ANR-10-LABX-19); Outgoing Ph.D. Fellowship, co-funded by a grant from the French government under the program “Investissements d’Avenir”.

References

- Apeti, D. A., Kim, Y., Lauenstein, G., Tull, J., Warner, R. 2014. Occurrence of Parasites and Diseases in Oysters and Mussels of the U.S. Coastal Waters. National Status and Trends, the Mussel Watch monitoring program. NOAA Technical Memorandum NOSS/NCCOS 182. Silver Spring, MD. pp 51.
- Baden, D. G., Bourdelais, A. J., Jacocks, H., Michelliza, S., Naar, J. 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ. Health Persp.* 113(5): 621-625.
- Baden, D.G., Fleming, L.E., Bean, J.A. 1995. Marine toxins. *Handbook of clinical neurology.* 21(65): Intoxications of the Nervous System, Part II. Elsevier Science B.V. pp. 141-175.
- Baden, D. G., Tomas, C. R. 1988. Variations in major toxin composition for six clones of *Ptychodiscus brevis*. *Toxicon.* 26(10): 961-963.
- Baden, D. G., Mende, T. J., Bikhazi, G., Leung, I. 1982. Bronchoconstriction caused by Florida red tide toxins. *Toxicon.* 20: 929–932.
- Barber, B. J., Blake, N. J. 1985. Intra-organ biochemical transformations associated with oogenesis in the bay scallop, *Argopecten irradians concentricus* (Say), as indicated by ¹⁴C incorporation. *Biol. Bull.* 168(1): 39-49.
- Benson, J. M., Gomez, A.P., Statom, G.L., Tibbetts, B.M., Fleming, L.E., Backer, L.C., Reich, A., Baden, D.G. 2006. Placental transport of brevetoxin-3 in CD-1 mice. *Toxicon.* 48(8): 1018-1026.
- Bricelj, V. M., Haubois, a. G., Sengco, M. R., Pierce, R. H., Culter, J. K., Anderson, D. M. 2012. Trophic transfer of brevetoxins to the benthic macrofaunal community during a bloom of the harmful dinoflagellate *Karenia brevis* in Sarasota Bay, Florida. *Harmful Algae.* 16: 27–34.
- Bricelj, V.M. 1979. Fecundity and related aspects of the hard clam (*Mercenaria mercenaria*) reproduction in Great South Bay, NY. MSc Thesis. State University of New York at Stony Brook.
- Brun, N. T., Ross, N. W., Boghen, A.D. 2000. Changes in the electrophoretic profiles of gill mucus proteases of the eastern oyster *Crassostrea virginica* in response to infection by the Turbellarian, *Urastoma cyprinae*. *J. Invert. Pathol.* 75(2): 163-170.
- Cake E. W., Menzel, R. W. 1980. Infections of *Tylocephalum metacestodes* in commercial oysters and three predaceous gastropods of the eastern Gulf of Mexico. *P. Nat. Shellfisheries Assoc.* 70(1): 94-104.
- Carriker, M.R., 2001. Embryogenesis and organogenesis of veligers and early juveniles. In: Kraeuter, J. N., Castagna, M. (Eds), *Biology of the hard clam.* Vol. 31. Gulf Professional Publishing. pp 77–115.
- Carriker, M.R., 1996. The shell and ligament. In: Kennedy, V.S. (Ed.) *The Eastern Oyster Crassostrea virginica.* Maryland Sea Grant College, University of Maryland, College Park, Maryland. pp. 75- 168.
- Chu, F. L., Soudant, P., Cruz-Rodríguez, L. A., Hale, R. C. 2000. PCB uptake and accumulation by oysters (*Crassostrea virginica*) exposed via a contaminated algal diet. *Mar. Environ. Res.* 50(1) : 217-221.
- da Silva, P. M., Hégaret, H., Lambert, C., Wikfors, G. H., Le Goïc, N., Shumway, S. E., Soudant, P. 2008. Immunological responses of the Manila clam (*Ruditapes philippinarum*) with varying parasite (*Perkinsus olseni*) burden, during a long-term exposure to the harmful alga, *Karenia selliformis*, and possible interactions. *Toxicon.* 51(4): 563–73.

- Delaporte, M., Soudant, P., Moal, J., Kraffe, E., Marty, Y., Samain, J.-F. 2005. Incorporation and modification of dietary fatty acids in gill polar lipids by two bivalve species *Crassostrea gigas* and *Ruditapes philippinarum*. *Comp. Biochem. Phys. A.* 140(4): 460–70.
- Delaporte, M., Soudant, P., Moal, J., Lambert, C., Quéré, C., Miner, P., Choquet, G., Paillard, C., Samain, J.-F. 2003. Effect of a mono-specific algal diet on immune functions in two bivalve species *Crassostrea gigas* and *Ruditapes philippinarum*. *J. Exp. Biol.* 206(17): 3053–3064.
- Desser, S. S., Bower, S. M., Hong, H. 1998. *Pseudoklossia semiluna* N. sp.(Apicomplexa: Aggregatidae): A coccidian parasite of the kidney of blue mussels, species of *Mytilus*, from British Columbia, Canada. *Parasite.* 5(1): 17-22.
- Dickey, R. W., Plakas, S. M., Jester, E. L. E., Said, K. R. El, Johannessen, J. N., Flewelling, L. J., Scott, P., Hammond, D. G., Van Dolah, F. M., Leighfield, T.A., Dachraoui, M. B., Ramsdell, J.S., Pierce, R. H., Henry, M. S., Poli, M. A., Walker, C., Kurtz, J., Naar, J., Baden, D. G., Musser, S. M., White, K. D., Truman, P., Miller, A., Hawryluk, T. P., Wekell, M. M., Stirling, D., Quilliam, M. A., Lee, J. K. .2004. Multi-Laboratory Study of Five Methods for the Determination of Brevetoxins in Shellfish Tissue Extracts. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A., (Eds). *Harmful Algae 2002. Proceedings of the Xth International Conference on Harmful Algae.* Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and the Intergovernmental Oceanographic Commission of UNESCO. St. Petersburg, FL, USA. pp. 300- 302.
- Dong, Q., Eudeline, B., Huang, C., Tiersch, T. R. 2005. Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg). *Aquac. Res.* 36(1): 86–93.
- Edmunds, J. S., McCarthy, R., Ramsdell, J. S. 1999. Ciguatera reduces larval survivability in finfish. *Toxicon.* 37(12): 1827–1832.
- Estrada, N., de Jesús Romero, M., Campa-Córdova, A., Luna, A., Ascencio, F. 2007. Effects of the toxic dinoflagellate, *Gymnodinium catenatum* on hydrolytic and antioxidant enzymes, in tissues of the giant lions-paw scallop *Nodipecten subnodosus*. *Comp. Biochem. Phys. C.* 146(4): 502–510.
- Eversole, A. G. 1997. Gametogenesis of *Mercenaria mercenaria*, *M. campechiensis* and their hybrids. *Nautilus.* 110(4): 107-110.
- Fisher, W. S., Winstead, J. T., Oliver, L. M., Edmiston, H. L., Bailey, G. O. 1996. Physiologic variability of eastern oysters from Apalachicola Bay, Florida. *J. Shellfish Res.* 15(3): 543-553.
- Fisher, W. S. 1992. Occurrence of agglutinins in the pallial cavity mucus of oysters. *J. Exp. Mar. Biol. Ecol.* 162(1): 1–13.
- Flewelling, L. J., Adams, D. H., Naar, J. P., Atwood, K. E., Granholm, A. A., O’Dea, S. N., Landsberg, J. H. 2010. Brevetoxins in sharks and rays (Chondrichthyes, Elasmobranchii) from Florida coastal waters. *Mar. Biol.* 157(9): 1937-1953.
- Friedman, C. S., Gardner, G. R., Hedrick, R. P., Stephenson, M., Cawthorn, R. J., Upton, S. J. 1995. *Pseudoklossia haliotis* sp. n.(Apicomplexa) from the Kidney of California Abalone, *Haliotis* spp.(Mollusca). *J. Invert. Path.* 66(1): 33-38.
- Galimany, E., Place, A. R., Ramón, M., Jutson, M., Pipe, R. K. 2008a. The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*. *Harmful Algae.* 7(1): 91–98.

- Galimany, E., Sunila, I., Hégaret, H., Ramón, M., Wikfors, G. H. 2008b. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: Histopathology, immune responses, and recovery. *Harmful Algae*. 7(5): 702–711.
- Galimany, E., Sunila, I., Hégaret, H., Ramón, M., Wikfors, G. H. 2008c. Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. *Harmful Algae*. 7(5): 630–638.
- Gallager, S. M., Mann, R. 1986. Growth and survival of larvae of *Mercenaria mercenaria* (L.) and *Crassostrea virginica* (Gmelin) relative to broodstock conditioning and lipid content of eggs. *Aquaculture*. 56(2): 105–121.
- Gallager, S. M., Mann, R., Sasaki, G.C. 1986. Lipid as an index of growth and viability in three species of bivalve larvae. *Aquaculture*. 56(2): 81-103.
- Gawley, R. E., Rein, K. S., Jeglitsch, G., Adams, D.J., Theodorakis, E.A., Tiebes, J., Nicolaou, K.C., Baden, D.G. 1995. The relationship of brevetoxin 'length' and A-ring functionality to binding and activity in neuronal sodium channels. *Chem. Biol.* 2(8): 533-541.
- Griffith, A. W., Shumway, S. E., Volety, A. K. 2013. Bioaccumulation and depuration of brevetoxins in the eastern oyster (*Crassostrea virginica*) and the northern quahog (= hard clam, *Mercenaria mercenaria*). *Toxicol.* 66: 75–81.
- Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P. 2010. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. *Harmful Algae*. 9(5): 427–439.
- Halliwell, B. 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol.* 141(2): 312-322.
- Hégaret, H., da Silva, P. M., Sunila, I., Shumway, S. E., Dixon, M. S., Alix, J., Wickfors, G.H., Soudant, P. 2009. Perkinsosis in the Manila clam *Ruditapes philippinarum* affects responses to the harmful-alga, *Prorocentrum minimum*. *J. Exp. Mar. Biol. Ecol.* 371(2): 112–120.
- Heil, C. A., Steidinger, K. A. 2009. Monitoring, management, and mitigation of *Karenia* blooms in the eastern Gulf of Mexico. *Harmful Algae*. 8(4): 611-617.
- Hesselman, D.M., Barber, B.J., Blake, N.J., 1989. The reproductive cycle of adult hard clams, *Mercenaria* spp. in the Indian River Lagoon, Florida. *J. Shellfish Res.* 8: 43–49.
- Howard, D.W., Lewis, E.J., Keller, B.J., Smith, C.S., 2004. *Histological Techniques for Marine Bivalve Mollusks and Crustaceans*. NOAA Technical Memorandum. NOSNCCOS, Oxford, MD, USA, pp. 218.
- Hu, C., Muller-Karger, F. E., Swarzenski, P. W. 2006. Hurricanes, submarine groundwater discharge, and Florida's red tides. *Geophys. Res. Lett.* 33: L11601.
- Humason, G. L. *Animal Tissue Techniques*, 1979. San Francisco, CA USA: WN Freeman and Company.
- Humphrey J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C., Creeper, J.H. 1998. Pearl oyster (*Pinctada maxima*) aquaculture: health survey of Northern Territory, Western Australia and Queensland pearl oyster beds and farms. FRDC Final Report 94. Fisheries Research Development Corporation, Canberra.
- International Mussel Watch Program (IMW). 1980. National Academy of Sciences, Washington D.C. pp 248.

- Jones, J. B., Crockford, M., Creeper, J., Stephens, F. 2010. Histopathology of oedema in pearl oysters *Pinctada maxima*. Dis. Aquat. Organ. 91(1): 67–73.
- Kang, D., Ahn, I., Choi, K.-S. 2003. Quantitative assessment of reproductive condition of the Antarctic clam, *Laternula elliptica* (King and Broderip), using image analysis. Invertebr. Reprod. Dev. 44(1): 71–78.
- Kimm-Brinson, K. L., Ramsdell, J. S. 2001. The red tide toxin, brevetoxin, induces embryo toxicity and developmental abnormalities. Environ. Health Perspect. 109(4): 377–81.
- Landsberg, J. H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev.Fish. Sci. 10(2): 113–390.
- Lauckner, G. 1983. Diseases of mollusca: bivalvia. In: O. Kinne, editor. Diseases of marine animals. Vol 2. Introduction: bivalvia to scaphopoda. Chichester, New York, Brisbane and Toronto: John Wiley and Sons. pp. 477-879
- Lassudrie, M., Soudant, P., Richard, G., Henry, N., Medhioub, W., da Silva, P. M., Donval, A., Bunel, M., Le Goïc, N., Lambert, C., de Montaudouin, X., Fabioux, C., Hégaret, H. 2014. Physiological responses of Manila clams *Venerupis (=Ruditapes) philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure. Aquat. Toxicol. 154: 27–38.
- Lee, R.F., Heffernan, P. B. 1991. Lipids and proteins in eggs of eastern oysters (*Crassostrea virginica* (Gmelin 1791)) and northern quahogs (*Mercenaria mercenaria* (Linnaeus, 1758)). J. Shellfish Res. 10(1): 203– 206.
- Le Goïc, N., Hégaret, H., Fabioux, C., Miner, P., Suquet, M., Lambert, C., Soudant, P. 2013a. Impact of the toxic dinoflagellate *Alexandrium catenella* on Pacific oyster reproductive output : application of flow cytometry assays on spermatozoa. Aquat. Living Resour. 26, 221–228.
- Le Goïc, N., Hégaret, H., Lambert, L., Fabioux, C., Soudant, P. 2013b. Cellular changes of *Crassostrea gigas* spermatozoa upon exposure to the toxic dinoflagellate *Alexandrium catenella*. Aquaculture 2013, Feb 21-25th 2013, Nashville, TN, U.S.A. Meeting abstract nr: 832, poster nr 209.
- Martoja, R., Martoja-Pierson, M. 1967. Initiation aux techniques de l'histologie animale, Paris. pp 1232.
- McFarren, E. F., Tanabe, H., Silva, F. J., Wilson, W. B., Campbell, J. E., Lewis, K. H. 1965. The occurrence of a ciguatera-like poison in oysters, clams and *Gymnodinium breve* cultures. Toxicol. 3:111–123.
- Miller, M. A. 1993. Maternal transfer of organochlorine compounds in salmonines to their eggs. Can. J. Fish. Aquatic Sci. 50(7): 1405-1413.
- Morado, J. F., Sparks, A. K., Reed, S. K. 1984. A coccidian infection of the kidney of the native littleneck clam, *Protothaca staminea*. J. Invert. Path. 43(2): 207-217.
- Morales-Alamo, R., Mann, R. 1989. Anatomical features in histological sections of *Crassostrea virginica* (Gmelin, 1791) as an aid in measurements of gonad area for reproductive assessment. J. Shellfish Res. 8(1): 71-82.
- Naar, J. P., Flewelling, L. J., Lenzi, A., Abbott, J. P., Granholm, A., Jacocks, H.M., Gannon, D., Henry, M., Pierce, R., Baden, D.G., Wolny, J., Landsberg, J. H. 2007. Brevetoxins, like ciguatoxins, are potent ichthyotoxic neurotoxins that accumulate in fish. Toxicol 50(5): 707-723.

- Naar, J., Kubanek, J., Weidner, A., Flewelling, L., Bourdelais, A., Steidinger, K., Baden, D. G. 2004. Brevetoxin Depuration in Shellfish via Production of Non-toxic Metabolites: Consequences for Seafood Safety and the Environmental Fate of Biotoxins, In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A., (Eds). Harmful Algae. 2002. Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and the Intergovernmental Oceanographic Commission of UNESCO. St. Petersburg, FL, USA. pp. 488- 490.
- Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P. L., Flewelling, L., Steidinger, K., Lancaster, J., Baden, D. G. 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ. Health Perspect. 110(2): 179–185.
- Normand, J., Ernande, B., Haure, J., McCombie, H., Boudry, P. 2009. Reproductive effort and growth in *Crassostrea gigas*: comparison of young diploid and triploid oysters issued from natural crosses or chemical induction. Aquat. Biol. 7(3): 229-241.
- NSSP, 2011. National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish 2011 Revision U. S. Department of Health and Human Services Public Health Service Food and Drug Administration Interstate Shellfish Sanitation Conference.
- Overstreet, R.S. 1978. Marine maladies? Worms, germs and other symbionts from the northern Gulf of Mexico. Mississippi. Alabama: Sea Grant Consortium Publication, MASGP-78-021
- Pierce, R. H., Henry, M., Blum, P. 2008. Brevetoxin abundance and composition during ECOHAB-Florida field monitoring cruises in the Gulf of Mexico. Continental Shelf Res. 28(1): 45-58.
- Pierce, R. H. , Henry, M. S., Blum, P. C., Plakas, S. M., Granade, H. R., Jester, E. L. E., El Said, K. R., Dickey, R.W., Steidinger, K.A., Scott, P.S., Flewelling, L.J., Wright, J. L. C. 2004. Comparison of methods for determination of brevetoxins and their metabolites in NSP-toxic bivalve molluscs. In: Proceedings of the Fifth International Conference on Molluscan Shellfish Safety, Galway Ireland, Current and Emerging Analytical Methods. pp 37– 42.
- Pierce, R. H., Henry, M. S., Blum, P. C., Lyons, J., Cheng, Y. S., Yazzie, D., Zhou, Y. 2003. Brevetoxin concentrations in marine aerosol: human exposure levels during a *Karenia brevis* harmful algal bloom. B. Environ. Contam. Tox. 70(1): 161-165.
- Plakas, S. M., Dickey, R. W. 2010. Advances in monitoring and toxicity assessment of brevetoxins in molluscan shellfish. Toxicon, 56(2): 137–149.
- Plakas, S. M., Jester, E.L.E., El Said, K.R., Granade, H.R., Abraham, A., Dickey, R.W., Scott, P.S., Flewelling, L.J., Henry, M., Blum, P., Pierce, R. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (*Crassostrea virginica*). Toxicon. 52(1): 32-38.
- Plakas, S.M., Wang, Z.H., El Said, K.R., Jester, E.L.E., Granade, H.R., Flewelling, L., Scott, P., Dickey, R.W. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon. 44: 677–685.
- Poli, M. A., Templeton, C. B., Thompson, W. L., Hewetson, J. F. 1990a. Distribution and elimination of brevetoxin PbTx-3 in rats. Toxicon. 28(8): 903-910.
- Poli, M. A., Templeton, C. B., Pace, J. G., Hines, H. B. 1990b. Detection, metabolism, and pathophysiology of brevetoxins. In ACS symposium series Oxford University Press. 418: 176-191.
- Poli, M. A., Mende, T. J., Baden, D. G. 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol. Pharmacol. 30(2): 129-135.

Riley, C. M., Holt, S. A., Holt, G. J., Edward, J., Arnold, C. R. 1989. Mortality of larval Red Drum (*Sciaenops ocellatus*) associated with *Ptychodiscus brevis* red tide. *Cont. Mar. Sci.* 31: 137– 146.

Roberts, B. S., G. E. Henderson, Medlyn, R. A. 1979. The effect of *Gymnodinium breve* toxin(s) on selected mollusks and crustaceans. In: Taylor, D. L., Seliger, H. H. (Eds.). *Toxic Dinoflagellate Blooms*. Elsevier, New York. pp. 419–424.

Rolton, A., Vignier, J., Soudant, P., Shumway, S. E., Bricelj, V. M., Volety, A. K. 2014. Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*. *Aquat. Toxicol.* 155: 199–206.

Rolton, A., Soudant, P., Vignier, J., Donaghy, L., Shumay, S. E., Bricelj, V. M., Pierce, R., Henry, M., Blum, P., Volety, A. K. in prep a. Exposure of adult *Crassostrea virginica* to *Karenia brevis*: Toxin accumulation and histopathological and cellular responses in adults and, effects of gametogenesis, gamete and larval quality.

Rolton, A., Vignier, J., Soudant, P., Shumway, S. E., Bricelj, V. M., Volety, A. K. in prep b. Effects of *Karenia brevis* exposure on adult *Mercenaria mercenaria*: Histopathological and cellular responses in adults and effects on gamete and larval quality.

Roszell, L. E., Schulman, L. S., Baden, D. G. 1990. Toxin profiles are dependent on growth stages in cultures *Phytodiscus brevis*. In: Graneli, E., Sundström, B., Elder, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton*. Elsevier, New York. pp 403-406.

Rounsefell, G. A., Nelson, W. R., 1966. Red-tide research summarized to 1964 including an annotated bibliography. In: United States Department of the interior Fish and Wildlife Service. Contribution number 20. Bureau of Commercial Fisheries Special Scientific Report. Fisheries number 535. Washington, DC. pp. 1–85.

Sharp, K. H., Eam, B., Faulkner, D. J., Haygood, M. G. 2007. Vertical transmission of diverse microbes in the tropical sponge *Corticium* sp. *Appl. Environ. Microb.* 73(2): 622–629.

Shaw, B. ., Battle, H. I. 1957. The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Can. J. Zool.* 325– 347.

Shumway, S. E., Cembella, A. D. 1993. The impact of toxic algae on scallop culture and fisheries. *Rev. Fish. Sci.* 1: 121–150.

Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aqua-culture. *J. World Aquac. Soc.* 21 (2), 65–104.

Shumway, S. E., Cucci, T. L. 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. *Aquat. Toxicol.* 10(1): 9-27.

Smolowitz, R., Shumway, S. E. 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve molluscs. *Aquacult. Int.* 5(4): 291–300.

Steidinger, K. A. 2009. Historical perspective on *Karenia brevis* red tide research in the Gulf of Mexico. *Harmful Algae.* 8(4): 549-561.

Steidinger, K. A., Wolny, J. L., Haywood, A. J., 2008. Identification of Kareninaceae (Dinophyceae) in the Gulf of Mexico. *Nova Hedwegia.* 133: 269–284.

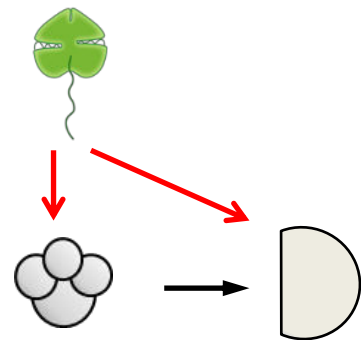
Steidinger, K. A., Carlson, P., Baden, D., Rodriguez, C., Seagle, J. 1998. Neurotoxic shellfish poisoning due to toxin retention in the clam *Chione cancellata*. In: Reguera, B., J. Blanco, M. Fernandez, L., Wyatt, T. (Eds). *Harmful Algae*. Paris: Xunta de Galicia, IOC. pp. 457–458.

- Steidinger, K. A. 1993. Some taxonomic and biological aspects of toxic dinoflagellates. In: Falconer, I. R. (Ed). *Algal Toxins in Seafood and Drinking Water*. London, Academic Press. pp. 1– 28.
- Steidinger, K. A., Burklew, M., Ingle, R. M. 1973. The effects of *Gymnodinium breve* toxin on estuarine animals. In: Martin, D. F., Padilla, G. M. (Eds). *Marine Pharmacognosy: Action of Marine Toxins at the Cellular Level*. New York: Academic Press. pp. 179–202.
- Summerson, H., Peterson, C. 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Ptychodiscus brevis*, outbreak recorded in North Carolina. *Estuaries*. 13(3): 322–331.
- Tester, P. A., Fowler, P. K. 1990. Brevetoxin contamination of *Mercenaria mercenaria* and *Crassostrea virginica*: A management issue. In: Graneli, E., Sundström, B., Edler, L., Anderson, D. M. (Eds). *Toxic marine phytoplankton*. Elsevier Science Publishing, New York. pp 499-503.
- Thompson, R. J., Newell, R. I. E., Kennedy, V. S., Mann, R. 1996. Reproductive processes and early development. In: Kennedy, V.S. (Ed.) *The Eastern Oyster Crassostrea virginica*. Maryland Sea Grant College, University of Maryland, College Park, Maryland, 335-370.
- Todd, C. D., Havenhand, J. N. 1983. Reproductive effort: its definition, measurement and interpretation in relation to molluscan life-history strategies. *J. Mollus. Stud.* 49(12): 203-208.
- Volety, A. K., Savarese, M., Tolley, S. G., Arnold, W. S., Sime, P., Goodman, P., Chamberlain, R. H., Doering, P. H., 2009. Eastern oysters (*Crassostrea virginica*) as an indicator for restoration of everglades ecosystems. *Ecol. Indic.* 9(6):120–136.
- Washburn, B. S., Baden, D. G., Gassman, N. J., Walsh, P. J. 1994. Brevetoxin: tissue distribution and effect on cytochrome P450 enzymes in fish. *Toxicol.* 32(7): 799–805.
- Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E., Kinne, R. K. H. 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev. Physiol. Biochem. Pharmacol.* 148: 1–80.
- Wikfors, G. H. 2005. A review and new analysis of trophic interactions between *Prorocentrum minimum* and clams, scallops, and oysters. *Harmful Algae*. 4(3): 585-592.
- Winstead, J. T., Volety, A. K., Tolley, S. G. 2004. Parasitic and symbiotic fauna in oysters (*Crassostrea virginica*) collected from the Caloosahatchee river and estuary in Florida. *J. Shellfish Res.* 23(3): 831– 840.

Chapter 4

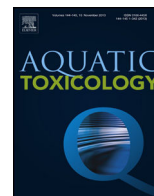
Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*.

Results from the previous chapter confirmed that PbTx was passed from the mother to the oocyte. The resulting offspring from these PbTx contaminated oocytes were hypothesized to have been affected throughout the lecithotrophic phase but beyond this, effects were not long lasting. In the field however, blooms may extend into periods of spawning. Thus, early life stages may be directly exposed to *K. brevis*. In this chapter, the effects of different cell preparations of *K. brevis* on early embryonic stages of *C. virginica* and *M. mercenaria* were studied.



The hypothesis tested was:

- Brevetoxin is the cause of toxic effects on early life stages during *K. brevis* exposure.



Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*



Anne Rolton^{a,b}, Julien Vignier^{a,b}, Philippe Soudant^b, Sandra E. Shumway^c, V. Monica Bricelj^d, Aswani K. Volety^{a,*}

^a Florida Gulf Coast University, Vester Marine Field Station, 5164 Bonita Beach Road, Bonita Springs, FL 34134, USA

^b LEMAR CNRS UMR 6539, Institut Universitaire Européen de la Mer—Université de Bretagne Occidentale, Place Nicolas Copernic, Technopôle Brest Iroise, 29280 Plouzané, France

^c Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340, USA

^d Institute of Marine and Coastal Sciences, Rutgers University, 71 Dudley Road, New Brunswick, NJ 0890, USA

ARTICLE INFO

Article history:

Received 7 April 2014

Received in revised form 26 June 2014

Accepted 27 June 2014

Available online 6 July 2014

Keywords:

Karenia brevis

Development

Larvae

Clams

Oysters

Brevetoxin

ABSTRACT

The brevetoxin-producing dinoflagellate, *Karenia brevis*, adversely affects many shellfish species including the commercially and ecologically important bivalve molluscs, the northern quahog (=hard clam) *Mercenaria mercenaria* and eastern oyster *Crassostrea virginica*, in the Gulf of Mexico, USA. This study assessed the effects of exposure of these bivalves to *K. brevis* during their early development. In separate experiments, embryos of 2–4 cell stage of *M. mercenaria* and *C. virginica* were exposed to both whole and lysed *K. brevis* cells isolated from Manasota Key, Florida. Low bloom concentrations of 500 to 3000 cells mL⁻¹ were simulated for 96 h. Shell length, percent abnormality (and normality), and percent mortality of resulting larvae were measured. Percentages were recorded after 6, 24, and 96 h of exposure; larval shell length was measured at 24 and 96 h. For both quahogs and oysters, the effects of exposing embryos to *K. brevis* on all larval responses were generally dose- and time-dependent. Percent mortalities and abnormalities of both clam and oyster embryos increased significantly after only 6 h of exposure to whole cells of *K. brevis*. For clams, these parameters were significantly higher in whole and lysed treatments (at 3000 cells mL⁻¹) than in controls. Percent mortalities of oysters were significantly higher in the whole-cell treatment (3000 cells mL⁻¹) than under control conditions. After 24 h of exposure, mean larval shell length of both bivalve species was significantly reduced relative to controls. This was evident for clam larvae in both the lysed treatment at 1500 cells mL⁻¹ and in whole and lysed treatments at 3000 cells mL⁻¹, and for oyster larvae in the lysed treatment at 3000 cells mL⁻¹. After 96 h, both species exposed to the lysed cell treatment at 3000 cells mL⁻¹ had significantly smaller larvae compared to those in the control. Overall, lysed cells of *K. brevis* had a more pronounced effect on shell length, percent abnormality, and mortality in both clams and oysters than did whole cells. Given the fact that blooms of *K. brevis* overlap with the spawning periods of these two bivalves, and that cells of this naked dinoflagellate are readily lysed by wave action, these results suggest that exposure to *K. brevis* during the early life history stages of clams and oysters could adversely affect their population recruitment. Further, the presence of whole or lysed cells of *K. brevis* in hatcheries could have a major negative impact on production.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Harmful algal blooms (HAB) can have deleterious, economic, environmental, and human health effects worldwide (e.g., Landsberg, 2002; Shumway, 1990; Smayda, 1990). In the US

alone, the cost of HAB to commercial fisheries, aquaculture, public health, tourism, monitoring and management has been estimated at \$82 million annually (Hoagland and Scatista, 2006). The most prevalent HAB species in the Gulf of Mexico is the neurotoxic dinoflagellate, *Karenia brevis*, a producer of brevetoxins (PbTx). The frequency of *K. brevis* blooms in the Gulf of Mexico appears to be increasing (Brand and Compton, 2007) and blooms, which typically form in the autumn, have been known to persist for up to 18 months (Gunter et al., 1948; Hu et al., 2006; Rounsefell and Nelson, 1966; Vargo et al., 2004). Blooms of *K. brevis* are characterised

* Corresponding author. Current address: University of North Carolina Wilmington, 601 S. College Rd, Wilmington, NC 28403. Tel.: +910 962 7232.

E-mail address: voletya@uncw.edu (A.K. Volety).

as above background concentrations of ≥ 1 cell mL⁻¹ (Finucane, 1964), but densities can vary up to thousands of cells per mL and frequently reach 10⁴ cells mL⁻¹ along the Florida (FL) coast (Steidinger, 2009; Tester and Steidinger, 1997). Blooms of *K. brevis* are often concentrated at the water surface and in nearshore areas, where wave action and turbulence may break the delicate, unarmored cells, releasing endogenous brevetoxins (Van Dolah, 2000). Between 9 and 14 lipophilic polyether brevetoxins are produced by *K. brevis* (Baden et al., 2005; Poli et al., 1986), which bind to voltage-sensitive sodium channels causing permanent excitation of the cell and thus interference with nerve transmission (Pierce and Henry, 2008). Multiple compounds other than PbTx, that are allelopathic, ichthyotoxic, and hemolytic, can also be produced by *K. brevis* and, together with PbTx, these compounds may add to the overall toxicity levels of blooms (Kubaneck et al., 2005, 2007; Neely and Campbell, 2006; Prince et al., 2010).

The northern quahog (=hard clam; *Mercenaria mercenaria*) and eastern oyster (*Crassostrea virginica*) contribute markedly to the local economy and ecosystem services in FL coastal waters: FL ranks second in the US for hard clam production and in 2007 clam and oyster aquaculture production for the state was valued at \$15.2 million (United States Department of Agriculture (US DOA), 2007). During the 2002–2003 HAB season, the FL shellfish aquaculture and oyster industries lost \$6 million in dockside sales alone, and up to 20% of planted clams were lost from harvesting areas (National Oceanic and Atmospheric Administration (NOAA), 2004), although this figure does not include the “halo” effect that affects all seafood sales (Conte, 1984; Hoagland and Scatasta, 2006; Jensen, 1975; Shumway, 1990; Steidinger, 2009). Both bivalve species also provide important ecosystem services in shallow estuaries, e.g., increased light penetration for submerged aquatic vegetation via their high filtration activity, and provision of secondary habitat (*sensu* Burkholder and Shumway, 2012; Newell, 2004; Wells, 1961).

The spawning periods of oysters and hard clams in FL often overlap with blooms of *K. brevis*. *C. virginica* in southwest FL spawn continuously (Volety et al., 2009), with peak spawning from May through October (Volety, 2008; Volety et al., 2009). Spawning of hard clams (*M. mercenaria* and *M. campechensis*) from the Indian River Lagoon, FL, is also essentially continuous with individuals in a spawning state in all months (Hesselman et al., 1989), thus resulting in co-occurrence of *Karenia* cells with gametes and larvae of these bivalve species in the water column.

Effects of toxic algae, including *K. brevis*, on the early developmental stages of bivalves remain poorly understood. In 1987, a bloom on one of the most productive beds of the bay scallop, *Argopecten irradians*, in North Carolina, resulted in almost total recruitment failure and low recruitment persisted the subsequent year (Summerson and Peterson, 1990). Shellfish stock enhancement programs are often conducted with limited understanding of the long-term impacts of *K. brevis* on recruitment success and thus population sustainability (Arnold, 2001; Leverone et al., 2010). Blooms can also negatively affect larval rearing in hatcheries that use seawater pumped from nearshore waters, as cells can be lysed by filtration systems, releasing PbTx (Deeds et al., 2002; Stoecker et al., 2008).

Early life cycle stages of bivalves have been shown to be highly susceptible to toxic algal exposure (Glibert et al., 2007; Yan et al., 2003). Susceptibility is species-specific (Glibert et al., 2007; Leverone et al., 2006; Shumway and Cucci, 1987), dependent upon developmental stage of the bivalve (Wang et al., 2006; Wikfors and Smolowitz, 1995; Yan et al., 2001), the duration of exposure (Basti et al., 2013; Leverone et al., 2006), the chemical structure and mode of action of associated toxins, and experimental conditions, including the concentration, mode of delivery, and growth stage of the algae (Basti et al., 2011, 2013; Glibert et al., 2007; Yan et al., 2001).

Leverone et al. (2006) exposed 3- and 7-day-old larvae of *C. virginica*, *M. mercenaria*, and *A. irradians* to concentrations of up to 5000 *K. brevis* cells mL⁻¹ for 3 and 7 days. Survival of 3-day-old larvae was dose-dependent and significantly lower at 1000 cells mL⁻¹ than in the control for all 3 species. Survival of 7-day-old larvae at 5000 cells mL⁻¹ was significantly lower than in the control for *A. irradians* and *C. virginica* indicating that the 3-day-old larvae were more susceptible to exposure than 7-day-old larvae for these two species. For *M. mercenaria*, survival was significantly lower than in the control at 1000 cells mL⁻¹ revealing differences in sensitivity between species. Exposure of the pre-feeding and embryonic stages was not investigated by Leverone et al. (2006). Embryogenesis is a period of rapid cellular differentiation and thus of high vulnerability to biotic and abiotic interferences (Carriker, 2001; Pechenik, 1987; Thompson et al., 1996). Bivalve larvae exposed during embryogenesis to the toxic dinoflagellate *Heterocapsa circularisquama* and the raphidophyte *Heterosigma akashiwo* were found to be more sensitive than hatched larvae (Basti et al., 2011, 2013; Wang et al., 2006). In contrast, Wikfors and Smolowitz (1995) showed that embryonic development of *C. virginica* was not affected by exposure to the thecate dinoflagellate *Prorocentrum minimum*.

Measurement of sub-lethal effects such as morphological abnormalities and growth inhibition throughout the period of exposure may provide a better understanding of stage-specific susceptibility, and may help determine the mode of action of the algae. Abnormalities interfere with larval feeding, swimming, and development to metamorphosis, thereby increasing the likelihood of mortalities. Reduction in shell growth rates increases the planktonic larval period and thus the vulnerability to predation (Calabrese et al., 1973; Strathmann, 1985).

The objective of this study was to determine the short-term effects (up to 96 h) of exposure of embryonic *M. mercenaria* and *C. virginica* to whole and lysed *K. brevis* cells over a range of bloom concentrations, on size and developmental success. Results will serve to: (1) improve our understanding of the mode of action of *K. brevis*, and (2) assist in making informed decisions on suitable sites for aquaculture, restoration, and enhancement efforts in areas affected by *K. brevis*.

2. Materials and methods

2.1. Broodstock and spawning

In October 2011, eastern oysters, *C. virginica*, were collected from Estero Bay, Bonita Springs, FL (Lat. 26.3484, Long. -81.8421) and northern quahogs, *M. mercenaria*, were obtained from Cut-throat Clams, Pine Island, FL (Lat. 26.6, Long. -82.153), to be used as broodstock. Adults were acclimatized for 7 days in 400-L tanks at the Vester Marine Field Station, Florida Gulf Coast University (FGCU), prior to induction of spawning. Oysters were held at a salinity of 25 and temperature of 23 °C (± 1) and clams were held at a salinity of 28 (± 2), and 23 °C (± 1). Both species were fed a mixed diet of *Tisochrysis lutea*, formerly *Isochrysis affinis galbana* Tahitian clone, (here named T-Iso; CCMP 1324), *Chaetoceros muelleri* (CCMP 1318), *Chaetoceros calcitrans* (CCMP 1315), *Tetraselmis chui* (CCMP 882) and *Pavlova lutheri* (CCMP 1325) *ad libitum*. All stock cultures were obtained from the Provasoli-Guillard National Center for Marine Microalgae and Microbiota (NCMA), Bigelow Laboratory, Maine (previously the CCMP). Prior to experimental exposure, 3 oysters and 3 clams were selected randomly and sacrificed to verify ripeness.

Spawning of broodstock was induced by thermal cycling between 30 °C and 18 °C in filtered seawater (0.1 μ m) every 30–45 min. Salinity was 30 for clams and 25 for oysters. Spawning individuals were removed from the tank, rinsed in filtered seawater and individually transferred to separate 1-L containers with

0.1 μm filtered and UV-sterilized seawater to complete spawning and prevent gamete contamination or fertilization. Once spawning had finished, gamete quality was assessed under the microscope. Motile sperm from 3 males were filtered through a 55 μm mesh⁻¹ and pooled in a sterile 1-L beaker. Oocytes from 4 females were filtered first through a 150 μm mesh to remove debris and then retained on a smaller mesh for washing before stocking. Oocytes of *C. virginica* were retained on a 20 μm mesh⁻¹ and those of *M. mercenaria* oocytes on a 35 μm mesh⁻¹. Washed oocytes were pooled in a sterile 2-L beaker to give a range of 500–2500 oocytes mL⁻¹. Sperm was added to reach an oocyte:sperm ratio of 1:3, mixed gently, and left until polar body–2-cell stage was reached.

2.2. Algal cultures and preparation

The unicellular alga *T. lutea* (T-Iso, equivalent spherical diameter, ESD = 5 μm) was grown in CELL-HI WP culture medium based on Walnes medium (Varicon Aqua) at a salinity of 30. Flagellates, *T. chui* (ESD = 10 μm) and *P. lutheri* (ESD = 5 μm) were grown in CELL-HI F2P based on Guillard F/2 medium (Varicon Aqua) at a salinity of 30. Diatoms, *C. muelleri* (ESD = 6 μm) and *C. calcitrans* (ESD = 6 μm) were grown in CELL-HI F2P based on Guillard F/2 medium and Si (Varicon Aqua) at a salinity of 24. All non-toxic algae were batch-cultured in aerated 90-L and 20-L capacity containers.

The toxic dinoflagellate, *K. brevis* (Manasota Key strain, mean ESD = 30.7 μm \pm SD = 5.5 μm ; $n = 40$) was obtained from the Mote Marine Laboratory, Sarasota, FL. Batches were grown in 1-L Erlenmeyer flasks without aeration. Algae were maintained in L1 medium prepared from 0.1 μm -filtered and UV-treated seawater from Estero Bay which had been autoclaved, allowed to cool, and L1 nutrients (NCMA) added aseptically. Cultures were kept at a salinity of 29 (± 2) and temperature of 22 $^{\circ}\text{C}$ (± 1) on a 12:12 light:dark cycle. Growth and purity of cultures were monitored microscopically and algae were sub-cultured every 2 weeks (once stationary phase had been reached and typically at a density of 1.5 $\times 10^4$ –2.5 $\times 10^4$ cells mL⁻¹).

Treatments of both whole and lysed cells of *K. brevis* were prepared at 500 cells mL⁻¹, 1500 cells mL⁻¹ and 3000 cells mL⁻¹ (referred to as low, moderate, and high, respectively). Control embryos were placed in seawater without algae. For embryonic exposures, cultures of *K. brevis* were used at the early stationary phase as this is the stage at which they are generally most toxic (Roth et al., 2007; Van Dolah et al., 2009). Cells of *K. brevis* were harvested at 2.3 $\times 10^4$ cells mL⁻¹ for the clam exposure and at 2.1 $\times 10^4$ cells mL⁻¹ for the oyster exposure. Triplicate cell counts of stock cultures were performed using a nanoplankton chamber (PhycoTech Inc.) and aliquots added to the glass exposure beakers to reach the desired final concentration. Varying proportions of PbTx are extracellular (Pierce et al., 2001), and the remaining intracellular toxins were released using a sonic dismembrator, (model 500, Fisher Scientific) set at continuous sonication for 10 min to prepare lysed *K. brevis* treatments according to Leverone et al. (2006). Sub-samples were observed under the microscope to ensure cells had been lysed and were then added to the exposure beakers at the same concentration as whole *Karenia* treatments.

2.3. Toxin analysis of *Karenia brevis* cultures

To ensure that the culture of *K. brevis* used in experiments had a toxin profile consistent with previously measured toxin profiles of *K. brevis* and had not been altered under culture conditions prior to experiments, whole cells of *K. brevis* were analysed for PbTx content by liquid chromatography, tandem mass spectrometry (LC–MS/MS) at the Mote Marine Laboratory, Sarasota, FL, USA, according to the method of Pierce et al. (2005).

2.4. Exposure of embryos

In separate experiments, embryos of *C. virginica* and *M. mercenaria* at the 2–4 cell stage were exposed to whole and lysed *K. brevis* at 500, 1500 and 3000 cells mL⁻¹ concentrations for 96 h.

Once embryos had reached the 2–4 cell stage in the stock solution (approximately 45 min post-fertilization), embryos in 3 aliquots of 0.1 mL stock solution were counted microscopically using a Sedgewick-Rafter cell. Embryos (~8000) were added to each beaker already containing the appropriate treatment of *K. brevis* to give a final concentration of 20 embryos mL⁻¹. A final volume of 400 mL was reached by adding 0.1 μm filtered and UV-treated seawater (salinity was 30 for *M. mercenaria* and 27 for *C. virginica*). Beakers were exposed to natural daylight, but kept away from direct sunlight during exposures. Four replicates were prepared per treatment. Temperature, dissolved oxygen (DO), pH and salinity were monitored daily using a Pro ODO optic probe (YSI), 'Pinpoint' pH monitor (American Marine Inc.) and a refractometer (Aquatic ecosystems), and temperature was maintained at 24 ± 1 $^{\circ}\text{C}$ for both *M. mercenaria* and *C. virginica* experiments. Gentle aeration (~5 bubbles s⁻¹) and daily 'swirling' of the uncovered beakers helped maintain DO levels and keep the *Karenia* cells well mixed. After sampling for mortality, abnormality and shell lengths at 24 h, newly hatched larvae were fed with clone T-Iso and *C. muelleri* at 5 $\times 10^4$ cells mL⁻¹. At 72 h T-Iso was again added at 5 $\times 10^4$ cells mL⁻¹ and *C. muelleri* at 3.5 $\times 10^4$ cells mL⁻¹ (total cell density = 10⁵ cells mL⁻¹ [T-Iso cell volume-equivalent]).

2.5. Larval mortality and prevalence of abnormalities

Sub-samples (10 mL) were taken from each beaker at 6 h, 24 h and 96 h and fixed in 0.1% buffered formalin. Numbers of normal, abnormal (presumably live) and dead (no visible cell contents) embryos/larvae were counted using a light microscope and a Sedgewick Rafter cell according to Carriker (2001). At 6 h, abnormal embryos were considered as those which had not reached the blastula stage (Carriker, 2001; Thompson et al., 1996), those which were unfertilized, and those with abnormally shaped cells. At 24 h, abnormal larvae were considered as those which had not reached the veliger/D-shape stage and those with shell deformities and/or mantle abnormalities. At 96 h, abnormal larvae were considered as those which had not reached veliger/D-shape stage or early umbo veliger stage and those with shell deformities and/or mantle abnormalities (Fig. 1). Percent mortality was calculated as

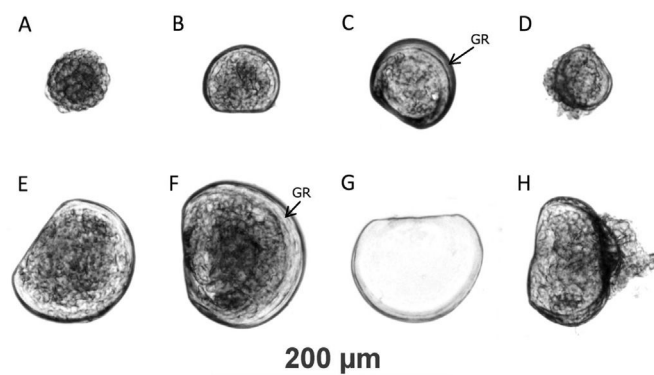


Fig. 1. Examples of normal, abnormal, and dead embryos/larvae. (A) 6 h: normal blastula stage of *Crassostrea virginica*. (B) 24 h: normal D-stage/veliger *C. virginica* larva. (C) 96 h: normal late D-stage/veliger *C. virginica* (growth ring: GR). (D) Mantle abnormality of *C. virginica*. (E) 24 h: normal D-stage *Mercenaria mercenaria*. (F) 96 h: normal late D-stage *M. mercenaria* (growth ring: GR). (G) Dead larvae of *M. mercenaria* showing no visible cell contents. (H) Mantle abnormality and shell deformity (saddle-shape) of *M. mercenaria*.

the number of presumed dead embryos/larvae divided by the total number of embryos/larvae observed [normal (live), abnormal (live) and dead]. Percent abnormality was calculated as the number of abnormal (live) embryos/larvae divided by the total number of embryos/larvae observed [normal (live), abnormal (live) and dead]. Percent normality was calculated as the number of normal (live) embryos/larvae divided by the total number of embryos/larvae observed [normal (live), abnormal (live) and dead].

2.6. Larval shell measurements

Twenty live larvae from each replicate were randomly measured at 24 and 96 h, with a micrometer on a compound light microscope. The mean shell length (greatest antero-posterior dimension) for each of the four replicates per treatment was used to plot graphs with standard deviations.

2.7. Statistical analysis

Results for percent normality, abnormality, and mortality were arcsine square root transformed to normalize distributions. Mean shell length data and transformed normality, abnormality and mortality data were compared using one-way analysis of variance (ANOVA), with *T*-tests between different sampling times and linear regressions of each metric vs. *K. brevis* cell density for both whole-cell and lysed treatments. When requirements of homogeneity were met (Levene's test), Tukey's multiple comparison post-hoc tests were used to identify differences between the 7 treatments. Dunnett's-T3 post hoc tests were used to identify differences between the 7 treatments when homogeneity requirements were not met (Levene's test). Statistical tests were run using SPSS 16 statistical software.

3. Results

3.1. Toxin analysis of *Karenia brevis* cultures

The total brevetoxin content of *K. brevis* was $9.66 \text{ pg cell}^{-1} \pm 1.03$ (Table 1) with PbTx-2 being the most abundant toxin in the culture ($7.35 \text{ pg cell}^{-1} \pm 0.58$). These results are consistent with earlier laboratory and field studies by Baden and Thomas (1989), Leverone et al. (2006), Pierce et al. (2001, 2005) and Pierce and Henry (2008). The low amount of the PbTx antagonist brevenal ($0.77 \text{ pg cell}^{-1} \pm 0.82$) is also in agreement with previous studies using this species.

3.2. Morphology and survival of bivalve larvae upon *Karenia brevis* exposure

For both clams and oysters, embryonic exposure to *K. brevis* significantly increased larval abnormalities and mortality (Figs. 2 and 3; Table 2). Mortalities in the controls were low (<5%) at all 3 sampling points for both species. Percent abnormalities and mortalities showed strong dose-dependence when embryos of both species were exposed to lysed *K. brevis* cells for 96 h, as determined by linear regressions (not shown). Dose-dependence was also evident in treatments of clams exposed to intact *K. brevis* cells ($R^2 = 0.995$) but not in those of oysters ($R^2 = 0.283$).

Table 1

Brevetoxin (PbTx) concentrations (pg cell⁻¹ and nM) of representative 14 day-old whole cell cultures of *Karenia brevis* (Manasota Key strain) harvested in October 2011, March and September 2012 for embryonic exposures. Data are presented as mean ± standard deviation, n = 3.

PbTx conjugate	PbTx-1	PbTx-2	PbTx-3	PbTx-carboxylic acid	Brevenal	Total
Concentration (pg cell ⁻¹)	0.72 ± 0.35	7.35 ± 0.58	0.59 ± 0.32	0.22 ± 0.18	0.77 ± 0.82	9.66 ± 1.03
Concentration (nM)	11.32 ± 5.63	111.05 ± 6.42	9.04 ± 5.03	3.36 ± 2.72	15.67 ± 16.22	150.43 ± 15.17

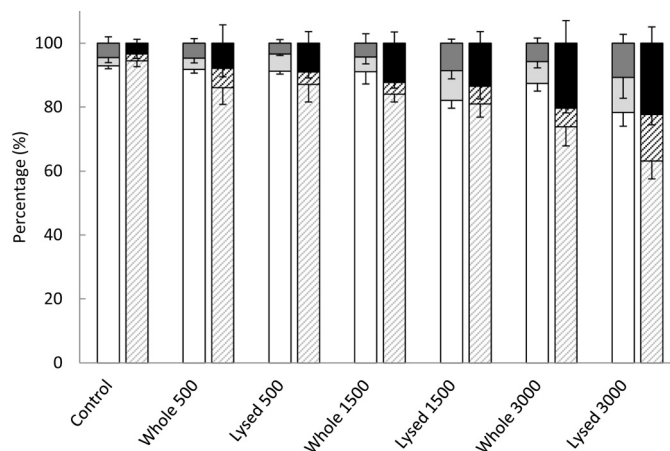


Fig. 2. Percent of normal clam larvae (white), percent of abnormal clam larvae (light grey), percent mortality of clam larvae (dark grey), percent of normal oyster larvae (diagonal lines), percent of abnormal oyster larvae (cross-hatched) and percent mortality of oyster larvae (black) after 96 h-exposure of *Mercenaria mercenaria* and *Crassostrea virginica* embryos to whole and lysed preparations of *Karenia brevis* at differing concentrations (500, 1500 and 3000 cells mL⁻¹). Data are presented as mean ± standard deviation, n = 3.

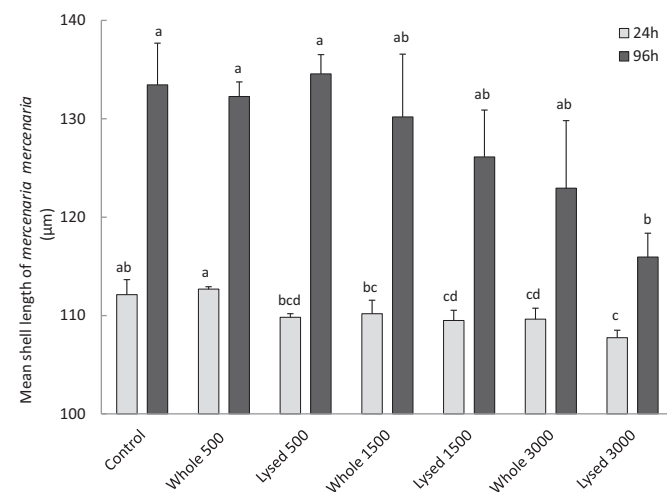


Fig. 3. Comparison of larval shell length of *Mercenaria mercenaria* after 24 h and 96 h of exposure to whole and lysed preparations of *Karenia brevis* at 500, 1500 and 3000 cells mL⁻¹. Treatments with the same letter were not significantly different ($p > 0.05$). Data are presented as mean ± standard deviation, n = 3.

3.2.1. *Mercenaria mercenaria*

Following only 6 h-exposure of embryos to *K. brevis*, the highest dose of whole cell treatment contained significantly fewer normal, and more dead embryos than in the control ($p < 0.02$ and $p < 0.04$, respectively). The treatment concentration of 3000 lysed cells mL⁻¹ resulted in significantly more abnormal embryos than in the control ($p < 0.03$).

At 24 h, there was no effect of either lysed or whole cells at any concentrations on the percentage of dead larvae. Nevertheless, the highest lysed cell treatment contained significantly fewer normal larvae than the control, 500 cells mL⁻¹, and whole 1500 cells mL⁻¹

Table 2
Percent normality, abnormality, and mortality values for *Mercenaria mercenaria* and *Crassostrea virginica* exposed for 6, 24, and 96 h to three treatments of *Karenia brevis* (500, 1500 and 3000 cells mL⁻¹), n = 3. Significance levels: *** p ≤ 0.001; ** 0.001 < p ≤ 0.01; * 0.01 < p ≤ 0.05. At each time-point, treatments with the same letter were not significantly different (p > 0.05).

	Sampling	time point								Significance
		Control	Whole 500	Lysed 500	Whole 1500	Lysed 1500	Whole 3000	Lysed 3000		
<i>Mercenaria mercenaria</i>	% Normal	94.2 ^a	86.3 ^{ab}	82.4 ^{ab}	88.3 ^{ab}	82.5 ^{ab}	78.6 ^b	81.1 ^{ab}	*	
		91.9 ^a	93.6 ^a	91.7 ^a	91.7 ^a	89.7 ^{ab}	90.6 ^{ab}	85.3 ^b	**	
	% Abnormal (live)	92.9 ^a	91.8 ^a	91.2 ^a	91.0 ^a	82.1 ^{bc}	87.4 ^{ab}	78.3 ^c	***	
		3.6 ^b	9.8 ^{ab}	12.3 ^{ab}	7.0 ^{ab}	12.3 ^{ab}	11.7 ^{ab}	14.4 ^a	*	
	% Mortality	3.8 ^b	4.3 ^b	5.8 ^{ab}	4.9 ^b	7.7 ^{ab}	5.8 ^{ab}	10.7 ^a	**	
		2.6 ^b	3.5 ^{ab}	5.4 ^{ab}	4.6 ^{ab}	9.3 ^a	6.9 ^{ab}	11.0 ^a	**	
<i>Crassostrea virginica</i>	% Normal	2.2 ^b	3.9 ^{ab}	5.3 ^{ab}	4.7 ^{ab}	5.2 ^{ab}	9.8 ^a	4.4 ^{ab}	*	
		4.2	2.0	2.5	3.3	2.6	3.6	4.0	NS	
	% Abnormal (live)	4.5 ^{bc}	4.7 ^{bc}	3.4 ^c	4.4 ^{bc}	8.6 ^{ab}	5.8 ^{abc}	10.7 ^a	***	
		85.7 ^a	85.0 ^a	85.2 ^a	84.4 ^a	80.1 ^a	83.2 ^a	83.2 ^a	***	
	% Mortality	97.0 ^a	93.4 ^{ab}	87.7 ^b	94.3 ^{ab}	86.6 ^{bc}	85.9 ^{bc}	77.0 ^c	***	
		94.5 ^a	86.1 ^b	87.1 ^b	84.0 ^{bc}	81.0 ^{bc}	73.8 ^{cd}	63.1 ^d	***	
<i>Crassostrea virginica</i>	% Abnormal (live)	6.2 ^{ab}	8.8 ^{ab}	8.1 ^b	8.1 ^{ab}	10.6 ^b	21.6 ^a	8.4 ^b	***	
		2.5 ^b	5.4 ^{ab}	10.3 ^{ab}	2.8 ^b	5.9 ^b	7.8 ^{ab}	14.7 ^a	***	
	% Mortality	2.2 ^b	6.0 ^b	3.9 ^b	3.7 ^b	5.5 ^b	5.8 ^b	14.6 ^a	***	
		4.1 ^b	6.3 ^{bc}	6.7 ^{bc}	7.5 ^{bc}	9.2 ^{ac}	13.4 ^a	8.4 ^{ab}	***	
		0.5 ^c	1.2 ^{bc}	2.0 ^{bc}	3.0 ^{abc}	7.5 ^{ab}	6.3 ^{ab}	8.3 ^a	***	
		3.4 ^d	7.9 ^{cd}	9.0 ^{bcd}	12.3 ^{abc}	13.5 ^{abc}	20.4 ^{ab}	22.3 ^a	***	

treatments ($p < 0.003$; Table 2). The highest lysed cell concentration treatment also resulted in significantly more abnormal larvae than the control, whole 500 and 1500 cells mL⁻¹ treatments ($p < 0.002$). From 24 to 96 h, the mean percent mortality significantly increased (from 3.8 to 10.7% at 24 h and from 2.6 to 11% at 96 h) while the percentage of abnormality remained in the same range.

After 96 h-exposure, the lysed cell treatment at 1500 and 3000 cells mL⁻¹ contained significantly fewer normal larvae than the control and all other treatments ($p < 0.001$; Table 2 and Fig. 2); the prevalence of abnormal larvae was significantly higher in the two highest lysed treatments compared to the control and all other treatments ($p < 0.01$). Larval mortalities were significantly higher (up to 10.7% in the highest lysed cell treatment) than in the control, low, and moderate whole-cell treatments ($p < 0.001$).

3.2.2. *Crassostrea virginica*

Six hours after exposure, the treatment with the highest whole cell concentration contained significantly fewer normal embryos than all other treatments and the control ($p < 0.001$; Table 2). The same high whole cell treatment also contained significantly more abnormal embryos than found in any of the lysed cell treatments ($p < 0.001$; Table 2) and higher mortalities compared to the control, low, and moderate whole cell treatments ($p < 0.001$).

After 24 h-exposure, all lysed treatments and the treatment at the highest whole-cell concentration contained significantly fewer normal larvae than found in the control. The lysed treatment at the highest concentration contained more abnormal larvae than the control and treatment with moderate cell concentrations ($p < 0.001$), and the treatments at the highest cell concentration and lysed (1500 cells mL⁻¹) resulted in higher mortalities than found in the control ($p < 0.001$). The highest lysed-cell treatment (3000 cells mL⁻¹) showed the lowest percentage of normal larvae (77%) and the highest percentage of abnormal (14.7%) and dead (8.3%) larvae (Table 2).

The mean larval mortalities for all *K. brevis* treatments increased significantly between 24 and 96 h while the percentage of abnormalities remained in the same range (2.5 to 14.7% at 24 h and 2.2 to 14.6% at 96 h). After 96 h of exposure, all treatments contained significantly fewer normal larvae than the control. The highest lysed-cell treatment showed a pronounced and significant increase in percent abnormalities compared to all other treatments and the control ($p < 0.001$). Mortalities were highest in the moderate and high cell treatments, reaching 22.3% in the lysed 3000 cells mL⁻¹ treatment (Fig. 2, Table 2).

3.3. Larval shell length

3.3.1. *Mercenaria mercenaria*

Exposure to *K. brevis* significantly reduced mean larval shell lengths and differences in size among treatments were more pronounced at 96 h than at 24 h (Fig. 3, Table 3). Linear regressions suggest an overall dose-dependent response (Table 3) particularly evident at 96 h in treatments of clams exposed to both whole and lysed cells ($R^2 = 0.960$ and 0.957, respectively).

After 24 h, embryos exposed to the moderate-lysed-cell and high-cell concentrations attained a significantly smaller larval size than in the control ($p < 0.001$). By 96 h, embryos exposed to the highest lysed cell treatment reached a significantly smaller larval size than those exposed to the low cell concentrations and the control ($p < 0.001$). At 96 h, the smallest larvae were found in the treatment with the highest lysed cell concentration (116 μm vs. 134 μm in the control).

3.3.2. *Crassostrea virginica*

Generally, the effects of exposure of embryos to *K. brevis* on larval *C. virginica* were not as pronounced as for larval *M. mercenaria*.

Table 3
Linear regression equations and R^2 values for mean larval size data of *Mercenaria mercenaria* and *Crassostrea virginica* exposed to whole and lysed treatments of *Karenia brevis* at 24 and 96 h, $n = 3$.

		Sampling time point	Regression equation	R^2 value
<i>Mercenaria mercenaria</i>	Whole cells	24	$-0.001x + 112.41$	0.808
		96	$-0.0035x + 134.06$	0.96
	Lysed cells	24	$-0.0013x + 111.36$	0.85
		96	$-0.0063x + 135.44$	0.957
<i>Crassostrea virginica</i>	Whole cells	24	$-0.0009x + 69.28$	0.751
		96	$-0.0012x + 73.537$	0.503
	Lysed cells	24	$-0.0012x + 69.028$	0.958
		96	$-0.001x + 73.265$	0.312

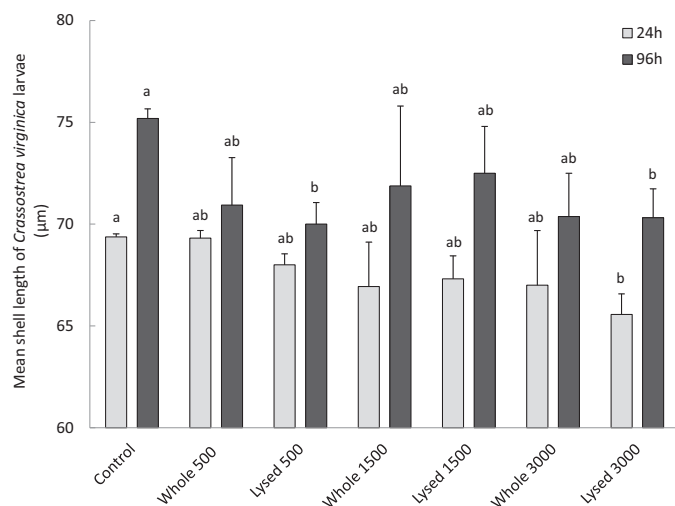


Fig. 4. Comparison of larval shell length of *Crassostrea virginica* after 24 h and 96 h of exposure to whole and lysed preparations of *Karenia brevis* at 500, 1500 and 3000 cells mL^{-1} . Treatments with the same letter were not significantly different ($p > 0.05$). Data are presented as mean \pm standard deviation.

The larvae exposed to the highest lysed cell treatment were significantly smaller than those in the control at 24 h ($p < 0.04$). At 96 h, larvae were significantly smaller in the treatments containing lysed 500 and 3000 cells mL^{-1} compared to the control ($p < 0.04$, Fig. 4). The larvae in controls were the largest (mean 75.2 μm) and those exposed to the lysed-500 cells mL^{-1} treatment were the smallest (70 μm), although they did not differ significantly in size from those occurring at the moderate- and high-lysed treatments.

4. Discussion

Results presented here clearly demonstrate significant negative effects of short-term exposure of *C. virginica* and *M. mercenaria* embryos to the toxic dinoflagellate *K. brevis* at relatively low concentrations (≤ 3000 cells mL^{-1}).

The dose-dependent effects on larval mortality shown here are in agreement with results of Leverone et al. (2006) who found that survival of 3- and 7-day-old *C. virginica*, *M. mercenaria* and *A. irradians* larvae exposed for 3 and 7 days to *K. brevis* concentrations of up to 5000 cells mL^{-1} were dose-dependent. In the present study, exposure to *Karenia* cells began at the embryonic stage, when larvae are particularly vulnerable to environmental and biotic interferences. Abnormalities and larval size were determined in addition to mortalities throughout exposure. This revealed that exposure to *K. brevis* caused sub-lethal, as well as lethal effects following a very short exposure time (6 h). Dose-dependent, sub-lethal and lethal effects were shown by Basti et al. (2011) when exposing trocophore and D-larvae of the pearl oyster, *Pinctada fucata martensii*, to the thecated dinoflagellate *H. circularisquama* for 72 h. For this

algal species, cytotoxicity was attributed to direct contact of the toxic algae with the soft tissues of the larvae, and effects on activity, development, prevalence of abnormalities, and survival were directly related to the concentration of toxic algae.

It is unclear exactly how brevetoxins affect the early life cycle stages of bivalves, or if other uncharacterized extracellular metabolites and allelopathic compounds contribute to the effects observed. In the present study, both bivalve species were exposed from a pre-feeding through a first-feeding stage (D-stage, veliger larva). Larvae do not develop the ability to feed until 24–36 h after fertilization (Carriker, 2001; Galtsoff, 1964; Kraeuter et al., 1981; Newell and Langdon, 1996) and thereafter, *K. brevis* cells ($\sim 30 \mu\text{m}$ ESD) are too large to be ingested until larvae are at least $>200 \mu\text{m}$ in length (Baldwin and Newell, 1995). Neither bivalve species in this study attained this size by 96 h post-fertilization. It is possible however, that higher densities of whole or lysed *K. brevis* cells could disturb larval feeding on the smaller, non-toxic algae T-Iso and *C. muelleri*. Although not measured quantitatively in the present study, larvae without food in their gut were observed at the highest concentrations of whole and lysed *K. brevis*. Leverone et al. (2006) also suggested that interference by *K. brevis* negatively affected feeding of 7 day-old larvae on non-toxic, smaller algae.

The ratio of intracellular to extracellular brevetoxins changes depending on the algal growth stage of *K. brevis*, with older cultures having a higher proportion of extracellular PbTx (Pierce et al., 2001; Pierce and Henry, 2008). Thus, lysis of *Karenia* cells is expected to increase the release of PbTx to the surrounding water (Leverone et al., 2006). The cultures used in the present study were grown under standardized conditions, and consistently harvested in early stationary phase (at ~ 14 days); thus, we assume that the ratio of intracellular to extracellular toxins of whole cells was consistent for all exposures. Although the impact of lysed cells was hypothesized to be greater than that of whole cells, differences between whole and lysed cell treatments remained modest and were not always consistent. This suggests that physical contact with the live algal cells is not needed to elicit deleterious effects. Thus, observed effects on both clam and oyster larvae in this study are likely related to exposure to exogenous PbTx and/or to other chemically uncharacterized compounds that can be allelopathic, ichthyotoxic and/or hemolytic (Kubanek et al., 2005, 2007; Marshall et al., 2005; Neely and Campbell, 2006). These compounds, possibly together with PbTx, may induce sublethal or lethal effects during early larval development upon exposure to blooms of *K. brevis* (whole or lysed) and act as a feeding deterrent on other nutritious algae at the time of first feeding.

The response of the larvae based on the duration of experimental exposure and/or their developmental stage are difficult to separate because early non-feeding embryonic stages progressed to first feeding D-stage larvae during the course of the experiment. In the present study, concentrations of *K. brevis* significantly affected survival following only 6 h of exposure and there was a significant increase in deleterious effects with increasing duration of exposure, from 6 h to 96 h. Similarly, when challenging trocophore

and D-larvae of *P. fucata martensii* with *H. circularisquama*, Basti et al. (2011) found that sub-lethal effects increased with increasing duration of exposure. The swimming activity of D-larvae was inhibited at higher concentrations ($>10^3$ cells mL⁻¹) after 3 h and at progressively lower algal concentrations at 48 and 72 h (500 and 100 cells mL⁻¹, respectively). Little is known about the stability of PbTx and/or other allelopathic or toxic compounds in the field. In two separate experiments conducted under similar conditions to those of the present study, *C. virginica* larvae were exposed to treatments of *K. brevis* for 4 days. Analysis of the seawater that had contained the oyster larvae (before being passed through a 20 µm filter), revealed that total PbTx concentrations in µg L⁻¹ remained present after 4 days of exposure although at a lower concentration (40–53% and 65–83% of initial concentration after 2 and 4 days, respectively, unpublished data). We therefore assume that larvae in the present study were continuously exposed to declining PbTx concentrations over time. This decline may be caused by bacterial degradation and/or toxin conversion. Roth et al. (2007) exposed *K. brevis* to algicidal bacteria and confirmed lysis of *K. brevis* cells and a rapid reduction in the proportion of the more potent PbTx congeners. Future tracking of the PbTx content and composition as well as of the bacterial communities throughout exposure would help to elucidate these effects. As larvae start to feed between 24 and 36 h, their feeding activity would likely increase their exposure to toxins either by ingesting them via incorporation in non-toxic algae, or by accumulation from contaminated water. More larvae without food in their gut were qualitatively observed at higher concentrations of whole and lysed *K. brevis* which suggests that sub-lethal and lethal effects at 96 h result from a combination of these effects.

The possibility that our results also reflect some stage-dependent sensitivity cannot be excluded. Embryos and actively feeding larvae may exhibit different responses to toxic compounds. On one hand, embryogenesis is an especially sensitive stage; on the other hand, as indicated above, feeding activity of veliger larvae may enhance or increase their exposure to toxic compounds. Other bivalve species show differential susceptibility to toxic algae during development. Bricelj et al. (2010) demonstrated that, a highly toxic *Alexandrium* sp., producer of paralytic shellfish poisoning (PSP) toxins, elicited no adverse effects on 1 week-old softshell clams, *Mya arenaria*, but significantly reduced growth and survival rates of post-metamorphic stages. This was attributed to the fact that *Alexandrium* spp. produce endogenous toxins and at ~35 µm in cell diameter, are too large to be ingested during early veliger stages. Wang et al. (2006) challenged different larval stages of *A. irradians*, with *H. akashiwo* and found that embryos and eye-spot larvae were more sensitive than veliger larvae and juveniles. Swimming activity of veligers, developmental rate of embryos, and survival of veligers and eye-spot larvae, were all negatively impacted by toxic algal exposure. Although the toxicological mechanism of *H. akashiwo* remains unidentified, algal cells were observed attached to the single-cell membrane of trochophore larvae, suggesting that algae may have released endogenous toxic substances (e.g., ROS) by direct contact, making exposed embryos and the exposed gills of eye spot larvae vulnerable. Veliger larvae have a larval shell that can protect their gill epithelia from direct contact with the harmful algae.

Responses of bivalves exposed to thecate dinoflagellates of the *Alexandrium tamarense/fundyense* complex, producers of PSP toxins, are known to be highly species-specific (e.g., Bricelj and Shumway, 1998). In the present study, both species were sensitive to exposure to *K. brevis* but, there was no clear difference between the responses of *C. virginica* and *M. mercenaria*. Percent abnormality and mortality results suggest that the former were more sensitive, whereas analysis of larval shell size and growth responses indicate that clam larvae were more sensitive to exposure to *K. brevis*. Results of this study thus do not conclusively demonstrate

species-specific differences in susceptibility to *K. brevis*. This is consistent with results of Leverone et al. (2006), who found that survival of 3-day-old larvae of *M. mercenaria*, *C. virginica* and *A. irradians* exposed to *K. brevis* for 3-days was similar and significantly lower at 1000 cells mL⁻¹ concentration compared to the control for all 3 species.

Blooms of *K. brevis* may last many months, co-occurring with both oyster and hard clam spawning and early larval development, so it is likely that the entire period of pelagic early ontogeny is affected and this may have serious consequences for recruitment success of both natural and cultured populations of these two ecologically and economically important bivalve species. Larvae may also be stressed by other synergistic factors, including low DO (Brand et al., 2012). The present study, in which high levels of DO were maintained and exposure lasted only 4 days may underestimate deleterious effects compared to those encountered in the field. The impacts and implications of these chronic exposures should be taken into account when establishing aquaculture sites, hatcheries, or restoration efforts.

Acknowledgments

We thank Richard Pierce and the team at the Mote Marine Laboratory, FL, for providing cultures of the Manasota Key strain of *K. brevis* and for LC-MS/MS toxin analysis of cultures. We also thank Fu-Lin Chu for critical review of an earlier version of the manuscript and Andrew Griffith, Maud Baudequin and the staff from FGCU for their help initiating the culture of *K. brevis* and microscopic analysis of clam and oyster larvae. This collaborative work was partially supported by Université de Bretagne Occidentale, and by “Laboratoire d’Excellence” LabexMER (ANR-10-LABX-19); Outgoing Ph. D. Fellowship, co-funded by a grant from the French government under the program “Investissements d’Avenir”.

Appendix A. Supplementary data

Supplementary, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2014.06.023>.

References

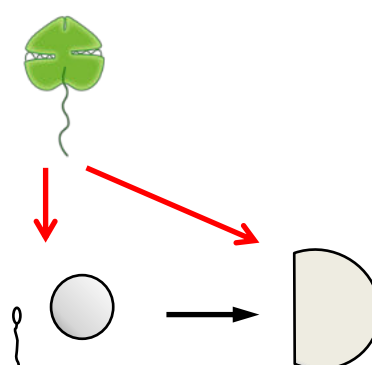
- Arnold, W.S., 2001. Bivalve enhancement and restoration strategies in Florida, U.S.A. *Hydrobiologia* 465, 7–19.
- Baden, D.G., Bourdelais, A.J., Jacocks, H., Michelliza, S., Naar, J., 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ. Health Perspect.* 113 (5), 621–625.
- Baden, D.G., Thomas, C.R., 1989. Variations in major toxin composition for six clones of *Ptychodiscus brevis*. In: Okaichi, T., Anderson, D.M., Nemoto, T. (Eds.), *Red Tides. Biology, Environmental Science and Toxicology*. Elsevier, Amsterdam, pp. 415–418.
- Baldwin, B.S., Newell, R.I.E., 1995. Relative importance of different size food particles in the natural diet of oyster larvae *Crassostrea virginica*. *Mar. Ecol. Prog. Ser.* 120 (1), 135–145.
- Basti, L., Go, J., Higuchi, K., Nagai, K., Segawa, S., 2011. Effects of the toxic dinoflagellate *Heterocapsa circularisquama* on larvae of the pearl oyster *Pinctada fucata martensii* (Dunker, 1873). *J. Shellfish Res.* 30 (1), 177–186.
- Basti, L., Nagai, K., Tanaka, Y., Segawa, S., 2013. Sensitivity of gametes, fertilization, and embryo development of the Japanese pearl oyster, *Pinctada fucata martensii*, to the harmful dinoflagellate, *Heterocapsa circularisquama*. *Mar. Biol.* 160 (1), 211–219.
- Brand, L.E., Compton, A., 2007. Long-term increase in *Karenia brevis* abundance along the Southwest Florida Coast. *Harmful Algae* 6 (2), 232–252.
- Brand, L.E., Campbell, L., Bresnan, E., 2012. *Karenia*: the biology and ecology of a toxic genus. *Harmful Algae* 14, 156–178.
- Bricelj, V.M., Shumway, S.E., 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics and biotransformation. *Rev. Fish. Sci.* 6 (4), 315–383.
- Bricelj, V.M., MacQuarrie, S.P., Doane, J.A.E., Connell, L.B., 2010. Evidence of selection for resistance to paralytic shellfish toxins during the early life history of soft-shell clam (*Mya arenaria*) populations. *Limnol. Oceanogr.* 55 (6), 2463–2475.
- Burkholder, J.M., Shumway, S.E., 2012. Bivalve shellfish aquaculture and eutrophication. In: Shumway, S.E. (Ed.), *Shellfish Aquaculture and the Environment*. Wiley and Sons Inc., Hoboken, NJ, pp. 155–215.

- Calabrese, A., Collier, R.S., Nelson, D.A., MacInnes, J.R., 1973. The toxicity of heavy metals to embryos of the American oyster *Crassostrea virginica*. *Mar. Biol.* 18 (3), 162–166.
- Carriker, M.R., 2001. Embryogenesis and organogenesis of veligers and early juveniles. *Dev. Aquac. Fish. Sci.* 31, 77–115.
- Conte, F., 1984. Economic impact of paralytic shellfish poison on the oyster industry in the Pacific United States. *Aquaculture* 39, 331–343.
- Deeds, J.R., Terlizzi, D.E., Adolf, J.E., Stoecker, D.K., Place, A.R., 2002. Toxic activity from cultures of *Karlodinium micrum* (= *Gyrodinium galatheanum*) (Dinophyceae) a dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. *Harmful Algae* 1 (2), 169–189.
- Finucane, J.H., 1964. Distribution and seasonal occurrence on *Gymnodinium breve* on the West Coast of Florida, 1954–57. In: United States Fish and Wildlife Service Special Scientific Report. Fisheries No. 487.
- Galtsoff, P.S., 1964. The American oyster, *Crassostrea virginica* Gmelin. *U.S. Fish. Bull.* 64, 1–480.
- Glibert, P.M., Alexander, J., Meritt, D.W., North, E.W., Stoecker, D.K., 2007. Harmful algae pose additional challenges for oyster restoration: impacts of the harmful algae *Karlodinium veneficum* and *Prorocentrum minimum* on early life stages of the oysters *Crassostrea virginica* and *Crassostrea ariakensis*. *J. Shellfish Res.* 26 (4), 919–925.
- Gunter, G., Williams, R.H., Davis, C.C., Smith, F.W., 1948. Catastrophic mass mortality of marine animals and coincident phytoplankton bloom on the west coast of Florida, November 1946 to August 1947. *Ecol. Monogr.* 18, 310–324.
- Hesselman, D.M., Barber, B.J., Blake, N.J., 1989. The reproductive cycle of adult hard clams, *Mercenaria* spp. in the Indian River Lagoon, Florida. *J. Shellfish Res.* 8, 43–49.
- Hoagland, P., Scatista, S., 2006. The economic effects of harmful algal blooms. In: Graneli, E., Turner, J.T. (Eds.), *Ecology of Harmful Algae*. Springer-Verlag, Berlin, pp. 391–401.
- Hu, C., Muller-Karger, F.E., Swarzenski, P.W., 2006. Hurricanes, submarine ground-water discharge, and Florida's red tides. *Geophys. Res. Lett.* 33, L11601.
- Jensen, A.C., 1975. The economic halo of a red tide. In: LoCicero, V.R. (Ed.), *Proceedings of the First International Conference on Toxic Dinoflagellate Blooms*. Wakefield, MA. MIT Science and Technology Foundation, pp. 507–516.
- Kraeuter, J.N., Castagna, M., Van Dessel, R., 1981. Egg size and larval survival of *Mercenaria mercenaria* and *Argopecten irradians* (Lamarck). *J. Exp. Mar. Biol. Ecol.* 56 (1), 3–8.
- Kubaneck, J., Hicks, M.K., Naar, J., Villareal, T.A., 2005. Does the red tide dinoflagellate *Karenia brevis* use allelopathy to outcompete other phytoplankton? *Limnol. Oceanogr.* 50 (3), 883–895.
- Kubaneck, J., Snell, T.W., Pirkle, C., 2007. Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing. *Limnol. Oceanogr.* 52 (3), 1026–1035.
- Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. *Rev. Fish. Sci.* 10 (2), 113–390.
- Leverone, J.R., Blake, N.J., Pierce, R.H., Shumway, S.E., 2006. Effects of the dinoflagellate *Karenia brevis* on larval development in three species of bivalve mollusc from Florida. *Toxicol.* 48, 75–84.
- Leverone, J.R., Geiger, S.P., Stephenson, S.P., Arnold, W.S., 2010. Increase in Bay scallop (*Argopecten irradians*) populations following releases of competent larvae in two west Florida estuaries. *J. Shellfish Res.* 29 (2), 395–406.
- Marshall, J., Ross, T., Pyecroft, S., Hallegraef, G., 2005. Superoxide production by marine microalgae 2: Towards understanding ecological consequences and possible functions. *Mar. Biol.* 147, 541–549.
- National Oceanic and Atmospheric Administration (NOAA), 2004. Action Plan for Harmful Algal Blooms and the Gulf of Mexico Coastal Ocean Observing System: Results from a Regional Workshop. NOAA Coastal Services Center. NOAA/CSC/20516-PUB. 27. NOAA.
- Neely, T., Campbell, L., 2006. A modified assay to determine hemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae* 5, 592–598.
- Newell, R.I.E., 2004. Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. *J. Shellfish Res.* 23 (1), 51–62.
- Newell, R.I.E., Langdon, C.J., 1996. Mechanisms and physiology of adult and larval feeding. In: Kennedy, V.S., Newell, R.I.E., Able, A. (Eds.), *The eastern oyster *Crassostrea virginica**. Maryland Sea Grant College, College Park, MD, pp. 185–229.
- Pechenik, J.A., 1987. Environmental influences on larval survival and development. In: Giese, A.C., Pearse, J.S., Pearse, V.B. (Eds.), *Reproduction of Marine Invertebrates*. Blackwell Scientific Publications, Palo Alto, CA, pp. 551–608.
- Pierce, R.H., Henry, M., 2008. Harmful algal toxins of the Florida red tide (*Karenia brevis*): natural chemical stressors in South Florida coastal ecosystems. *Ecotoxicology* 17 (7), 623–631.
- Pierce, R.H., Henry, M.S., Blum, P.C., Hamel, S.L., Kirkpatrick, B., Cheng, Y.S., Zhou, Y., Irvin, C.M., Naar, J., Weidner, A., Fleming, L.E., Backer, L.C., Baden, D.G., 2005. Brevetoxin composition in water and marine aerosol along a Florida beach: assessing potential human exposure to marine biotoxins. *Harmful Algae* 4, 965–972.
- Pierce, R.H., Henry, M., Blum, P., Payne, S., 2001. *Gymnodinium breve* toxins without cells: intra-cellular and extra-cellular toxins. In: Hallegraef, G., Blackburn, S., Bolch, C., Lewis, R. (Eds.), *Harmful Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO, pp. 421–424.
- Poli, M.A., Mende, T.J., Baden, D.G., 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol. Pharmacol.* 30 (2), 129–135.
- Prince, E.K., Poulson, K.L., Myers, T.L., Sieg, R.D., Kubaneck, J., 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. *Harmful Algae* 10, 39–48.
- Rounsefell, G.A., Nelson, W.R., 1966. Red-tide research summarized to 1964 including an annotated bibliography. In: United States Department of the interior Fish and Wildlife Service. Contribution number 20. Bureau of Commercial Fisheries Special Scientific Report. Fisheries number 535. Washington, DC, pp. 1–85.
- Roth, P.B., Twiner, M.J., Wang, Z., Dechraoui, M.B., Doucette, G.J., 2007. Fate and distribution of brevetoxin (PbTx) following lysis of *Karenia brevis* by algicidal bacteria, including analysis of open A-ring derivatives. *Toxicol.* 50, 1175–1191.
- Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquac. Soc.* 21 (2), 65–104.
- Shumway, S.E., Cucci, T.L., 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. *Aquat. Toxicol.* 10 (1), 9–27.
- Smayda, T.J., 1990. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton*. Elsevier, New York, NY, pp. 29–40.
- Steidinger, K.A., 2009. Historical perspective on *Karenia brevis* red tide research in the Gulf of Mexico. *Harmful Algae* 8 (4), 549–561.
- Stoecker, D.K., Adolf, J.E., Place, A.R., Glibert, P.M., Meritt, D.W., 2008. Effects of the dinoflagellates *Karlodinium veneficum* and *Prorocentrum minimum* on early life history stages of the eastern oyster (*Crassostrea virginica*). *Mar. Biol.* 154 (1), 81–90.
- Strathmann, R.R., 1985. Feeding and non-feeding larval development and life-history evolution in marine invertebrates. *Annu. Rev. Ecol. Syst.* 16, 339–361.
- Summerson, H., Peterson, C., 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Ptychodiscus brevis*, outbreak recorded in North Carolina. *Estuaries* 13 (3), 322–331.
- Tester, P.A., Steidinger, K.A., 1997. *Gymnodinium breve* red tide blooms: Initiation, transport, and consequences of surface circulation. *Limnol. Oceanogr.* 42 (5), 1039–1051.
- Thompson, R.J., Newell, R.I.E., Kennedy, V.S., Mann, R., 1996. Reproductive processes and early development. In: Kennedy, V.S., Newell, R.I.E., Eble, A.F. (Eds.), *The Eastern Oyster: *Crassostrea virginica**. Maryland Sea Grant College, College Park, MD, pp. 335–370.
- United States Department of Agriculture, 2007. *Census of Agriculture: United States Summary and State Data*. Geographic Area Series. Part 51 AC-07-A-51, vol 1. United States Department of Agriculture, Washington, D.C., pp. 438–439.
- Van Dolah, F.M., 2000. Marine algal toxins: origins, health effects, and their increased occurrence. *Environ. Health Perspect.* 108 (1), 133–141.
- Van Dolah, F.M., Lidie, K.B., Monroe, E.A., Bhattacharya, D., Campbell, L., Doucette, G.J., Kamykowski, D., 2009. The Florida red tide dinoflagellate *Karenia brevis*: new insights into cellular and molecular processes underlying bloom dynamics. *Harmful Algae* 8 (4), 562–572.
- Vargo, G.A., Heil, C.A., Ault, D.N., Neely, M.B., Murasko, S., Havens, J., Lester, K.M., Dixon, L.K., Merkt, R., Walsh, J., Weisberg, R., Steidinger, K.A., 2004. Four *Karenia brevis* blooms: a comparative analysis. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), *Harmful Algae 2002: Proceedings of the Xth International Conference on Harmful Algae*. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and the Intergovernmental Oceanographic Commission of UNESCO. St Petersburg, FL, pp. 14–16.
- Volety, A.K., 2008. Effects of salinity, heavy metals and pesticides on health and physiology of oysters in the Caloosahatchee Estuary, Florida. *Ecotoxicology* 17, 579–590.
- Volety, A.K., Savarese, M., Tolley, S.G., Arnold, W.S., Sime, P., Goodman, P., Chamberlain, R.H., Doering, P.H., 2009. Eastern oysters (*Crassostrea virginica*) as an indicator for restoration of everglades ecosystems. *Ecol. Indic.* 9 (6), 120–136.
- Wang, L., Yan, T., Zhou, M., 2006. Impacts of HAB species *Heterosigma akashiwo* on early development of the scallop *Argopecten irradians* Lamarck. *Aquaculture* 255 (1–4), 374–383.
- Wells, H.W., 1961. The fauna of oyster beds, with special reference to the salinity factor. *Ecol. Monogr.* 31 (3), 239–266.
- Wikfors, G.H., Smolowitz, R.M., 1995. Experimental and histological studies of four life-history stages of the eastern oyster, *Crassostrea virginica*, exposed to a cultured strain of the dinoflagellate *Prorocentrum minimum*. *Biol. Bull.* 188, 313–328.
- Yan, T., Zhou, M., Fu, M., Yu, R., Wang, Y., Li, J., 2003. Effects of the dinoflagellate *Alexandrium tamarensis* on early development of the scallop *Argopecten irradians concentricus*. *Aquaculture* 217 (1), 167–178.
- Yan, T., Zhou, M., Fu, M., Wang, Y., Yu, R., Li, J., 2001. Inhibition of egg hatching success and larvae survival of the scallop, *Chlamysfareri*, associated with exposure to cells and cell fragments of the dinoflagellate *Alexandrium tamarensis*. *Toxicol.* 39 (8), 1239–1244.

Chapter 5

Susceptibility of gametes and embryos of the eastern oyster, *Crassostrea virginica*, to *Karenia brevis* and its toxins.

The modest and not-always significant differences in the impacts of whole-cell and cell-lysate of *K. brevis* on early stages alluded to the involvement of other bioactive compounds. This was further studied in the following chapter. Gametes and early embryonic stages of *C. virginica* were exposed to whole-cell and culture filtrate of *K. brevis*. This was followed by a 'recovery' period, during which the development of larvae was monitored.



Hypotheses tested were:

- Brevetoxin is the cause of toxic effects on early life stages during *K. brevis* exposure.
- Sensitivity to *K. brevis* exposure is not life cycle stage dependent.
- Sperm and oocytes do not differ in their sensitivity to *K. brevis* exposure.
- Deleterious effects on early life stages are not long lasting after an exposure has finished.



Contents lists available at ScienceDirect

Toxicol

journal homepage: www.elsevier.com/locate/toxicol

Susceptibility of gametes and embryos of the eastern oyster, *Crassostrea virginica*, to *Karenia brevis* and its toxins



Anne Rolton^{a, b, *}, Philippe Soudant^b, Julien Vignier^{a, b}, Richard Pierce^c, Michael Henry^c, Sandra E. Shumway^d, V. Monica Bricelj^e, Aswani K. Volety^{a, 1}

^a Florida Gulf Coast University, College of Arts and Sciences, 10501 FGCU Blvd South, Fort Myers, FL 33965, USA

^b LEMAR CNRS UMR 6539, Institut Universitaire Européen de la Mer – Université de Bretagne Occidentale, Place Nicolas Copernic, Technopôle Brest Iroise, 29280 Plouzané, France

^c Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, FL 34236, USA

^d Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340, USA

^e Department of Marine and Coastal Sciences and Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA

ARTICLE INFO

Article history:

Received 26 November 2014

Received in revised form

2 March 2015

Accepted 4 March 2015

Available online 12 March 2015

Keywords:

Crassostrea virginica

Karenia brevis

Brevetoxin

Gametes

Development

ABSTRACT

The bivalve mollusc, *Crassostrea virginica*, is frequently exposed to blooms of *Karenia brevis* along the west coast of Florida during periods of spawning and early larval development. A continuous 4-day exposure of gametes and 2–4 cell stage embryos of *C. virginica* to whole-cell and culture filtrate of *K. brevis* at 500 and 5000 cells mL⁻¹, was followed by a 4-day 'recovery' period. Larval growth, percent of normal, abnormal and dead larvae, and the presence of food in the larval gut were measured throughout the exposure period. Results suggest that negative effects mainly occur during embryogenesis and early development. Damage to feeding apparatus/gut may occur during embryonic development or exposure to toxins may act as a feeding deterrent on non-toxic algae. Following 2-h *in vitro* exposure of gametes, differences in oocyte and sperm cell parameters were investigated using flow cytometry. The reduced sperm viability in the whole-cell 5000 cells mL⁻¹ treatment suggests the involvement of extracellular brevetoxins (PbTx) and perhaps other harmful, uncharacterized compounds associated with the *K. brevis* cell membrane. The cumulative effects of reduced sperm viability, fertilization success, embryonic and larval survival, and the near-annual exposure to blooms of *K. brevis* could cause significant bottlenecks on oyster recruitment.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Harmful algal blooms (HAB) affect co-occurring organisms by the production of toxins, physical contact, and/or increased biomass (Anderson et al., 2002; Landsberg, 2002). This can lead to mass mortalities of fish and shellfish, including marine bivalve molluscs, which can also act as vectors of toxins to humans (Gunter et al., 1948; Landsberg, 2002; Shumway, 1990). The effects of HAB on the early life history stages of many bivalve species are largely unknown; however, recent studies suggest that some HAB can

* Corresponding author. LEMAR CNRS UMR 6539, Institut Universitaire Européen de la Mer – Université de Bretagne Occidentale, Place Nicolas Copernic, Technopôle Brest Iroise, 29280 Plouzané, France.

E-mail address: annerolton@gmail.com (A. Rolton).

¹ Present address: University of North Carolina Wilmington, 601 S. College Rd, Wilmington, NC 28403, USA.

affect fertilization, growth, development, and survivorship of bivalve larvae (Basti et al., 2011, 2013, 2015; Bricelj and MacQuarrie, 2007; Glibert et al., 2007; Leverone et al., 2006; Padilla et al., 2006; Rolton et al., 2014).

The toxic, un-theated dinoflagellate, *Karenia brevis*, is the primary HAB-forming species in the Gulf of Mexico (Brand et al., 2012; Geesey and Tester, 1993; Tester and Steidinger, 1997) and produces between 9 and 14 intracellular lipophilic polyether brevetoxins (PbTx), that are released during cell lysis (Pierce et al., 2001). Once released from *K. brevis* cells, PbTx bind to voltage-gated sodium channels of cell membranes causing permanent excitation of the cell and interference with nerve transmission (Baden et al., 2005; Huang et al., 1984; Poli et al., 1986). Other characterized and uncharacterized bioactive compounds that can be allelopathic, ichthyotoxic, and hemolytic, can also be produced by *K. brevis* and together with PbTx, may contribute to the overall toxicity of blooms (Kubaneck et al., 2005, 2007; Leblond et al., 2003; Mooney et al.,

2007; Neely and Campbell, 2006; Prince et al., 2010; Sipler et al., 2014).

Blooms of *K. brevis* occur on a near-annual basis along the coast of southwest Florida (SW FL) (Heil et al., 2014; Stumpf et al., 2003). They initiate offshore and are advected to near-shore areas and estuaries where the cells can concentrate (Brand and Compton, 2007; Steidinger, 2009; Tester and Steidinger, 1997). Peak cell densities of 1.5×10^5 cells mL⁻¹ were recorded around Estero Bay and the Caloosahatchee estuary during the 2012–2013 *K. brevis* bloom in SW FL, although cell densities $>10^4$ cells mL⁻¹ were more commonly reached in this area (Florida Fish and Wildlife Conservation Commission, FWC, 2013, data not shown). Oysters found within shallow estuaries along the SW FL coast provide important ecosystem services (*sensu* Burkholder and Shumway, 2012; Newell, 2004; Wells, 1961) and populations may be at risk from exposure to blooms of *K. brevis*. The spawning period of *Crassostrea virginica* in SW FL often overlaps with blooms of *K. brevis* (Brand and Compton, 2007; Volety, 2008); thus, gametes and larvae may come into direct contact with *K. brevis* cells and associated toxins. Gametes and early larval stages are critical phases in the life history of marine bivalves and are highly vulnerable to abiotic and biotic interferences such as HAB (Gosselin and Qian, 1997; Pechenik, 1987; Thompson et al., 1996; Thorson, 1950). Exposure of these early life stages to *K. brevis* could pose a significant bottleneck to recruitment of natural and cultured bivalve populations (Padilla et al., 2006; Schneider et al., 2003; Summerson and Peterson, 1990).

Previous exposures of early stage embryos of *C. virginica* and the northern quahog (hard clam), *Mercenaria mercenaria*, to whole-cell and lysed preparations of *K. brevis* (≤ 3000 cells mL⁻¹) were found to significantly reduce larval size, and increase the prevalence of larval abnormalities and mortalities following only a few hours of exposure (Rolton et al., 2014). This prior study did not consider the effects of direct exposure of the gametes which may result in additional negative effects on larval survival, as has been shown with other algal and bivalve species (Basti et al., 2013; Granmo et al., 1988). The former showed that exposure of the Japanese pearl oyster, *Pinctada fucata martensii*, to whole cells of the toxic dinoflagellate, *Heterocapsa circularisquama*, resulted in significant detrimental effects on the viability of the oocytes and sperm as well as fertilization rate within minutes of exposure. Deleterious effects on fertilization and embryonic development of blue mussels, *Mytilus edulis*, also occurred when gametes were exposed to the haptophyte, *Chrysochromulina polylepis* (Granmo et al., 1988).

An important question raised by Rolton et al.'s (2014) study, was whether other harmful, chemically uncharacterised compounds were also associated with the observed negative effects on bivalve larvae. In that study, the impact of lysed cells was hypothesized to be greater than that of whole cells due to the increased release of PbTx from *K. brevis* cells; however, differences between whole- and lysed-cell treatments were modest and not always consistent. This suggested that exposure of bivalve larvae to other harmful bioactives together with extracellular PbTx, may have induced negative effects during early larval development. Observations also suggested that exposure to higher densities of whole and lysed *K. brevis* cells caused feeding disturbance of bivalve larvae, although this effect was not quantified (Rolton et al., 2014).

It is not known if exposure of early life stages of bivalves to *K. brevis* has long-term effects on their development; however, previous studies have shown that exposure to toxic algae may result in long-term negative effects. Bricelj and MacQuarrie (2007) exposed 1-day-old *M. mercenaria* larvae to 800 cells μL^{-1} of the brown tide alga, *Aureococcus anophagefferens*, for 15 days and found that previously exposed larvae showed no growth during a 12 day-recovery period. It was suggested that larval feeding inhibition may have resulted from contact with bioactive compounds produced by

A. anophagefferens following ingestion (Bricelj and MacQuarrie, 2007).

The present study was designed to help identify when the harmful effects of exposing early life-cycle stages of *C. virginica* to *K. brevis* occur, and to determine if other unknown toxic compounds as well as PbTx are associated with the negative effects observed. The effects of 4-day exposure to *K. brevis* (whole-cell and culture filtrate) followed by a 4-day 'recovery' period on gamete cellular parameters, fertilization success, embryonic and subsequent larval development of *C. virginica* were examined. One experiment started from pre-fertilization (exposure of gametes) and the other from post-fertilization (exposure of 2–4 cell stage embryos). Differences in gamete and embryo susceptibility, responses to different preparations of *K. brevis* cell treatments, as well as any prolonged effects after exposure has ended, may help to identify the harmful mechanisms of this alga against early stages of bivalves.

2. Methods

2.1. Broodstock

Broodstock oysters, *C. virginica* [collected from Estero Bay, Bonita Springs, Florida (26.3484, -81.8421) in September 2013] were acclimated for 7 days in 400 L tanks at the Vester Marine Field Station, FGCU. Oysters were held at a salinity of 25, temperature of 23 °C (± 1) and were fed a mixed diet of *Tisochrysis lutea* sp. nov (T-Iso; CCMP 1324), *Chaetoceros muelleri* (CCMP 1318), *Tetraselmis chui* (CCMP 882) *ad libitum*. Algal stock cultures were obtained from the Provasoli-Guillard National Center for Marine Microalgae and Microbiota (NCMA, previously the CCMP), Bigelow, Maine. Prior to spawning and experimental exposure, 5 oysters were randomly selected and sacrificed to verify ripeness.

2.2. Algal cultures and preparation

The flagellates *Tisochrysis lutea* sp. nov (T-Iso) and *Tetraselmis chui* were grown at a salinity of 30 in CELL-HI F2P based on Guillard F/2 medium (Varicon Aqua). The diatom *C. muelleri* was grown in CELL-HI F2P based on Guillard F/2 medium and Si at a salinity of 24.

The toxic dinoflagellate, *Karenia brevis* (Manasota Key strain) was obtained from the Mote Marine Laboratory, Sarasota, FL. Cultures were grown and maintained according to Rolton et al. (2014). Whole-cell (representing extracellular and intracellular PbTx) and culture filtrate (representing only extracellular PbTx) treatments of *K. brevis* were prepared at 500 cells mL⁻¹ and 5000 cells mL⁻¹ (referred to as low and high, respectively). Controls were prepared with no *K. brevis*. *Karenia* cultures in early stationary phase, (14-days-old at $\sim 1.3 \times 10^4$ cells mL⁻¹), were used for all exposures, as *K. brevis* cells are typically most toxic at this stage (Baden and Thomas, 1989). Triplicate cell counts of stock cultures were performed using a nanoplankton chamber (PhycoTech Inc.) and aliquots added to either glass beakers or plastic microwell plates to reach the desired final concentration for exposure trials. Culture filtrate treatments were prepared by centrifuging the *K. brevis* cells at 800 rpm for 5 min at 18 °C, before slowly filtering the supernatant through a 13 μm Nitex mesh. Subsamples were observed under the microscope to ensure cell removal, before the treatment was added to the experimental containers at the same concentration as whole *K. brevis* treatments.

2.3. Oyster spawning

All subsequent exposures were performed on the same batch of offspring. Broodstock oysters were placed in a tank of 0.1 μm

filtered seawater (FSW) at a salinity of 25 and temperature of 18 °C for 30 min, before slowly raising the temperature to 30 °C and maintaining it for 30 min to induce spawning. Spawning individuals were removed from the tank, rinsed with FSW and transferred to individual 1 L containers containing a small amount of FSW to complete spawning and ensure gametes were not contaminated or fertilized. Spawning was considered finished 30 min after the first individual had started spawning. Gamete quality was assessed microscopically and the highest quality gametes (dense, motile (>90%) sperm, and dense, round, full, ~60 µm diameter oocytes) were used for fertilization. Sperm from 4 males were individually sieved through a 55 µm Nitex mesh and kept separate. Oocytes from 4 females were individually sieved first through a 150 µm Nitex mesh to remove debris, and then washed with FSW and retained on a 20 µm mesh, re-suspended in FSW, kept separate and counted using a Sedgwick-Rafter counting chamber and dissecting microscope. Oocytes and spermatozoa were sampled for flow cytometric analysis (described below).

2.4. Experimental design

Oocytes rinsed with FSW from individual females ($n = 4$) were pooled in a sterile 2 L beaker to yield a concentration of ~2500 oocytes mL⁻¹. Sperm were also pooled from individual males ($n = 4$) and the concentration was determined by spectrophotometric absorbance at 581 nm (Dong et al., 2005).

2.4.1. Four-day exposure experiment

Four replicates per treatment were prepared for both pre-fertilization and post-fertilization exposures using FSW at a salinity of 27.

Gamete exposure (pre-fertilization group): Aliquots of pooled oocytes were transferred to glass beakers already containing 300 mL of the appropriate *K. brevis* treatment to obtain a final concentration of 20 oocytes mL⁻¹. In separate 60 mL glass beakers, 1 mL of sperm (at ~2.5 × 10⁷ sperm mL⁻¹) was added to 49 mL of the appropriate *K. brevis* treatment. Exposure of separated gametes lasted ~30 min before 10 mL of the *K. brevis* treated sperm was added to the beaker containing oocytes treated with *K. brevis*, thus yielding ~500–800 sperm oocyte⁻¹. One hour after fertilization, 10 mL subsamples were taken from each beaker and fixed in 0.1% buffered formalin (Fig. 1).

Embryonic exposure (post-fertilization group): Pooled sperm were added to the oocyte suspension to obtain 500–800 sperm oocyte⁻¹ and mixed gently. Once embryos had reached the 2–4 cell stage in the stock solution (~45 min post-fertilization), embryos in 3 aliquots of 0.1 mL of stock solution were counted as described above for oocytes. Embryos were added to each beaker containing the appropriate *K. brevis* treatment to obtain a final concentration of 20 embryos mL⁻¹ (Fig. 1).

Pre- and post-fertilization groups were maintained for 4 days in

contact with *K. brevis* treatments. Temperature, dissolved oxygen, pH and salinity were monitored and maintained in exposure beakers according to Rolton et al. (2014).

At Day 1 (after sampling), newly hatched larvae were fed clone T-Iso and *C. muelleri* at 2.5 × 10⁴ cells mL⁻¹ and 1.75 × 10⁴ cells mL⁻¹, respectively, providing a ration of 5 × 10⁴ T-Iso volume equivalent (eq.) cells mL⁻¹. At Day 3 this ration was increased to 10⁵ T-Iso eq. cells mL⁻¹. At 6 h, Day 1 and Day 4, 10 mL sub-samples were taken from each beaker from both the pre- and post-fertilization exposures and fixed in 0.1% buffered formalin (Fig. 1).

2.4.2. Four-day 'recovery' after exposure

On Day 4 of exposure to *K. brevis* treatments, larvae from both treatments were sieved through a 20 µm Nitex mesh screen to remove *K. brevis* cells and media. Beakers were rinsed and wiped clean and new 0.1 µm-filtered and UV-treated seawater at a salinity of 27 was added. Larvae were restocked without grading and fed with the same ration of algal food as on Day 3. Feeding on Day 6 was also the same as on Day 3. On Day 8, larvae from both exposure treatments were sieved through a 20 µm mesh, re-suspended in clean seawater, and preserved as above (Fig. 1).

2.5. Analysis of larval and embryonic development

2.5.1. Fertilization success, larval mortalities, and abnormalities

From the 1 h pre-fertilization exposure samples, the numbers of fertilized (polar body up to 4-cell stage) and unfertilized (no polar body) oocytes were counted using a light microscope and a Sedgwick-Rafter counting chamber. At least 30 oocytes were counted per replicate; data were expressed as the mean percent (%) fertilization success ± standard deviation (SD).

For both pre- and post-fertilization exposures, numbers of normal, abnormal (live) and dead embryos/larvae were counted at 6 h, Day 1, Day 4 and Day 8 according to Rolton et al. (2014). On Day 8, abnormal larvae were considered those which had not reached early umbo veliger stage and those with shell deformities and/or mantle abnormalities. Results were expressed as mortality, abnormality and normality percentages (Rolton et al., 2014).

2.5.2. Larval shell and gut fullness measurements

On Day 1, 4, and 8, the shell length (SL) of 30 live larvae from each replicate of both pre- and post-fertilization exposures were measured using an Olympus Inverted IX73 microscope equipped with a DP73 Olympus camera and 'Cell sense' software. The mean larval growth in µm day⁻¹ for days 1–4 and days 4–8 (±SD) was calculated. The presence of food in the gut of the same 30 larvae measured for SL was also recorded at Day 4 and 8 and data expressed as percent (%) of larvae feeding.

2.6. Flow-cytometric analysis

Morphological and physiological characteristics of oocytes and sperm were assessed using an EasyCyte HT flow cytometer (Guava Technologies, Millipore, Billerica, Massachusetts), equipped with a 488-nm argon laser and 3 fluorescence detectors; green (525 ± 15 nm), yellow (583 ± 13 nm), and red (680 ± 15 nm). Incubation of samples with fluorescent dyes were performed in the dark at room temperature prior to flow cytometric analysis. Unless stated, relative fluorescence values were expressed in arbitrary units. Cellular assays performed on gametes are described below and data were analysed using InCyte software (Millipore).

2.6.1. Oocyte viability

Viability was measured using propidium iodide (PI, Sigma, St

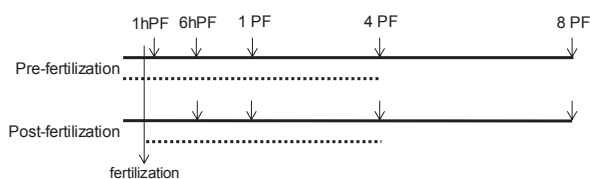


Fig. 1. Experimental design of the 4-day exposure of gametes or embryos to *Karenia brevis* followed by a 4-day 'recovery' period. One experiment started from pre-fertilization (gametes) and the other from post-fertilization (2–4 cell stage embryos). —: indicates time starting from spawning;: indicates exposure to *K. brevis* treatments; ↓ indicates sampling times at 1 h post-fertilization (PF), 6 h PF, Day 1, Day 4, and Day 8.

Quentin Fallavier, France), which penetrates cells with compromised membranes and emits in the red fluorescence range (550–600 nm). Aliquots of 200 μl oocyte suspension (of 2×10^4 to 5×10^4 cells mL^{-1}) were stained with PI (final concentration $10 \mu\text{g mL}^{-1}$) for 10 min (Le Goïc et al., 2014). Results were expressed as percentages of live cells.

2.6.2. Oocyte ROS production

Production of ROS was measured using non-fluorescent, cell-permeable dye, 2',7'-dichlorofluorescein diacetate DCFH-DA (Sigma, St Quentin Fallavier, France); DCFH-DA is hydrolysed intracellularly to form DCFH which turns to highly green fluorescent 2',7'-dichlorofluorescein (DCF) upon oxidation by ROS in a quantitative manner. Upon addition of 2 μl of DCFH-DA (final concentration $10 \mu\text{M}$) on 200 μl oocyte suspensions, samples were incubated for 30 min (Le Goïc et al., 2014).

2.6.3. Sperm viability

Viability was measured according to Le Goïc et al. (2013a) using dual staining with SYBR-14 (which penetrates only cells with intact membranes) and propidium iodide (PI), which penetrates cells with damaged membranes) (Live/dead[®] Sperm Viability kit – Molecular Probes). An aliquot of 200 μl of sperm diluted at 2×10^5 cell mL^{-1} in sterile FSW was stained with both SYBR-14 and PI (final concentrations of $1 \mu\text{M}$ and $10 \mu\text{g mL}^{-1}$, respectively, for 10 min. Results were expressed as percentages of live cells (viability).

2.6.4. Sperm mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was measured using the potential-dependent J-aggregate-forming delocalized lipophilic cation, 5,5',6,6'-tetrachloro-1,1',3,3'-tetra-ethylbenzimidazol carbocyanine iodide (JC-10, ATT Bioquest Inc.). The dye, JC-10, can selectively penetrate mitochondria and reversibly change color from green to orange as the membrane potential increases. An aliquot of 200 μl sperm samples at 2×10^5 cell mL^{-1} were stained with JC-10 (final concentration $5 \mu\text{M}$), incubated 5 min (Le Goïc et al., 2013a). Membrane potential of active cells was estimated by the ratio of aggregate/monomer, i.e. orange/green fluorescence ratio.

2.6.5. Spermatozoa ROS production

Production of ROS by sperm was measured according to Le Goïc et al. (2013b) using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular probes, Invitrogen) at a final concentration of $10 \mu\text{M}$. Sperm and control treatments were incubated for 30 min.

2.7. Toxin analysis of *Karenia brevis* cultures

The culture of *Karenia brevis* used for all exposures was sampled at Day 0. To determine PbTx content and profile throughout the 4-day exposure of pre- and post-fertilization groups, a 30 mL sample was taken from one of the four replicate beakers per treatment on Days 1 and 4. Samples were extracted and analysed for PbTx content and their toxin profile determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Mote Marine Laboratory, Sarasota, FL, USA, according to methods of Abraham et al. (2008) and Plakas et al. (2008).

2.8. Statistical analyses

To normalize distributions, results for % fertilization success, % normality, abnormality, mortality and % of larvae feeding as well as percent or ratio data recorded by flow cytometry were arcsine square root transformed. Mean larval growth data and transformed

percentages were compared using independent t-tests to determine differences among treatments. When variance homogeneity requirements were not met (Levene's test), Welch's correction was used to identify differences between the 5 treatments. The Mann-Whitney-U Test was used for any data that did not meet normality or homogeneity requirements. Statistical tests were run using SPSS 19 statistical software.

3. Results

3.1. Toxin analysis of *Karenia brevis*

Toxin profiles of the *K. brevis* used for all exposure assays show that gametes, embryos, and larvae of *Crassostrea virginica* were exposed to detectable levels of PbTx (Table 1) and that control treatments contained no detectable toxin.

Total PbTx concentrations were $\sim 3\times$ higher in whole-cell treatments than in corresponding culture filtrate treatments on Day 0. The whole-cell *K. brevis* treatments at the low and high concentrations contained 16.3 and 143.5 $\mu\text{g L}^{-1}$ compared to 5.0 and 51.0 $\mu\text{g L}^{-1}$ total PbTx in culture filtrate treatments. Total PbTx concentrations in the high, whole-cell *K. brevis* treatment remained high for the pre- and post-fertilization assays throughout the 4-day exposure. In this treatment, total PbTx increased slightly on Day 1 and returned at Day 4 to values similar to those determined on Day 0. In the low, whole cell treatment, total PbTx decreased to 65–83% of initial content on Day 1 and dropped to 42–53% of initial content on Day 4. The total PbTx concentration declined faster in culture filtrate treatments than in whole-cell treatments during the exposure, especially between Day 1 and Day 4. The culture filtrate treatment at the high concentration dropped to 17–20% of the initial total PbTx content and as low as 5–6% of the initial level at the low concentration. The toxin profiles for the whole-cell *K. brevis* treatments changed little over the 4 days, with lower proportions of the parent toxins PbTx-1 and PbTx-2 and the PbTx antagonist, brevenal, as well as higher proportions of the PbTx-2 reduction conjugate, PbTx-3, by Day 4. In the high concentration culture filtrate treatment, the proportions of PbTx-3 and PbTx-CA (the oxidized PbTx-2 conjugate with the end aldehyde group converted to a carboxylic group), by Day 4 were much higher than on Day 0, while PbTx-1 and brevenal dropped below detection level. Detected PbTx in the low concentration culture filtrate treatment were too low to allow interpretation.

3.2. Gamete exposure

Fertilization success was high in all treatments ($\geq 94\%$); however, the fertilization success of the high density, whole-cell treatment was significantly lower compared to the control (99%) and low-density, whole-cell treatment (97%) (Fig. 2).

3.3. Four-day exposure experiment

Negative effects on embryonic development of both pre- and post-fertilization groups at 6 h were greater in the high, whole-cell treatment than in the culture filtrate treatment (Table 2). Percent normality was 80.0% and 83.6% in the high concentration, whole-cell treatment for the pre- and post-fertilization groups, respectively, and 83.3% and 88.1% in the high concentration, culture filtrate treatment.

Larvae from both pre- and post-fertilization groups exposed to the high-density *Karenia brevis* treatments showed significant increases in mortality compared to their respective controls and low *K. brevis* concentration treatments from 6 h exposure (Table 2, Fig. 3).

Table 1
Brevetoxin (PbTx) concentrations ($\mu\text{g L}^{-1}$) of the 14-day-old *Karenia brevis* (Manasota Key strain) whole-cell and culture filtrate treatments of *Karenia brevis* at two concentrations (500 and 5000 cells mL^{-1}) used for all exposures. The measurement at Day 0 was common to gametes and embryos from all exposure assays. Day 1 and Day 4 measurements were taken from one of the four replicate beakers in each treatment.

PbTx conjugate			PbTx-1	PbTx-2	PbTx-3	PbTx-carboxylic acid	Brevenal	Total
Pre-fertilization	Day 0	Whole 500	1.1	14.7	0.5	<0.01	<0.01	16.3
		Filtrate 500	<0.01	2.7	1.0	1.4	<0.01	5.0
		Whole 5000	8.8	98.7	6.2	1.5	28.4	143.5
		Filtrate 5000	1.8	28.6	5.0	9.5	6.1	51.0
	Day 1	Whole 500	1.0	12.0	0.5	<0.01	<0.01	13.5
		Filtrate 500	0.2	2.3	0.3	0.1	<0.01	3.0
		Whole 5000	15.0	138.8	14.7	1.5	39.3	209.2
		Filtrate 5000	1.0	24.1	4.5	5.1	<0.01	34.6
	Day 4	Whole 500	0.4	7.5	0.8	<0.01	<0.01	8.6
		Filtrate 500	<0.01	0.1	0.2	<0.01	<0.01	0.3
		Whole 5000	9.7	108.0	28.6	1.4	24.2	171.8
		Filtrate 5000	<0.01	3.1	4.3	2.9	<0.01	10.4
Post-fertilization	Day 1	Whole 500	0.7	9.7	0.3	<0.01	<0.01	10.7
		Filtrate 500	<0.01	2.9	0.4	0.1	<0.01	3.4
		Whole 5000	9.9	105.7	6.2	1.4	30.2	153.5
		Filtrate 5000	1.1	21.6	4.2	4.4	<0.01	31.2
	Day 4	Whole 500	0.2	5.5	0.9	0.1	<0.01	6.8
		Filtrate 500	<0.01	0.1	0.2	<0.01	<0.01	0.2
		Whole 5000	5.5	65.1	15.7	1.4	15.5	103.2
		Filtrate 5000	<0.01	3.02	3.42	2.30	<0.01	8.73

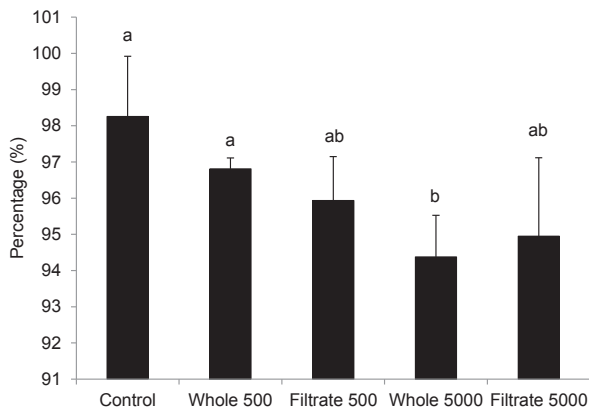


Fig. 2. One-hour fertilization success (%) of sperm and oocytes of *Crassostrea virginica* previously exposed for 30 min prior to fertilization to whole-cell and culture filtrate preparations of *Karenia brevis* at two concentrations (500 and 5000 cells mL^{-1}). Data are presented as mean \pm SD, $n = 4$. Treatments with the same letter were not significantly different ($p > 0.05$).

After 4 days of exposure to high doses of *K. brevis* in both whole-cell and culture filtrate treatments, both pre- and post-fertilization larval groups showed significantly higher mortalities compared to controls (Fig. 3). Mortalities continued to increase after exposure terminated (Fig. 3). By Day 8, larvae in the high *K. brevis*

Table 2
Percent normality, abnormality, and mortality values for embryos of *Crassostrea virginica* exposed from pre-fertilization (gametes) and post-fertilization (2–4 cell stage embryos) for 6 h to whole-cell and culture filtrate preparations of *Karenia brevis* at two concentrations (500 and 5000 cells mL^{-1}). Treatments with the same letter were not significantly different ($p > 0.05$).

		Control	Whole 500	Filtrate 500	Whole 5000	Filtrate 5000
Pre-fertilization	% normal	97.7 ^a	88.0 ^{abc}	92.3 ^{ab}	80.0 ^c	83.3 ^{bc}
	% abnormal (live)	2.3 ^b	12.0 ^{ab}	2.6 ^b	10.0 ^a	7.4 ^{ab}
	% mortality	0.0 ^b	0.0 ^b	5.1 ^{ab}	10.0 ^a	9.3 ^a
Post-fertilization	% normal	95.2 ^a	82.1 ^{ab}	88.9 ^{ab}	83.6 ^b	88.1 ^{ab}
	% abnormal (live)	2.4 ^b	14.3 ^{ab}	11.1 ^{ab}	11.5 ^a	7.5 ^{ab}
	% mortality	2.4 ^{ab}	3.6 ^{ab}	0.0 ^b	4.9 ^a	4.5 ^a

concentrations from both pre- and post-fertilization groups showed significantly higher mortalities than controls or the low concentration for both whole-cell and filtrate treatments. Mortalities were 84.3% and 72.5% in the high-density, whole-cell treatment from the pre- and post-fertilization groups, respectively compared to 14.2% and 15.2% mortality in their respective controls.

In both pre- and post-fertilization groups, growth of oysters exposed to the high concentration of whole cells and filtrate *K. brevis* treatments were significantly lower after 4 days of larval rearing. In the pre-fertilization exposed group, larvae in the high-density, *K. brevis* filtrate treatment grew from Day 1 to Day 4 at $0.7 \mu\text{m day}^{-1}$ compared to $2.8 \mu\text{m day}^{-1}$ in the control. In the post-fertilization exposed group, larvae in the high-density, whole-cell *K. brevis* treatment grew at $1.2 \mu\text{m day}^{-1}$ compared to $2.4 \mu\text{m day}^{-1}$ in the control over the same period. Although not systemically significant, the overall effect is clear (Fig. 4). During the recovery phase from Day 4 to Day 8, larvae at the high *K. brevis* concentration continued to exhibit a significantly lower growth rate ($0.3 \mu\text{m day}^{-1}$ and $0.4 \mu\text{m day}^{-1}$ for pre- and post-fertilization groups, respectively) than in controls and low-density, filtrate concentration ($1.3 \mu\text{m day}^{-1}$ for all).

Larval feeding in the highest *K. brevis* concentration treatments (whole-cell and culture filtrate) was significantly lower than in controls and low concentration treatments on both Day 4 and Day 8 (Fig. 3). By Day 8, only 6.3% and 13.8% of larvae in the high-density, whole-cell treatments and 0% and 2.5% of larvae in the high-density, filtrate treatments were feeding compared to 86.3% and 85% in the controls for the pre- and post-fertilization assays respectively. The mean size of the larvae showed a positive, significant relationship to larval feeding, with smaller larvae containing less food. At Day 4, R^2 values from both pre- and post-fertilization were above 0.78 and at day 8, were above 0.94.

Overall there were few significant differences between the larvae that were exposed at pre- or post-fertilization stages to *K. brevis* treatments (Table 2, Figs. 3 and 4). In high-density *K. brevis* treatments at Day 1, larvae that were exposed from pre-fertilization showed higher abnormalities ($p = 0.037$ and $p = 0.006$ for whole-cell and filtrate treatments, respectively) than those exposed from post-fertilization. At Day 4, larvae that were exposed to the high-density, whole-cell *K. brevis* treatment from pre-fertilization showed significantly higher mortalities than those exposed from

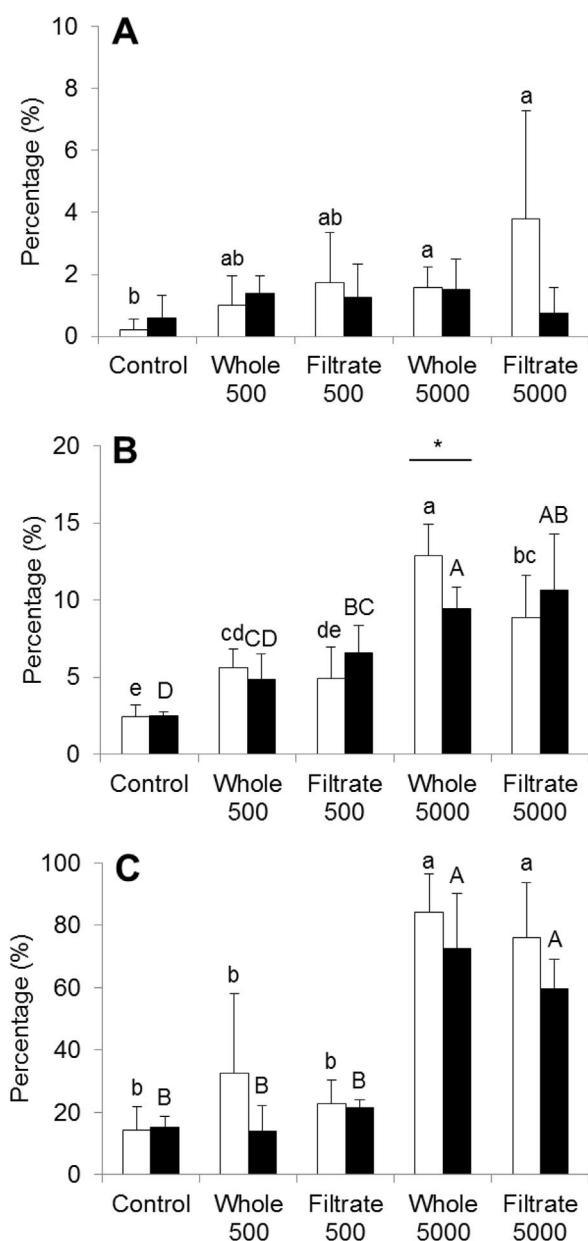


Fig. 3. Percent mortality of oyster larvae exposed from pre- (□) and post-fertilization stages (■) after A: 1 day and; B: 4 days of exposure to whole-cell and culture filtrate preparations of *Karenia brevis* at low and high concentrations (500 and 5000 cells mL⁻¹, respectively) and; C: after exposure on Day 8. Within letters of the same case, treatments with the same letter were not significantly different ($p > 0.05$). Lines with * above bars indicate significant differences between pre- and post-fertilization groups ($p \leq 0.05$). All data are presented as mean \pm SD, $n = 4$.

post-fertilization stage ($p = 0.022$, Fig. 3). On Day 8, larvae exposed from pre-fertilization to the low *K. brevis* concentration treatments had less food in their gut than those exposed post-fertilization (significant difference at $p = 0.002$ and $p = 0.019$ for whole-cell and filtrate treatments, respectively, Fig. 4).

3.4. Flow cytometric assay

There were significantly fewer viable sperm cells after exposure to the high-density, whole-cell *Karenia brevis* concentration (62%) compared to all other treatments (e.g. 83% in the control, shown in Table 3). No significant changes were recorded in oocyte

morphology, viability, or ROS production between treatments.

4. Discussion

Gametes and early larval stages are critical phases in marine invertebrate life histories which are highly vulnerable to abiotic and biotic interferences such as HAB (Gosselin and Qian, 1997; Pechenik, 1987; Thompson et al., 1996; Thorson, 1950). This study has shown for the first time that i) exposure of gametes (sperm and oocytes) and larvae of the eastern oyster to *Karenia brevis* reduced gamete quality, fertilization success, larval growth, and feeding (as evident by assessment of the presence of food in the larval gut), and ii) this initial exposure had irreversible, prolonged effects on larvae that extended beyond the end of exposure (at higher bloom concentrations, 5000 cells mL⁻¹).

4.1. Deleterious effects

Dose-dependent lethal and sub-lethal effects were observed for both gametes and embryos following only a few hours of exposure to treatments of *K. brevis*. This is consistent with results of prior studies which found deleterious effects on early life stages of *Crassostrea virginica*, *Mercenaria mercenaria*, and the bay scallop, *Argopecten irradians*, following relatively short exposures to *K. brevis* (Leverone et al., 2006; Rolton et al., 2014) and, on *Pinctada fucata martensii* exposed to the harmful algae, *Heterocapsa circularisquama* and *Karenia mikimotoi* (Basti et al., 2013, 2015). After exposure had ended, larvae which had been exposed to the high *K. brevis* concentration continued to exhibit increased mortalities, growth inhibition and reduced larval feeding, than larvae which had been exposed to low concentrations of *K. brevis* and controls. Similarly, no growth recovery of 1-day-old *M. mercenaria* larvae previously exposed to a unialgal suspension of *Aureococcus anophagefferens* at 800 cells μL^{-1} for 15 days, followed by a 12 day-recovery period was observed by Bricelj and MacQuarrie (2007). *Aureococcus anophagefferens* toxins have not been chemically characterized; larval feeding inhibition, however, may have resulted from contact with bioactive compounds associated with extracellular polysaccharide substances (EPS) following cell capture by the gills and subsequent ingestion (Bricelj and MacQuarrie, 2007; Gainey and Sumway, 1991). In the present study, the lack of food in the larval gut on Day 4 implies that larvae were starving and thus did not increase their intake of PbTx and/or other harmful compounds, although larvae normally start to feed between 24 and 36 h post-fertilization (Carriker, 2001; Kraeuter et al., 1981). This suggests that irreversible damage may occur during embryogenesis, in which the feeding apparatus and/or gut development are impaired by exposure to PbTx and/or other toxic compounds, or, these compounds act as a long-term feeding deterrent on other nutritious algae.

4.2. Toxicity of extracellular brevetoxin

There was little difference in the susceptibility of both embryos and larvae that were exposed to either whole-cell or culture filtrate preparations of *K. brevis* at the same cell concentration. Prior studies showed that lysed cells of *K. brevis* had similar or slightly increased negative effects on larvae when compared with whole-cells at the same concentration (Leverone et al., 2006; Rolton et al., 2014). The latter hypothesized that due to the modest negative impacts of lysed cells over whole cells and inconsistent differences between whole-cell and lysed treatments, the observed effects on *C. virginica* larvae might be related to exposure to extracellular PbTx and/or other unknown bioactives. The present study served to clarify that major deleterious effects occur in the

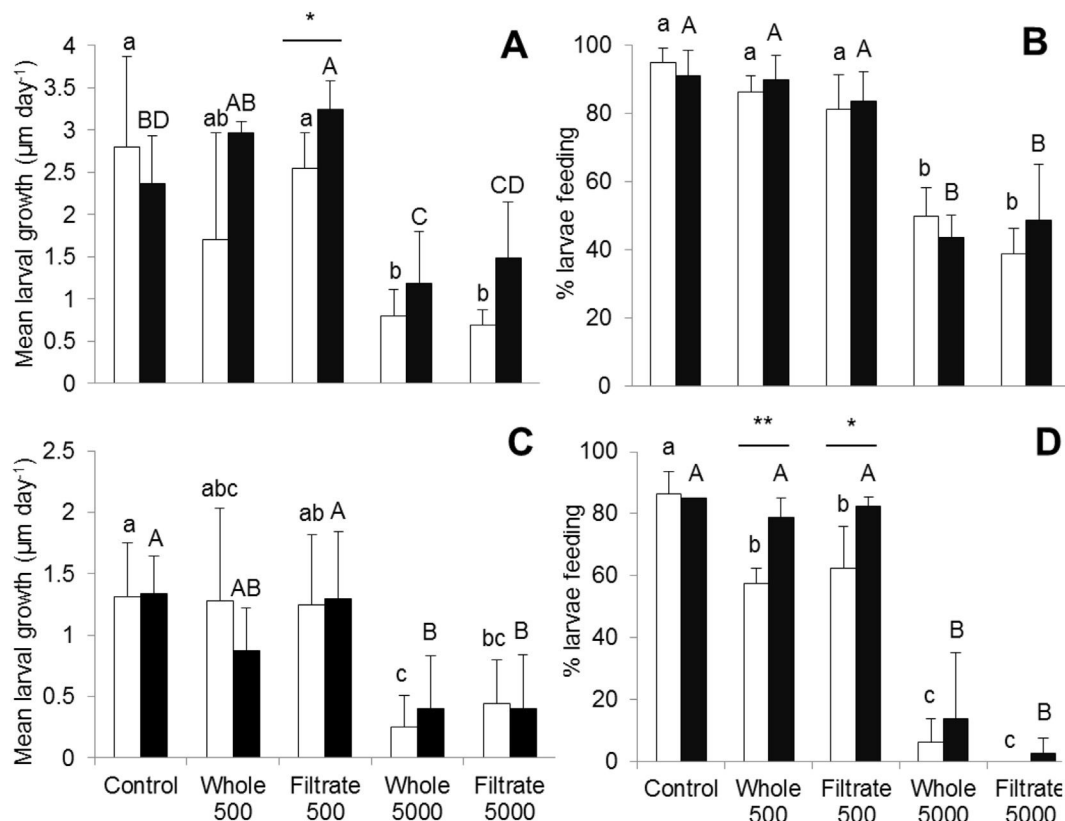


Fig. 4. Growth (left) and feeding responses (right) of *Crassostrea virginica* larvae exposed to whole-cell and culture filtrate preparations of *Karenia brevis* at 500 and 5000 cells mL⁻¹ from pre- (□) and post-fertilization (■) stages. A: Larval growth between days 1 and 4 ($\mu\text{m day}^{-1}$); B: percentage of larvae feeding after 4 days of exposure; C: larval growth between days 4–8 ($\mu\text{m day}^{-1}$), and D: percentage of larvae feeding after 4 days exposure followed by a 4 day 'recovery' period. Within letters of the same case, treatments with the same letter were not significantly different ($p > 0.05$). Lines with * above bars indicate significant differences between mortalities from pre- and post-fertilization treatments. Significance levels: ** $0.001 < p \leq 0.01$, * $0.01 < p \leq 0.05$. All data are presented as mean \pm SD, $n = 4$.

absence of *K. brevis* cells. However, although the negative effects on larvae correlate well with *K. brevis* cell concentrations, the contribution of PbTx to the observed negative effects remains difficult to assess.

Total PbTx concentrations in the whole-cell treatments represent the extracellular PbTx to which larvae are exposed, plus the endogenous PbTx to which larvae are not exposed. The total PbTx in the culture filtrate treatments, represents only the extracellular PbTx (as the whole cells were removed); thus, we hypothesized that at the beginning of exposure, *C. virginica* should be exposed to the same amount of extracellular PbTx in both whole-cell and filtrate treatments at the same cell density. During culture filtrate preparation however, *K. brevis* cells may have lysed or been stressed during removal which may have resulted in more extracellular PbTx being available to the gametes and early life stages than in the whole treatment (as shown by a higher proportion of PbTx-3 and PbTx-CA relative to PbTx-2 in this treatment). This hypothesized increase in PbTx available to larvae did not however correspond to

an increased negative effect compared to larvae in the whole-cell treatment. This suggests that other compounds in addition to PbTx may be involved in negative effects. Also, the increase in total PbTx concentrations in the high-density, whole-cell *K. brevis* treatments measured on Day 1 suggests that some *K. brevis* cells were growing and dividing during larval exposure. This maintained a higher level of the most potent PbTx-1 toxin (Baden, 1989) in the medium which although considered to be mainly intracellular (Lekan and Tomas, 2010), can also be extracellular and thus available to larvae.

After 4 days of exposure, the increase in the proportion of PbTx-3 relative to PbTx-2 in all treatments indicates that the cells were lysing and a larger portion of the toxin was becoming extracellular (Pierce et al., 2001, 2008; Roszell et al., 1990; Tester et al., 2008). Cell lysis generally results in less potent derivatives from hydrolysis of highly potent parent molecules (e.g. PbTx-1) (Roth et al., 2007). Thus, the similar response of larvae exposed to whole-cell and filtrate preparations after 4 days of exposure, when total PbTx in

Table 3
Viability, ROS production (arbitrary units, A.U.) of *Crassostrea virginica* spermatozoa and oocytes, and mitochondrial membrane potential of sperm after 2-h exposure to whole-cell and culture filtrate preparations of *Karenia brevis* at two concentrations (500 and 5000 cells mL⁻¹). Data are presented as mean \pm SD, $n = 4$.

		Control	Whole 500	Filtrate 500	Whole 5000	Filtrate 5000
Sperm	Viability %	82.8 \pm 4.7 ^a	81.5 \pm 5.1 ^a	80.8 \pm 8.6 ^a	62.3 \pm 11.8 ^b	79.7 \pm 5.6 ^a
	ROS production	13.5 \pm 1.5	14.1 \pm 1.6	14.3 \pm 2.6	17.5 \pm 3.1	15.2 \pm 3.6
	Mitochondrial membrane potential	0.1 \pm 0.03	0.1 \pm 0.02	0.1 \pm 0.03	0.12 \pm 0.04	0.13 \pm 0.04
Oocytes	Viability %	98.7 \pm 1.2	99.1 \pm 0.2	98.9 \pm 1.0	96.1 \pm 6.1	96.8 \pm 2.9
	ROS production	2593.9 \pm 1946.2	2019.0 \pm 1660.9	1605.0 \pm 1525.4	1356.5 \pm 1541.2	888.5 \pm 275.8

the culture filtrate treatment is very low, and more of the less potent PbTx metabolites are present, again suggests that PbTx are only partially if at all responsible for the deleterious effects observed, in agreement with Rolton et al. (2014). Early embryonic and larval stages of *P. fucata martensii* were found to be sensitive to bioactives produced by the similar, non- PbTx-producing *Karenia* species, *K. mikimotoi* and *Karenia papilionacea* (Basti et al., 2015). In addition to PbTx, other chemically uncharacterized compounds that can be allelopathic, ichthyotoxic, or hemolytic are known to be produced by *K. brevis* (Kubaneck et al., 2005, 2007; Leblond et al., 2003; Marshall et al., 2005; Neely and Campbell, 2006). Allelochemicals and cellular organic extracts produced by *K. brevis* are polar compounds which are often unstable and short-lived, and thus difficult to study under experimental conditions (Kubaneck et al., 2005, 2007; Prince et al., 2010). By day 4, in the filtrate treatment, PbTx were hydrolyzed into less potent derivatives, and were almost totally transformed. It can thus be hypothesized that allelochemicals may have at least partially undergone the same fate. It is assumed that whole-cell preparations of *K. brevis* maintained their production of allelopathic compounds as they did for PbTx during the first days of exposure, although this needs to be confirmed once other bioactive compounds are identified. Future studies exposing early developmental stages of bivalve molluscs directly to PbTx and/or to toxin-free cultures of *K. brevis* would help to elucidate the relative contribution made by exposure to PbTx vs. that to unknown harmful compounds to the negative effects observed.

4.3. Toxins associated with cell membranes?

Although results of this study have clarified that deleterious effects occur without the presence of *K. brevis* cells, the presence of whole cells caused some additional effects. Negative effects on sperm viability were greater in the high-density, whole-cell treatment than in the filtrate treatment. This suggests that gametes were not only negatively affected by extracellular products of *K. brevis* but, also by direct contact with *K. brevis* cells and any harmful compounds associated with the *K. brevis* cell surface. Similarly, Yan et al. (2001) revealed that unknown toxins which are associated with the cell membrane of the paralytic shellfish toxin-producing algae, *Alexandrium tamarense*, impaired the egg hatching rate of the scallop, *Chlamys farreri*. Phytoplankton produce various types of harmful compounds, such as glycoprotein and carbohydrates, on the cell surface (Costas and Rodas, 1994; Waite et al., 1995; Yokote and Honjo, 1985), and these EPS are produced by many HAB species such as *A. anophagefferens* (Gainey and Shumway, 1991) and *Aureoumbra lagunensis* (Liu and Buskey, 2000). As yet chemically uncharacterized compounds may be associated with the *K. brevis* cell membrane. Direct contact with whole, live cells of *K. mikimotoi* was needed to cause mortality of the rotifer, *Brachionus plicatilis* (Zou et al., 2010). Cells of *K. mikimotoi* are surrounded by a layer of fatty acids that are responsible for ichthyotoxic effects (Parrish et al., 1998) and also display autotoxicity, releasing toxic compounds in close proximity to the cell, which are only active briefly (Gentien et al., 2007). Previous studies have shown that *K. brevis* also produces hemolytic toxins however, contact with the *K. brevis* cell or cell fragments resulting from sonication is required to elicit these effects (Neely and Campbell, 2006; Tatters et al., 2010). Nevertheless, it remains puzzling that in the present study the whole-cell treatment which produced more extracellular PbTx and bioactives, did not further impact larvae between Day 1 and Day 4 than in the culture filtrate treatment. The lack of a difference between the two treatments may be related to the fact that observed deleterious effects are larval-stage dependent.

4.4. Developmental stage-dependent effects

Most larvae tested in this study were D-stage by Day 1 and may have had more protection from direct contact with algal bioactives by the larval shell, in contrast to the earlier, pre-shelled stages. Similarly, Wang et al. (2006) suggested that the reduced vulnerability of veliger larvae of *A. irradians*, exposed to *Heterosigma akashiwo* cells was due to the protection of the gill epithelia from direct contact with harmful algae afforded by the larval shell. Developing embryos are metabolically active and impacts from unknown deleterious compounds and/or a dramatic change in membrane ion permeability, such as permanent Na⁺ channel activation by PbTx, could reduce survival (Fitzpatrick et al., 2008; Franchet et al., 1997). Stage-dependent susceptibility to *K. brevis* exposure during embryonic development was previously demonstrated by Moon and Morrill (1976). In their study, gametes and embryos of the sea urchin, *Lytechinus variegatus*, were exposed to lysates of *K. brevis*, both throughout larval development, and just prior to hatching. Development of the larvae was not impacted until gastrula stage, indicating that the lysate disturbed one or more developmental processes, such as ion flux and/or respiration in the embryos, prior to gastrulation.

4.5. Gamete quality

In the present study, some differences in the larval response to *K. brevis* exposure between those exposed from pre-fertilization and those exposed from post-fertilization were recorded prior to Day 1, confirming that gametes were affected by exposure to *K. brevis*. Indeed, the 2 h exposure assay showed a reduction in sperm viability (but no effect on oocytes) which may have resulted in the reduced fertilization success recorded in the high-density, whole-cell treatment, as sperm quality influences successful fertilization in free-swimming invertebrates (Nice, 2005). Sperm are motile and may therefore encounter more cells, toxin, and/or harmful compounds than oocytes. Oocytes may be more protected from physical and chemical damage from harmful compounds than sperm because of their peri-vitelline envelope (Galtsoff, 1964). The “quality” of this membrane may partially explain the high variability observed in oocyte responses upon exposure to *K. brevis*; however, the presence of the fertilization membrane did not isolate urchin embryos from the toxic effects of the *K. brevis* lysate (Moon and Morrill, 1976). Similarly, the vitelline and fertilization membrane did not protect oocytes of the scallop, *Pinctada fucata martensii*, exposed to the toxic alga *H. circularisquama* (Basti et al., 2013). In their study, both sperm and oocytes of scallops showed severe negative impacts within minutes of exposure to 10 cells mL⁻¹ of *H. circularisquama* (Basti et al., 2013), due to direct contact with the toxins on the cell surface of this alga (Matsuyama et al., 1997, 2001). Our results also suggest direct contact with harmful compounds on or close to the surface of the algal cell; however, in contrast to the above study, our results indicate that sperm were more sensitive to *K. brevis* exposure, possibly due to a higher encounter probability with harmful compounds than oocytes.

5. Conclusions

A continuous 4-day exposure of pre-fertilization (gametes) and post-fertilization stages (2–4 cell stage embryos) of *C. virginica* to *K. brevis* revealed dose-dependent negative effects on larvae. Following a 4 day ‘recovery’ period, larvae previously exposed to 500 cells mL⁻¹ were able to recover from some effects; however, at the 5000 cells mL⁻¹ concentration, there was no scope for recovery. Results suggest that negative effects occurred mainly during

embryogenesis and early development. We hypothesize that there may be damage to the gut/feeding apparatus during embryonic development and/or exposure to *K. brevis* toxins (PbTx and/or unknown bioactives) act as a feeding deterrent on non-toxic algae and larvae subsequently starve. There may also be additional involvement of other harmful, chemically uncharacterized compounds associated with the *K. brevis* cell membrane, which can affect the sperm prior to fertilization. The presence of other unidentified bioactives, in addition to PbTx should be considered when assessing the effects of *K. brevis* blooms on early life stages of bivalves. The cumulative negative effects observed throughout early larval development combined with the near-annual exposure to bloom concentrations of *K. brevis* as observed along the SW FL coast, could pose a significant bottleneck on recruitment of bivalves.

Acknowledgements

We thank Patricia Blum, Mote Marine Laboratory, FL, for LC-MS/MS toxin analysis; Nelly LeGoïc from the Institut Universitaire Européen de la Mer, France, for flow cytometry training, and Kelsey McEachern and staff from FGCU for their technical assistance. Thanks also to Diane Blackwood from the FWC for provision of *Karenia brevis* concentration data for Lee County, FL, and Héléne Hégaret for a review of an earlier version of the manuscript. This collaborative work was partially supported by Université de Bretagne Occidentale, by “Laboratoire d’Excellence” LabexMER (ANR-10-LABX-19), an Outgoing Ph.D. Fellowship, co-funded by a grant from the French government under the program “Investissements d’Avenir”.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicon.2015.03.002>.

References

- Abraham, A., Plakas, S.M., Flewelling, L.J., El Said, K.R., Jester, E.L.E., Granade, H.R., White, K.D., Dickey, R.W., 2008. Biomarkers of neurotoxic shellfish poisoning. *Toxicon* 52 (2), 237–245.
- Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication nutrient sources, composition, and consequences. *Estuaries* 25 (4b), 704–726.
- Baden, D.G., Bourdelais, A.J., Jacocks, H., Michelliza, S., Naar, J., 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ. Health Perspect.* 113 (5), 621–625.
- Baden, D.G., 1989. Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J.* 3, 1807–1817.
- Baden, D.G., Thomas, C.R., 1989. Variations in major toxin composition for six clones of *Ptychodiscus brevis*. In: Okaichi, T., Anderson, D.M., Nemoto, T. (Eds.), *Red Tides. Biology, Environmental Science and Toxicology*. Elsevier, Amsterdam, pp. 415–418.
- Basti, L., Nagai, S., Go, J., Okano, S., Nagai, K., Watanabe, R., Suzuki, T., Tanaka, Y., 2015. Differential inimical effects of *Alexandrium* spp. and *Karenia* spp. on cleavage, hatching, and two larval stages of the Japanese pearl oyster, *Pinctada fucata martensii*. *Harmful Algae* 43, 1–12.
- Basti, L., Nagai, K., Tanaka, Y., Segawa, S., 2013. Sensitivity of gametes, fertilization, and embryo development of the Japanese pearl oyster, *Pinctada fucata martensii*, to the harmful dinoflagellate, *Heterocapsa circularisquama*. *Mar. Biol.* 160 (1), 211–219.
- Basti, L., Go, J., Higuchi, K., Nagai, K., Segawa, S., 2011. Effects of the toxic dinoflagellate *Heterocapsa circularisquama* on larvae of the pearl oyster *Pinctada fucata martensii* (Dunker, 1873). *J. Shellfish Res.* 30 (1), 177–186.
- Brand, L.E., Campbell, L., Bresnan, E., 2012. *Karenia*: the biology and ecology of a toxic genus. *Harmful Algae* 14, 156–178.
- Brand, L.E., Compton, A., 2007. Long-term increase in *Karenia brevis* abundance along the Southwest Florida Coast. *Harmful Algae* 6 (2), 232–252.
- Bricelj, V.M., MacQuarrie, S.P., 2007. Effects of brown tide (*Aureococcus anophagefferens*) on hard clam *Mercenaria mercenaria* larvae and implications for benthic recruitment. *Mar. Ecol. Prog. Ser.* 331, 147–159.
- Burkholder, J.M., Shumway, S.E., 2012. Bivalve shellfish aquaculture and eutrophication. In: Shumway, S.E. (Ed.), *Shellfish Aquaculture and the Environment*. Wiley and Sons Inc, Hoboken, New Jersey, pp. 155–215.
- Carriker, M.R., 2001. Embryogenesis and organogenesis of veligers and early juveniles. *Dev. Aquac. Fish. Sci.* 31, 77–115.
- Costas, E., Rodas, V.L., 1994. Identification of marine dinoflagellates using fluorescent lectins I. *J. Phycol.* 30 (6), 987–990.
- Dong, Q., Eudeline, B., Huang, C., Tiersch, T.R., 2005. Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg). *Aquac. Res.* 36, 86–93.
- Fitzpatrick, J.L., Nadella, S., Bucking, C., Balshine, S., Wood, C.M., 2008. The relative sensitivity of sperm, eggs and embryos to copper in the blue mussel (*Mytilus trossulus*). *Comp. Biochem. Physiol. Part C* 147, 441–449.
- Franchet, C., Goudeau, M., Goudeau, H., 1997. Mercuric ions impair the fertilization potential, the resumption of meiosis, the formation of male pronucleus, and increase polyspermy, in the egg of the ascidian *Phallusia mammillata*. *J. Exp. Zool.* 278 (4), 255–272.
- Gainey, L.F., Shumway, S.E., 1991. The physiological effect of *Aureococcus anophagefferens* (“brown tide”) on the lateral cilia of bivalve mollusks. *Biol. Bull.* 181 (2), 298–306.
- Galtsoff, P.S., 1964. The American oyster, *Crassostrea virginica* Gmelin. *U. S. Fish. Bull.* 64, 1–480.
- Geesey, M., Tester, P.A., 1993. *Gymnodinium breve*: Ubiquitous in Gulf of Mexico waters? In: Smayda, T.J., Shimizu, Y. (Eds.), *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam, pp. 251–255.
- Gentien, P., Lunven, M., Lazure, P., Youenou, A., Crassous, M.P., 2007. Motility and autotoxicity in *Karenia mikimotoi* (Dinophyceae). *Phil. Trans. R. Soc. B* 362, 1937–1946.
- Glibert, P.M., Alexander, J., Meritt, D.W., North, E.W., Stoecker, D.K., 2007. Harmful algae pose additional challenges for oyster restoration: impacts of the harmful algae *Karlodinium veneficum* and *Prorocentrum minimum* on early life stages of the oysters *Crassostrea virginica* and *Crassostrea ariakensis*. *J. Shellfish Res.* 26 (4), 919–925.
- Gosselin, L.A., Qian, P., 1997. Juvenile mortality in benthic marine invertebrates. *Mar. Ecol. Prog. Ser.* 146 (1), 265–282.
- Granmo, A., Havenhand, J., Magnusson, K., Svane, I., 1988. Effects of the planktonic flagellate *Chrysochromulina polylepis* (Manton et Park) on fertilization and early development of the ascidian *Ciona intestinalis* (L.) and the blue mussel *Mytilus edulis* L. *J. Exp. Mar. Biol. Ecol.* 124, 65–71.
- Gunter, G., Williams, R.H., Davis, C.C., Smith, F.W., 1948. Catastrophic mass mortality of marine animals and coincident phytoplankton bloom on the west coast of Florida, November 1946 to August 1947. *Ecol. Monogr.* 18, 310–324.
- Heil, C.A., Bronk, D.A., Dixon, L.K., Hitchcock, G.L., Kirkpatrick, G.J., Mulholland, M.R., O’Neil, J.M., Walsh, J.J., Weisberg, R., Garrett, M., 2014. The Gulf of Mexico ECOHAB: *Karenia* Program 2006–2012. *Harmful Algae* 38, 3–7.
- Huang, J.M.C., Wu, C.H., Baden, D.G., 1984. Depolarizing action of red tide dinoflagellate brevetoxin on axonal membranes. *J. Pharmacol. Exp. Ther.* 299, 615–621.
- Krauter, J.N., Castagna, M., Van Dessel, R., 1981. Egg size and larval survival of *Mercenaria mercenaria* and *Argopecten irradians* (Lamarck). *J. Exp. Mar. Biol. Ecol.* 56 (1), 3–8.
- Kubaneck, J., Hicks, M.K., Naar, J., Villareal, T.A., 2005. Does the red tide dinoflagellate *Karenia brevis* use allelopathy to outcompete other phytoplankton? *Limnol. Oceanogr.* 50 (3), 883–895.
- Kubaneck, J., Snell, T.W., Pirkle, C., 2007. Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing. *Limnol. Oceanogr.* 52 (3), 1026–1035.
- Landsberg, J.H., 2002. The effects of harmful algal blooms on Aquatic organisms. *Rev. Fish. Sci.* 10 (2), 113–390.
- Le Goïc, N., Hégaret, H., Fabioux, C., Miner, P., Suquet, M., Lambert, C., Soudant, P., 2013a. Impact of the toxic dinoflagellate *Alexandrium catenella* on Pacific oyster reproductive output: application of flow cytometry assays on spermatozoa. *Aquat. Living Resour.* 26 (3), 221–228.
- Le Goïc, N., Hégaret, H., Lambert, L., Fabioux, C., Soudant, P., 2013b. Cellular changes of *Crassostrea gigas* spermatozoa upon exposure to the toxic dinoflagellate *Alexandrium catenella*. In: *Aquaculture 2013*, Feb 21–25th 2013, Nashville, TN, U.S.A. Meeting abstract nr: 832, poster nr 209.
- Le Goïc, N., Hégaret, H., Boulais, M., Béguel, J., Lambert, C., Fabioux, C., Soudant, P., 2014. Flow cytometric assessment of morphology, viability, and production of reactive oxygen species of *Crassostrea gigas* oocytes. Application to toxic dinoflagellate (*Alexandrium minutum*) exposure. *Cytom. Part A* 85 (12), 1049–1056.
- Leblond, J.D., Evans, T.J., Chapman, P.J., 2003. The biochemistry of dinoflagellate lipids, with particular reference to the fatty acid and sterol composition of a *Karenia brevis* bloom. *Phycologia* 42 (4), 324–331.
- Lekan, D.K., Tomas, C.R., 2010. The brevetoxin and brevenal composition of three *Karenia brevis* clones at different salinities and nutrient conditions. *Harmful Algae* 9 (1), 39–47.
- Leverone, J.R., Blake, N.J., Pierce, R.H., Shumway, S.E., 2006. Effects of the dinoflagellate *Karenia brevis* on larval development in three species of bivalve mollusc from Florida. *Toxicon* 48, 75–84.
- Liu, H., Buskey, E.J., 2000. The exopolymer secretions (EPS) layer surrounding *Aureobamba lagunensis* cells affects growth, grazing, and behavior of protozoa. *Limnol. Oceanogr.* 45 (5), 1187–1191.
- Marshall, J., Ross, T., Pyecroft, S., Hallegraef, G., 2005. Superoxide production by marine microalgae 2: towards understanding ecological consequences and possible functions. *Mar. Biol.* 147, 541–549.
- Matsuyama, Y., Uchida, T., Honjo, T., 1997. Toxic effects of the dinoflagellate *Heterocapsa circularisquama* on clearance rate of the blue mussel *Mytilus galloprovincialis*. *Mar. Ecol. Prog. Ser.* 146, 73–80.

- Matsuyama, Y., Uchida, T., Honjo, T., Shumway, S.E., 2001. Impacts of the harmful dinoflagellate *Heterocapsa circularisquama* on shellfish aquaculture in Japan. *J. Shellfish Res.* 20 (3), 1269–1272.
- Moon, R.T., Morrill, J.B., 1976. The effects of *Gymnodinium breve* lysate on the larval development of the sea urchin *Lytechinus variegatus*. *J. Environ. Sci. Health A* 11, 673–683.
- Mooney, B.D., Nichols, P.D., de Salas, M.F., Hallegraeff, G.M., 2007. Lipid, fatty acid, and sterol composition of eight species of *Karenia* (dinophyta): Chemotaxonomy and putative lipid phycotoxins. *J. Phycol.* 43, 101–111.
- Neely, T., Campbell, L., 2006. A modified assay to determine hemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae* 5, 592–598.
- Newell, R.I.E., 2004. Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. *J. Shellfish Res.* 23 (1), 51–62.
- Nice, H.E., 2005. Sperm motility in the Pacific oyster (*Crassostrea gigas*) is affected by nonylphenol. *Mar. Pollut. Bull.* 50, 1668–1674.
- Padilla, D.K., Doall, M.H., Gobler, C.J., Hartson, A., O'Boyle, K., 2006. Brown tide alga, *Aureococcus anophagefferens*, can affect growth but not survivorship of *Mercenaria mercenaria* larvae. *Harmful Algae* 5, 736–748.
- Parrish, C.C., Bodennec, G., Gentien, P., 1998. Haemolytic glycolipids from *Gymnodinium* species. *Phytochemistry* 47 (5), 783–787. [http://dx.doi.org/10.1016/S0031-9422\(97\)00661-4](http://dx.doi.org/10.1016/S0031-9422(97)00661-4).
- Pechenik, J.A., 1987. Environmental influences on larval survival and development. In: Giese, A.C., Pearse, J.S., Pearse, V.B. (Eds.), *Reproduction of Marine Invertebrates*. Blackwell Scientific Publications, Palo Alto, California, pp. 551–608.
- Pierce, R.H., Henry, M., Blum, P., 2008. Brevetoxin abundance and composition during ECOHAB-Florida field monitoring cruises in the Gulf of Mexico. *Cont. Shelf Res.* 28 (1), 45–58.
- Pierce, R., Henry, M., Blum, P., Payne, S., 2001. *Gymnodinium breve* toxins without cells: intra-cellular and extra-cellular toxins. In: Hallegraeff, G., Blackburn, S., Bolch, C., Lewis, R. (Eds.), *Harmful Algal Blooms*, Intergovernmental Oceanographic Commission of UNESCO, pp. 421–424.
- Plakas, S.M., Jester, E.L.E., ElSaid, K.R., Granade, H.R., Abraham, A., Dickey, R.W., Scott, P.S., Flewelling, L.J., Henry, M., Blum, P., Pierce, R., 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (*Crassostrea virginica*). *Toxicon* 52, 32–38.
- Poli, M.A., Mende, T.J., Baden, D.G., 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol. Pharmacol.* 30 (2), 129–135.
- Prince, E.K., Poulson, K.L., Myers, T.L., Sieg, R.D., Kubanek, J., 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. *Harmful Algae* 10, 39–48.
- Rolton, A., Vignier, J., Soudant, P., Shumway, S.E., Bricej, V.M., Volety, A.K., 2014. Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*. *Aquat. Toxicol.* 155, 199–206.
- Roszell, L.E., Schulman, L.S., Baden, D.G., 1990. Toxin profiles are dependent on growth stages in cultures *Pyrodiscus brevis*. In: Graneli, E., Sundstrom, B., Elder, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton*. Elsevier, pp. 403–406.
- Roth, P.B., Twiner, M.J., Wang, Z., Dechraoui, M.B., Doucette, G.J., 2007. Fate and distribution of brevetoxin (PbTx) following lysis of *Karenia brevis* by algicidal bacteria, including analysis of open A-ring derivatives. *Toxicon* 50, 1175–1191.
- Schneider, K.R., Pierce, R.H., Rodrick, G.E., 2003. The degradation of *Karenia brevis* toxins utilizing ozonated seawater. *Harmful Algae* 2, 101–107.
- Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquac. Soc.* 21 (2), 65–104.
- Sipler, R.E., McGuinness, L.R., Kirkpatrick, G.J., Kerkhof, L.J., Schofield, O.M., 2014. Bacteriocidal effects of brevetoxin on natural microbial communities. *Harmful Algae* 38, 101–109.
- Steidinger, K.A., 2009. Historical perspective on *Karenia brevis* red tide research in the Gulf of Mexico. *Harmful Algae* 8 (4), 549–561.
- Stumpf, R.P., Culver, M.E., Tester, P.A., Tomlinson, M., Kirkpatrick, G.J., Pederson, B.A., Truby, E., Ransibrahmanakul, V., Soracco, M., 2003. Monitoring *Karenia brevis* blooms in the Gulf of Mexico using satellite ocean color imagery and other data. *Harmful Algae* 2, 147–160.
- Summerson, H., Peterson, C., 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Pyrodiscus brevis*, outbreak recorded in North Carolina. *Estuaries* 13 (3), 322–331 (Bottom of Form).
- Tatters, A.O., Muhlstein, H.I., Tomas, C.R., 2010. The hemolytic activity of *Karenia selliformis* and two clones of *Karenia brevis* throughout a growth cycle. *J. Appl. Phycol.* 22, 435–442.
- Tester, P.A., Shea, D., Kibler, S.R., Varnam, S.M., Black, M.D., Litaker, R.W., 2008. Relationships among water column toxins, cell abundance and chlorophyll concentrations during *Karenia brevis* blooms. *Cont. Shelf Res.* 28 (1), 59–72.
- Tester, P.A., Steidinger, K.A., 1997. *Gymnodinium breve* red tide blooms: Initiation, transport, and consequences of surface circulation. *Limnol. Oceanogr.* 42 (5), 1039–1051.
- Thompson, R.J., Newell, R.I.E., Kennedy, V.S., Mann, R., 1996. Reproductive processes and early development. In: Kennedy, V.S., Newell, R.I.E., Eble, A.F. (Eds.), *The Eastern Oyster: Crassostrea virginica*. Maryland Sea Grant College, College Park, Maryland, pp. 335–370.
- Thorson, G., 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biol. Rev.* 25 (1), 1–45.
- Volety, A.K., 2008. Effects of salinity, heavy metals and pesticides on health and physiology of oysters in the Caloosahatchee Estuary, Florida. *Ecotoxicol* 17, 579–590.
- Waite, A.M., Olson, R.J., Dam, H.G., Passow, U., 1995. Sugar- containing compounds on the cell surfaces of marine diatoms measured using concanavalin A and flow cytometry. *J. Phycol.* 31 (6), 925–933.
- Wang, L., Yan, T., Zhou, M., 2006. Impacts of HAB species *Heterosigma akashiwo* on early development of the scallop *Argopecten irradians* Lamarck. *Aquaculture* 255, 374–383.
- Wells, H.W., 1961. The fauna of oyster beds, with special reference to the salinity factor. *Ecol. Monogr.* 31 (3), 239–266.
- Yan, T., Zhou, M., Fu, M., Wang, Y., Yu, R., Li, J., 2001. Inhibition of egg hatching success and larvae survival of the scallop, *Chlamys farreri*, associated with exposure to cells and cell fragments of the dinoflagellate *Alexandrium tamarense*. *Toxicon* 39, 1239–1244.
- Yokote, M., Honjo, T., 1985. Morphological and histochemical demonstration of a glycocalyx on the cell surface of *Chattonella antiqua*, a 'naked flagellate'. *Experientia* 41 (9), 1143–1145.
- Zou, Y., Yamasaki, Y., Matsuyama, Y., Yamaguchi, K., Honjo, T., Oda, T., 2010. Possible involvement of hemolytic activity in the contact-dependent lethal effects of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. *Harmful Algae* 9, 367–373.

Discussion

Discussion

1. Introduction

The global increase in the occurrence, severity and range expansion of toxic algal blooms and, identification of new toxic algal species, are of worldwide concern due to the associated negative environmental and economic impacts (Hallegraeff, 1993; Shumway, 1990). The brevetoxin (PbTx) producing dinoflagellate, *Karenia brevis* is one such species, with blooms that cause human health, marine animal and wider reaching environmental and economic problems reportedly increasing in frequency (Brand and Compton, 2007; Landsberg, 2002; Poli et al., 2000). Eastern oysters (*Crassostrea virginica*) and hard clams (= northern quahog, *Mercenaria mercenaria*) are economically and environmentally important species not only along the Gulf coast of Florida but also, throughout their native range along the eastern coast of north America and around the world. These two bivalve species are frequently exposed to blooms of *K. brevis* along the SW FL coast which may result not only in NSP in humans and trophic transfer of PbTx, but also a reduction in essential ecosystem services (i.e. reduced filtration, nutrient cycling and secondary habitat for other species) and, negatively affect important fisheries, aquaculture and species restoration projects. Mixed reports on the lethal and sublethal effects of *K. brevis* blooms on bivalves have highlighted the need for further research into this area. Previous laboratory studies investigating the effects of *K. brevis* on adult bivalves have been relatively short- term and focused on behavioral and feeding responses of these species (Leverone, 2007; Leverone et al., 2007; Ray and Aldrich, 1967; Sievers, 1969). Reproductive and related physiological effects of longer-term sublethal *K. brevis* exposure on these 2 bivalve species (more realistic to how *C. virginica* and *M. mercenaria* are exposed in the field) were unknown. As the reproductive periods of these bivalve species overlap with blooms of *K. brevis*, these parameters may be affected. Spawning periods may be affected by *K. brevis* and bivalve gametes, embryos and early larval stages may also be directly exposed to *K. brevis*. Although previous work identified larval stages as vulnerable to exposure (Leverone et al., 2006), the underlying toxicological mechanism(s) remained elusive. This project aimed to determine the impacts of *K. brevis* on reproductive success and related physiological processes of these two bivalve species.

2. Adults

2.1 Toxin accumulation

Both adult *C. virginica* and *M. mercenaria* in this study accumulated high levels of PbTx following 10 week and 10 day laboratory exposures and long-term field exposure (Table 1). Experimental exposure of adults to bloom concentrations of *K. brevis* did not result in mortality of these species. Clams and oysters accumulated different amounts of PbTx when exposed under the same conditions (Table 1), agreeing with previous studies showing PbTx accumulation to differ between species (Bricelj et al., 2012; Griffith et al., 2013). Clams accumulated around four-fold less PbTx than did oysters (Table 1). Marine biotoxin assimilated by bivalves depends on several factors including toxic algal cell concentration, the duration of exposure, bivalve filtration/ingestion rates, bivalve species and population differences and even variation between individual bivalves from the same population (Bricelj et al., 2005; Bricelj and Shumway, 1998; Haberkorn et al., 2010, 2011).

Clams may assimilate and/ or depurate PbTx differently to oysters. Naar et al. (2007) recorded differences in PbTx assimilation in different fish tissues. A 'threshold' of PbTx accumulation was reached in the muscle tissue of planktivorous fish exposed to *K. brevis* but not in the viscera. This was thought either to be a 'saturation effect' or due to a change in the kinetics of accumulation in the muscle at higher *K. brevis* concentrations. Brevetoxin concentrations up to hundreds of thousands of ng g⁻¹ PbTx have been recorded in molluscs (species not specified) exposed naturally in the field (Landsberg, 2009). Further studies are required to establish if any maximum 'threshold' at which no more PbTx is accumulated exists.

Oysters may be less sensitive to PbTx than clams. Following *in vitro* exposure of nerves of different bivalve species (including *M. mercenaria* and *C. virginica*) to the voltage sensitive sodium channel (VSSC) blocking toxin, saxitoxin (STX), Twarog et al. (1972) determined that differences in PST accumulation among bivalve species were correlated with their sensitivity to STX. In their study, hard clam nerves were less sensitive to STX than eastern oyster nerves. This was hypothesized to be due to species specific binding characteristics of STX on binding site of the VSSC. It was postulated that although both species would be exposed to STX- producing *Alexandrium* sp. in the field, oysters had developed behavioral modifications (i.e. valve closures) to protect themselves from contact with toxic cells (Twarog et al., 1972).

Although not quantitatively measured in the present study, pseudofaeces production was noted for clams but not for oysters. Such selective strategies for limiting exposure to toxic algae (i.e. shell/ valve closure, pseudofaeces production, reducing filtration rate) have been shown for other bivalve and toxic algal species (i.e. Bricelj and Shumway, 1998; Gaaney and Shumway, 1988; Hégaret et al, 2007a; Shumway and Cucci, 1987; Shumway et al., 1985; Ward et al., 1997; Ward and Shumway, 2004). Indeed, Leverone et al. (2007) observed that juvenile *C. virginica* filtered significantly more whole-cells of *K. brevis* and T-Iso in 1 h than did juvenile *M. mercenaria*, agreeing with the suggestion that oysters may be less sensitive to PbTx than clams. As hypothesized by Twarog et al. (1972), this may be due to differing characteristics of PbTx binding/ different VSSC structure between species. Future electrophysiological studies testing the *in vitro* sensitivity of the nerves of *C. virginica* and *M. mercenaria* to PbTx would allude to this.

However, results of *in vitro* tests may not apply to the whole organism. Although hard clam nerves were insensitive to STX, Bricelj et al. (1991) found clams only accumulated low levels of PST and showed significant reductions in feeding and increased shell closure following exposure to highly toxic *Alexandrium* sp. Bivalves have a relatively simple bilaterally symmetrical nervous system composed of paired cerebral, visceral and pedal ganglia, and several pairs of nerves; however, we know little about the nervous system control of various tissue functions of *C. virginica* and *M. mercenaria* (Carroll and Catapane, 2007; Catapane, 1983; Paparo, 1986a,b). Leverone (2007) hypothesized that PbTx [which causes permanent activation of VSSC in nerve cells, disrupting nervous transmission] may suppress gill ciliary activity in the scallop (*A. irradians*) leading to a reduction of oxygen uptake and subsequently death. Previous studies have shown other neurotoxin producing algal species to have impacts on tissues controlled by the nervous system (i.e. Bricelj et al., 1996, 2005). Future studies investigating the effects of *K. brevis*/ PbTx exposure on tissue functions controlled by the nervous system (such as gill ciliary activity/ feeding processes) in bivalves using a video-endoscope may help determine if nervous transmission in the whole organism is affected. Multiple biotoxins from *K. brevis* in addition to PbTx may interact not only with nervous but also pulmonary and enzymatic regulatory systems. This may cause a combination of negative effects on organisms and complicate interpretation of any results (Baden et al., 2005; Fleming et al., 2011).

The high levels of PbTx accumulated in both oysters and to a lesser degree, clams, suggest a degree of insensitivity to PbTx; however, significant negative effects were recorded in adult tissues and in subsequently produced offspring (Table 1). This indicates that PbTx/ *K. brevis* cells/ other bioactive compounds do affect these bivalves.

2.2 Physiological effects

Significant inflammatory responses (such as hemocyte infiltration, diapedesis and occluded hemolymph vessels) and increases in the presence of edema and mucus were recorded in the tissues of clams and oysters exposed in the laboratory and in the field to bloom concentrations of *K. brevis* (Table 1).

Hemocyte infiltration is a commonly reported response to toxic algal exposure in bivalves (e.g. Wikfors and Smolowitz, 1993; Estrada et al., 2007; Galimany et al., 2008a,b,c; Hégaret et al., 2009; Haberkorn et al., 2010; Leverone, 2007; Lassudrie et al., 2014; Smolowitz and Shumway, 1997). Following a 2 week exposure of the bay scallop (*Argopecten irradians*) to whole- and lysed- cell preparations of *K. brevis* at 500 cells mL⁻¹, Leverone (2007) recorded a higher incidence of hemocytic infiltrations in the digestive diverticula in individuals either starved or exposed to lysed culture of *K. brevis*. A combination of poor nutrition and cytotoxicity were suggested as the reasons for observed effects. In the present study, although not showing signs of starvation (i.e. thin digestive tubules), control oysters showed an increase of diapedesis and hemocyte infiltration in the stomach/ intestine compared to the start of exposure (Chapter 1). This suggests that in addition to toxic algal exposure, experimental conditions may also have been stressful. Nonetheless, the recorded significant increase in the presence of hemocyte infiltration in the digestive gland of exposed oysters as compared to controls is consistent with the pathology reported by Leverone (2007) and suggests that exposure to whole cells of *K. brevis* and/ or associated toxins is causing an effect on oysters.

Hemocytes involved in the recorded inflammatory responses may be involved in toxin ingestion and removal (i.e. Franchini, 2003) or, repair of tissue damage, caused by exposure to toxins (i.e. Grzebyk et al., 1997; Hégaret, 2008; Wikfors and Smolowitz, 1993). Indeed the corresponding significant increase in total circulating

hemocytes as recorded in the 10 day oyster exposure (Table 1, Chapter 1) indicate that more new hemocytes may be produced and move to sites of inflammation within tissues or, are produced in tissues near the site of inflammation and are moving into circulation.

Differing to oysters, no effect on the hemocyte variables were recorded in the 10 day clam exposure (Table 1). The hemocytes of these species are morphologically different and may also differ in their functional abilities (Soudant et al., 2013). Previous studies have recorded significant pathological changes in the tissues of bivalves exposed to toxic algae yet comparatively little effect on hemocytes (da Silva et al., 2008; Hégaret et al., 2007b,c; Lassudrie et al., 2014). Circulating hemocytes contribute not only to immune functions but also, possibly more significantly, to homeostasis (Donaghy et al., 2009). For clams, homeostasis may not have been jeopardized and the cellular parameters of hemocytes were therefore not altered. Indeed, compared to oysters, overall fewer clam tissues were affected by exposure and fewer histological features were recorded (Table 1/ Chapter 1 and 2).

The tissues affected in the present study were those in direct contact with *K. brevis* cells and/ or associated toxins (mantle, gills, stomach/ intestine, connective tissue, Table 1). This suggests that effects may have been caused at least in part by contact with the *K. brevis* cell and/or associated toxins (PbTx) in *K. brevis* cultures. Both *K. brevis* and PbTx have been shown to cause negative effects *in vivo* and *in vitro* and it is possible that one or both are involved in the recorded negative effects herein. Indeed, in addition to intracellular PbTx, cells of *K. brevis* produce ichthyotoxic, allelopathic and hemolytic compounds (Kubanek et al., 2005, 2007; Marshall et al., 2005; Neeley and Campbell, 2006; Prince et al., 2010; Sipler et al., 2014; Tatters et al., 2010) and may also produce other as-yet uncharacterized bioactive compounds (Chapter 5) which could elicit deleterious effects in bivalve tissues. Although PbTx- 2 was shown not to be cytotoxic against *Crassostrea gigas* hemocytes following *in vitro* exposure (Mello et al., 2012), PbTx-1 was ichthyotoxic against Medaka fish (*Oryzias latipes*) embryos (Kimm- Brinson and Ramsdell, 2001), demonstrating that brevetoxin alone may cause deleterious impacts.

Mucus contains lytic enzymes and is produced both under normal physiological conditions and as a response to dissolved organic material and organic particles in

seawater (ie toxic algal exposure) (Brun et al., 2000; Fisher, 1992; Galimany et al., 2008a,b,c). Therefore, contact with cells of *K. brevis* and /or associated toxins may elicit such a response.

Once ingested, the *K. brevis* cells lyse during digestion, releasing intracellular PbTx. The proportion of intracellular to extracellular toxin is variable depending on the age of the *K. brevis* culture. Pierce et al. (2001) showed ~40% of toxin in cultures of *K. brevis* were intracellular. Thus, the stomach/ intestine may be exposed to a considerable increase in PbTx (compared to gill and mantle). This released PbTx is thought to be rapidly metabolized into many non-polar and polar compounds, the composition of which vary between species and in their toxicity (Abraham et al., 2006, 2012; Dickey et al., 1999; Plakas et al., 2002, 2004; Poli et al., 2000; Wang et al., 2004). Although the brevetoxin ELISA detects congeners with type 2 (PbTx-2) backbone structures (i.e. PbTx-2, PbTx-3, and metabolites, Naar et al., 2002), the exact PbTx metabolites nor the toxic potency of PbTx cannot be determined using this assay (Echevarria et al., 2012). From previous studies however, PbTx metabolites accumulated in clams have been shown to be non- toxic to fish (Naar et al., 2007). Indeed, PbTx accumulated and stored in oysters and clams may be a less potent form of PbTx (i.e. cysteine conjugates) than in culture. Yet, these conjugates are still capable of binding to the VSSC and as they are highly concentrated in tissues, may still affect these bivalves.

Therefore the present hemocytic, edematous and mucus responses in tissues may be caused by a combination of contact with *K. brevis* cells and associated toxins in culture, followed by additional exposure to previously intracellular PbTx when cells lyse in the stomach/ intestine and, further exposure to accumulated metabolites of PbTx (Figure 1).

Feeding bivalves with non-toxic algae 'infused' with PbTx may help determine if recorded pathologies are caused by exposure to other bioactives produced by *K. brevis*.

2.2.1 Feeding processes

Although this study was not designed to investigate differences in filtration/ digestion, our results indicate that in oysters, there may be modifications in these processes in response to *K. brevis* exposure. Oysters exposed to blooms of *K. brevis* for 10 days showed a significantly lower presence of food/ debris in the stomach/ intestine compared to controls (Chapter 1). This difference was not noted however in the longer 10 week exposure to 500 cells mL⁻¹ *K. brevis* (Chapter 1). Clams similarly exposed to high bloom concentrations of *K. brevis* for 10 days in contrast to oysters, showed no significant difference in the presence of food/ debris in the stomach/ intestine between treatments. Thus, the presence of food in the gut may differ between species, *K. brevis* concentration and duration of exposure. Toxic algal effects on the feeding processes of bivalves have previously been shown to differ between species (Hégaret et al., 2007a; Leverone et al., 2007; Shumway and Cucci, 1987).

Oysters exposed to *K. brevis* in the 10 day exposure may have fed more slowly than non-exposed. Leverone et al. (2007) found that the clearance rate of juvenile oysters exposed to *K. brevis* at 1000 cells mL⁻¹ for 1 hour was significantly reduced. Alternatively, the response may be a feedback loop, where oysters filter high amounts of *K. brevis* initially, and accumulate high concentrations of PbTx. Once a 'threshold' is reached however, they may slow or stop feeding. Oysters exposed over a 10 week period (in which these effects were not observed) may have acclimated to exposure or, there may be a 'threshold' *K. brevis*/ PbTx concentration at which negative effects are seen on the feeding process.

These possible changes warrant further investigation. The need for nutritional input (by feeding of harmful algal cells, and/or co-occurring, nutritious algae) may outweigh the cost of any harmful impacts from *K. brevis* (Hégaret, 2008). The alteration of feeding and digestive processes indicates that PbTx may not only cause inflammatory responses in bivalve tissues but, may also affect processes controlled by the nervous system. Previous exposure of these bivalve species to *K. brevis* has been short in duration (< 2 days; Ray and Aldrich, 1967; Sievers, 1969). Long-term exposures measuring filtration rate and pseudofaeces production with flow- through systems rather than the static exposure as used herein would allude to this.

2.3 Reproductive effects

Significant and non-significant trends were observed on reproductive parameters. Although non-significant (Table 1), the gonadal stage of clams and oysters exposed to high bloom concentrations of *K. brevis* over a 10 day and 10 week period (Chapters 1 and 2) and those exposed naturally to field blooms (Chapter 3) were lower than for non-exposed bivalves. For the longer 10 week oyster exposure (Chapter 1) and field exposures for both clams and oysters (Chapter 3), a non-significant (10 week) and significant (field) increase in the number of males was observed compared to non-exposed populations. Finally, significant (10 day oyster, Chapter 1) and non-significant (clam, Chapter 2; and oyster 10 week, Chapter 1) reductions in ripeness and spawning frequency were observed in exposed bivalves compared to non-exposed.

An increase in the male to female ratio supports the hypothesis of an energy cost/depletion in response to *K. brevis* exposure. The tissue and cellular changes recorded in the adult (i.e. increased hemocyte infiltration) may cause more energy to be channeled into tissue repair/homeostasis, making less energy available for reproduction. Davis and Hillmand (1971) observed an increase in the number of male *C. virginica*, the shells of which had been damaged by weekly filing, compared to non-injured oysters. The energy needed for shell repair was hypothesized to have caused a shift towards an excess of male oysters in injured populations.

Impacts on reproductive parameters in adults may also be due to the high level of PbTx accumulated within the bivalve. The brevetoxin metabolites accumulated in the bivalve may follow the flux of lipid from the digestive gland [where the majority of PbTx is thought to accumulate, Landsberg, 2002; Bricelj and Shumway, 1998] to the gonad (Figure 1, Barber and Blake, 1985). These metabolites (the majority of which are thought to be cysteine conjugates, Plakas et al., 2002, 2004) may be less potent/polar than parent toxins and other metabolites. Yet, these conjugates are still capable of binding to the VSSC and as they are highly concentrated in tissues, may still affect these bivalves.

Following ejection of gametes, further differences between offspring from exposed and non-exposed adults of both species were observed. These may be due to the transfer of PbTx (and other toxic compounds?) to the gametes. Between 16- 30% of the adult PbTx content was detected in sperm and eggs of clams and oysters

exposed to a field bloom of *K. brevis* (Chapter 3). The gametes and early life stages of bivalves are sensitive to environmental stressors (Carriker, 2001; Fitzpatrick et al., 2008; Thompson et al., 1996; Thorson, 1950). Differences in sperm parameters recorded following field exposure for both clams and oysters (Table 1, Chapter 3), and for the 10 day clam trial (including the later ejection of lesser quality sperm, Chapter 2) showed a significant reduction in ROS production. This may indicate a reduction in cellular respiration which may lead to low energy production, motility and, finally viability in sperm. Low energy (ATP) production and motility were observed by Haberkorn et al. (2010) following a 4-day continuous exposure of adult *C. gigas* to PST producing *A. minutum*. In the present study however, sperm were not significantly impacted so as to see corresponding changes in the mitochondrial membrane potential or viability.

A negative effect on the fertilization success was observed for exposed clams (Chapters 2 and 3) but not in oysters. This may be due to the higher concentration of PbTx estimated to be present per clam egg than per oyster egg. Clams may transfer comparatively more PbTx to eggs and/ or the larger size of clam eggs may mean comparatively more PbTx is accumulated compared to oyster eggs. However, this difference may also be due to the higher concentration of PbTx recorded in adult clams at the time of sampling from the field. We speculate that this PbTx in clam and oyster oocytes is associated with the lipid within the oocyte and is utilized by larvae during the lecithotrophic larval phase (Figure 1). These early life stages are vulnerable to abiotic and biotic stressors and the low body mass to high toxin load per embryo may disturb developmental processes.

Once fertilized, the embryos of both species undergo a period of high metabolic activity during cell division. Just as adults metabolize PbTx into a suite of conjugates, the active division of young stages may further alter the PbTx conjugates stored in the oocytes. These PbTx analogs/ conjugates may vary in potency and may be more bioavailable to the larvae. Furthermore, these early stages may not have developed detoxification systems to cope with newly metabolized PbTx.

Negative effects were recorded for up to 6 days following fertilization in oysters (Chapter 3) and 4 days for clams (Chapters 2 and 3). Differences in when these effects on larvae end could be due to the time these species utilize the parentally derived endogenous lipid (Figure 1). Gallager et al. (1986) found that *C. virginica* stop utilizing oocyte lipid reserves within 4 days of the first feeding, whereas lipid

droplets from parents of *M. mercenaria* larvae disappeared within 2 days of first feeding. This roughly fits with the length of the negative effect from the field trial (Chapter 3, Figure 1) for both species. Future studies assessing not just PbTx presence but, toxin profile and toxicity in oocytes, embryos and larvae throughout these first few days could confirm this.

As *K. brevis* blooms will likely continue into periods of spawning, the additional stress of direct exposure of gametes and early stages to *K. brevis* was also considered.

Discussion

Table 1: Summary of the physiological and reproductive effects on adult *Crassostrea virginica* and *Mercenaria mercenaria* exposed to *Karenia brevis*. NS= non-significant (p> 0.05), * p< 0.05, ** p< 0.01, *** p< 0.001. Popn.= population, CI= condition index, GS= gonadal stage, M/A/N= percent mortality, abnormality, normality, CT= connective tissue, PF= post-fertilization, ROS= reactive oxygen species production, comp.= complexity, conc.= concentration.

		Differences recorded after exposure														
Exposure duration	Ripeness at start of exposure	Popn.	Exposure conc. (cell mL ⁻¹)	PbTx accumulated (ng g ⁻¹)	CI	Ripe/spawn freq.	GS	Sex	Sperm	Fertilization	Larval M/A/N	Larval size	Histopathology		Hemocyte	
													Tissue	Effect		
<i>Crassostrea virginica</i>	10 days	Ripe	Estero Bay	1000, 5000	4645, 21880	NS	*	NS	NS	NS	NS	NS				
	10 days	Unripe	Iona Cove	1000, 5000	5535, 22009	NS							intestine, gill, mantle, CT	hemocyte infiltration, edema	* hemocyte concentration	
	10 weeks	Unripe	Iona Cove	500	10800	NS	NS	NS	NS	NS			intestine	hemocyte infiltration		
	Field (months)		Cedar Key/ Iona Cove	1- 150,000	922	**		NS	*	↓ ROS**, size*	NS	* to *** (6 Day PF)	* to *** (6 Day PF)	intestine, mantle	hemocyte infiltration, edema, mucus	
<i>Mercenaria mercenaria</i>	10 days	Ripe	Cedar Key	1000, 5000	1444, 5229	NS	NS	NS	NS	***	*** (4 Day PF)	NS	intestine	hemocyte infiltration		
	10 days	Ripe	Pine Island	1000, 5000	1228, 4604	NS	No spawn	NS					intestine, gill	hemocyte infiltration	NS	
	Field (months)		Cedar Key/ Pine Island	1- 150,000	1508	*		NS	**	↓ ROS**, comp.*	**	NS	** (1 Day PF)	intestine, gill	edema, mucus	

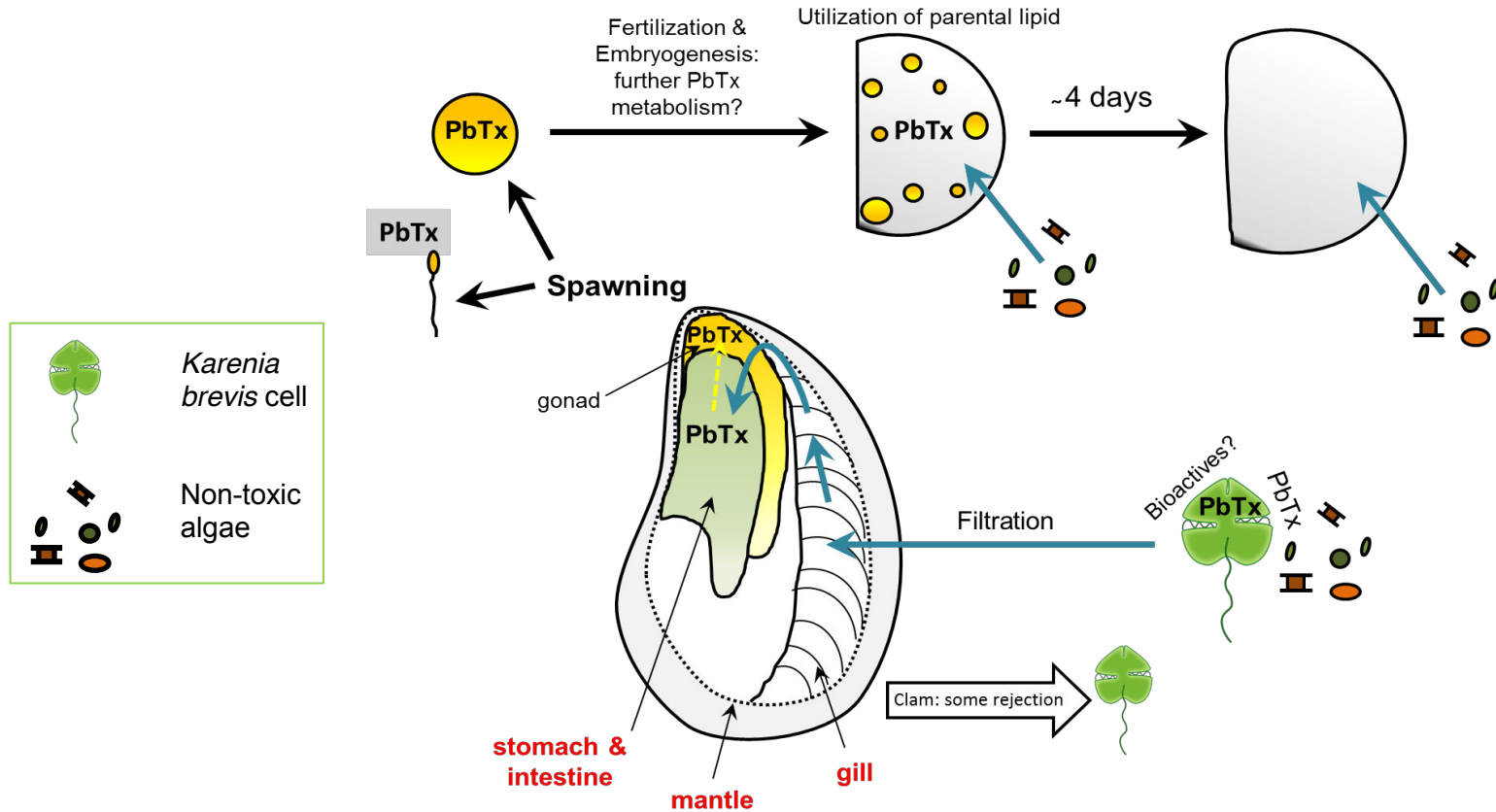


Figure 1: Proposed series of effects of *Karenia brevis* exposure on adult *Crassostrea virginica* and *Mercenaria mercenaria*. Bivalves filter *K. brevis* cells (there may be some rejection of *K. brevis* by *M. mercenaria*). Cells of *K. brevis*/ associated toxins are in contact with the **mantle** and **gills** before being passed into the **stomach and digestive areas** (—). In the stomach/intestine, *K. brevis* cells lyse releasing additional PbTx from within the cell. Contact with cells and associated toxins elicits negative effects on **these tissues**. PbTx may be metabolized and accumulated in the digestive gland. PbTx may follow the flux of lipid from the digestive gland to the gonad (---), where it is passed on to the oocytes and sperm. This may cause a decrease in sperm ROS production. PbTx (assumed to be inside) the oocyte is passed on to offspring and embryos and larvae continue to be negatively affected until they finish utilizing the lipid laid down in the oocyte from the mother (~2 (clam) and 4 days (oyster) after first- feeding). Higher PbTx concentrations in the oocyte may result in longer-term effects after the lecithotrophic phase.

3. Early stages

Exposure of the vulnerable early life stages of *C. virginica* and *M. mercenaria* to different cell preparations of *K. brevis* for 4 days caused a significant negative effect on their development (Figure 2). Exposure of 2- 4 cell stage embryos of *C. virginica* and *M. mercenaria* revealed dose- and time- dependent sub-lethal and lethal effects within 6 hours of exposure to whole cells of *K. brevis* at 3000 cells mL⁻¹ (Chapter 4). Clams were also significantly impacted by the lysed- cell preparation at this concentration. This is in agreement with a previous study exposing older larval stages of the same bivalve species (Leverone et al., 2006).

Neither contact with whole cells of *K. brevis*, nor ingestion of *K. brevis* cells was necessary to elicit deleterious effects on larvae. This contrasts with studies on later larval stages of these species exposed to the same cell preparations of *K. brevis*, in which ingestion of whole cells may have contributed to negative effects (Leverone et al., 2006).

Although both species responded slightly differently to exposure, neither species was determined to be more sensitive than the other. Mortality and abnormality data implied oysters were more sensitive, whereas mean larval shell size implied clams were more sensitive. These early stages may not yet have developed species specific processes/ functions to 'cope' with stressors which adults of both species may have.

Overall, lysed cells of *K. brevis* had a more pronounced negative effect on shell length, percent abnormality, and mortality in both clams and oysters than did whole cells; however, differences were modest and not always significant, which suggested the involvement of other bioactive compounds in addition to PbTx. Using the non-toxic 'Wilson' strain of *K. brevis*, Lauritano et al. (2013) and Turner et al. (2012) recorded an increase in the expression of stress genes and a reduction in egg hatching success of the Mediterranean copepod, *Calanus helgolandicus*, suggesting the involvement of other harmful metabolites.

In light of this, an additional exposure of gametes and embryos of *C. virginica* to whole-cell and culture filtrate of *K. brevis* at environmentally relevant concentrations (500 and 5000 cells mL⁻¹) was performed to further allude to the mechanism of toxicity (Chapter 5). Toxic effects were observed in the absence of cell membranes or fragments indicating that PbTx and other bioactives are free in the water.

The similar response of larvae exposed to whole-cell and culture filtrate of *K. brevis* after several days of exposure (when total brevetoxin, PbTx, concentration in the filtrate treatment was lower and more of the less potent PbTx metabolites were present) and between *C. virginica* exposed from before or after fertilization, suggested that negative effects of exposure mainly occur during embryogenesis and early development, agreeing with previous findings (Moon and Morrill, 1976, Figure 2).

Early stages exposed to higher concentrations (5000 cells mL⁻¹) showed no scope for recovery (Figure 2) indicating that irreversible damage may occur during embryonic gut development or act as a long-term feeding deterrent on non-toxic nutritious algae (Bricelj and MacQuarrie, 2007).

The viability of sperm exposed to the 5000 cell mL⁻¹, whole-cell *K. brevis* treatment was reduced more than that in the corresponding filtrate treatment following 2 h *in vitro* exposure of gametes (Figure 2). This suggests that contact with *K. brevis* cells does elicit additional negative effects (to the involvement of extracellular PbTx/ bioactives) and perhaps other harmful, chemically uncharacterized compounds are associated with the *K. brevis* cell membrane. Similarly Yan et al. (2001) revealed unknown toxins associated with the membrane of *Alexandrium tamarense* cells inhibited the egg hatching rate of *Chlamys farreri*. Other harmful compounds have been identified on the EPS layer of toxic algal cells (Bricelj and Lonsdale, 1997; Gaaney and Shumway, 1988; Parrish et al., 1998).

Fertilization success was significantly reduced in the same treatment of the 4 day exposure assay and is likely a consequence of reduced sperm viability (Figure 2). High individual variation was observed between batches of oocytes from different oysters, which may have masked any significant effects; however, sperm are motile and may encounter more *K. brevis* cells/ toxin than do oocytes and may be more vulnerable to exposure.

Identification of these as yet uncharacterized bioactives is needed to determine the respective contribution of PbTx and other bioactives to these negative effects on early (and adult) bivalve stages. As yet, identification of other harmful compounds has been elusive due to the often unstable nature of these compounds.

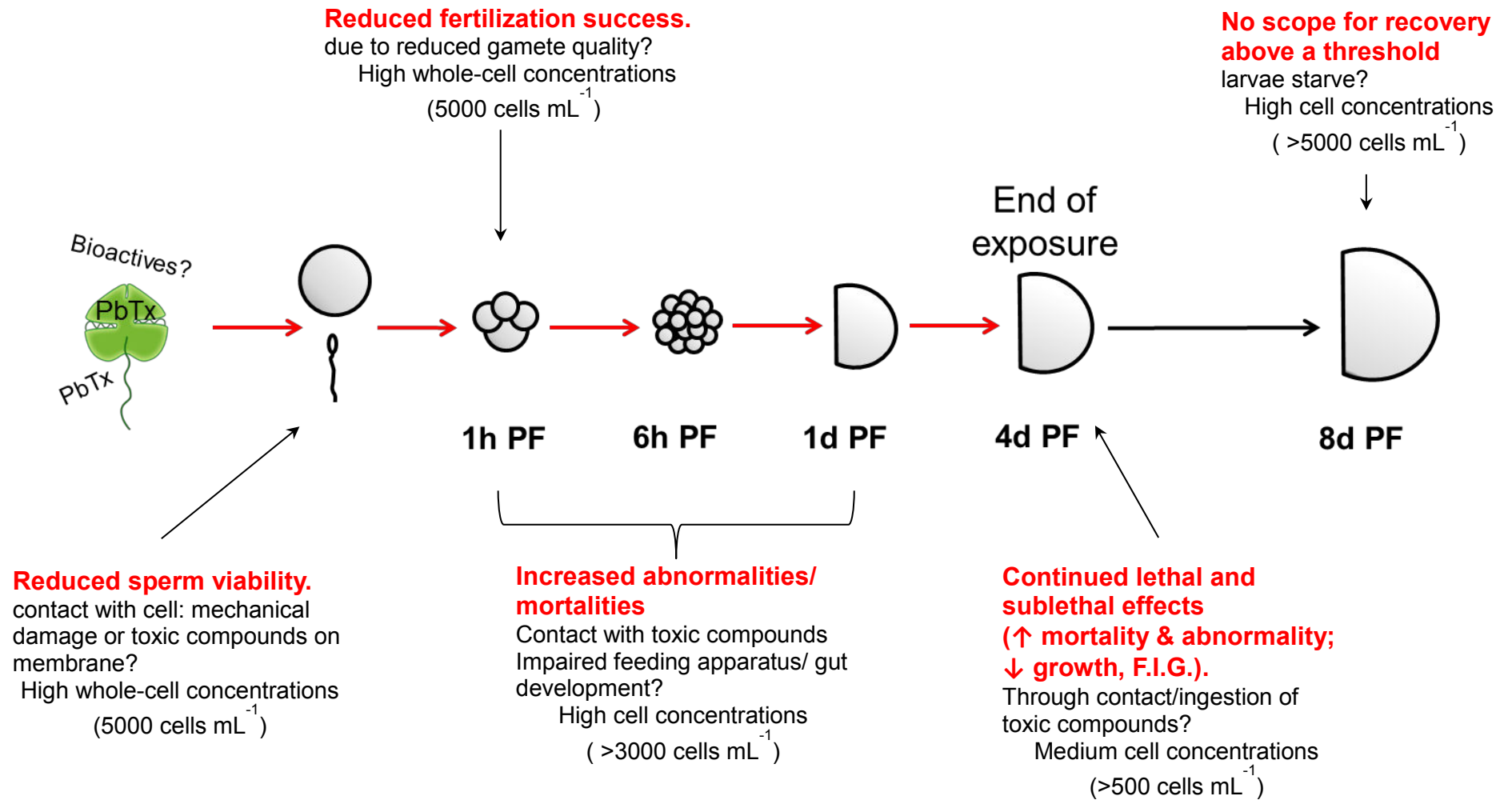


Figure 2: Effects of *Karenia brevis* exposure on gametes and early life stages of *Crassostrea virginica*, showing at what concentrations effects were recorded. F.I.G.= food in gut.

4. Summary

The accumulation of PbTx to such high levels in hard clams and eastern oysters indicates a degree of 'insensitivity'; yet, both species were significantly affected by direct and indirect exposure to *K. brevis*/ PbTx/ other associated bioactive compounds. Non-specific pathologies were recorded in adult clam and oyster tissues (mantle, gill and stomach/ intestine), indicating activation of defense mechanisms (mucus/ edema/ hemocyte infiltration), possibly in response to contact with *K. brevis* cells / PbTx / associated toxins and/ or accumulated PbTx.

Brevetoxin is considered a neurotoxin however, inflammatory responses, edema and mucus production recorded in adult tissues allude to an effect on processes not controlled by the nervous system. Indeed, pulmonary/ enzymatic systems may be affected by PbTx, and other bioactive compounds associated with *K. brevis* have been implicated in negative effects (Baden et al., 2005; Kubanek, 2005, 2007; Prince et al., 2010).

Results also alluded to possible behavioral and digestive responses in adults. Clams may exhibit pseudofaeces production to limit exposure to *K. brevis*. Exposure to *K. brevis* at different concentrations may cause a change in feeding/ digestion processes, dependent on bivalve species. These processes may be controlled by the nervous system.

The fecundity and resulting gamete and larval quality were reduced following exposure of adult *C. virginica* and *M. mercenaria* to bloom concentrations of *K. brevis*. The presence of PbTx in the sperm may have caused the observed reduction in ROS production (which may lead to reduced energy content of the sperm in both species). Adults transferred PbTx and possibly other toxins to offspring via the oocytes which may have caused significant negative impacts on larval development but, only up to the end of the phase at which larvae utilize lipid reserves in the oocyte laid down by the adult.

Following direct exposures on early life stages, effects on gametes and embryos were found to be dose dependent and suggest that other toxic compounds in

addition to PbTx are involved in toxicity. Sperm were found to be more susceptible to exposure, possibly due to increased contact with *K. brevis* cells/ toxin; however, the high variability between oocytes from different individuals may have masked any impacts on oocytes. Larvae of oysters exposed from gamete and early embryonic stages responded similarly in terms of sensitivity, implying that the majority of negative effects occur during embryonic divisions (developmental damage?). At lower *K. brevis* concentrations, larvae showed the potential to recover from sublethal effects.

5. Implications

Adult *C. virginica* and *M. mercenaria* are more susceptible to *K. brevis* exposure than previously realised. During persistently high bloom concentrations of *K. brevis*, reproductive and other physiological processes may be significantly compromised. Although, persistent high bloom concentrations are not as frequent as near-annual *K. brevis* blooms, their effects are much more severe. One severe bloom in North Carolina resulted in recruitment failure of the bay scallop, *A. irradians*, causing long-lasting effects (> 1 year) on population dynamics (Summerson and Peterson, 1990).

Direct exposure of early life stages to high bloom concentrations, even for a short time (< day), can cause cumulative effects of reduced sperm viability, fertilization success, embryonic and larval survival. These significant lethal and sub-lethal effects on early stages should be considered in addition to any negative effects which may have already occurred following adult exposure. However, caution should be taken with generalising these results on early life stages. High variation in the toxin profile and total PbTx (and other harmful compounds?) between different strains of *K. brevis* has been recorded (Lekan and Tomas, 2010; Loret et al., 2002) and may alter the effects recorded.

These findings should be considered when searching for suitable areas for oyster reef and scallop bed restoration projects in SW FL. Not only do adult oysters appear sensitive to exposure (Table 1), any successful restoration would involve reproduction in these newly established populations. Reproductive output and early life stages have both been shown to be negatively affected by *K. brevis* exposure.

Indeed, even brief exposure to higher concentrations of *K. brevis* may significantly affect larvae in direct contact with the bloom. Thus, restoration sites should be placed ideally in areas less prone to blooms and avoid enclosed bays and other places where cells could concentrate.

The valuable clam aquaculture industry in SW FL is already significantly affected by blooms of *K. brevis*. As farmed clams are produced in hatcheries, the negative effects of direct exposure on the vulnerable early stages during spawning may not have severe repercussions on early stage survival. Occasionally however, blooms of *K. brevis* do impact areas where hatchery production occurs. Using activated carbon filtration in these hatcheries would help remove PbTx from waters used for spawning and larval rearing (Pierce et al., 1992). The accumulation of high levels of PbTx in clams exposed to *K. brevis* and potential for persistence of PbTx in tissues (as shown in oyster tissue for ≥ 10 weeks, Chapter 1), will result in closure of clam harvest areas and therefore cause significant economic impacts to clam farmers. To help minimize these impacts, planting clams in lease areas frequented by *K. brevis* blooms should be avoided where possible. The joint use of leases in *K. brevis* free areas (such as Cedar Key) and/or depuration facilities in addition to leases in *K. brevis* 'affected' areas, would help ensure PbTx- free clams could be sold year-round.

Results herein highlight that bivalve responses to *K. brevis* exposure differ between bivalve species. Therefore the potential effects of *K. brevis* exposure on new species promising for aquaculture in SW FL, such as the Venus sunray clam (*Macrocallista nimbosa*), should be investigated.

6. Additional considerations

The impacts of additional environmental stressors should also be considered. Many microbiological interactions happen within the same individual and it is unknown how other stressors such as the oyster parasite *Perkinsus marinus* (abundant in SW FL) or environmental contaminants such as oil or heavy metal exposure (i.e. Haberkorn et al., 2014) could compound these *K. brevis* related effects. Previous studies have shown that parasites cause complex responses and may increase or decrease

susceptibility of bivalves to toxic algal exposure (da Silva et al., 2008; Hégaret et al., 2009; Lassudrie et al., 2014). The combined effects of *Prorocentrum minimum* and *Perkinsus olseni* exposure on adult Manila clams (*Ruditapes philippinarum*) led to increased negative impacts on the gonad compared with exposure of clams to *P. minimum* or *P. olseni* alone (Hégaret et al., 2009).

The persistence of lower levels of PbTx as shown in oyster tissues (Chapter 1), may also result in trophic transfer and bio-magnification of brevetoxins throughout the food web (Bricelj et al, 2012; Echevarria et al., 2012; Kirkpatrick et al., 2004; Landsberg et al, 2009; Naar et al, 2007; Poli et al., 2000). Chronic dietary exposure to brevetoxins has been shown to have lethal or sub-lethal effects at all trophic levels (Landsberg, 2002).

7. Conclusion

Hard clams and eastern oysters are more susceptible to *K. brevis* exposure than previously realised. This study has highlighted the importance of considering the entire reproductive process of bivalves in response to toxic algal exposure. During persistently high bloom concentrations of *K. brevis*, reproductive and related physiological processes in adults of both species may be significantly compromised. Direct exposure of early life stages to high bloom concentrations, even for a short time, can significantly reduce larval survival.

The identification/ confirmation of *K. brevis* associated bioactives is required to determine the relative contribution of PbTx and other bioactives to observed negative effects. The specific responses and multiple modes of action alluded to in this study require further research into the full spectrum of effects of PbTx and other bioactives on bivalves. Furthermore, biological interactions between the bivalve, *K. brevis* and other stressors (such as bacteria/parasite/heavy metal/oil) undoubtedly occur in nature and should be investigated.

References

- Abraham, A., Wang, Y., El Said, K. R., Plakas, S. M. 2012. Characterization of brevetoxin metabolism in *Karenia brevis* bloom-exposed clams (*Mercenaria* sp.) by LC-MS/MS. *Toxicon*. 60(6): 1030-1040.
- Abraham, A., Plakas, S.M., Wang, Z., Jester, E.L.E., El Said, K.R., Granade, H.R., Henry, M.S., Blum, P.C., Pierce, R.H., Dickey, R.W. 2006. Characterization of polar brevetoxin derivatives isolated from *Karenia brevis* cultures and natural blooms. *Toxicon*. 48(1): 104-115.
- Anderson, P. A., Roberts-Misterly, J., Greenberg, R. M. 2005. The evolution of voltage-gated sodium channels: were algal toxins involved?. *Harmful Algae*. 4(1): 95-107.
- Baden, D. G., Bourdelais, A. J., Jacocks, H., Michelliza, S., Naar, J. 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ. Health Persp.* 113(5): 621-625.
- Barber, B. J., Blake, N. J. 1985. Intra-organ biochemical transformations associated with oogenesis in the bay scallop, *Argopecten irradians concentricus* (Say), as indicated by ¹⁴C incorporation. *Biol. Bull.* 168(1): 39-49.
- Brand, L.E. Compton, A. 2007. Long-term increase in *Karenia brevis* abundance along the Southwest Florida Coast. *Harmful Algae*. 6(2): 232-252.
- Bricelj, V. M., Haubois, a. G., Sengco, M. R., Pierce, R. H., Culter, J. K., Anderson, D. M. 2012. Trophic transfer of brevetoxins to the benthic macrofaunal community during a bloom of the harmful dinoflagellate *Karenia brevis* in Sarasota Bay, Florida. *Harmful Algae*. 16: 27–34.
- Bricelj, V.M., MacQuarrie, S.P., 2007. Effects of brown tide (*Aureococcus anophagefferens*) on hard clam *Mercenaria mercenaria* larvae and implications for benthic recruitment. *Mar. Ecol. Prog. Ser.* 331: 147-159.
- Bricelj, V. M., Connell, L., Konoki, K., MacQuarrie, S.P., Scheuer, T., Catterall, W.A., Trainer, V.L. 2005. Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP. *Nature*. 434(7034): 763-767.
- Bricelj, V. M., Shumway, S. E. 1998. Paralytic Shellfish Toxins in Bivalve Molluscs: Occurrence, Transfer Kinetics, and Biotransformation. *Rev. Fish. Sci.* 6(4): 315–383.
- Bricelj, V. M., Lonsdale, D. J. 1997. *Aureococcus anophagefferens*: causes and ecological consequences of brown tides in US mid-Atlantic coastal waters. *Limnol. Oceanogr.* 42(5): 1023-1038.
- Bricelj, V. M., Cembella, A., Laby, D., Shumway, S. E., Cucci, T. L. 1996. Comparative physiological and behavioral responses to PSP toxins in two bivalve molluscs, the softshell clam, *Mya arenaria*, and surfclam, *Spisula solidissima*. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), *Harmful and Toxic Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO, Paris. pp 405-408.
- Bricelj, V. M., Lee, J. H., Cembella, A. 1991. Influence of dinoflagellate cell toxicity on uptake and loss of paralytic shellfish toxins in the northern quahog *Mercenaria mercenaria*. *Mar. Ecol. Prog. Ser.* 74: 33-46.
- Brun, Nicole T., Neil W. Ross, and Andrew D. Boghen. 2000. Changes in the electrophoretic profiles of gill mucus proteases of the eastern oyster *Crassostrea virginica* in response to infection by the Turbellarian *Urastoma cyprinae*. *J. Invert. Pathol.* 75(2): 163-170.

- Carriker, M.R., 2001. Embryogenesis and organogenesis of veligers and early juveniles. In: Kraeuter, J. N., Castagna, M. (Eds), *Biology of the hard clam*. Vol. 31. Gulf Professional Publishing. pp 77- 115.
- Carroll, M. A., Catapane, E. J. 2007. The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comp. Biochem. Phys. A*. 148(2): 445-450.
- Catapane, E.J., 1983. Comparative study of the lateral ciliary activity in marine bivalves. *Comp. Biochem. Physiol. C*. 75: 403–405.
- da Silva, P. M., Hégaret, H., Lambert, C., Wikfors, G. H., Le Goïc, N., Shumway, S. E., Soudant, P. 2008. Immunological responses of the Manila clam (*Ruditapes philippinarum*) with varying parasite (*Perkinsus olseni*) burden, during a long-term exposure to the harmful alga, *Karenia selliformis*, and possible interactions. *Toxicon*. 51(4): 563–73.
- Davis, N. W., Hillman, R. E. 1971. Effect of artificial shell damage on sex determination in oysters. *Proc. Natl. Shellfish Assoc.* 61: 2.
- Dickey, R.E., Jester, R.G., Mowdy, D., Moncreiff, C., Rebarchik, D., Robl, M., Musser, S., Poli, M. 1999. Monitoring brevetoxins during a *Gymnodinium breve* red tide: comparison of sodium channel specific cytotoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. *Natural Toxins*. 7(4): 157-165.
- Donaghy, L., Lambert, C., Choi, K. S., Soudant, P. 2009. Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): Current knowledge and future prospects. *Aquaculture*. 297(1): 10-24.
- Echevarria, M., Naar, J.P., Tomas, C., Pawlik, J.R. 2012. Effects of *Karenia brevis* on clearance rates and bioaccumulation of brevetoxins in benthic suspension feeding invertebrates. *Aquat. Toxicol.* 106: 85-94.
- Estrada, N., de Jesús Romero, M., Campa-Córdova, A., Luna, A., Ascencio, F. 2007. Effects of the toxic dinoflagellate, *Gymnodinium catenatum* on hydrolytic and antioxidant enzymes, in tissues of the giant lions-paw scallop *Nodipecten subnodosus*. *Comp. Biochem. Phys. C*. 146(4): 502–510.
- Fisher, W. S. 1992. Occurrence of agglutinins in the pallial cavity mucus of oysters. *J. Exp. Mar. Biol. Ecol.* 162(1): 1–13.
- Fitzpatrick, J.L, Nadella, S., Bucking, C., Balshine, S., Wood, C.M. 2008. The relative sensitivity of sperm, eggs and embryos to copper in the blue mussel (*Mytilus trossulus*). *Comp. Biochem. Physiol., Part C*. 147: 441–449.
- Fleming, L. E., Kirkpatrick, B., Backer, L. C., Walsh, C. J., Nierenberg, K., Clark, J., Reich, A., Hollenbeck, J., Benson, J., Cheng, Y.S., Naar, J., Pierce, R., Bourdelais, A.J., Abraham, W.M., Kirkpatrick, G., Zaias, J., Wanner, A., Mendes, E., Shalat, S., Hoagland, P., Stephan, W., Bean, J., Watkins, S., Clarke, T., Byrne, M., Baden, D. G. 2011. Review of Florida Red Tide and Human Health Effects. *Harmful Algae*. 10(2): 224–233.
- Franchini, A., Milandri, A., Poletti, R., Ottaviani, E. 2003. Immunolocalization of yessotoxins in the mussel *Mytilus galloprovincialis*. *Toxicon*. 41: 967–970.
- Gainey, L. F., Shumway, S. E., 1988. A compendium of the responses of bivalve molluscs to toxic dinoflagellates. *J. Shellfish Res.* 7(4): 623-628.

- Galimany, E., Place, A. R., Ramón, M., Jutson, M., Pipe, R. K. 2008a. The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*. *Harmful Algae*. 7(1): 91–98.
- Galimany, E., Sunila, I., Hégaret, H., Ramón, M., Wikfors, G. H. 2008b. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: Histopathology, immune responses, and recovery. *Harmful Algae*. 7(5): 702–711.
- Galimany, E., Sunila, I., Hégaret, H., Ramón, M., Wikfors, G. H. 2008c. Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. *Harmful Algae*. 7(5): 630–638.
- Gallager, S. M., Mann, R., Sasaki, G. C. 1986. Lipid as an index of growth and viability in three species of bivalve larvae. *Aquaculture*. 56(2): 81-103.
- Griffith, A. W., Shumway, S. E., Volety, A. K. 2013. Bioaccumulation and depuration of brevetoxins in the eastern oyster (*Crassostrea virginica*) and the northern quahog (= hard clam, *Mercenaria mercenaria*). *Toxicon*. 66: 75–81.
- Grzebyk, D., Denardou, A., Berland, B., Pouchus, Y. F. 1997. Evidence of a new toxin in the red-tide dinoflagellate *Prorocentrum minimum*. *J. Plankton Res.* 19(8): 1111-1124.
- Haberkorn, H., Lambert, C., Le Goïc, N., Quéré, C., Bruneau, A., Riso, R., Auffret, M. Soudant, P. 2014. Cellular and biochemical responses of the oyster *Crassostrea gigas* to controlled exposures to metals and *Alexandrium minutum*. *Aquatic Toxicology*, 147, 158-167.
- Haberkorn, H., Tran, D., Massabuau, J. C., Ciret, P., Savar, V., Soudant, P. 2011. Relationship between valve activity, microalgae concentration in the water and toxin accumulation in the digestive gland of the Pacific oyster *Crassostrea gigas* exposed to *Alexandrium minutum*. *Mar. Pollut. Bull.* 62(6): 1191-1197.
- Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P. 2010. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. *Harmful Algae*. 9(5): 427–439.
- Hallegraeff, G. M. 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia*. 32(2): 79-99.
- Hégaret, H., da Silva, P.M., Sunila, I., Shumway, S.E., Dixon, M.S., Alix, J., Wikfors, G.H., Soudant, P. 2009. Perkinsosis in the Manila clam *Ruditapes philippinarum* affects responses to the harmful-alga, *Prorocentrum minimum*. *J. Exp. Mar. Biol. Ecol.* 371(2): 112-120.
- Hégaret, H. 2008. Impacts of harmful algal blooms on physiological and cellular processes of bivalve molluscs. ProQuest.
- Hégaret, H., Wikfors, G. H., Shumway, S. E. 2007a. Diverse feeding responses of five species of bivalve mollusc when exposed to three species of harmful algae. *J. Shellfish Res.* 26(2): 549-559.
- Hégaret, H., da Silva, P. M., Wikfors, G. H., Lambert, C., De Bettignies, T., Shumway, S. E., Soudant, P. 2007b. Hemocyte responses of Manila clams, *Ruditapes philippinarum*, with varying parasite, *Perkinsus olseni*, severity to toxic-algal exposures. *Aquat. Toxicol.* 84(4): 469–479.

- Hégaret, H., Wikfors, G. H., Soudant, P., Lambert, C., Shumway, S. E., Bérard, J. B., Lassus, P. 2007c. Toxic dinoflagellates (*Alexandrium fundyense* and *A. catenella*) have minimal apparent effects on oyster hemocytes. *Mar. Biol.* 152(2): 441-447.
- Jones, J. B., Crockford, M., Creeper, J., Stephens, F. 2010. Histopathology of oedema in pearl oysters *Pinctada maxima*. *Dis. Aquat. Organ.* 91(1): 67-73.
- Kimm-Brinson, K. L., Ramsdell, J. S. 2001. The red tide toxin, brevetoxin, induces embryo toxicity and developmental abnormalities. *Environ. Health Persp.* 109(4): 377- 381.
- Kirkpatrick, B., Fleming, L.E., Squicciarini, D., Backer, L.C., Clark, R., Abraham, W., Benson, J., Cheng, Y.S., Johnson, D., Pierce, R., Zaias, J., Bossart, G.D., Baden, D.G. 2004. Literature review of Florida red tide: implications for human health effects. *Harmful algae.* 3(2): 99-115.
- Kubanek, J., Hicks, M.K., Naar, J., Villareal, T.A. 2005. Does the red tide dinoflagellate *Karenia brevis* use allelopathy to outcompete other phytoplankton? *Limnol. Oceanogr.* 50(3): 883-895.
- Kubanek, J., Snell, T.W., Pirkle, C. 2007. Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing. *Limnol. Oceanogr.* 52(3): 1026-1035.
- Landsberg, J. H., Flewelling, L. J., Naar, J. 2009. *Karenia brevis* red tides, brevetoxins in the food web, and impacts on natural resources: Decadal advancements. *Harmful Algae.* 8(4): 598-607.
- Landsberg, J.H. 2002. The Effects of Harmful Algal Blooms on Aquatic Organisms. *Rev. Fish. Sci.* 10(2): 113-390.
- Lassudrie, M., Soudant, P., Richard, G., Henry, N., Medhioub, W., da Silva, P. M., Donval, A., Bunel, M., Le Goïc, N., Lambert, C., de Montaudouin, X., Fabioux, C., Hégaret, H. 2014. Physiological responses of Manila clams *Venerupis (=Ruditapes) philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure. *Aquat. Toxicol.* 154: 27-38.
- Lauritano, C., Carotenuto, Y., Procaccini, G., Turner, J. T., Ianora, A. 2013. Changes in expression of stress genes in copepods feeding upon a non-brevetoxin-producing strain of the dinoflagellate *Karenia brevis*. *Harmful Algae.* 28: 23-30.
- Lekan, D.K., Tomas, C.R., 2010. The brevetoxin and brevenal composition of three *Karenia brevis* clones at different salinities and nutrient conditions. *Harmful Algae.* 9(1): 39-47.
- Leverone, J. R. 2007. Comparative effects of the toxic dinoflagellate, *Karenia brevis*, on bivalve molluscs from Florida (Doctoral dissertation, University of South Florida).
- Leverone, J. R., Shumway, S. E., Blake, N. J. 2007. Comparative effects of the toxic dinoflagellate *Karenia brevis* on clearance rates in juveniles of four bivalve molluscs from Florida, U.S. *Toxicon.* 49(5): 634-645.
- Leverone, J. R., Blake, N. J., Pierce, R. H., Shumway, S. E. 2006. Effects of the dinoflagellate *Karenia brevis* on larval development in three species of bivalve mollusc from Florida. *Toxicon.* 48: 75-84.
- Loret, P., Tengs, T. Villareal, T. A. Singler, H. Richardson, B. McGuire, P. Morton, S. Busman, M. Campbell, L. 2002. No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. *J. Plankton Res.* 24(7): 735-739.

- Marshall, J., Ross, T., Pyecroft, S., Hallegraef, G., 2005. Superoxide production by marine microalgae 2: Towards understanding ecological consequences and possible functions. *Mar. Biol.* 147: 541–549.
- Mello, D. F., De Oliveira, E. S., Vieira, R. C., Simoes, E., Trevisan, R., Dafre, A. L., Barracco, M. A. 2012. Cellular and transcriptional responses of *Crassostrea gigas* hemocytes exposed in vitro to brevetoxin (PbTx-2). *Mar. Drugs.* 10(3): 583-597.
- Moon, R.T., Morrill, J.B. 1976. The effects of *Gymnodinium breve* lysate on the larval development of the sea urchin *Lytechinus variegatus*. *J. Environ. Sci. Health A.* 11:673–683.
- Naar, J. P., Flewelling, L.J., Lenzi, A., Abbott, J.P., Granholm, A., Jacocks, H.M., Gannon, D., Henry, M., Pierce, R., Baden, D.G., Wolny, J., Landsberg, J.H. 2007. Brevetoxins, like ciguatoxins, are potent ichthyotoxic neurotoxins that accumulate in fish. *Toxicon.* 50(5): 707-723.
- Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P. L., Flewelling, L., Steidinger, K., Lancaster, J., Baden, D. G. 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. *Environ. Health Perspect.* 110(2): 179–185.
- Neely, T., Campbell, L. 2006. A modified assay to determine hemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae.* 5(5): 592–598.
- Paparo, A., 1986a. Neuroregulatory activities and potassium enhancement of lateral ctenidial beating in *Crassostrea virginica*. *Comp. Biochem. Physiol. A* 84: 585–588.
- Paparo, A., 1986b. DOPA decarboxylase activities and potassium stimulation of lateral cilia on the ctenidium of *Crassostrea virginica* (Gmelin). *J. Exp. Mar. Biol. Ecol.* 95: 225–231.
- Parrish, C. C., Bodennec, G., Gentien, P. 1998. Haemolytic glycolipids from *Gymnodinium* species. *Phytochemistry.* 47(5): 783–787.
- Pierce, R., Henry, M., Blum, P., Payne, S., 2001. *Gymnodinium breve* toxins without cells: intra-cellular and extra-cellular toxins. In: Hallegraef, G., Blackburn, S., Bolch, C., Lewis, R. (Eds.), *Harmful Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO, pp. 421–424.
- Pierce, R. H., Henry, M. S., Proffitt, L. S., de Rosset, A. J. 1992. Evaluation of Solid Sorbents for the Recovery of Polyether Toxins (Brevetoxins) in Seawater. *Bull. Environ. Contain. Toxicol.* 49:479-484.
- Plakas, S.M., Wang, Z.H., El Said, K.R., Jester, E.L.E., Granade, H.R., Flewelling, L., Scott, P., Dickey, R.W. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. *Toxicon.* 44: 677–685.
- Plakas, S.M., El Said, K.R., Jester, E.L.E., Granade, H.R., Musser, S.M., Dickey, R.W. 2002. Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. *Toxicon.* 40: 721–729.
- Poli, M. A., Musser, S. M., Dickey, R. W., Eilers, P. P., Hall, S. 2000. Neurotoxic shellfish poisoning and brevetoxin metabolites: A case study from Florida. *Toxicon.* 38(7): 981–993.
- Prince, E. K., Poulson, K. L., Myers, T. L., Sieg, R. D., Kubanek, J. 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. *Harmful Algae.* 10(1): 39–48.

- Ray, S. M., Aldrich, D.V. 1967. Ecological interactions of toxic dinoflagellates and molluscs in the Gulf of Mexico. In: Russell, F., Saunders, P. (Eds.). *Animal Toxins*. Oxford: Pergamon. pp. 75–83.
- Shumway, S.E. 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquac. Soc.* 21 (2): 65–104.
- Shumway, S. E., Cucci, T. L. 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. *Aquat. Toxicol.* 10(1): 9-27.
- Shumway, S. E., Cucci, T. L., Gainey, L., Yentsch, C. M. 1985. A preliminary study of the behavioral and physiological effects of *Gonyaulax tamarensis* on bivalve molluscs. *Toxic Dinoflagellates*. 389-394.
- Sievers, A. M. 1969. Comparative toxicity of *Gonyaulax monilata* and *Gymnodinium breve* to annelids, crustaceans, molluscs and a fish. *J. Protozool.* 16(3): 401-404.
- Sipler, R. E., McGuinness, L. R., Kirkpatrick, G. J., Kerkhof, L. J., Schofield, O. M. 2014. Bacteriocidal effects of brevetoxin on natural microbial communities. *Harmful Algae*. 38: 101-109.
- Smolowitz, R., Shumway, S. E. 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve molluscs. *Aquacult. Int.* 5(4): 291–300.
- Soudant, P., Chu, F. L. E., Volety, A. 2013. Host–parasite interactions: Marine bivalve molluscs and protozoan parasites, Perkinsus species. *J. Invert. Pathol.* 114(2): 196-216.
- Summerson, H., Peterson, C., 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Ptychodiscus brevis*, outbreak recorded in North Carolina. *Estuaries*. 13(3): 322–331.
- Tatters, A.O, Muhlstein, H.I., Tomas, C.R. 2010. The hemolytic activity of *Karenia selliformis* and two clones of *Karenia brevis* throughout a growth cycle. *J. Appl. Phycol.* 22: 435–442.
- Thompson, R.J., Newell, R.I.E., Kennedy, V.S., Mann, R. 1996. Reproductive processes and early development. In: Kennedy, V.S., Newell, R.I.E., Eble, A.F. (Eds.), *The eastern oyster: Crassostrea virginica*, Maryland Sea Grant College, College Park, Maryland. pp 335-370.
- Thorson, G. 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biol. Rev.* 25(1): 1-45.
- Turner, J.T., Roncalli, V., Camarillo, P., Dell'Aversano, C., Fattorusso, E., Tartaglione, L., Carotenuto, Y., Romano, G., Esposito, F., Miralto, A., Ianora, A., 2012. Biogeographic effects of the Gulf of Mexico red tide dinoflagellate *Karenia brevis* on Mediterranean copepods. *Harmful Algae*. 16: 63–73.
- Twarog, B. M., Hidaka, T., Yamaguchi, H. 1972. Resistance to tetrodotoxin and saxitoxin in nerves of bivalve molluscs: a possible correlation with paralytic shellfish poisoning. *Toxicon*. 10(3): 273-278.
- Wang, Z., Plakas, S. M., El Said, K. R., Jester, E. L. E., Granade, H. R., Dickey, R. W. 2004. LC/MS analysis of brevetoxin metabolites in the Eastern oyster (*Crassostrea virginica*). *Toxicon*. 43(4): 455–65.
- Ward, J. E., Levinton, J. S., Shumway, S. E., Cucci, T. 1997. Site of particle selection in a bivalve mollusc. *Nature*. 390(6656): 131-132.

Discussion

Ward, J. E., Shumway, S. E. 2004. Separating the grain from the chaff: particle selection in suspension-and deposit-feeding bivalves. *Journal of Experimental Marine Biology and Ecology*. 300(1): 83-130.

Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E., Kinne, R. K. H. 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev. Physiol. Biochem. Pharmacol.* 148: 1–80.

Wikfors, G. H., Smolowitz, R. M. 1993. Detrimental effects of a *Prorocentrum* isolate upon hard clams and bay scallops in laboratory feeding studies. *Dev. Mar. Biol.* 1993.

Yan, T., Zhou, M., Fu, M., Wang, Y., Yu, R., Li, J. 2001. Inhibition of egg hatching success and larvae survival of the scallop, *Chlamys farreri*, associated with exposure to cells and cell fragments of the dinoflagellate *Alexandrium tamareense*. *Toxicon*. 39: 1239-1244.

Publications and communications

Publications

- **Rolton**, A., Soudant, P., Vignier, J., Pierce, R., Henry, M., Shumway, S. E., Bricelj, V. M., Volety, A. K. submitted. Susceptibility of gametes and embryos of the eastern oyster, *Crassostrea virginica*, to *Karenia brevis* and its toxins. *Toxicon*.
- **Rolton**, A., Vignier, J., Soudant, P., Shumway, S. E., Bricelj, V. M., and Volety, A. K. (2014). Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and northern quahog *Mercenaria mercenaria*. *Aquatic Toxicology* 155: 199–206
- Treasurer, J., Atack, T., **Rolton**, A., Walton, J., Bickerdike, R. 2011. Social, stocking density and dietary effects on the failure of farmed cod, *Gadus morhua*. *Aquaculture*. 322: 241-248.

Foreseen Publications

- **Rolton**, A., Vignier, J., Soudant, P., Donaghy, L., Shumway, S., Bricelj, V.M., Volety, A.K. In prep a. Effects of *Karenia brevis* exposure on the reproductive and related physiological processes of *Crassostrea virginica*.
- **Rolton**, A., Vignier, J., Soudant, P., Donaghy, L., Shumway, S., Bricelj, V.M., Volety, A.K. In prep b. Effects of *Karenia brevis* exposure on adult *Mercenaria mercenaria*: Histopathological responses in adults and effects on gamete and larval quality.

Communications

- **Rolton**, J. Vignier, A. Volety, L. Donaghy, S. Shumway, M. Bricelj, R. Pierce, M. Henry and P. Soudant (2013) Effects of *Karenia brevis* exposure on the reproductive and related physiological processes of *Crassostrea virginica*. PHYCOTOX, Brest, France, March 31st – April 2nd, 2015.
- **Rolton**, J. Vignier, A. Barbe, P. Soudant, R. Pierce, S. Shumway, M. Bricelj and A. Volety (2013) The effects of field exposure of *Crassostrea virginica* to *Karenia brevis*: Histopathology and brevetoxin accumulation in gametes. 7th Symposium on harmful Algae, Sarasota, Florida, USA, Oct 27-31st 2013.
- **Rolton**, J. Vignier, P. Soudant, A. Volety, L. Donaghy and S. Shumway (2013) Effects of *Karenia brevis* exposure on the physiology and reproductive performance of *Crassostrea virginica* and the quality of gametes that are produced. *Aquaculture* 2013, Nashville, Tennessee, USA, Feb 21-25th 2013.

- **Rolton**, L. Donaghy, N. Le Goïc, P. Soudant and A. Volety (2012) Effects of the toxic dinoflagellate *Karenia brevis* on the cellular physiology of sperm and hemocytes from *Mercenaria mercenaria* and *Crassostrea virginica*. 15th International conference on harmful algae, Changwon Gyeongnam, South Korea, Oct 29th- Nov 2nd 2012.
- **Rolton**, P. Soudant, A. Volety, S. Shumway and M. Bricelj (2012) Impacts of the redtide organism *Karenia brevis* on the early life stages of oysters *Crassostrea virginica* and clams *Mercenaria mercenaria*. 104th National Shellfisheries association meeting, Seattle, Washington, USA, March 25-29th 2012.
- Volety, J. Vignier, K. McEachern, J.P. Roberts, E. Nickols, A. **Rolton**, L. Haynes, P. Soudant, F.L. Chu, J.M. Morris, M.W. Carney, J. Lipton, D. Cacela, M. Krasnec, C. Lay, Stratus Consulting Inc., Boulder, Colorado. Impacts of Deepwater Horizon oil and dispersants on the metamorphosis and settlement success of Eastern oysters *Crassostrea virginica*. SETAC 2014. Vancouver.
- J. Vignier, A. **Rolton**, A. Volety, R. Robert, P. Soudant, S. LeFloch, J. Guyomarch, F. L. Chu, J. M. Morris, M. W. Carney, J. Lipton, D. Cacela, C. Lay, M. Krasnec. (2014) Sub-lethal effects of Deepwater Horizon oil on *Crassostrea virginica* larvae using dietary pathways. EAS 2014, San Sebastian, Spain, 14-17 October 2014.
- J. Vignier, R. Ketover, J. Divine, A. **Rolton**, A. Volety, R. Robert, P. Soudant, S. LeFloch, J. Guyomarch, F.L. Chu, J. M. Morris, M. W Carney, J. Lipton, D. Cacela, C. Lay, M. Krasnec. (2013) Sub-lethal effects of DWH oil on *Crassostrea virginica* using dietary pathways. Symposium on Environmental Toxicity and Chemistry, SETAC 2013, Nashville, TN, U.S.A. 17- 21 November 2013.

Other

- Session chair for 'General Shellfish contributed', Aquaculture 2013, Nashville, Tennessee, USA, Feb 21-25th 2013.
- Winner of the Thurlow C. Nelson Award, given for the outstanding oral presentation of research that represents a distinctive and valuable contribution to shellfisheries science at NSA/WAS 2013 in Nashville, TN, U.S.A.

Impacts of *Karenia brevis* on bivalve reproduction and early life history

Anne ROLTON

Abstract

The brevetoxin (PbTx) producing dinoflagellate, *Karenia brevis* is the most prevalent harmful algal bloom species in the Gulf of Mexico. The effects of this alga on *Mercenaria mercenaria* and *Crassostrea virginica* are poorly understood yet, blooms typically overlap with periods of reproduction and spawning in these species.

The aims of this project were to determine the effects of i) laboratory and field exposure of *K. brevis* on the reproductive and related physiological processes of adult *M. mercenaria* and *C. virginica* and the quality of the offspring that were produced and ii) *K. brevis* exposure on gamete, embryo and larval development in these species.

Following exposure of adult clams and oysters to *K. brevis*, negative effects were recorded on reproductive and physiological parameters. PbTx was recorded in gamete tissues, and maternal transfer of this PbTx to the offspring via the oocytes, may have resulted in the significant negative effects recorded on larval development up to the end of the lecithotrophic phase.

The similar dose-dependent negative effects caused by direct exposure of gamete and early life stages to different cell preparations of *K. brevis* suggests that other toxic compounds in addition to PbTx may be involved in toxicity and, that the majority of negative effects occur during embryonic divisions.

Hard clams and eastern oysters are susceptible to *K. brevis* exposure. The negative effects on adult and early life stages combined with the near- annual exposure to blooms of *K. brevis* could cause significant bottle-necks on the recruitment and population dynamics of these important species and, have wider reaching environmental and economic impacts.

Keywords : *Karenia brevis*, brevetoxin, *Crassostrea virginica*, *Mercenaria mercenaria*, reproduction, gametes, larval development, histopathology, flow cytometry.

Impacte de *Karenia brevis* sur la reproduction et les stades de vie précoces des bivalves.

Resumé

Karenia brevis, le dinoflagellé produisant des brevetoxines (PbTx), est la principale espèce d'efflorescences d'algues toxiques dans le Golfe du Mexique. Les effets de cette algue sur *Mercenaria mercenaria* et *Crassostrea virginica* sont méconnus tandis que les efflorescences coïncident avec la période de reproduction de ces espèces.

Ce projet avait pour but de déterminer les effets i) d'une exposition à *K. brevis* en laboratoire et naturelle de terrain sur les processus physiologiques associés à la reproduction de *M. mercenaria* et *C. virginica*, et ii) d'une exposition à *K. brevis* sur la qualité et le développement des gamètes, embryons et larve de ces espèces.

Suite à l'exposition des adultes de clams et d'huîtres à *K. brevis*, les paramètres physiologiques de la reproduction ont été affectés. La présence de PbTx dans les tissus des gamètes et le potentiel transfert maternel de PbTx à la progénie *via* les ovocytes, pourraient avoir entraîné les effets négatifs observés lors du développement larvaire.

Les effets négatifs similaires causés par l'exposition des stades précoces à différentes préparations de cellules de *K. brevis* suggèrent que d'autres composés toxiques, en plus de PbTx, pourraient être impliqués dans la toxicité et, que la majorité des effets délétères se produisent durant les divisions embryonnaires.

Le clam et l'huître américaine sont sensibles à *K. brevis*. Les effets négatifs sur les adultes et jeunes stades de vie, combinés à une exposition quasi-annuelle aux efflorescences de *K. brevis*, pourraient engendrer des perturbations majeures sur le recrutement des populations de ces espèces importantes, et avoir des répercussions environnementales et économiques.

Mots clés : *Karenia brevis*, brevetoxine, *Crassostrea virginica*, *Mercenaria mercenaria*, reproduction, gamètes, développement larvaire