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The current and future risk of harmful algal blooms in the North Sea

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“In the oceanic world of plankton, there is light where there are no nutrients. Equally, for terrestrial plants, light and CO_2 are in the sky, nutrients are in the soil. The world appears to be made of misplaced things”

Ramon Margalef

CONTENTS

ABBREVIATIONS

A

ADME	Adsorption, Distribution, Metabolization and Excretion
ASP	Amnesic Shellfish Poisoning
ASTM	American Society for Testing and Materials
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning

B

BPNS	Belgian Part of the North Sea
BTX	Brevetoxin

C

CCAP	Culture Collection
CCMP	Center for Marine Algae and Microbiota (now NCMA)
CEFAS	Centre for Environment, Fisheries and Aquaculture
CF	Concentration factor (% of L1 medium nutrients)
CFU	Colony Forming Units
CFP	Ciguatera Fish Poisoning
CRM	Certified Reference Material
CRLMB	Community Reference Laboratory for Marine Biotoxins

D

DA	Domoic acid
DO	Dissolved oxygen
DSP	Diarrhetic Shellfish Poisoning
DTX	Dinophysistoxin

E

EC_{50}	Effect Concentration for 50% of the individuals
EC_x	Effect Concentration for x % of the individuals
EDTA	Ethylenediaminetetraacetic acid
EEZ	Economic Exclusion Zone
EFSA	European Food Safety Agency
EurOBIS	European Node of OBIS

F

f2	An algal culture medium used for diatoms
FAO	Food and Agricultural Organization of the United Nations
FDA	Food and Drug Administration
FLTR	From Left to Right
FOD	Federale Overheidsdienst

G

GES	Good Environmental Status
GTX	Gonyautoxin

H

HA	Harmful algae
HAB	Harmful Algal Bloom
HRMS	High Resolution Mass Spectrometry
HSP	Hepatotoxic Shellfish Poisoning

I

ICES	International Council for the Exploration of the Sea
IPCC	International Panel for Climate Change

K

KW	Kruskal-Wallis H test

L

L1	An algal culture medium used for dinoflagellates
LC_{50}	Lethal Concentration for 50% of the individuals
L-DOPA	L-3,4-dihydroxyphenylalanine
LMBT	Lipophilic Marine Biotoxins
LSD	Least Significant Differences test

M

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSFD	Marine Strategy Framework Directive (2008/56/EC)
MWU	Mann-Whitney U test

N

NAO	North Atlantic Oscillation
NSP	Neurotoxic Shellfish poisoning

O

OA	Okadaic acid
OBIS	the international Ocean Biogeographic Information System
OD	Optical Density

P

pNPP	para-nitrophenylphosphate
PO	Phenoloxidase, an immunological enzyme
PP	Protein Phosphatase
PSP	Paralytic Shellfish Poisoning
PSU	Practical Salinity Unit (equivalent to ‰ or $g.kg^{-1}$)
PTX	Pectenotoxins

Q

QSR	Quality Status Report of the North Sea

S

SCCAP	Scandinavian Culture Collection of Algae and Protozoa
SPE	Solid Phase Extraction
SPX	Spirolides
SSP	Spiroimine Shellfish Poisoning
STX	Saxitoxin

U

UHPLC	Ultra-High-Performance Liquid Chromatography

W

WFD	EU Water Framework Directive (2000/60/EC)

Y

YTX	Yessotoxin

DANKWOORD

In loving memory of Gerard Van Hessche, a humble man who taught me to rely on my instincts and follow my heart.

Dit zijn ze dan, de laatste pagina's van mijn doctoraat, de bekroning van meer dan vijf noeste jaren in dienst van het lab en de Universiteit. Clichématig weet ik als een echte, emotioneel geconstipeerde West-Vlaming niet waar te beginnen. Niet zozeer uit gebrek aan inspiratie, en al helemaal niet uit ondankbaarheid, maar uit een vrees om te kort te schieten. Om in enkele knullige zinnetjes niet uit te kunnen drukken wat ieder van jullie voor me hebben gedaan of betekend. Dat, en de angst om iemand te vergeten. Vind je jezelf niet in de lijst, of is uw persoonlijk stukje net dat extra beetje oppervlakkigheid? Kom gerust klagen, want met emotionele chantage krijg je veel BBQs georganiseerd (#ThingsKarelTaughtUs).

De veiligste manier om niemand te vergeten, lijkt me een chronologische volgorde. Maar hoe ver ga je terug? En wat is significant? Eline Dekeyster die me door de helft van de groepswerkjes en examenperiodes van de bachelor biologie heeft geloosd? Patrick Bollengier die met engelengeduld acht uur wiskunde in wispelturige pubers kon lepelen? Of Gerry Seynaeve, die tegen het voltallig lerarenkorps verkondigde dat ik niet dom maar lui was, en zo eigenhandig mijn heroriëntatie van het ASO naar het TSO vermeed. De waarheid is dat heel veel mensen, waarvan ik de helft vergeet, me brachten tot dit summum van mijn academische opleiding.

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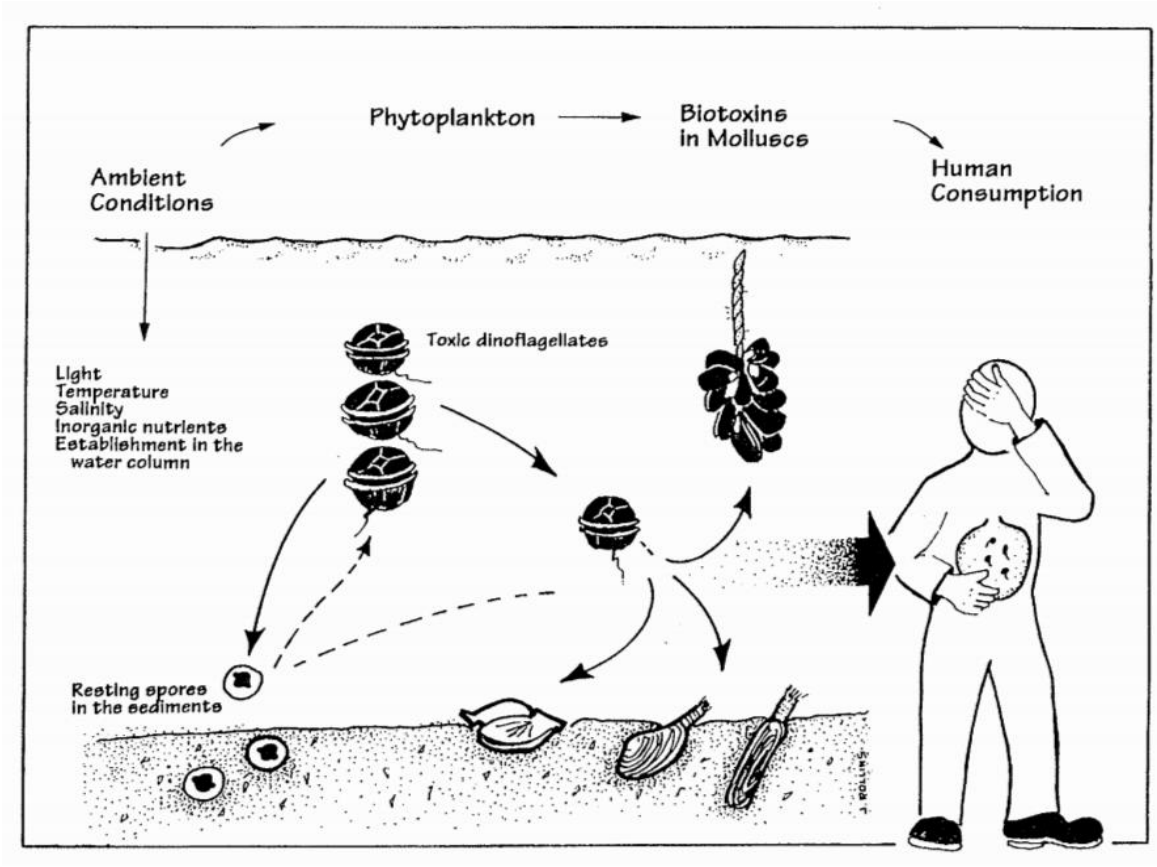
1

General introduction and outline



Credit: Shumway (1995)

1. Global phenomena of harmful algal blooms

The number of human cosmonauts on Fuller's spaceship Earth (ref. Fuller, 1969) has drastically changed since the Industrial Revolution. Going from 1.5 to 6.1 billion, the human population growth of the 20th century was three times greater than that of the entire preceding history of mankind (Kremer, 1993). Over 6.5% of all humans ever born, are alive today (Haub, 1995). Still, this number continues to climb as the global population is set to surpass 10 billion by the end of this century (UN, 2015). Consequently, planet Earth has irrevocably entered a new geological time period – colloquially called "the Anthropocene" – in which mankind's influence on the environment rivals the scale, power and universality of the greater forces of Earth (Crutzen, 2002; Ellis and Ramankutty, 2008; Waters et al., 2016).

Population growth, as well as changes in per capita income, increase the demand for plant- and animal-based food (Alexandratos and Bruinsma, 2012; Speedy, 2003). Anthropologists estimate that the current global population consumes over 40% and 35% of the total primary productivity of terrestrial and coastal systems, respectively (Pauly and Christensen, 1995; Rojstaczer et al., 2001). To produce food, water and shelter for all of mankind, we have transformed vast portions of the planet's surface. Although the land-use changes vary between regions, the transformation generally entailed extensive deforestation in favour of agricultural biomes and urban centres (Ellis and Ramankutty, 2008; Foley et al., 2005). Pastures and fields are now among the most common habitat types in the world (Foley et al., 2005; Matson et al., 1997). Yet, while surface expansions contributed to the success of modern agriculture, most of the production gains were brought about by the introduction of chemical fertilizers, high-yield cultivars, (automated) irrigation and biocides (i.e. the Green Revolution).

Fertilizers, whose worldwide use increased by well over 700% in the last 50 years, cause extensive environmental damage (Tilman et al., 2001). Most long-term records show distinct increases in the nitrogen (N) and phosphorus (P) loading of coastal ecosystems (Brush, 2008; Clarke et al., 2006; Cloern, 2001). These decadal changes in the availability of nutrients have profound effects on the composition and dynamics of phytoplankton assemblages, ultimately increasing the likelihood of harmful algal blooms (HABs) (Anderson et al., 2002; Davidson et al., 2014; Glibert et al., 2014; Granéli et al., 2008b; Heisler et al., 2008; Hodgkiss and Ho, 1997; Paerl et al., 2014). HABs have steadily increased in frequency, scale and geographical distribution during the past decades (Anderson et al., 2012). Never before have we seen this much food web disruption caused by this many harmful algae, nor have we suffered as much economic losses, in this many locations, as today.

2. Harmful mechanisms

The term “harmful algal bloom” invokes images of extensive accumulations of pelagic, unicellular phytoplankton which cause a clear discoloration of the water, as seen during classical “red tides”. In reality, however, HAB is an umbrella term which encompasses a wide range of harmful events. Not all HAB species are unicellular. HABs can be composed of colony-forming (e.g. *Phaeocystis* spp.), chain-forming (e.g. *Pseudo-nitzschia* spp.) and even multicellular organisms (e.g. *Ulva* spp.). HABs are not “extensive blooms” per se (e.g. low-biomass bloomers & epibenthic species), nor are they necessarily made by algae (e.g. cyanobacteria and euglenoids). Among the microalgae, HAB species are found across the Chrysophyceae, Cryptophyceae, Bacillariophyceae, Dinophyceae, Prymnesiophyceae and the Raphidophyceae. Generally speaking, “harmful” is the only common denominator. Any organism can be a “HAB species” as long as its proliferation can be associated with debilitating effects on the environment, human health or economies (Granéli and Turner, 2006). The vast majority of the ± 300 known bloom-forming algae are, in fact, not inherently harmful but cause environmental damage through indirect effects such as anoxia, shading and starvation (Anderson et al., 2002; Hallegraeff, 1993).

2.1 Anoxia, shading & starvation

Oxygen depletion (i.e. hypoxia to anoxia) is a growing threat to marine ecosystems (Diaz and Rosenberg, 2008). Ten years after the introduction of artificial fertilizers, coastal dissolved oxygen (DO) levels started to decrease (Galloway et al., 2008). This is often caused by an interplay of thermal stratification – the restricted mixing of deep water layers with oxygen-rich surface water during summer (Gehrke, 1916) – and excessive plankton growth. Algal blooms developing in the warm surface layers may reduce the DO levels of the surface layers by their respiratory requirements (Anderson et al., 2002; Pitcher and Probyn, 2016). More commonly, however, dead cells sink to the bottom where they fuel microbial respiration which depletes the DO of bottom layers (Baird et al., 2004). The resulting hypoxia inhibits nitrification and triggers the release of phosphorus from the sediment, providing a double feedback mechanism for the bloom (Kemp et al., 1990). Based on the severity of the hypoxia, the duration of the event, and the degree of ecosystem degradation, these HABs affect benthic life of all trophic levels (Hallegraeff, 1993; O’Boyle and Nolan, 2011), as well as zooplanktonic grazers (Grodzins et al., 2016). The resulting “dead zones” take years to recover and have been reported in over 400 systems, spanning a total area of more than 245.000 km² across the globe (Diaz and Rosenberg, 2008).

Prolonged hypoxia affects the food web structure of coastal systems profoundly. The loss of invertebrate populations and vegetation removes shelter and prey from the ecosystem and, hence, debilitates the entire marine food web (Baird et al., 2004). Similar trophic cascades can be seen when vegetation is killed by light attenuation (Bonsdorff et al., 1997; Hauxwell et al., 2003; Lee et al., 2007). HABs may also be inedible or unpalatable, deadlocking nutrients in biomass that contributes little to the secondary productivity of the ecosystem (Stolte et al., 2007). A well-known example of this inedibility is *Phaeocystis* spp. which has a gelatinous morphology that protects against predation and viral infections (Verity et al., 2007). *Phaeocystis* spp. blooms are also notorious for the odorous foam on beaches that affect the coastal tourism. Likewise, decaying *Ulva* spp. blooms can harm tourism-based economies.

2.2 Morphology

Chain and colony formation are alternative behavioural responses to planktonic grazers which balance nutrient efficiency against the protection against predation (Bergkvist et al., 2012; Bjærke et al., 2015; van Donk et al., 1999; Verity and Medlin, 2003). Another feature that discourages grazers, is the formation of protruding spines (Pondaven et al., 2007; Tillmann, 2004). This trait is only found in taxa with a rigid outer skeleton such as diatoms (e.g. *Chaetoceros* spp. and *Skeletonema* spp.) and dinoflagellates (e.g. *Ceratium* spp. and *Peridinium* spp.). Once embedded in the soft tissues of fish gills, spines cause irritation, lesions and excessive mucus production (Albright et al., 1993; Bell, 1961; Kent et al., 1995; Mamcarz and Worniallo, 1986). Blood hypoxia usually starts within 12 hours, followed by necrosis of the gut and liver. Blooms of spine-carrying species can decimate entire farms of caged finfish where fish are unable to display natural avoidance behaviour. In 2003, for instance, a bloom of *Chaetoceros wighami* killed off 170 tonnes of Atlantic salmon – net worth in excess of 500m € – in a single Scottish sea loch (Treasurer et al., 2003).

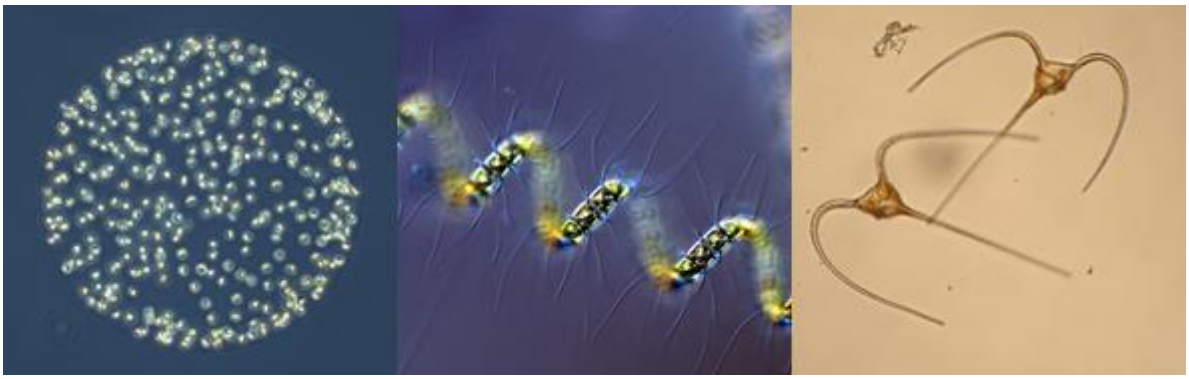


Figure 1.1: (FLTR) *Phaeocystis globosa*, *Chaetoceros debilis & Ceratium macroceros*.

2.3 Toxins

The most infamous HABs produce potent toxins that affect life of all trophic levels, incl. man. Phycotoxins accumulate in seafood, causing gastrointestinal problems as well as neurologic disorders that range from motoric weakness, headache, dizziness, disorientation and confusion to memory loss, paralysis and death (FAO, 2004). Blooms of *Karenia* spp. and *Ostreopsis* spp. are also known to aerosolize toxins, causing severe respiratory distress (Ciminiello et al., 2014; Fleming et al., 2011). Roughly a third of all HAB species, spread across all major HAB taxa, possess toxins (Chomérat et al., 2016). Marine toxins are very diverse in their function and structure. Most have a unique biological activity which, in some cases, can be extremely lethal. Saxitoxin and maitotoxin are, for instance, 50 and a 1000 times more deadly than the notorious nerve gas sarin (Dixit et al., 2005; Halstead, 2002). Toxins are often classified by their effectiveness (fast vs. slow-acting), certain chemical properties (e.g. lipophilicity), their mode of action, structural similarity or their tissue specificity. Most commonly, however, toxins are grouped by human diseases, of which 10 are linked to HABs (Botana and Alfonso, 2015; Cembella, 2003; Chomérat et al., 2016; FAO, 2004; Khora, 2015; Landsberg, 2002; Martínez et al., 2015).

Table 1.1: Known HAB-related diseases and their respective causative agents.

Syndrome	Toxins	Producers
Amnesic Shellfish Poisoning (ASP)	Domoic acid (DA)	*Pseudo-nitzschia* spp., *Nitzschia* spp.
Azaspiracid Shellfish Poisoning (AZP)	Azaspiracid (AZA)	*Azadinium* spp., *Protoperidinium* spp.
Ciguatera Fish Poisoning (CFP)	Cigua- and maitotoxin Cooliatoxin, OTX, PTX, OA	*Gambierdiscus* spp., *Coolia* spp., *Ostreopsis* spp., *Prorocentrum* spp.
Diarrhetic Shellfish Poisoning (DSP)	Okadaic acid (OA), Pectenotoxins (PTX) Dinophysistoxins (DTX)	*Dinophysis* spp., *Prorocentrum* spp.
Hepatotoxic Shellfish Poisoning (HSP)	Microcystins, Nodularins, Cylindrospermopsins, Anatoxins,	Cyanobacteria (*Microcystis* spp., *Anabaena* spp., etc.)
Neurotoxic Shellfish Poisoning (NSP)	Brevetoxins (BTX)	*Karenia brevis*, *Chattonella* spp., *Heterosigma* spp., *Fibrocapsa* spp.
Paralytic Shellfish Poisoning (PSP)	Saxitoxins (STX) Gonyautoxins (GTX)	*Alexandrium* spp., *Gymnodinium* spp., *Pyrodinium* spp.
Palytoxin Poisoning	Palytoxins	*Ostreopsis* spp.
Spiroimine Shellfish Poisoning (SSP)	Spirolides (SPX)	*Alexandrium* spp.
Yessotoxin poisoning	Yessotoxin (YTX)	*Protoceratium* spp., *Lingulodinium* spp., *Gonyaulax* spp.

3. Evolutionary ecology

At this point, it should be clear that the field of HAB research is rather complex. HAB species are distributed among several taxa with different ecological niches, often possess multiple mechanisms which may or may not come into play at once, and affect systems that range from fresh and brackish water to eutrophic coastal zones and oligotrophic oceans. Due to this diversity, there is no single answer to HABs or HAB research. Choices are inevitable. The focus of this work was mostly on dinoflagellates and the high-biomass blooming dinoflagellates that cause “red tides”.

> “…all the waters that were in the river were turned to blood. And the fish that were in the river died; and the river became foul, and the Egyptians could not drink water from the river“ Exodus 7: 20-21

This Bible verse, detailing the first Plague of Egypt, is thought to be the oldest written record of a red tide (Hallegraeff, 1993; Hort, 1957). Despite recent concerns, HABs have been around for ages. The fossil record shows that dinoflagellates first appeared during the Precambrian, 1.8 billion years ago, and speciated during the Mesozoic (Fensome et al., 1996; Meng et al., 2005). For the last 200 million years, dinoflagellates have been a major component of the phytoplankton, depositing both their cysts as well as their victims (e.g. whale graveyards) into the sedimentary rock (Dale, 2001; Pyenson et al., 2014).

Throughout their history, dinoflagellates have amassed numerous adaptations to cope with their environment. Still, they are usually slow-growing, poor competitors (Smayda, 1997). Margalef (1978), inspired by the famous r/K selection paradigm of MacArthur and Wilson (1967), considered them to be true *K*-strategists. Similarly, the CSR classification of Smayda and Reynolds (2001) defined them as nutrient efficient and stress-tolerant (S) species, placing them alongside faster growing, *r*-selected colonist (C) species that should displace them when sharing a niche. Yet, despite the competitive exclusion principle (Hardin, 1960), a remarkable variety of phytoplankton species can co-exist on a finite number of resources in perpetual non-equilibrium. This “paradox of the plankton” can be explained by resource partitioning, species interactions, weather-driven fluctuations, seasonal cycles, and chaotic oscillations (Huisman et al., 2002; Hutchinson, 1961; Richerson et al., 1970; Stewart and Levin, 1973; Tilman, 1977). In addition, dinoflagellates can escape competitive exclusion by toxicity-mediated allelopathy, grazer deterrence and mixotrophy (Chakraborty et al., 2015; Crane and Grover, 2010; Gross, 2003; Roy and Chattopadhyay, 2007).

3.1 Allelopathy

The ancient Greek and Roman philosophers already knew that terrestrial plants may interfere with the growth of neighbouring plants (Willis, 2007). This process was later dubbed “allelopathy” (Molisch, 1937). Over time, the definition was broadened to include stimulatory as well as inhibitory chemical-based interactions between plants, grazers and parasites (Rice, 1974). However, the resulting concept encroached on predator-prey relationships, making it difficult to separate allelopathy from species interactions such as grazer deterrence. For this reason, it was recently reversed back to its original essence i.e. “chemical interactions between plants, algae or bacteria which inhibit the growth of competitors” (Legrand et al., 2003; Willis, 2007). Here, allelopathy will only be discussed in this strict sense.

The ability to successfully compete for nutrients determines the bloom potential of a species. In *r*-selected species, this is achieved with fast nutrient uptake and high conversion rates. Other algae produce “allelochemicals” that suppress competitors. Such exudates are found in all major HAB taxa including diatoms, dinoflagellates cyanobacteria, prymnesiophytes and raphidophytes (Allen et al., 2016; Granéli and Hansen, 2006; Gross, 2003). These metabolites are usually hemolytic, perforating the membranes of both algae and grazers, though the inhibition of photosynthesis, cell-cycle progression and other enzymatic activities have also been observed (Granéli et al., 2008a; Legrand et al., 2003; Reigosa et al., 1999). Allelopathy can be tied to toxins associated with human poisoning (e.g. OA, DTX) peptides, fatty acids or other substances (e.g. reactive oxygen species).

Allelochemicals are thought to prevent the competitive exclusion of HA species, but this process is rather difficult to study in situ (Gross, 2003; Legrand et al., 2003). As a result, allelopathy is usually demonstrated in mixed batch cultures or with daily additions of cell-free filtrate (Granéli and Hansen, 2006). Co-culturing may, however, create false positives when pH conditions are generated that are fatal to competitors (Hansen, 2002; Lundholm et al., 2005; Møgelhøj et al., 2006). Filtrates, per contra, neglect labile toxins and changes in production and sensitivity under shared stress (Fistarol et al., 2004, 2005; Granéli and Johansson, 2003). Both approaches should be interpreted with great care. The strength of allelopathic interactions depends on conditions like temperature, light, pH, nutrients, densities and the species’ sensitivity (Fistarol et al., 2003, 2005; Schmidt and Hansen, 2001; Tillmann, 2003). Significant interactions are nearly always found at bloom concentrations (Jonsson et al., 2009). For this reason, it has been suggested that the role of allelochemicals is to prolong HABs, rather than establish them (Granéli et al., 2008a; Smayda, 1997).

3.2 Grazer deterrence & avoidance

HABs are biologically controlled by the combined grazing of microzooplankton, mesozooplankton and benthic filter-feeders (Smayda, 2008). Before the 1990's, most research focused on interactions between toxic algae and zooplanktonic grazers (Turner and Tester, 1997). Recently, though, we have come to understand that microzooplankton – i.e. heterotrophic dinoflagellates and ciliates - are as (if not more) important than mesozooplankton to control HABs (Tillmann, 2004; Turner, 2006). Together, copepods and benthic filter-feeders consume between 10-40% and 0-30% of the phytoplanktonic biomass. By contrast, microzooplankton consumes 60-70% of the primary production (Calbet, 2001; Calbet et al., 2003; Calbet and Landry, 2004). Microzooplanktonic grazers can reduce the growth of blooms but, in some cases, fall prey to potentially harmful heterotrophic dinoflagellates themselves, creating complex feedback loops which are not fully understood (Calbet et al., 2003; Calbet, 2008; Calbet and Saiz, 2005; Jeong et al., 2010; Sherr and Sherr, 2009, 2007). Mesozooplankton, on the other hand, is usually unable to prevent bloom proliferation, but can affect the initiation of blooms by consuming both toxic and non-toxic algae as well as parasites and microzooplanktonic grazers (Calbet et al., 2003; Turner, 2014; Turner and Anderson, 1983; Turner and Tester, 1997). While much still needs to be learned about these interactions, HABs generally reflect a lapse in grazing pressure (Smayda, 2008). To facilitate the breakdown in grazer control, dinoflagellates have developed mechanisms to avoid or deter grazers over the course of their evolution. These will be explained through the concept of the grazing pit, which is an extension of a three dimensional Lotka-Volterra predator-prey model (Messier, 1994).

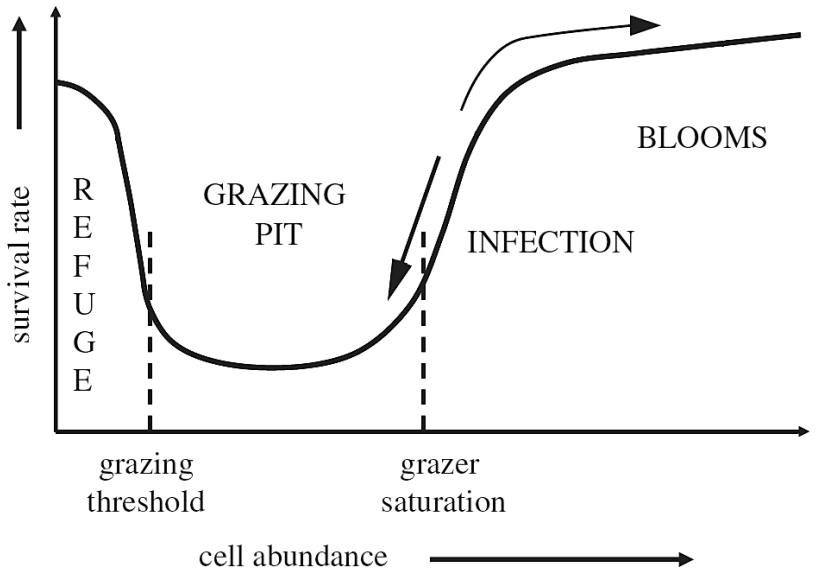


Figure 1.2 Messier's grazing pit, from Bakun (2006), Messier (1994) and Wyatt (2014).

Only a fraction of cells in natural assemblages are harmful algae (Turner, 2006). This rarity offers refuge from grazers and pathogens, as it is energetically inefficient to purposely target scarcely distributed cells. Rare cells are also less vulnerable to indiscriminate feeding. As the prey densities increase beyond a threshold, however, selective grazers will improve their uptake rates by purposely targeting the species. When prey abundances are sufficiently high, though, grazers can become saturated: i.e. they are unable to match their uptake to the availability of more prey within an ecologically relevant time-frame (Messier, 1994; Wyatt, 2014). Both equilibria are separated by a period of growth with unfavourably high mortality, i.e. the grazing pit, which harmful algae need to navigate in order to form a bloom (Bakun, 2006).

Small HAB species (e.g. cyanobacteria) simply outgrow the pressure of grazers (Domis et al., 2007). For others, size is similarly related to grazing like abundance (Kiørboe, 2008; Sournia, 1982). For this reason, colonies and chains can be formed or dissolved in response to grazers (Bergkvist et al., 2012; van Donk et al., 1999). The formation of spines or barbed setae may also be induced, but their function and efficiency is questionable (Gifford et al., 1981; Nguyen et al., 2011; Tillmann, 2004). Dinoflagellates, on the other hand, often produce chemicals which are detrimental to micro- and mesozooplanktonic grazers (Huntley et al., 1986; Teegarden, 1999; Teegarden et al., 2001; Tillmann et al., 2007). In addition, dinoflagellates can affect the fitness of grazers through their poor nutritional value (Cruz-Rivera and Hay, 2003; Prince et al., 2006; Vehmaa et al., 2012). Most grazers will, hence, not feed on toxic dinoflagellates when alternative prey is present. In other words, toxicity pushes the grazing threshold towards higher densities while, at the same time, inducing rapid grazer saturation by reducing the fertility and survival of grazers.

By removing competitors, grazers prevent the exclusion of HA and facilitate the onset of blooms (Chakraborty et al., 2012; Solé et al., 2006; Teramoto et al., 1979). Yet, despite deleterious effects, most grazers will eventually consume toxic algae if other prey becomes scarce (Cruz-Rivera and Hay, 2003; Prince et al., 2006; Schultz and Kiorboe, 2009; Teegarden, 1999). Due to the nutritional quality of dinoflagellates, they even tend to increase their uptake rates. As competitors divert grazing pressure from HA, evolution may select against strong allelopathic interactions (Flynn, 2008). Natural selection should also benefit grazers that are less susceptible to the toxins. HAB exposed copepod populations are, indeed, more resilient than naive populations (Colin and Dam, 2005, 2007; Dam and Haley, 2011). As a result, the introduction of toxic HA in non-native areas by ballast water dispersal decreases grazing pressure, enhancing the competitive success of the invasive, toxic species in a similar fashion as is described by the enemy release hypothesis (Keane and Crawley, 2002).

While algal blooms facilitate sexual reproduction, enhance the formation of cysts and offer shelter against grazing, they also promote grazer adaptation (Wyatt, 2014). To avoid the coevolution of grazers, it is advantageous for HABs to be randomly distributed in space and time. Irregular escapes from rarity instil ecological and evolutionary advantages, and should be viewed as a distinct demographic strategy (Bakun, 2006; Lewis, 1977). Blooms also need to be ephemeral: critical densities need to be attained before grazers have the opportunity to respond, and the life-cycle should be completed as quickly as possible. This is done through mass excystment, thin layer formation, and physical aggregation.

Dinoflagellate blooms often accumulate faster than regular growth would allow. Mass excystment of “seed banks” provides one way to rapidly escape (pseudo-)rarity (Wyatt, 2014). Around 10% of all marine dinoflagellates is able to produce cysts (Dale, 2001; Dodge and Harland, 1991). In coastal environments, around 20 to 50% of all motile dinoflagellates is a cyst-producer (Anderson et al., 1985; Dale, 1976). Cysts formation can be used to temporarily escape grazing pressure or unfavourable conditions by diapause (e.g. pellicle cyst), or to provide a long-term survival strategy (Bravo and Figueroa, 2014; Smayda and Trainer, 2010). Dormant resting cysts that have a thick dinosporin cell wall remain viable for months to a hundred years on end (Bravo and Figueroa, 2014; Lundholm et al., 2011). When sufficient numbers of cysts hatch simultaneously, dinoflagellates may skip the grazing pit entirely.

Harmful algae, like all phytoplankton, are influenced by the local hydrodynamics. In a lot of cases, HABs are initiated offshore – where the nutrient-poor conditions benefit the K-selected species – and are then pushed into nutrient-rich coastal waters by the wind-driven currents (McGillicuddy et al., 2003; Ruiz-de la Torre et al., 2013). Contrarily, developing blooms can be disrupted by Eddy diffusion, vertical shear gradients and small-scale turbulent motions (Estrada et al., 1987; Margalef, 1978). To some extent, certain dinoflagellates are able to reduce the effect of these physical processes by secreting extracellular polysaccharides that change their hydrodynamic environment (Cheriton et al., 2009; Wyatt and Horwood, 1973). More often, though, they use their exceptional motility to counter physical drift. Dinoflagellates swim up to 16m a day (Eppley et al., 1968; Smayda, 2002). Using geotaxis and chemotaxis, they perform daily vertical migrations for the acquisition of nutrients and to avoid grazers (Harvey and Menden-Deuer, 2012; Kamykowski et al., 1998). In addition, they can use their mobility to accumulate in 1m thick subsurface layers wherein their densities exceed grazer saturation (Cheriton et al., 2009; Margalef, 1978; Smayda, 2002).

Table 1.2: Life-history characteristics of the harmful algae used in this work. Traits with ? have been suggested, but need verification.

Species	Class	Zone	Size (µm)	Toxins	Allelopathy	Grazer deterrence	Mixotrophy	Motility	Cyst
Alexandrium minutum	Dinophyceae	Pelagic	15-30	STX, GTX	✔	✔	✔	High	✔
Alexandrium ostenfeldii	Dinophyceae	Pelagic	40-50	SPX, STX	✔	✔	✔	High	✔
Karenia mikimotoi	Dinophyceae	Pelagic	15-40	Gymnocins	✔	✔	✔	High	✘
Prorocentrum cordatum	Dinophyceae	Pelagic	10-20	Unknown	✔	✘	✔	High	✘
Prorocentrum lima	Dinophyceae	Benthic	30-50	OA, DTX, PTX	✔	✔	?	Low	?
Prorocentrum micans	Dinophyceae	Pelagic	20-70	Unknown	✔	✘	✔	High	?
Protoceratium reticulatum	Dinophyceae	Pelagic	25-40	YTX	✔	✔	✔	High	✔
Pseudo-nitzschia multiseries	Bacillariophyceae	Pelagic	40-175	DA	✔	✔	✘	Low	✘
Scrippsiella trochoidea	Dinophyceae	Pelagic	20-30	Unknown	✔	✘	✔	High	✔

Based on studies by Ajuzie (2007), Barreiro et al. (2006), Colin and Dam (2005), Dang et al. (2015), Dutra (2009), Estrada et al. (2010), Fistarol et al. (2004b), Granéli et al. (2008a), Hakanen et al. (2014), Harðardóttir et al. (2015), He et al. (2016), Heil et al. (2005), Jacobson and Anderson (1996), Jeong et al. (2005), Ji et al. (2011), Lelong et al. (2011), Mackenzie et al. (1996), Makino et al. (2008), Matsuoka et al. (1997), Nielsen and Kiørboe (2015), Sala-Pérez et al. (2016), Sopanen et al. (2011), Tameishi et al. (2009), Tillmann et al. (2007), Wang and Tang (2008), Xu et al. (2015), Yang et al. (2008), and Zhang et al. (2011)

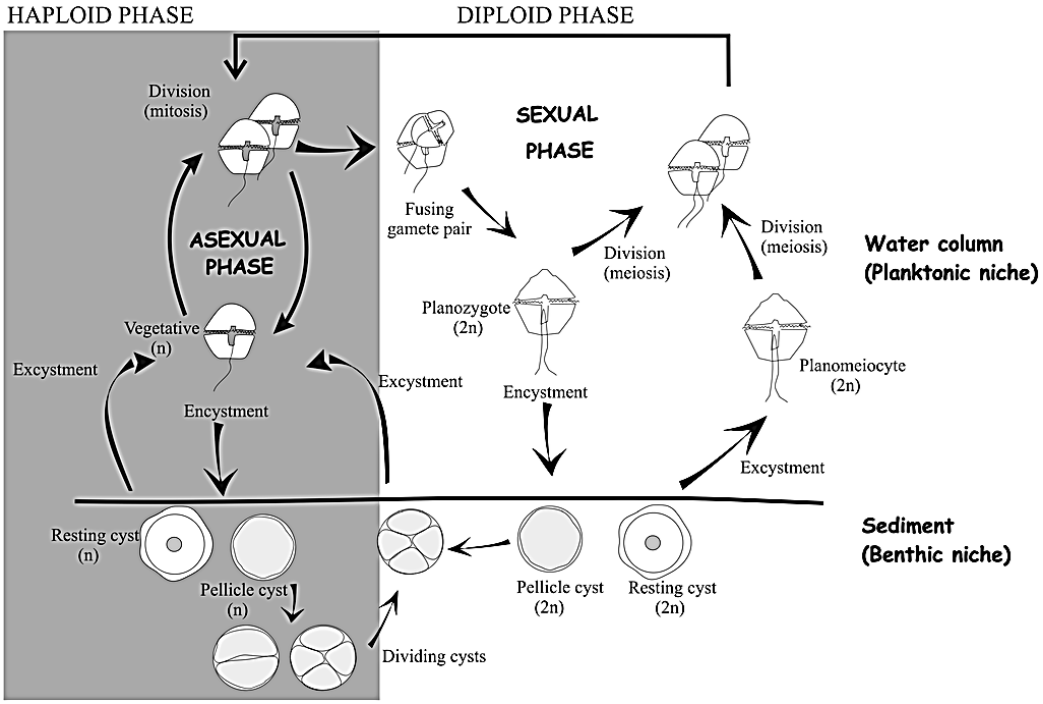


Figure 1.3 The life-cycle of cyst-forming dinoflagellates (Bravo and Figueroa, 2014).

3.3 Mixotrophy

The competitive success of toxic dinoflagellates is also commonly bolstered by their ability to obtain nutrients from both photosynthesis as well as phagocytosis (Dagenais-Bellefeuille and Morse, 2013; Jeong et al., 2010). Mixotrophy is found in all major dinoflagellate taxa (Stoecker, 1999). The vast majority are facultative heterotrophs: i.e. they consume external organic matter, but need light to grow (Stoecker, 1998). Extreme cases, e.g. *Dinophysis* spp., are obligate heterotrophs: they do not own chloroplasts, but utilize chloroplasts “borrowed” from cryptophytic, haptophytic or cyanobacterial prey (Qiu et al., 2011). Less than 10% are “ideal” mixotrophs that grow equally well on photosynthesis or heterotrophy (Hansen, 2011). Mixotrophic dinoflagellates often feed on bacteria, pico-eukaryotes, nanoflagellates, cryptophytes, haptophytes, raphidophytes, prasinophytes, ciliates, diatoms and other dinoflagellates (Jeong et al., 2010 and references therein). Feeding strategies include active hunting (raptorial feeding), generating water movement towards their uptake site (filter feeding) and passively waiting for prey uptake (diffusion feeding). Prey particles can be engulfed (phagocytosis), captured by a veil (pallium feeding), or siphoned out (peduncle feeding) (Jeong et al., 2010). The consumption of N_2-fixing cyanobacteria, in particular, is thought to promote the development of HABs in nutrient-poor waters (Glibert et al., 2009; Jeong et al., 2005).

Box 1.1 Observations on the role of benthic grazers

Most commonly farmed shellfish species, i.e. mussels, oysters, scallops, cockles and clams, readily feed on harmful algae. This creates the risk of human intoxication during both blooms as well as periods of low abundance (FAO, 2004; Turner, 2006). Through accumulation and biotransformation, bivalves accumulate toxin profiles with completely different toxic effects. While the speed of these processes varies among bivalve species and toxin classes, bivalves will vector toxins to higher trophic levels (e.g. gastropods, cephalopods, crustaceans, finfish, mammals & humans) based on their position in the food web (Shumway, 1995). Yet, due to their small contribution to the grazing pressure on HABs, the effect of benthic grazers on bloom development is often overlooked (Calbet, 2001; Calbet et al., 2003; Calbet and Landry, 2004).

The increasing occurrence of harmful brown tides (*Aureococcus anophagefferens*) along the coast of New Jersey has been linked to eutrophication as well as the loss of benthic filter-feeders during the 1980's (Cerrato et al., 2004; Gastrich et al., 2004). The overexploitation of the Northern quahog *Mercenaria mercenaria* is thought to have increased the relative importance of planktonic grazers, which are often more efficient at selective feeding than bivalves (Gobler and Sunda, 2006). This shifting grazer pressure has contributed to the success of *Aureococcus* spp. by removing their competitors (Gobler and Sunda, 2012). Now, the blooms of *Aureococcus* spp. prevent the restoration of the quahog population and cause mortality and recruitment failures in bay scallops *Argopecten irradians* and seagrass beds *Zostera marina*, resulting in a complete disruption of the ecosystem.

The case of *Aureococcus* spp. highlights the need to understand the interactions between HABs and benthic filter feeders. Bivalves often seem to be immune to toxins that are lethal to copepods, fish, birds or even whales. In part, their resilience is due to their lack of a central nervous system, a common target of marine toxins. Adult bivalves also possess a remarkable innate immune system, and can respond to toxic HA by reducing their filtration rates, producing mucosal pseudofaeces and closing their shells (Manfrin et al., 2012). Bivalve larvae are, however, more vulnerable to environmental stress (ASTM, 2004). This frail pelagic life-stage is essential for the recruitment of next generations of both natural and commercial bivalve populations (Seitz et al., 2001; Smaal, 2002). A number of recent studies have shown that harmful dinoflagellates affect the viability and development of these early life-stages (Jeong et al., 2004; Padilla et al., 2006; Rolton et al., 2014). To prevent the collapse of benthic communities (cfr. New Jersey) and protect the exploitation of shellfish, more research into the direct and indirect effects of HABs on larvae is still needed.

4. Environmental control of blooms

4.1 Windows of opportunity

HABs are a departure from the norm, an exceptional succession of phytoplankton that requires certain environmental qualities. Identifying the specific combinations of biotic and abiotic conditions that enable the initiation and development of HABs, dubbed “windows of opportunity” (Stoecker et al., 2008b), has been the holy grail of HAB research for decades. Phytoplankton communities are continuously structured by nutrient availability, biotic interactions (grazing, allelopathy, competition), abiotic variability (wind, light, temperature, turbulence, etc.) and a degree of randomness (Armstrong, 1979; Chesson, 1994; Richerson et al., 1970; Tilman, 1977, 1982). This complexity gave HABs the allure of unpredictability which, as discussed, is enforced by their biological traits (Sweeney, 1975, 1978). Still, some environmental conditions are essential to their development and persistence.

Ramon Margalef (1978) was among the first to try to strip away the HAB ecology and identify some key features that make dinoflagellate blooms “optional”. In his now iconic “mandala” (Figure 1.4), he found that nutrient availability and turbulent energy determine the succession of phytoplankton groups and, hence, the likelihood of HAB development. In this simple bottom-up model, individual species are sorted, based on their life cycles and the “selective properties of the environment” (Margalef, 1978). While species may occur outside their designated zones by chance, they will be at a competitive disadvantage there as they are not adapted to the prevailing conditions. Due to seasonal weather changes, nutrient and turbulence conditions can shift in any direction, promoting one over another species.

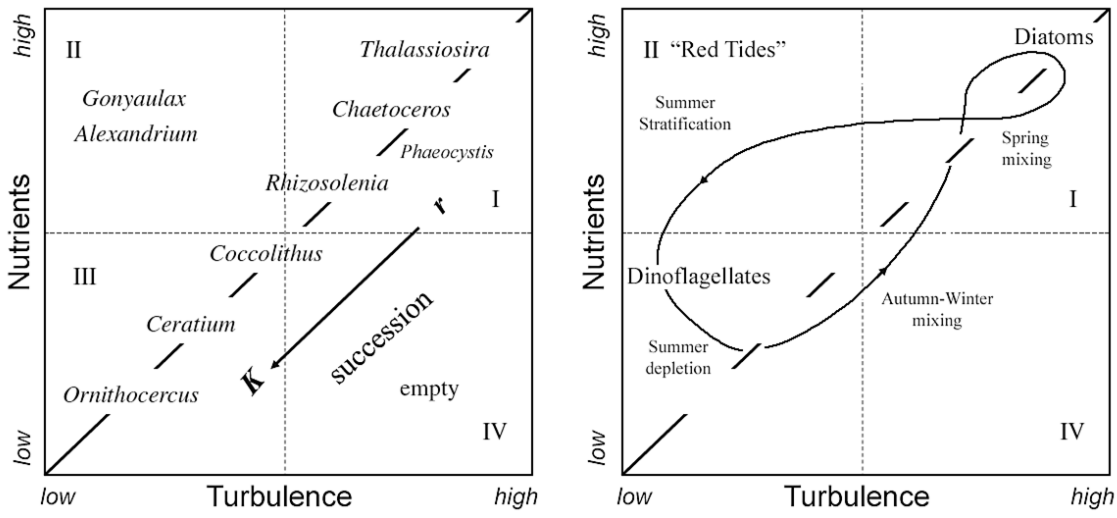


Figure 1.4 Margalef’s mandala (Wyatt, 2014)

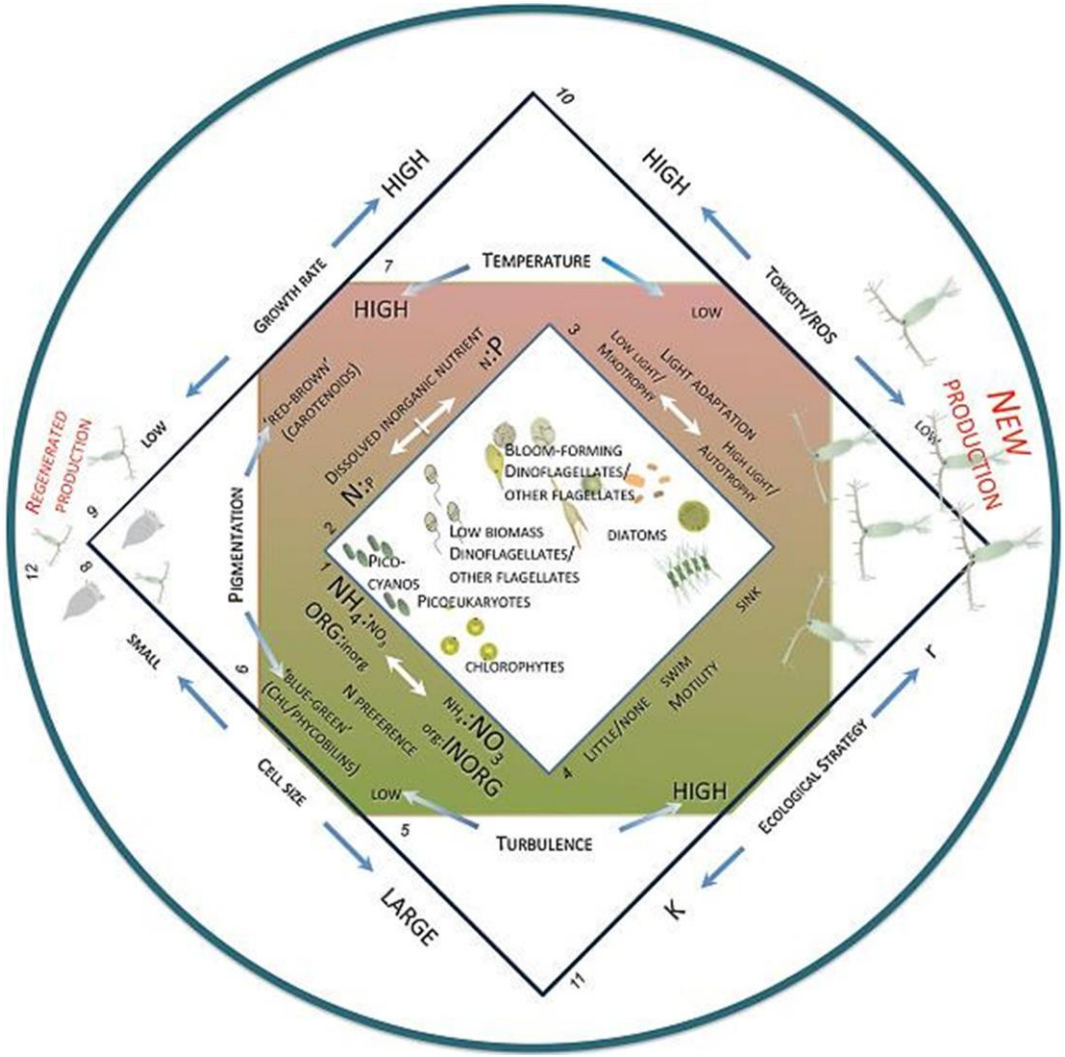


Figure 1.5 The latest incarnation of Margalef's Mandala by Glibert (2016), linking phytoplankton succession to twelve essential variables. i.e. turbulence, nutrient ratios, temperature, r vs K strategy, cell size, cell motility, relative growth rate, pigmentation, autotrophic or mixotrophic preference, the production of toxins or reactive oxygen species, grazing pressure and the chemical form of nitrogen.

The mandala is divided into four domains (I-IV) that represent the annual cycle of phytoplankton biomass (ref. Winder and Cloern, 2010). From late autumn onwards, remineralization and vertical mixing releases nutrients (i.e. N, P, Si) into the water, but temperatures are too low for substantial algal growth. For this reason, domain IV is considered “empty”. Next, r-selected diatoms deplete most nutrients during the spring blooms (domain I). Later, summer temperatures create thermal stratification which reduces vertical mixing and enhances the depletion of nutrients. At that time, the abundance of zooplankton increases. These conditions promote grazer resistant and nutrient efficient K-selected species. Ideally, the system produces a harmless peak of non-siliceous algae during summer (domain III). Yet, when sufficient nutrients remain in the system, red tides may develop instead (domain II).

The mandala covers much of the abiotic control over phytoplankton communities. Slobodkin (1989) later wrote: ‘‘any body of water that meets the criterion of relatively constant conditions and a low mixing rate will tend towards a monoculture bloom’’. According to the mandala, sufficiently stable conditions will indeed create a bloom of whichever species is best adapted to the prevailing conditions. Yet, it still overcomes Hutchinson’s paradox by incorporating constant changes in nutrients and turbulence. The mandala is neither absolute nor unidirectional, as time and time-related factors (e.g. light, temperature) are rather implicit. These strengths made Margalef’s work a cornerstone in phytoplankton ecology. As Margalef put it: “there is no paradox of the plankton, but we are excessively myopic in the perception of the many possibilities of spatial and temporal organization’’ (Margalef, 1978).

His mandala was nonetheless a product of its time. Our understanding of red tides has progressed tremendously since its publication. Adaptive traits and strategies (e.g. allelopathy, grazer deterrence, cysts, motility, internal nutrient storage) which let HAB species cope with diverse environments are now given more credit than before. We now know that grazing and eutrophication are both crucial for HAB development (Chakraborty and Feudel, 2014; Mitra and Flynn, 2006; Sunda and Shertzer, 2014), which cannot be included in a simple bottom-up model (Glibert, 2016; Wyatt, 2014). Even so, the enigmatic nature of Margalef’s mandala continues to inspire authors to adapt the framework to the new insights and groups (Balch, 2004; Cullen et al., 2007; Smayda and Reynolds, 2001). In its latest incarnation (ref. Glibert, 2016; Fig. 1.5), the mandala has become a twelve dimensional “map” that contains (1) turbulence, (2) nutrient ratios, (3) temperature, (4) r vs K strategies, (5) cell size, (6) cell motility, (7) relative growth rate, (8) pigmentation, (9) autotrophic or mixotrophic preference, (10) the production of toxins or reactive oxygen species, (11) grazing pressure and (12) the chemical form of nitrogen

4.2 Fishing and shipping

Most axes of the revised mandala are intrinsic species properties. Other axes are directly affected by anthropogenic activities. As already discussed, benthic grazing is susceptible to overfishing, leading to relatively more pelagic grazing which is also affected by fishing activities. Fisheries have generally depleted the stocks of large piscivorous fish (FAO, 2016), increasing the number of small planktivorous fish that remove zooplanktonic grazers and, hence, modify the grazing pressure of HABs (Anderson et al., 2012). These trophic cascades indirectly influence the grazing pit, and may be enhanced by the involuntary introduction of toxic algae in locations with naïve grazer populations through ballast water dispersal and shellfish stock vectoring (Colin and Dam, 2005, 2007; Dam and Haley, 2011; Smayda, 2007).

4.3 Eutrophication

Coastal eutrophication has been linked to the increasing frequency of HABs across the globe (Anderson et al., 2002, 2012; Cloern, 2001; Davidson et al., 2014; Glibert et al., 2014). The predominant underlying cause is rather straight-forward. The Green Revolution has increased the nutrient availability of most aquatic systems. The industrial production rates of N and P vastly exceed the natural weathering rates of these elements (Carpenter and Bennett, 2011; Galloway et al., 2008). By contrast, the natural availability of silicon has changed little (Tréguer and De La Rocha, 2013). Nearshore diatom communities tend to be silicon-limited (e.g. Burson et al., 2016), leaving more nitrogen and phosphorus to fuel blooms of non-siliceous phytoplankton (Graneli et al., 1999; Officer and Ryther, 1980; Radach et al., 1990; Riegman et al., 1992; Roberts, 2003; Schöllhorn and Granéli, 1996; Smayda, 1989; Sommer, 1994).

Changes in the relative availability of nutrients are commonly expressed as ratios. Shifts in ambient nutrient ratios may move co-existing phytoplankton species towards competitive exclusion when each is limited by another resource (Tilman, 1977, 1980). In the case of silicious vs. non-silicious phytoplankton, it is clear that the N:Si or the P:Si ratio has an important structuring role. Still, eutrophication has another effect. While nitrogen fertilizers are still extensively used, phosphorus emissions have been severely restricted by legislation (e.g. Clean Water Act, Water Framework Directive). In addition, wastewater treatment plants remove P more efficiently than N, causing riverine inputs to be richer in N than P (Glibert et al., 2014; Van Drecht et al., 2009). As a result of this imbalance, marine systems now vary between extreme N-limitation (e.g. 1:1) and extreme P-limitation (e.g. 375:1) on varying spatial and temporal scales (Burson et al., 2016; Conley et al., 2009; Elser et al., 2007).

For want of an atmospheric component, P is held to be the most limiting nutrient on the geological time-scale (Conley et al., 2009; Elser et al., 2007; Tyrrell, 1999). Conversely, N is thought to be self-regulating. Redfield (1934) observed that oceans have an average N:P ratio of 16:1. This ratio is balanced by nitrogen homeostasis. Higher ratios inhibit nitrogen fixation, while lower ratios promote nitrogen fixation (Redfield, 1958; Tyrrell, 1999). This was recently attributed to a homeostatic balance between protein and RNA synthesis, as the 16:1 protein:RNA ratio is found in both prokaryotic and eukaryotic organisms (Daines et al., 2014; Loladze and Elser, 2011). The Redfield ratio is, hence, firmly rooted in the fundamental structure of life.

Recent measurements have shown that the global oceanic mean is currently 22:1 (Martiny et al., 2014). Imbalanced supplies of N, P and C affect the nutritional quality of phytoplankton, causing grazers to increase their consumption rates, increase their retention efficiency or decrease their body sizes (Malzahn et al., 2010; Malzahn and Boersma, 2012; Schoo et al., 2009, 2014; Sterner, 1990; Vanni and McIntyre, 2016). These trade-offs affect the food web at all trophic levels (Philippart et al., 2007). Shifting N:P ratios have, hence, also been associated with an increased risk of HABs (Heil et al., 2007; Hodgkiss and Ho, 1997; Lagus, 2004). While there is little evidence that N:P ratios play a significant role in the bottom-up control of toxic red tides (Davidson et al., 2012; Flynn, 2010), nutrient stoichiometry may indirectly affect the top-down control of HABs through changes in food quality or the production of toxins (Granéli and Flynn, 2006; Malzahn et al., 2010; Schoo et al., 2009). Yet, there is no reason to suggest that the N:P ratio selects for red tides by itself.

Associating red tides to the recent deviations from the Redfield ratio harkens back to the succesful association of N:Si ratios and blooms of non-siliceous phytoplankton. At first glance, there is even a theoretical framework to support the HAB-N:P theory. The Redfield ratio is not a universal optimum for all algae (Klausmeier et al., 2004). Fast-growing r-strategist cells have a greater energy allocation to P-rich ribosomes, and are more attuned to low N:P ratios, than slow-growing K-strategists which have more N-rich proteins and, hence, grow well at high N:P ratios. Given a sustantial shift in N:P ratio, Tilman's resource ratio hypothesis predicts the competitive displacement of one group by the other (Tilman, 1977). While this could explain the recent success of cyanobacterial HABs or *Phaeocystis* spp., it fails to address the issue of red tides. There is no ecological theory which supports the notion that nutrient ratios can select between closely related species with resembling nutrient requirements. Nevertheless, the link between N:P ratios and red tides is frequently reiterated without this nuance (Glibert et al., 2014; Glibert, 2016; Heisler et al., 2008).

4.4 Climate change

Besides our influence on the global nitrogen cycle, human activities have also transgressed the planetary boundaries of climate change (Rockström et al., 2009). Anthropogenic global change is now beyond dispute (Cook et al., 2013; IPCC, 2014). Relative to 1986-2005, the global mean surface temperatures will have increased by 0.3 (best case) to 4.8°C (worst case) by the end of the 21st century (IPCC, 2014). Even though the Paris climate agreement intends to hold global warming below 2°C, the proposed climate efforts could still result in a warming of 2.6°C to 3.1°C by 2100 (Rogelj et al., 2016). Marine ecosystems will have higher sea surface temperatures, sea level rise by thermal expansion and ice melt, deoxygenation, ocean acidification, changes in continental runoff, and an increased frequency of extreme events such as storms (Pörtner et al., 2014). Several of these effects may promote the occurrence of HABs in the future oceans (Anderson et al., 2012; Hallegraeff, 2010).

Long-term changes in the phytoplanktonic biomass production of the North Sea are linked to eutrophication and regional changes in temperature (Edwards, 2001). Though cell growth is generally positively correlated with temperature (Eppley, 1972), dinoflagellates are already more adapted to higher temperatures than other algae, giving them more chance to grow under climate change conditions than competitors (Smayda and Smayda, 2015). Range expansions of harmful species, together with range contractions of competitors and grazers, will determine the risk of future HABs (Anderson et al., 2012; Chevin et al., 2010; Wells et al., 2015). Climate change may also increase the production during winter and affect the timing of peak productivity, leading to trophic mismatches between producers, grazers and higher trophic levels (Anderson et al., 2012; Edwards, 2001; Edwards and Richardson, 2004).

On a regional scale, changes in precipitation and continental runoff will enhance both the thermal and haline stratification (Holt et al., 2014; Hordoir and Meier, 2012). Dinoflagellate blooms are often associated with persistently stratified environments, as they promote nutrient efficiency, mixotrophy, cell motility, and the accumulation of biomass into thin layers (Berdalet et al., 2014; Huisman et al., 2004; Smayda, 2002). Stratification also severely reduces the competitive effectiveness of their main rivals. Diatoms, which tend to outgrow other phytoplankton groups, need upward advection or mixing to maintain a depth with favourable light conditions (Huisman et al., 2002). As a result, climate chance is believed to increase the windows of opportunity for HAB development. Little is known, however, about the effects of temperature and pH on nutrient competition and toxin-mediated processes such as allelopathy and grazer deterrence (Wells et al., 2015).

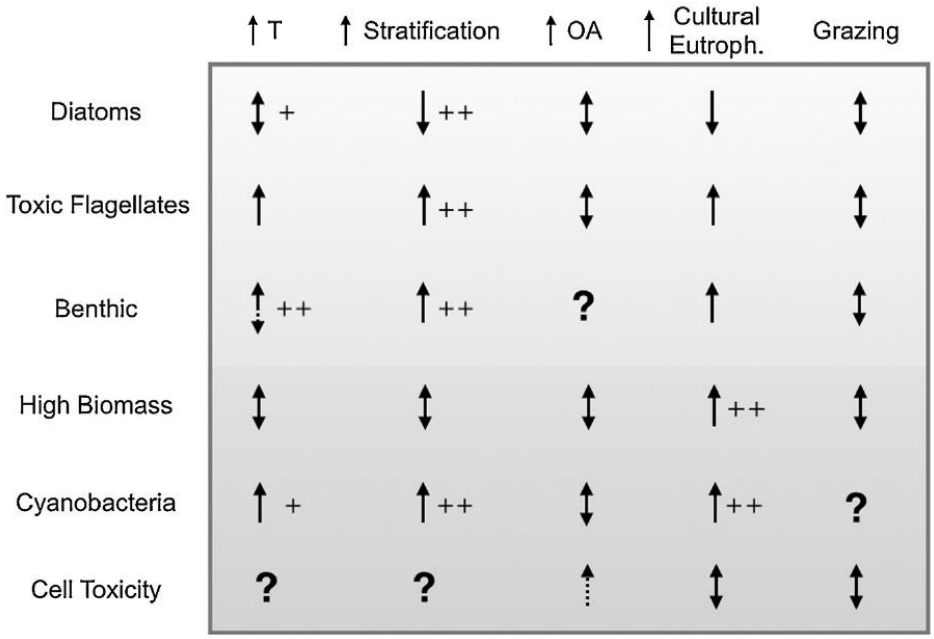


Figure 1.6 The predicted effect of climate-change related stressors, like temperature (T), ocean acidification (OA) and stratification, as well as the future impact of eutrophication and grazing on different phytoplankton groups. Arrows indicate inhibition, stimulation or species-dependent effects (double arrow). Questions marks are insufficiently studied to predict. Symbols denote confidence: likely (+) and very likely (++). Credit to Wells et al. (2015).

Climate change will also affect the mechanisms that can induce bloom termination. Most commonly, senescent blooms dissipate by weather-driven changes in currents (pushed offshore) and waves (turbulence or loss of stratification). Long-term records of the North Sea have, for instance, detected decadal changes in the wind forcing, showing an increase in mean wind speeds and a higher occurrence of westerly winds (Siegismund and Schrum, 2001). Ultimately, these changes determine the likelihood of bloom termination by weather patterns. Another potential way of bloom termination is nutrient depletion. As the growth rates increase with temperature (Eppley, 1972), blooms may deplete nutrients more rapidly, potentially making them more ephemeral. This process could be enforced further by an increase in thermal stratification which will prevent mixing with deeper, nutrient-rich waters. Climate change will, however, also have an effect on nutrient remineralisation (Segschneider and Bendtsen, 2013), which could perhaps negate the rapid depletion of available nutrients to some extent. Lastly, established blooms are known to succumb to viral, bacterial and fungal lysis (Bidle and Vardi, 2011; Frenken et al., 2016; Imai et al., 1998; Mayali et al., 2008). The linkage between algal blooms and these parasitic organisms will evolve as each clade adapts to the global change conditions (temperature, pH, etc.).

Box 1.2 The complex interactions between bacteria and algae

To bridge Messier's grazing pit, algae need to overcome the mortality induced by viral and bacterial infections, that shunt about 20% of all the produced biomass away from higher trophic levels (Cole et al., 1988; Fuhrman and Noble, 1995; Wyatt, 2014). For the most part, this mortality can be linked to *Roseobacter* clade bacteria, though *Flavobacter* clade bacteria are also positively related to the density of phytoplankton (Buchan et al., 2014; Cole, 1982; Mayali et al., 2008). The interactions between HA and their bacteria can vary from commensalism to parasitism (Ramanan et al., 2016), and change over time as the organic matter excreted by the algae bottom-up induces bacterial succession which affects the functional traits of the associated community (Riemann et al., 2000; Teeling et al., 2012).

Little is known, though, about the role of bacteria in controlling HABs. Infections both promote and inhibit bloom formation (e.g. Ferrier et al., 2002; Imai et al., 1995), creating unstable trade-offs between the uptake of nutrients and the risk of infection (Menge and Weitz, 2009). In certain cases, the bacterial diversity in the phycosphere may also act as both the target and mediator of allelopathic interactions between HA (Hulot and Huisman, 2004; Weissbach et al., 2011), further complicating their role in the formation and maintenance of HABs. In a few rare cases, the production of toxins (e.g. DA) of the algae has been linked to the presence of certain associated bacteria (Bates et al., 2004; Stewart et al., 1997).

While we still do not completely understand the role of bacteria in HAB dynamics, climate change is predicted to have a tremendous impact on prokaryotic communities (Vezzulli et al., 2012). Crucially, bacteriologists fear that global warming will increase the occurrence of pathogens, such as *Vibrio splendidus* and *Vibrio coralliilyticus*, which are found in bloom-associated communities (Barlaan et al., 2007; Burge et al., 2014; Eiler et al., 2006; Mourino-Perez et al., 2003; Vezzulli et al., 2016). If and when this happens, the effects of HA may work in tandem with the virulence of pathogens, enhancing the environmental impact of HABs. Up to now, only a handful of studies have looked at the interactive effects of HABs and pathogens on benthic grazers, and none have investigated this mixed toxicity in pelagic grazers. In adult bivalves, both synergistic and antagonistic interactions between HA and pathogens were found (Basti et al., 2015a; da Silva et al., 2008; Hégaret et al., 2010). To date, however, not a single study has looked into the potential for mixed toxicity effects in bivalve larvae, which are usually more susceptible to environmental stress. To truly understand the entire impact of HABs on (benthic) grazing communities (ref. box 1.1), these mixed toxicity effects need to be evaluated further.

5. North Sea status

The North Sea was recently identified as a global “hot spot” of climate change (Holt et al., 2014; Pinnegar et al., 2016). In the past, the North Sea was a “hot spot” for eutrophication (QSR, 1987; Fig. 1.7), and decades of overfishing that changed the food web towards a new ecosystem regime that favours above normal Chl A levels (McQuatters-Gollop et al., 2007). The Southern Bight of the North Sea, in particular, is highly eutrophied (Djambazov and Pericleous, 2015) and its nutrient stoichiometry is very heterogeneious (Burson et al., 2016), increasing the risk of HAB development.

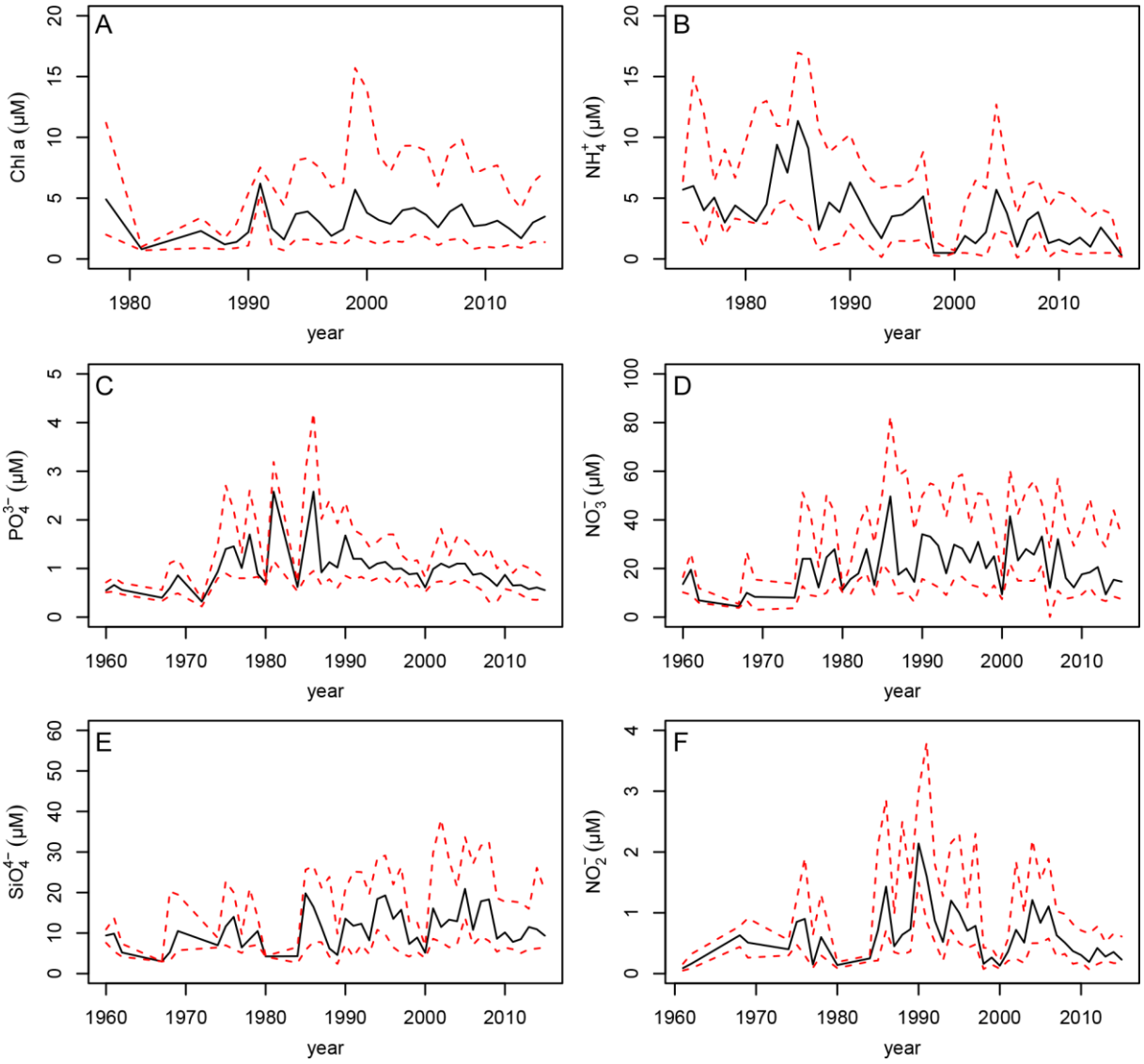


Figure 1.7: The evolution of the Chlorophyll A concentration of the English Channel and the Southern Bight of the North Sea (A), and the winter (December–February) concentrations of ammonium (B), phosphate (C), nitrate (D), silicate (E), and nitrite (F). Full black lines are the median values, dotted red lines represent the 25-75% quantiles. Monitoring data derived from the ICES data portal (ICES areas IVc and VIId).

Table 1.3: List of potentially harmful phytoplankton spotted inside the Belgian EEZ, based on a query of the EurOBIS database for the Belgian EEZ, and expanded with (grey) literature and personal observations.

Harmful species	Effect	References
Dinophysis acuta	Toxic (OA,DTX)	Bastin (1991) Van Wichelen et al. (2008)
Dinophysis acuminata	Toxic (OA,DTX)	Louis et al. (1974) Bastin (1991) M'harzi (1999) Denys and Maeckelberghe (2002) Van Wichelen et al. (2008)
Dinophysis norvegica	Toxic (OA,DTX)	Louis et al. (1974)
Dinophysis rotundata	Toxic (OA,DTX)	Müller (2004) personal observations
Protoceratium reticulatum	Toxic (YTX)	M'harzi (1999); *Gonyaulax grindleyii*
Karenia mikimotoi	Fish kills Hypoxia Toxic (Gym)	Parke and Dixon (1976); *Gymnodinium breve* Louis and Petes (1979); *Gymnodinium aureum* personal observations
Scrippsiella trochoidea	Hypoxia	Louis and Petes (1979); *Scrippsiella faeroeense* personal observations
Phaeocystis globosa	Hypoxia	Baumann et al. (1994) personal observations
Phaeocystis pouchetii	Hypoxia	De Pauw (1975) Lancelot and Mathot (1985) Müller (2004)
Prorocentrum lima	Toxic (OA,DTX)	Leloup and Miller (1940) personal observations
Prorocentrum micans	Hypoxia	De Pauw (1975) Louis and Petes (1979) Bastin (1991) Van Wichelen et al. (2008) personal observations
Pseudo-nitzschia seriata	Toxic(DA)	M'harzi (1999) Denys and Maeckelberghe (2002)
Alexandrium ostenfeldii	Toxic (SPX, STX)	Woloszynska & Conrad (1939); *Pyrodinium phoneus* Van Wichelen et al. (2008)
Skeletonema costatum	Fish kills (spines)	Reid et al. (1990) M'harzi (1999) Rousseau et al. (2002)
Heterocapsa rotundata	Hypoxia	Leloup and Miller (1940) Mommaerts-Billiet et al. (1974) Van Wichelen et al. (2008)
Heterocapsa triquetra	Hypoxia	Louis and Petes (1979); *Peridinium triquetra* Conrad (1939); *Gonyaulax. triacantha*

In recent years, though, the nutrient loading of the Southern Bight of the North Sea is gradually decreasing (ref. ICES data; Fig. 1.7). First, the phosphate levels dropped to stagnant levels because of the voluntary agreements with the detergent industries and a European-wide ban on phosphates in household detergents. Now, the nitrogen levels are plummeting as the combined result of a multitude of European legislations (i.e. the Nitrates Directive, the Urban Waste Water Directive, the Water Framework Directive, the Marine Strategy Framework Directive etc.). Despite this progress, the nutrient inputs are still too high and unbalanced, causing a shift in the natural nutrient stoichiometry of the Southern Bight of the North Sea. The average N:P ratio of the Belgian Part of the North Sea (BPNS) is, for instance, between 12 and 14, though extremer cases of N-limitation (e.g.1:1) and P-limitation (365:1) have also been found (Brion et al., 2004; Burson et al., 2016). Assuming that HAB occurrences can indeed be linked to changes in the N:P ratio, these shifts could have increased the risk of HAB development in the BPNS.

Because of eutrophication, overfishing and climate change, scientists believe that more HABs will develop in the North Sea (Peperzak, 2005; Friocourt et al., 2011). Some of the countries adjacent to the Southern Bight of the North Sea, like France, the Netherlands and the UK have, in fact, already found an increase in HABs within their EEZs (Figure 1.8). Yet, despite the number of known harmful algae which were spotted within the Belgian EEZ (Table 1.3), Belgium still needs to start monitoring for HAB events. As a result, we know little about the risk of HABs inside the BPNS.

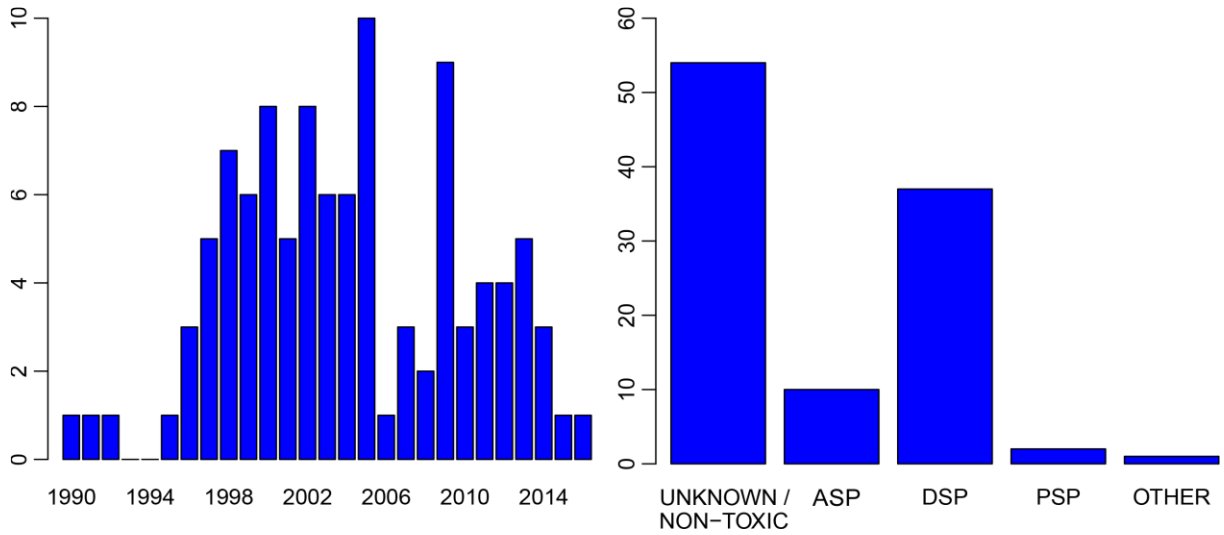


Figure 1.8: The yearly occurrence of HABs in the Southern Bight of the North Sea (left), and the predominant toxin groups that occur during these blooms (right). Data extracted from the Harmful Algal Event Database (HAEDAT), using a subset of zones (FR01, FR02, FR03, GB03, GB04, GB06, GB07, NL03) that coincide with ICES areas IVc and VIId.

6. Objectives

While all of the neighbouring countries have detected various HAB species in their economic exclusion zones, ref. reports by Rijkswaterstaat (NL), Ifremer (France), and CEFAS (UK), Belgium remains ignorant of the human health risks that lurk within its coastal waters. Despite growing evidence that dinoflagellate blooms are gaining in importance on a global scale, our environmental monitoring report is still based on broad parameters such as chlorophyll-A, total inorganic nutrient concentrations, and the abundance of a single known harmful species i.e. *Phaeocystis* spp. (FOD, 2015). Contrary to most other European countries, the historical collapse of our aquaculture industry robbed us of a compelling reason to monitor beyond what was required by European laws against eutrophication (e.g. Water Framework Directive 2000/60/EC). Now, though, the Marine Strategy Framework Directive (2008/56/EC) requires us to include indicators that reveal changes in the phytoplanktonic community composition, with increased attention to the presence of HAB species, and recommends research into the effects of nutrient ratios and nutrient loads on the structure and function of the planktonic food web in relation to other trophic levels.

The **main objective of this thesis** was to assess whether the ongoing chances in the environmental conditions of the BPNS have any effect on the risk of toxic HABs. Based on the knowledge gaps that were identified in the general introduction, and the recommendations of the EU Marine Strategy Framework Directive, we formulated **five research questions** which were addressed throughout the chapters of this PhD. Each question was used to improve our understanding of HAB dynamics and the associated effects on the food web, so that we could formulate a suitable answer to the main objective.

1. Do shifting N:P ratios affect the competitive traits of dinoflagellates?
2. Do nutrient load reductions affect the competitive traits of dinoflagellates?
3. Will global change affect the competitive traits of dinoflagellates?
4. Do toxic HABs affect populations of keystone bivalve species?
5. What is the risk of HABs in the BPNS?

7. Rationale and scope of this work

As the world plunges ever-faster towards the point of no return for climate change, the chance to avoid a major escalation of HABs worldwide is gradually slipping away. Now more than ever, policymakers need to comprehend the dangers of ecosystem disruption by HABs, and get a clear signal which areas to prioritize in order to avoid this catastrophe (Chomérat et al., 2016). There is, however, a lack of experimental evidence that global change will affect HABs (Wells et al., 2015). While the rate of temperature change is already accelerating in some regions (Smith et al., 2015), HAB scientists are still discussing the fundamental ecological importance of key traits (e.g. allelopathy) and environmental constraints (e.g. N:P ratio) in HAB development. Critically, we need to identify and employ model species in cross-validated, long-term multifactorial studies with co-occurring species to rapidly progress our understanding of HAB and non-HAB physiological plasticity to climate stress (Wells et al., 2015). These efforts should, however, not preclude the search for "windows of opportunity" and "black swans" in HAB ecology. Quantifying the effects on the socioeconomic well-being of our species, by continuing to look for new HAB species, new toxins, newly affected locations and previously unknown toxic effects on key organisms, should not be impeded either, as this information is key to persuade policy makers.

The first two chapters of this dissertation aim to improve our understanding of two age-old questions which often detract from the bigger environmental issues at hand: the N:P ratio and allelopathy. **Chapter 2** investigates how nutrient stoichiometry and temperature affect monocultures and mixed cultures of four dinoflagellates. While the success of dinoflagellate blooms is often ascribed to these factors, few studies have simultaneously investigated their effect on closely related species. We hypothesized that temperature would affect growth rates and, hence, nutrient consumption, but that changes in nutrient stoichiometry would not be able to affect the species' dominance because of the resemblance in nutrient requirements of these species.

Chapter 3 builds further on chapter 2 to elucidate the roles of nutrient competition, allelopathy and relative densities during bloom initiation. Several theoretical studies suggest that allelopathy is dysfunctional at low densities, but experimental studies often fail to elucidate nutrient competition from allelopathic interactions. We believe that allelopathy plays no significant role during the first stage of bloom development. To demonstrate this, and verify the role of the N:P ratios, we grew communities of three dinoflagellates under different temperatures, N:P ratios and initial densities. Using mechanistic modelling, we then identified the predominant mechanism which determines interspecific competition in mixed laboratory cultures.

The second half of this dissertation looks at the effect of red tides on bivalves. These bioturbating or reef-forming organisms are ecosystem engineers and, hence, are often keystone species in temperate marine ecosystems such as the North Sea. In shallow systems with a high biomass to water volume ratio (e.g. Eastern Scheldt), bivalves may have a strong influence on phytoplankton abundance (Dame, 2011). Yet, despite of this, their role in HAB ecology has long been overlooked. Recently, self-sustaining brown tides have been linked to the overexploitation of bivalves (Gobler and Sunda, 2012). This “black swan” event (i.e. an unexpected event with major ramifications that is often overly rationalized later with the benefit of hindsight) encourages renewed research efforts into interactions between HABs and bivalves.

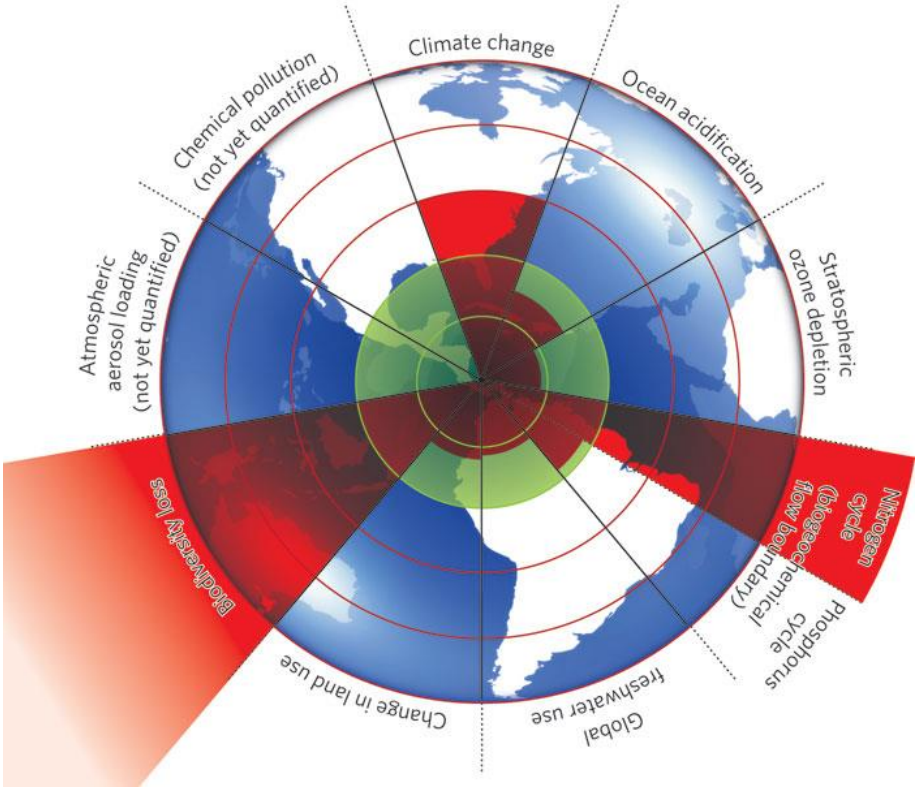
Adult bivalves are known to sequester and transform various chemical pollutants. Marine toxins are frequently found at remarkably high concentrations without any apparent effect on the mussel. Despite the risk to human consumers, little is known about the accumulation and detoxification mechanisms of bivalves. The simultaneous exposure to multiple toxic algae, in particular, is yet to be studied. For this reason, **chapter 4** looks at the absorption, distribution, metabolization and excretion kinetics of two emerging toxin groups (OA & SPX) in the common mussel *Mytilus edulis* by HPLC-MS/MS. We knew that mussels would accumulate toxins by feeding on the two toxic dinoflagellates, and assumed that the combined exposure to both would reduce the accumulation of toxins due to an increase in avoidance behaviour.

As both HABs and pathogens will become more prevalent in the near future, marine bivalves will increasingly have to face both stressors at once. Because of this, **chapter 5** explored the potential for interactive toxicity effects in their most sensitive life-stage i.e. the larvae. Beforehand, we reasoned that the exposure to okadaic acid and domoic acid, the two most common toxins in Europe, may affect the viability and immunological resilience of *M. edulis* larvae. We expanded on the latter hypothesis by simultaneously exposing mussel larvae to marine pathogens and a whole range of previously unstudied toxic dinoflagellates in **chapter 6**.

The final part of this thesis, **chapter 7**, summarizes the results and conclusions of this work, and frames them within the current state of the art of the HAB field. Suggestions for future research are then provided alongside recommendations for the regional management of the Belgian Part of the North Sea (BPNS).

2

The effect of temperature and nutrient stoichiometry on the performance of potentially harmful dinoflagellates



Credit: Rockström et al. (2009)

Abstract

Over the last sixty years, eutrophication has gradually upset the biogeochemical balance of the world's oceans. Shifts in the relative abundance of growth-limiting nutrients such as nitrogen and phosphorus have been observed across the world. The resource ratio (i.e. nutrient stoichiometry) can affect the biological success of competing taxa when species are limited by different nutrients. As a result, there is a persistent believe that the imbalance in N:P ratios is linked to the growing success of dinoflagellate blooms. Yet, to date, there is a lack of experimental evidence that changes in the external nutrient stoichiometry promote harmful dinoflagellates over co-occurring benign species. Here, four dinoflagellates of the Belgian Part of the North Sea (*Prorocentrum micans*, *Prorocentrum lima*, *Protoceratium reticulatum* & *Scrippsiella trochoidea*) were grown in 300 single and mixed batch cultures to explore the effect of nutrient stoichiometry on the outcome of interspecific competition across 10 N:P ratios (between 8 and 24) and two temperatures (20°C and 24°C). Cell counts, nutrient measurements and toxin extractions were then used to determine the individual performance of each dinoflagellate. Overall, the results of this study indicate that the N:P ratio has no structuring role in the competition between dinoflagellates. Observed patterns between growth rates and N:P ratios either failed to replicate themselves at another temperature, or vanished in mixed cultures. For this reason, this study warns against the use of the N:P ratio as a key predictor of the risk of HAB development in coastal areas such as the BPNS.

1. Introduction

Since the 1950's, mankind has left enough distinctive geochemical signatures in the geological record to warrant the creation of a new epoch: the Anthropocene (Crutzen, 2002; Waters et al., 2016). Among these signs is the gradual increase in the availability of growth-limiting nutrients. The industrial production of nitrogen and phosphorus vastly exceeds the natural weathering rates of these structural elements (Carpenter and Bennett, 2011; Galloway et al., 2008). While our influence on the silicon cycle is limited (Tréguer and De La Rocha, 2013), the N and P cycles have drastically changed since the Green Revolution. Coastal eutrophication is detected in all long-term monitoring records (Brush, 2008; Clarke et al., 2006; Cloern, 2001). Changes in the relative and total abundance of N and P are linked to the increase in harmful algal blooms (HABs) across the globe (Anderson et al., 2002, 2008; Cloern, 2001; Davidson et al., 2014; Glibert et al., 2014; Paerl et al., 2014; Smith, 2003). Yet, it is still not fully understood how nutrient availability and nutrient stoichiometry affect HAB species (Wells et al., 2015).

Due to the extensive use of N fertilizers in agriculture, the high emission of N-rich waste by lifestock production, the higher removal efficiency of P in wastewater treatment plants and the strict legislation concerning P in detergents, anthropogenic emissions tend to be richer in N than P (Glibert et al., 2014; Van Drecht et al., 2009). Predominantly N-rich riverine inputs were fed into the world's oceans for decades. Now, the mean N:P ratio of marine systems has evolved from 16:1 in the 1930's - the famous, biogeochemically balanced Redfield ratio (Redfield, 1958) – to 22:1 today (Martiny et al., 2014; Redfield, 1934). Changes in nutrient ratios can drive co-existing phytoplankters towards competitive exclusion when each species is limited by a different resource (Tilman, 1977, 1980). Nutrient ratios determine the phytoplankton community composition and, hence, can be linked to predator-prey relationships (Malzahn et al., 2010; Philippart et al., 2007; Schoo et al., 2009) and an increased risk of HAB development (Heil et al., 2007; Hodgkiss and Ho, 1997; Lagus, 2004). The change from diatoms to potentially harmful non-siliceous phytoplankton groups (e.g. cyanobacteria, dinoflagellates) has, for instance, been linked to shifting N:Si or P:Si ratios in both laboratory and field studies (Graneli et al., 1999; Officer and Ryther, 1980; Paerl et al., 2014; Radach et al., 1990; Riegman et al., 1992; Roberts, 2003; Schöllhorn and Granéli, 1996; Smayda, 1989; Sommer, 1994). Nevertheless, there is no real evidence that the N:Si or P:Si ratio promotes harmful species per se (Davidson et al., 2012; Gilpin et al., 2004). In fact, no ecological theory supports the notion that nutrient ratios may select between species with closely resembling nutrient requirements.

Inspired by the success of the N:Si ratio, the increase in dinoflagellate blooms is frequently attributed to the recent changes in the N:P ratio (Glibert et al., 2012; Handy et al., 2008; Heisler et al., 2008; Hodgkiss and Ho, 1997; Li et al., 2009). While understandably attractive, this approach may be dysfunctional in HAB ecology. While nutrient stoichiometry certainly affects the top-down control of HABs, through changes in the nutritional quality or toxin production (Granéli and Flynn, 2006; Malzahn et al., 2010; Philippart et al., 2007; Schoo et al., 2009), its role in bottom-up control appears to be limited at best (Davidson et al., 2012; Flynn, 2010). There is, however, a lack of experimental studies that use mixed cultures to grow co-existing species under various environmental conditions. Now, as HABs stand to gain from global change, this hampers our understanding of the current and future effect of nutrient stoichiometry on interspecific competition (Wells et al., 2015).

Here, we performed a growth experiment of 300 cultures to explore the effect of nutrient stoichiometry on the performance of four common North Sea dinoflagellates, and the competition between them. To this end, two non-toxic species: *P. micans* Ehrenberg 1834 and *Scrippsiella trochoidea* (Stein) Loeblich III 1976 and two toxic species: the benthic *Prorocentrum lima* (Ehrenberg) F. Stein 1878 and the pelagic *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli 1885 were grown at ten N:P ratios, ranging between 8 and 24, and two temperatures: 20°C and 24°C. Cell counts and nutrient measurements were used to determine growth rates, nutrient cell quota and the presumed internal N:P ratio of each species. Intracellular toxin concentrations were detected through ultra-high-performance liquid chromatography coupled to high-resolution Orbitrap mass spectrometry (UHPLC-MS).

2. Material & Methods

2.1 Algal cultures

Prorocentrum lima (CCAP1136/9) and *P. micans* (CCAP1136/20) were bought at the Culture Collection of Algae and Protozoa (Oban, Scotland), while *Protoceratium reticulatum* (SCCAP K-1478) came from the Scandinavian Culture Collection of Algae and Protozoa (Copenhagen, Denmark). *Scrippsiella trochoidea* was taken from the Belgian Part of the North Sea (BPNS) and identified by electron microscopy (Vergucht et al., 2015). Algae were grown in L1 medium (32 PSU, pH 8), prepared with artificial seawater (Instant Ocean^{tm}, Belcopet, Belgium) as dictated by Guillard and Hargraves (1993). Stock cultures were grown at 20°C with a 12 h light-dark cycle (20-40 µmol m^{-2} s^{-1}). Roughly 80% of the culture media was replaced every 2 weeks.

2.2 Experimental design

Regular L1 medium contains 882 µM NO_3^- and 36.2 µM PO_4^{3-}, or a N:P ratio of 24. To get media with different N:P ratios, the NO_3^- content of the recipe was changed. Other medium ingredients were added at the regular dose. Ten media were prepared (i.e. N:P 8, 10, 12, 13, 14, 15, 16, 18, 20, 24), centered around the mean N:P ratios of the BPNS. Monocultures of each dinoflagellate were made in each of the media by adding 100 cells ml^{-1} to Erlenmeyer flasks filled with 50 ml of medium. Mixed batch cultures were set up by adding 100 cells ml^{-1} of each species to 50 ml of a medium. Treatments were replicated six times: three flasks were grown at 20°C for 35 days, three were set at 24°C for 28 days. Both climate rooms had a 12-hour photoperiod of 20±5 µmol m^{-2} s^{-1} (cool white light). Twice a week, 1 ml was taken from each flask, fixed with 100 µl of 12% formaldehyde and counted with a Sedgewick-Rafter counting chamber and a Nikon TMS-F light microscope (10x10). In addition to the initial media (day 0), 7 ml samples were taken on day 14 and day 28 for toxin and nutrient analyses. Replicates were pooled, filtered (Ø 0.2 µm) and measured with standard colorimetric tests for NO_3^- and PO_4^{3-} (Hansen and Koroleff, 1999) using spectrophotometric kits (Merck Millipore, Darmstadt, Germany) and an Aquamate spectrophotometer (Thermo Scientific, San Jose, USA).

2.3 Toxin analyses

Additional 3 ml samples were taken on day 14 and day 28 for toxin analyses. Replicates were pooled and processed using the glass bead extraction method of Orellana et al. (2015). Analytical grade methanol (VWR, Leuven, Belgium), 0.5 mm glass beads (Thistle Scientific Ltd, Glasgow, UK) and Millex-GV 0.22 µm PVDF syringe filters (Millipore, Darmstadt, Germany) were used during this process. Certified reference material of okadaic acid (CRM-OA-c), 13-desmethyl spirolide C (CRM-SPX-1), azaspiracid-1 (CRM-AZA-1), yessotoxin (CRM-YTX) pectenotoxin-2 (CRM-PTX-2) and dinophysistoxin-1 (CRM-DTX-1) were obtained from the Canadian National Research Council (Ottawa, Canada) to create a multitoxin standard. LC-MS grade methanol, acetonitrile, as well as Milli-Q water, were used for UHPLC-MS (Merck, Darmstadt, Germany). A Thermo Fisher Scientific (San Jose, CA, USA) UHPLC-MS consisting of an Accela UHPLC pump, an Accela Autosampler/Degasser and an Exactive^{TM} benchtop Orbitrap mass spectrometer was fitted with a Nucleodur C18 Gravity column (1.8 µm, 50x2 mm, Macherey-Nagel, Düren, Germany) and a heated electrospray ionization probe (HESI-II), operating in switching polarity mode. Instrument settings were adopted from Orellana et al. (2015). Quantification was based on 9-point calibration curves of the multitoxin standard.

2.4 Data treatment

Cell densities were converted to biovolume (μm^3) according to Olenina (2006). Next, logistic or exponential growth models were fitted to the data with the R package "nlstools" (Baty et al., 2015) to estimate growth rates (μ) and carrying capacities (K). Confidence intervals were obtained by Monte Carlo simulations. Then, nutrient quota (Q_N, Q_P) were calculated for each species. The required volume for nutrient analyses was met by pooling the samples of biological replicates (as detailed in section 2.2). Nutrient quota were, hence, calculated by dividing the amount of consumed nutrients – i.e. the initial concentrations $[\ ]_{d0}$ minus the residual concentrations $[\ ]_{d28}$ ($mg.l^{-1}$) – by the mean cell density of day 28 $\langle N_{d28} \rangle$ ($\mu m^3\ ml^{-1}$; Eq. 2.1-2.2). Using the molecular weights of phosphate and nitrate (M_w), the resulting quota were converted to moles, before being divided by each other to obtain the relative consumption of nutrients (RC; Eq. 2.3). Similarly, the toxin concentrations were measured on a pooled sample of biological replicates (ref. section 2.3). Toxin cell quota were, hence, calculated by dividing the toxin concentrations [TOX] ($\mu g.l^{-1}$) by the mean culture density ($cells.ml^{-1}$) of the respective day ‹N› to obtain toxin cell quota (Q_{Tox}).

$$(2.1)\ Q_N = \frac{[NO_3^-]_{d0} - [NO_3^-]_{d28}}{\langle N_{d28} \rangle}$$

$$(2.2)\ Q_P = \frac{[PO_4^{3-}]_{d0} - [PO_4^{3-}]_{d28}}{\langle N_{d28} \rangle}$$

$$(2.3)\ RC = \frac{Q_N * M_w(PO_4^{3-})}{Q_P * M_w(NO_3^-)}$$

$$(2.4)\ Q_{Tox} = \frac{[TOX]_{d28}}{\langle N_{d28} \rangle}$$

Multiple regression with backward elimination was used to examine the effects of temperature (T) and nutrient stoichiometry (N:P) on the growth rates of each species. The initial model included a quadratic nutrient ratio term and an interaction between ratios and temperature to allow non-linear behaviour (Eq. 2.5). Non-significant terms were eliminated one by one using a Bonferroni-adjusted stepwise approach to control the overall type I error (ref. Perrett et al., 2006). Common (CCC) and unique (UCC) commonality coefficients were used to determine the contribution of each predictor to the overall R^2, a proxy of effect size, using the R package "yhat" (Nimon et al., 2008). Similar regressions were used to investigate carrying capacities and toxin quota.

$$(2.5)\ \mu = b_0 + b_1 * T + b_2 * N{:}P + b_3 * T * NP + b_4 * (N{:}P)^2 + \varepsilon$$

In addition to F-tests, Mann-Whitney U (MWU) and Kruskal-Wallis H (KW) tests were used to compare growth rates, carrying capacities and cell quota after visually comparing the distributions. The Bonferroni correction was again used against the type I bias of k number of tests.

3. Results

3.1 Monocultures

Growth was observed in all treatments, but the cultures at 20°C had a remarkably longer lag phase than those at 24°C (Fig. A1-4). For this reason, none of the 20°C cultures reached stationary growth by the end of the experiment. The slow-growing, benthic *P. lima* never reached the plateau phase either. The data from all of these cultures was fitted with exponential growth models (Fig. 2.1). The three other species (i.e. *P. micans*, *P. reticulatum* and *S. trochoidea*) reached stationary growth at 24°C and, hence, were fitted with logistic growth models that estimate carrying capacities. These cultures also consumed sufficient nutrients to calculate cell quota (Table 2.1).

Temperature affected the growth of all dinoflagellates (Fig. 2.2; MWU $p < 0.001$). Though most grew faster at 24°C (Table 2.1), *P. lima* grew slower (MWU $p < 0.001$). *P. lima* was also the only species to produce quantifiable amounts of toxins. Both the production of OA and DTX-1 concentrations increased at 24°C (MWU $p < 0.001$), but were unaffected by the N:P ratio (KW $p > 0.05$). Nutrient stoichiometry did not really affect the growth of *P. lima* either (Table 2.2). While the regression analysis suggests curvature (quadratic term $p = 0.010$; $\alpha = 0.013$), the small contribution of the N:P ratio ($p = 0.016$; $\alpha = 0.013$) was dwarfed by the effect of temperature. While the variable density data of *P. lima* produced uncertainty ($R^2 = 0.60$), the role of temperature was confirmed by the other species' regressions which were markedly better ($R^2 > 0.9$).

N:P ratios did not affect the growth rate of *P. micans* and *S. trochoidea* ($p > 0.05$). Still, temperature and N:P ratio interactively affected *S. trochoidea* and *P. reticulatum* ($p < 0.01$). Uniquely, the growth rate of *P. reticulatum* was influenced by the N:P ratio ($p < 0.01$), but no toxins were detected. The carrying capacities of *P. micans* and *S. trochoidea* were affected by the N:P ratio (F-test $p < 0.001$), which was not seen in *P. reticulatum* (F-test $p = 0.03$; $\alpha = 0.013$). Similarly, the N:P ratio altered the N (+) and P (-) cell quota (Q_N, Q_P), as well as the relative consumption (RC), of *P. micans* and *S. trochoidea*, which was not seen in *P. reticulatum* (F-tests). *P. micans* and *S. trochoidea* also had similar carrying capacities (K; MWU $p > 0.05$), which were mostly lower than those of *P. reticulatum* (K; MWU $p < 0.001$). *P. reticulatum* needed significantly less P than its competitors (Q_P; MWU $p < 0.001$), but its N demand was similar to the Q_N of *P. micans* (Q_N; MWU $p > 0.05$). *S. trochoidea* had the highest Q_N (MWU $p < 0.001$), but its P demand was similar to that of *P. micans* (MWU $p > 0.05$). By virtue of its average nutrient quota, *P. micans* had the lowest relative consumption of nutrients (RC; MWU $p < 0.01$). *P. reticulatum* and *S. trochoidea* had similar relative consumptions (RC; MWU $p > 0.05$).

Table 2.1: The average growth rate, carrying capacity (K), and cell quota (Q_N, Q_P, Q_{TOX}) of *P. lima* (*P.l.*), *P. micans* (*P.m.*), *P. reticulatum* (*P.r.*) and *S. trochoidea* (*S.t.*) across all ratios.

Parameter	Species	Temp	Mean±SE (min-max)
Growth rate (μ)	*P.l.*	20°C	0.09±0.002 (0.07-0.11) day^{-1}
	P.l.	24°C	0.05±0.004 (0.02-0.11) day^{-1}
	P.m.	20°C	0.13±0.004 (0.09-0.19) day^{-1}
	P.m.	24°C	0.49±0.025 (0.31-0.61) day^{-1}
	P.r.	20°C	0.09±0.003 (0.07-0.13) day^{-1}
	P.r.	24°C	0.30±0.008 (0.23-0.37) day^{-1}
	S.t.	20°C	0.16±0.005 (0.10-0.22) day^{-1}
	S.t.	24°C	0.42±0.011 (0.30-0.55) day^{-1}
Carrying capacity (K)	*P.m.*	24°C	0.53±0.03 (0.24-0.93) 10^9 $\mu m^3.ml^{-1}$
	P.r.	24°C	0.80±0.06 (0.39-1.58) 10^9 $\mu m^3.ml^{-1}$
	S.t.	24°C	0.44±0.02 (0.29-0.67) 10^9 $\mu m^3.ml^{-1}$
Nitrogen cell quota (Q_N)	*P.m.*	24°C	1.66±0.07 (0.85-2.53) 10^{-5} $pg.\mu m^{-3}$
	P.r.	24°C	1.63±0.08 (1.05-3.31) 10^{-5} $pg.\mu m^{-3}$
	S.t.	24°C	2.05±0.09 (1.37-3.27) 10^{-5} $pg.\mu m^{-3}$
Phosphorus cell quota (Q_P)	*P.m.*	24°C	0.19±0.01 (0.10-0.36) 10^{-5} $pg.\mu m^{-3}$
	P.r.	24°C	0.13±0.01 (0.07-0.23) 10^{-5} $pg.\mu m^{-3}$
	S.t.	24°C	0.17±0.00 (0.12-0.22) 10^{-5} $pg.\mu m^{-3}$
Relative consumption (RC)	*P.m.*	24°C	14.4±0.70 (7.55-18.5) N:P
	P.r.	24°C	20.0±0.92 (12.1-29.7) N:P
	S.t.	24°C	18.3±0.73 (13.1-24.9) N:P
Toxin cell quota (Q_{OA})	*P.l.*	20°C	0.62±0.05 (0.22-1.33) $pg.cell^{-1}$
	P.l.	24°C	1.03±0.08 (0.46-1.80) $pg.cell^{-1}$
Toxin cell quota (Q_{DTX-1})	*P.l.*	20°C	0.71±0.06 (0.25-1.45) $pg.cell^{-1}$
	P.l.	24°C	1.24±0.09 (0.49-1.96) $pg.cell^{-1}$

All dinoflagellates were grown under similar conditions: an initial density of 100 $cells.ml^{-1}$ and 10 different N:P ratios between 8 and 24. Growth rate (μ) and carrying capacity (K) were estimated from exponential (20°C) or logistic equations (24°C). Cell quota and the relative consumption of nutrients (RC) were calculated as described in section 2.4.

Table 2.2: Regression coefficients of the backward selected multiple regression models, with unique (UCC), common (CCC) and total commonality coefficients (TCC) plus propability.

Species	Coef. (pred.)	Est.±SE	UCC	CCC	TCC	%R^2	p
P. lima	b_0 (intercept)	0.32±0.03	/	/	/	/	<0.001
R^2 = 0.5967	b_1 (T)	-94.4±10.4.10^{-4}	0.5607	0.0000	0.5607	96.16	<0.001
	b_2 (N:P)	-74.6±24.2.10^{-4}	0.0418	-0.0342	0.0076	1.30	0.016
	b_3 (T*N:P)	/	/	/	/	/	>0.050
	b_4 (N:P^2)	2.37±0.89.10^{-4}	0.0490	-0.0342	0.0148	2.54	0.010
P. micans	b_0 (intercept)	-1.69±0.09	/	/	/	/	<0.001
R^2 = 0.9039	b_1 (T)	0.91±0.04.10^{-1}	0.9055	0	0.9055	100	<0.001
	b_2 (N:P)	/	/	/	/	/	>0.050
P. reticulatum	b_0 (intercept)	-0.51±0.15	/	/	/	/	0.001
R^2 = 0.9258	b_1 (T)	0.32±0.07.10^{-1}	0.0287	0.8885	0.9172	91.62	<0.001
	b_2 (N:P)	-0.29±0.09.10^{-1}	0.0122	-0.0122	0.0000	0.00	0.003
	b_3 (T*N:P)	0.01±0.00.10^{-1}	0.0124	0.0715	0.0839	8.38	0.003
S. trochoidea	b_0 (intercept)	-1.63±0.21	/	/	/	/	<0.001
R^2 = 0.9119	b_1 (T)	0.90±0.09.10^{-1}	0.1444	0.7468	0.8912	96.10	<0.001
	b_2 (N:P)	0.33±0.13.10^{-1}	0.0104	0.0047	0.0151	1.63	0.017
	b_3 (T*N:P)	-0.01±0.00.10^{-1}	0.0127	0.0083	0.0210	2.26	0.008

3.2 Competition

At 20°C, all dinoflagellates grew slower than their monocultures (MWU $p < 0.001$), and displayed a similarly long lag phase (Fig. 2.1; Fig A5-8). By the end of the study, all four species were still growing exponentially at similar concentrations. By contrast, the 24°C cultures were quickly dominated by *P. micans* in each of the 10 N:P ratios. In these cultures, the initial growth of *S. trochoidea* and *P. reticulatum* was slower than the monocultures (MWU $p < 0.001$), and came to a complete stop after 14 days. By contrast, *P. lima* grew faster than its 24°C monocultures (MWU $p < 0.001$) and grew for the entirety of the experiment. The toxin production, however, decreased from 0.58±0.31 pg OA.$cell^{-1}$ and 0.66±0.36 pg DTX-1.$cell^{-1}$ at day 14 – which is similar to the 20°C monocultures and 20°C mixed batch cultures (MWU $p < 0.001$) - to 0.19±0.12 pg OA.cell-1 and 0.20±0.14 pg DTX-1.$cell^{-1}$ (MWU $p < 0.01$) at day 28.

Once again, neither the toxin production of *P. lima*, nor the growth rates of the four dinoflagellates were affected by the N:P ratio (F-test $p > 0.05$) in the mixed cultures. The carrying capacities of *P. micans* again increased with the N:P ratio (KW $p < 0.01$). The presence of competitors had no influence on the carrying capacity of *P. micans* (MWU $p > 0.05$). The nutrient indices, Q_N, Q_P and RC could, hence, not be discerned from those of the 24°C monocultures (F-tests). Consequently, these uptake indices are influenced by the N:P ratio as well (F-test $p < 0.001$). Overall, *P. micans* grew at about the same rate as it did in monoculture (MWU $p > 0.05$).

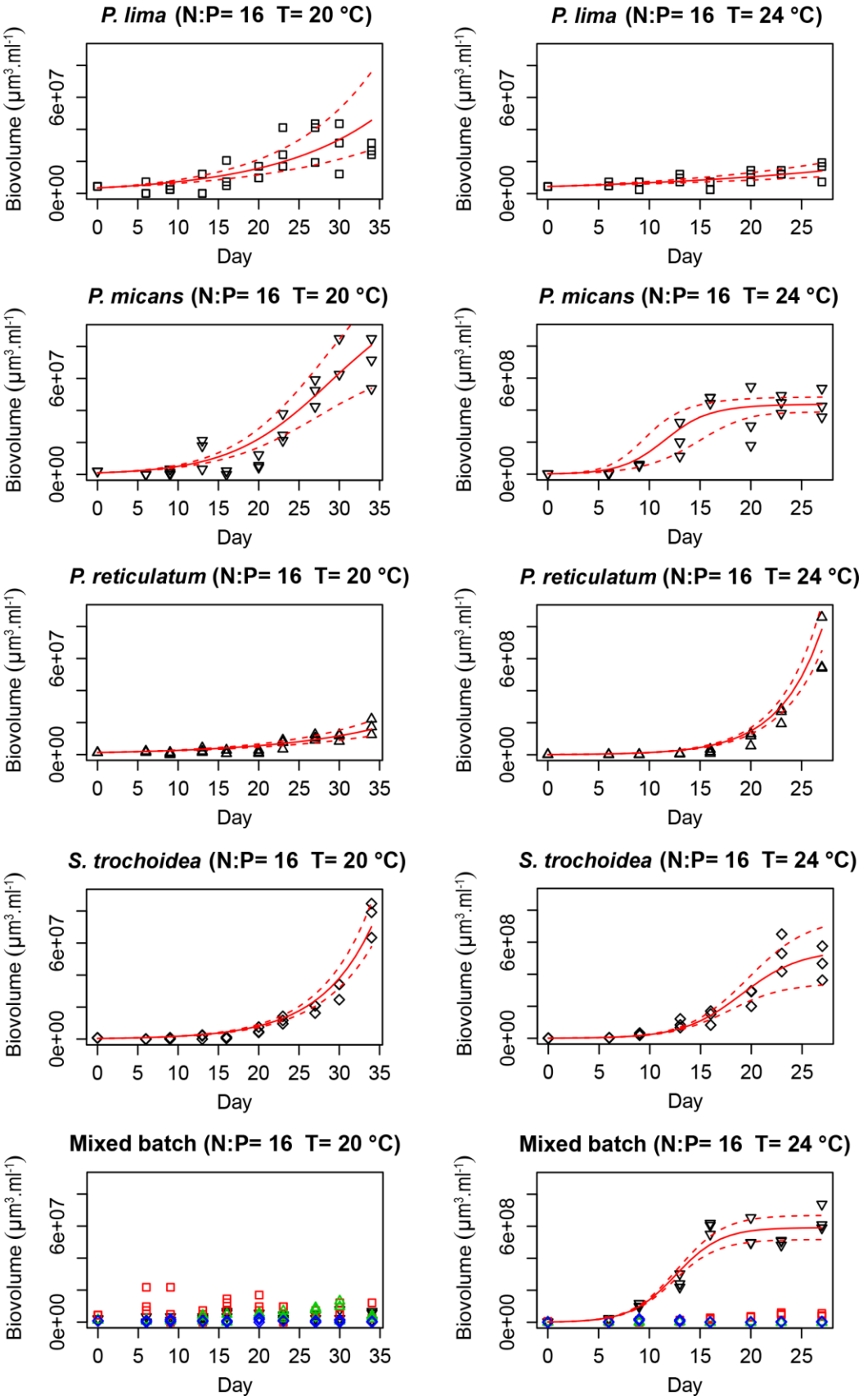


Figure 2.1: Culture growth at the Redfield ratio. Note the prolonged lag phase at 20°C. Cultures were fitted with either exponential or logistic growth models. Full lines represent the mean model prediction, dashed lines the 95% confidence interval.

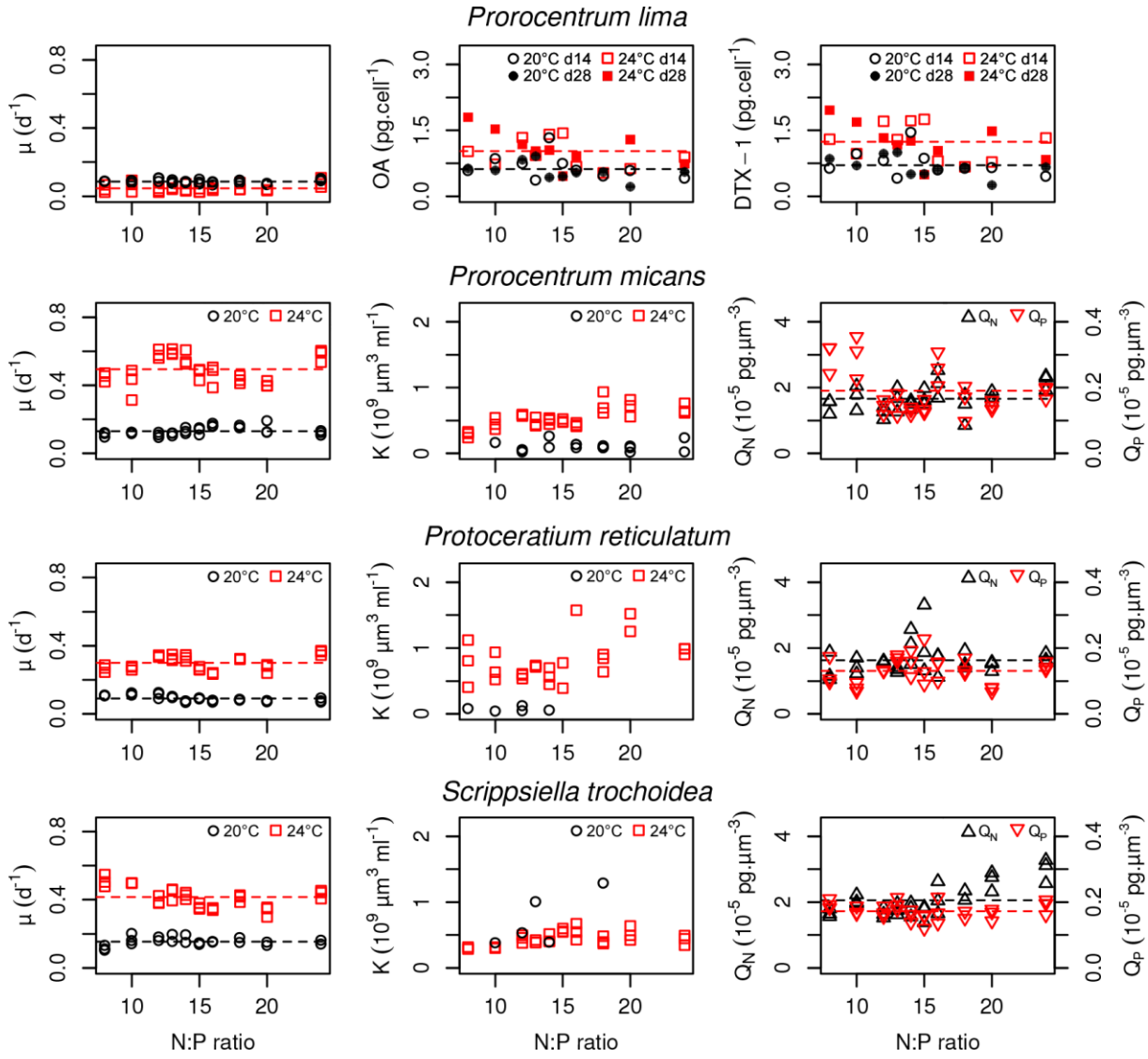


Figure 2.2: The growth rates (μ), carrying capacities (K) and cell quota of cultures of each dinoflagellate across 10 N:P ratios. Cell quota of okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are expressed as pg.cell^{-1}. Nitrogen (Q_N) and phosphorus cell quota (Q_P) are expressed as pg.μm^{-3}.

4. Discussion

Eutrophication has upset the biogeochemical balance of the marine environment. The perpetual changes in absolute and relative concentrations of macronutrients, such as phosphorus and nitrogen, have both been linked to the occurrence of HABs around the world (Glibert et al., 2014; Heisler et al., 2008). Yet, there is no evidence, nor an ecological theory, that suggests that changes in the relative availability of nutrients promotes HABs per se. According to Tilman's resource ratio hypothesis, the interspecific competition for resources is only influenced by the ratio of nutrients when each competitor is strictly limited by a different resource (Tilman, 1977, 1980). In other words, competing phytoplankton species need to have sufficiently different resource requirements before nutrient stoichiometry can matter. The applicability of Tilman's theory is obvious in the case of silicon, which is a key nutrient for diatoms but is not used by non-siliceous phytoplankton groups (Egge and Aksnes, 1992). Changing N:Si or P:Si ratios are, indeed, associated with shifts in the phytoplankton community (Roberts, 2003; Schöllhorn and Granéli, 1996; Sommer, 1994). Similarly, we have come to understand that the Redfield ratio is not an universal optimum for the growth of all phytoplankton (Klausmeier et al., 2004). As a result, large shifts in the N:P ratio can also be linked to the occurrence of blooms of cyanobacteria or prymnesiophytes such as *Phaeocystis* spp. (Paerl et al., 2014; Riegman et al., 1992). Yet, when looking at species with similar nutrient requirements like closely related dinoflagellate species, the N:P ratio is probably a dysfunctional and misleading metric (Davidson et al., 2012; Flynn, 2010).

Here, we grew four dinoflagellates with diverse traits and evolutionary adaptations (e.g. benthic vs. pelagic, toxic vs. non-toxic, small vs. large cells) under various temperature and nutrient stoichiometry scenarios to investigate the role of N:P ratios in HAB development. While we based our range of N:P ratios on the on-going shift in the mean ratio of the BPNS (ref. Brion et al. 2004), we also need to acknowledge that extremer cases of N-limitation and P-limitation (e.g. 1:1 to 375:1) are known to occur (Burson et al., 2016; Conley et al., 2009; Elser et al., 2007). Overall, the N:P ratio had little effect on the competitive traits of our dinoflagellates. Potential effects of the external nutrient stoichiometry on growth rate either disappeared in mixed cultures, or failed to replicate at another temperature. The OA and DTX-1 production of *P. lima* was not susceptible to the changes in the nutrient ratio either. Observed effects on carrying capacities were related to the increased absolute availability of nitrate rather than the N:P ratios. As a result, the nutrient stoichiometry was not found to affect the competitive outcome of our mixed batch cultures.

4.1 Nutrient stoichiometry

Unbalanced nutrient ratios affect the toxin production and the nutritional quality of algae, changing the likelihood of HAB development through effects on the top-down control of phytoplankton communities (Glibert et al., 2012; Hauss et al., 2012; Malzahn et al., 2010; Van de Waal et al., 2014). Moreover, it has been shown that HABs can sustaini themselves through mixotrophic and allelopathic interactions at non-Redfieldian ratios (Glibert et al., 2012; Heisler et al., 2008). There is, however, sizable doubt that the N:P ratio plays a key role in the bottom-up control of red tides (Davidson et al., 2012; Flynn, 2010). Experimental studies often find no relation between the growth of dinoflagellates and the relative availability of external nutrients (Chapter 3; Johansson and Granéli, 1999; John and Flynn, 2000; Li et al., 2012; Rhee, 1978; Varkitzi et al., 2010; this study). Cellular growth consumes intracellular reserves of nitrogen and phosphorus (Droop, 1974), that are taken up independently (Cembella et al., 1984; Dagenais-Bellefeuille and Morse, 2013). When the external nutrient supply is stoichiometrically unbalanced, cells alter their uptake efficiencies of each nutrient individually to maintain growth (Flynn, 2002; Klausmeier et al., 2007). Small changes in the N:P ratio are, hence, unlikely to affect interspecific competition (Flynn, 2010; Reynolds, 1999).

Intracellular nutrient ratios cannot be used to predict competitive advantages either (Terry et al., 1985). As a biological bet-hedging strategy against temporal variations in the supply of resources, as well as to deny competing species access to nutrients, steady state populations usually assimilate excess amounts of non-limiting nutrients (de Mazancourt and Schwartz, 2012; Droop, 1974). Due to this luxury consumption, intracellular N:P ratios tend to mimic the external nutrient ratio at low growth rates, but converge on species-specific values once the nutrient reserves are depleted at high growth rates (Goldman et al., 1979; Klausmeier et al., 2004; Rhee, 1978). These intrinsic values are the basis of the homeostatic nature of the oceans' N:P ratio. Under nutrient-replete conditions, the average intracellular N:P ratio of phytoplankton strives towards Redfield's ratio (Klausmeier et al., 2008; Redfield, 1958). Interspecific variation of these intracellular ratios, as described by Klausmeier et al. (2004), can be linked to life-history traits. Recently, it was shown that these intracellular N:P ratios are derived from an innate balance between N-rich proteins and P-rich ribosmal RNA (Loladze and Elser, 2011). Crucially, this new insight reveals that N-limitation inhibits protein synthesis and, hence, may benefit ribosome rich (i.e. fast-growing) species, while P-limitation restricts RNA transcription which is better endured by long-lived, biomass-conserving species such as dinoflagellates.

In this study, we calculated the relative consumption of nutrients as a measure of the internal nutrient ratios. According to these calculated values, *P. reticulatum* was predominantly P-limited (RC > 16:1), which is supported by the absence of biomass responses to additional nitrate and the literature (Gallardo Rodríguez et al., 2009). *P. micans*, by contrast, was the only species with a relative consumption below 16:1 (i.e. N-limited). Like Zhengbin et al. (2006) and Zheng-fang et al. (1995), we found that the stepwise addition of more nitrate (i.e. our N:P ratios) consistently increases the carrying capacity of *P. micans*. *S. trochoidea*, on the other hand, also increased its production at higher N:P ratios, despite appearing to be P-limited (i.e. RC > 16:1). A post-hoc analysis, in which we repeatedly used regression analyses after excluding the lowest N:P ratios in a stepwise manner, revealed that nitrate addition above 12:1 no longer provoked a response in additional biomass. Because of this, we believe that *S. trochoidea* switched between N-limitation and P-limitation or light limitation throughout our nutrient series. In literature, this species is also believed to be mostly N-limited (Cooper et al., 2016; Hoins et al., 2016; Xiao-ming et al., 1999). The high nitrogen cell quota of *S. trochoidea*, that led to the RC values found here, could have resulted from intracellular storage of excess nutrients, but little is known about luxury consumption in this species.

Luxury consumption is a major confounding factor when looking at the RC and the nutrient quota of algae. The uptake of excess nutrients by steady-state populations is in itself dependent on the external N:P ratio (Elrifi and Turpin, 1985), while the quota of non-steady-state populations may vary with the growth rate (Persson et al., 2010). As the 24°C cultures experienced both states, conclusions drawn from the RC or the nutrient quota should be interpreted with care.

In the experiments presented here, we manipulated the N:P ratio of the standard L1 growth medium for dinoflagellates by decreasing the amount of nitrate which was added during the preparation steps. The reason behind this methodology is two-fold. The normal N:P ratio of L1 medium is 24. To obtain N:P ratios below 24 by changes in the phosphate concentration, we would need to add above standard levels of PO_4^{3-} to the media, which could lead to phosphate precipitation in some of the treatments. By decreasing the nitrate concentrations instead, we can avoid this potential problem and mimic the current decrease in nitrogen concentrations in the natural environment (Fig. 1.7, p. 21). It should also be added that the alteration of nitrate concentrations is a common practice in literature (e.g. Zhengbin et al., 2006; Zheng-fang et al. 1995). Regardless of the literature, though, the results of this study suggest that the chosen approach would not matter for the individual performance of the algae.

4.2 Temperature

As temperature affects most key physiological processes of phytoplankton cells (e.g. motility, germination, photosynthesis and nutrient uptake), higher temperatures are usually associated with faster growth (Eppley, 1972). Even though we expected to see faster growth at higher temperatures, the difference between both temperature treatments seems to be out of proportion. While the 24°C growth rates of *P. micans*, *P. reticulatum* and *S. trochoidea* were similar to those found in literature, the similarity disappeared at 20°C (ref. Lee et al., 2005; Paz et al., 2006; Peperzak, 2003). Overall, the growth rates at 20°C were too low. In addition, we observed that all 20°C cultures experienced a rather lengthy lag phase, indicative of some form of shared stress. This experimental artefact was probably caused by unintentionally handling the algae at elevated temperatures while starting the experiment. A a posteriori investigation revealed that the prolonged use of the laminar flow cabinet – as was needed to simultaneously start 300 cultures – increases the temperature by several degrees. For this reason, we believe that 24°C cultures may have had the chance to gradually adapt to their new temperature, while the 20°C cultures were abruptly brought back down to their initial temperature at the start, effectively heat-shocking the cells. Steps were taken to avoid this problem in follow-up experiments (e.g. Chapter 3).

The light conditions used during the experiment could have enhanced the stress induced by the heat shock. Here, a restricted light intensity of 20±5 µmol m^{-2} s^{-1} was chosen to better mimic the light-limited conditions of the turbid waters of the BPNS. Between April and October, light penetrating the first 20 meters of the North Sea has an mean intensity of 75 µmol m^{-2} s^{-1} (Gröger et al., 2013). Beyond this euphotic zone, there is virtually no light (<1 µmol m^{-2} s^{-1} on average). As the water column of the North Sea is often fully mixed, the phytoplankton cells constantly move between the euphotic and aphotic zones. Without sufficiently strong compensatory adaptations to float or swim, and assuming a total water column depth of 30 to 40m, this means that North Sea phytoplankton is exposed to a mean light intensity of 37-50 µmol m^{-2} s^{-1}. These values are, however, derived for the entire North Sea. Because of suspended particles in the water column and the strong presence of so-called “yellow substance” (i.e. colored dissolved organic matter) the Southern Bight of the North Sea is known to have a very high light attenuation (Neukermans et al., 2012; Warnock et al., 2012). For this reason, we reduced the light intensity even further. Note, however, that some HABs develop in thin subsurface layers in the euphotic zone during certain periods of low turbulence, where they will receive a higher intensity of light.

4.3 Growth and toxin production of P. lima

Varkitzi et al. (2010) observed that *P. lima* produces OA at a fairly constant rate. Toxin quota are, hence, affected by the growth rate, as the intracellular toxins are "diluted" by divisions. This mechanism can clearly be seen in the results of this study. As the growth rate of *P. lima* decreased, e.g. the 20°C vs. 24°C monocultures, the toxin concentrations increased. The cellular concentrations of OA and DTX-1 were consistent with our work with this strain (chapter 5) as well as the available literature (Bravo et al., 2001; Koike et al., 1998; Nascimento et al., 2005; Vanucci et al., 2010). Remarkably, the growth-inhibition seen in 24°C monocultures was not observed in mixed cultures. In all likelihood, *P. lima* was able to benefit from the organic nutrients delivered by decaying pelagic competitors. The resulting changes in net growth may explain the difference in toxin content between day 14 and day 28.

Several studies have found relations between nutrient ratios and toxin production (Béchemin et al., 1999; John and Flynn, 2000; Lim et al., 2010; Murata et al., 2006). These interactions are, for instance, well described in *Alexandrium* spp. that produce N-rich toxins such as saxitoxin and gonyautoxins. More recently, Varkitzi et al. (2010) proposed a similar link between the N:P ratio and the production of OA by *P. lima*. Here, we found no effect of nutrient stoichiometry on the production of toxins by this species. Based on our results, the proposed correlation between toxin content and the N:P ratio seems improbable. Contrary to STX, OA and DTX-1 molecules do not contain nitrogen or phosphorus atoms. We do, however, know that nutrient stress may increase the accumulation of toxins within this species (Vanucci et al., 2010). More often than not, though, authors working on the production and release of toxins under nutrient stress (or other environmental stress for that matter) fail to recognise and report the reduced growth rate of the producer as the key determining factor of the observed trends in toxicity (Davidson et al., 2014).

4.4 Competition

As growth rates were unaffected by nutrient stoichiometry, we found no variation in the outcome of the interspecific competition either. *P. micans* dominated all mixed batch cultures at 24°C. While this species produces allelochemicals (Ji et al., 2011), this was most likely achieved through sheer speed (Chapter 3). *P. micans* consumed nutrients at such a tremendous rate, that its performance was indistinguishable from its growth in monoculture. Its pelagic competitors *S. trochoidea* and *P. reticulatum* could, by contrast, only divide three to four times before running out of reserves. At that point (day 14), virtually all of the nutrients were already gone from the medium.

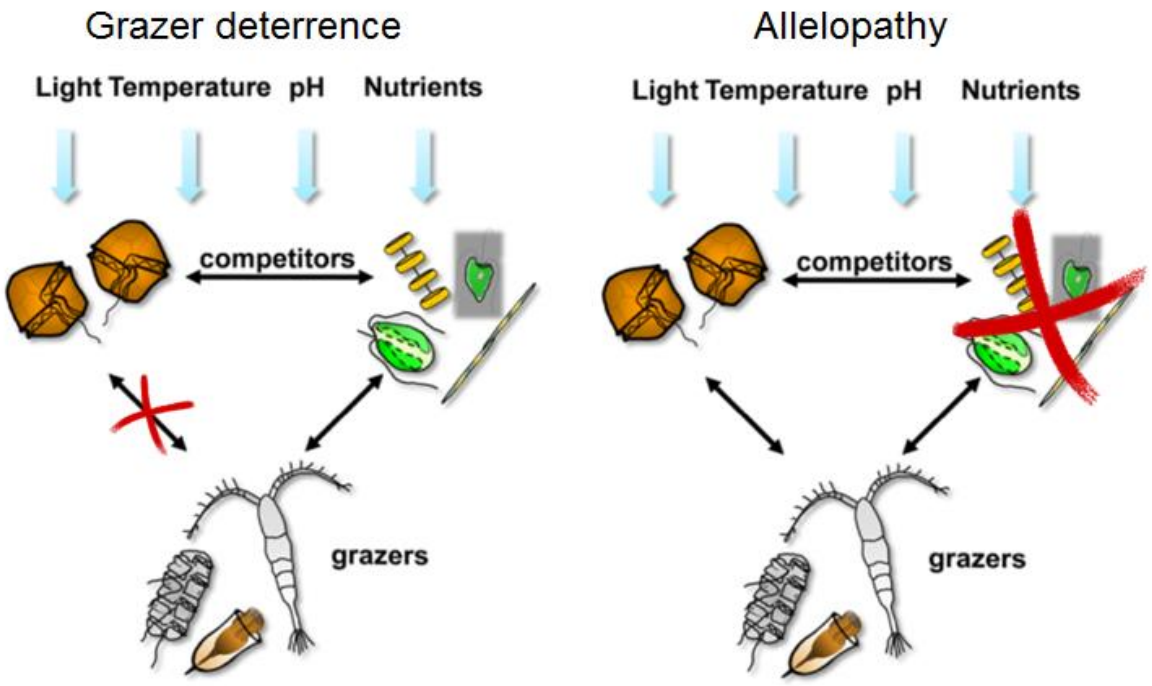
P. micans is a common, highly diversified dinoflagellate species (Dodge, 1975). Despite its remarkable competitiveness and ubiquitous occurrence across the globe, only a handful of *P. micans* blooms have ever been recorded. In part, these may be underreported as this species is often considered to be harmless or at least non-toxic (Glibert et al., 2012). High density blooms are, however, known to cause hypoxia with noticeable results (Pybus, 1990). It would be interesting to explore the processes that select against *P. micans* during the development of a toxic HAB in the same region. Top-down control needs to be the primary suspect, as some laboratory studies have shown that toxic dinoflagellates may enhance the grazing of copepods on *P. micans* (Barreiro et al., 2006; Guisande et al., 2002; Huntley et al., 1987). Alternatively, mixotrophy and the presence of other nitrogen sources (e.g. $NH4^+$ and urea) may provide competing species with additional means to avoid competitive exclusion (Burkholder et al., 2008; Glibert et al., 2008; Hansen, 2011; Kudela et al., 2008a; Lomas and Glibert, 1999, 2000).

5. Conclusions

Harmful algal blooms are known to result from complex interactions between both bottom-up and top-down processes, but the importance of the external resource ratio hypothesis (i.e. bottom-up) might be exaggerated. Here, we show that the N:P ratio has no significant role in the competition between harmful and benign dinoflagellates. The observed nutrient imbalance of the BPNS will, therefore, not directly increase the risk of toxic HAB development. Like Flynn (2010) and Davidson (2012), we urge HAB ecologists to reconsider the use of external resource ratios as a reliable measure of eutrophication. While nutrient stoichiometry certainly has an important structuring role in the environment, it only functions when taxa are limited by different resources. Direct effects of resource ratios on taxa with closely resembling nutrient requirements are, hence, unlikely. However, to attain a holistic understanding of the importance of the N:P ratio for HAB development, much may still be learned about the indirect effects on trophic dynamics.

3

The role of nutrient competition and abiotic variability during the development of potentially harmful dinoflagellate blooms



Abstract

As global change takes hold of the world's oceans, harmful algal blooms (HABs) are expected to change in frequency, scale and distribution. Anthropogenic changes in nutrient availability and water temperature are some of the main drivers of HABs. Yet, to date, it remains unclear how certain HAB species are able to outcompete similar non-HAB species during the development of blooms. Harmful dinoflagellates are thought to benefit from their ability to suppress or kill their competitors through allelopathy, but the function of allelochemicals during bloom initiation is still debated. Here, we set out to understand which factors and interactions determine the outcome of competition between three dinoflagellates under controlled laboratory conditions. To this end, co-occurring dinoflagellates of the North Sea (i.e. *Alexandrium minutum*, *Prorocentrum micans* and *Protoceratium reticulatum*) were cultured together in two large-scale multifactorial growth experiments. The outcome of competition and, thus, bloom development was studied under various scenarios: the first experiment used different macronutrient concentrations (0.1 to 100% of L1 medium) and N:P ratios (8, 16 and 24), while the second experiment varied the temperature (20°C vs. 24°C), N:P ratio (8 vs. 14) and initial species densities (0, 10 or 100 cells.ml^{-1}). The resulting community dynamics of both experiments could then be accurately predicted by the nutrient uptake rates, conversion efficiencies and the maintenance requirements of each species through MacArthur's resource competition model. As such, we found that the outcome of interspecific competition between dinoflagellates – in laboratory cultures - is mostly nutrient driven, leaving little room for allelopathy to play a vital role at pre-bloom concentrations.

1. Introduction

Ever since a series of harmful algal blooms (HABs) in the 1960s and 1970s, researchers have tried to understand and predict the spatiotemporal dynamics of dinoflagellate blooms. At first, red tides were thought to be inherently unpredictable due to the dynamic nature of marine ecosystems and the vast number of functional properties (nutrient uptake rates, internal storage, pigment composition etc.) and adaptive strategies (cyst production, shape, motility, thin layer formation, etc.) of harmful dinoflagellates (Sweeney, 1975, 1978). Today, phytoplankton communities are known to be structured by nutrient competition, direct species interactions (grazing, allelopathy), abiotic variables (light, temperature, turbulence etc.) and stochasticity (Armstrong, 1979; Eppley, 1972; Huisman and Weissing, 1994; Legrand et al., 2003; Margalef, 1978; Richerson et al., 1970; Smayda, 2008; Tilman, 1977). Yet, while it is clear that these dynamic main factors need to come together to create "windows of opportunity" for HABs, little is still known about the relative importance of nutrient competition, allelopathy and stochasticity during the initiation of blooms (Granéli and Turner, 2006; Stoecker et al., 2008b; Wells et al., 2015).

Ramon Margalef had observed that nutrient availability and the decay of turbulent energy are key to determine the succession of phytoplankton groups and, hence, the likelihood of bloom development (Margalef, 1978). In his "mandala", red tides can develop when the nutrient availability is high and the turbulent energy is restricted. While this window of opportunity was recently improved through the addition of functional properties, demographic strategies and the inclusion of novel HAB species (e.g. Allen and Polimene, 2011; Balch, 2004; Glibert, 2016), neither the mandala nor the recent renditions overcame the non-deterministic nature of this conceptual model. Blooms often fail to develop despite seemingly ideal conditions. To date, we are still unable to predict how changes in the relative and absolute availability of nutrients will affect the risk of HABs in any given phytoplankton community. While both episodic and chronic eutrophication are known to promote HAB development, there is little evidence that nutrients select for HABs, as the nutrient preferences and uptake kinetics of HAB species are no different from those of related non-HAB species (Anderson et al., 2002; Heisler et al., 2008; Wells et al., 2015). Dinoflagellates are poor competitors for nutrients and, hence, at constant risk of competitive exclusion (Smayda, 1997). In part, this risk can be reduced by toxicity-mediated allelopathy, grazer deterrence and mixotrophy (Chakraborty et al., 2015; Crane and Grover, 2010; Gross, 2003; Roy and Chattopadhyay, 2007).

Most dinoflagellates produce allelochemicals that cause nutrient leakage, inhibit photosynthesis, arrest cell-cycle progression, or affect other enzymatic activities of other algae (Granéli and Hansen, 2006; Legrand et al., 2003; Reigosa et al., 1999). The ability to suppress competitors gives a significant ecological advantage, and may be pivotal to maintain dinoflagellate blooms (Granéli et al., 2008a; Smayda, 1997). Most allelopathic effects are found at densities that typify well-developed HABs (Jonsson et al., 2009). Yet, to date, little is known about the importance of allelopathy during the initial stages of bloom development. While phytoplankton species are in a constant state of chaotic non-equilibrium, oscillating around an average density in a semi-random fashion due to weather-driven fluctuations and species interactions, the communities have a high degree of excitability. When some perturbation thresholds are exceeded, phytoplankton communities develop huge pulsed biomass responses (Truscott and Brindley, 1994). During these events, species dominance may depend upon the initial concentrations of each phytoplankter and, hence, predetermined by the presence of a holo- or meroplanktonic HAB inoculum (Granéli and Turner, 2006). Still, as blooms often fail to develop despite the presence of an inoculum, much could still be learned about this process (Smayda and Trainer, 2010).

Few mixed batch culture studies have been able to separate nutrient competition (i.e. indirect interactions) from direct interactions such as allelopathy and mixotrophy. Here, we aim to improve our understanding of the competition between toxic and non-toxic dinoflagellates. Specifically, this study investigates whether initial densities may predetermine the outcome of interspecific competition between dinoflagellates under laboratory conditions (1), and whether conditions such as temperature, nutrient availability and nutrient stoichiometry may change this outcome (2). To this end, three North Sea dinoflagellates: the non-toxic *Prorocentrum micans* Ehrenberg 1834 and the toxic *Alexandrium minutum* Halim 1960 and *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli 1885 were grown in two multifactorial batch culture experiments: one which explores the effect of the N:P ratio (8, 16 and 24) and total nutrient availability (0.1 to 100% L1 medium), and one which uses two nutrient regimes (N:P ratio 8 or 14), two climate scenarios (20 and 24°C) and three initial starting densities (0, 10 and 100 cells.ml^{-1}). MacArthur's (1970) consumer resource model was then used to accurately predict the outcome of each competition – under every set of conditions - between these three species.

2. Material and Methods

2.1 Algal cultures

Prorocentrum micans (CCAP1136/20) was obtained from the Culture Collection of Algae and Protozoa (Oban, Scotland). *Alexandrium minutum* (SCCAP K-0993) and *Protoceratium reticulatum* (SCCAP K-1478) were bought from the Scandinavian Culture Collection of Algae & Protozoa (Copenhagen, Denmark). Each dinoflagellate was grown in L1 medium (32 PSU, pH 8), prepared from autoclaved Instant Ocean^{tm} artificial seawater (Belcopet, Belgium) as advised by Guillard and Hargraves (1993). Around 80% of the culture medium was replaced every 2 weeks. Stock cultures were grown at 20°C, with a 12 hour light-dark cycle (20-40 µmol m^{-2} s^{-1}). Experiments were started from cultures in the exponential growth phase. Biovolumes were calculated using the methods of Olenina (2006).

2.2 Experiment 1: Nutrient availability & stoichiometry

To study the effect of nutrient stoichiometry and the availability of macronutrients on the interspecific competition between *A. minutum*, *P. reticulatum* and *P. micans*, L1 medium was prepared with nitrate and phosphate concentrations of four different orders of magnitude. These N and P concentrations matched with 0.1, 1, 10 or 100% of regular L1 medium and are hereafter called concentration factors (CF). In addition, the nitrate concentrations were modified to obtain three N:P ratios (8, 16 or 24) within each CF, resulting in twelve different media in total. Vitamins and trace elements were added at the regular dose of 100% L1 medium. In each of the twelve media, monocultures and a mixed batch culture of all three dinoflagellates were started by adding 100 cells.ml^{-1} (each) to 75 ml of medium placed in 100 ml Erlenmeyer flasks. Every treatment was replicated three times. Cultures were placed at 20 ± 1°C with a 12-hour photoperiod of 33±6 µmol m^{-2} s^{-1} for 54 days. Twice a week, cell counts were made on 1 ml of each flask, fixed with 100 µl of 12% formaldehyde and stored at 4°C. Densities were determined with a Sedgewick-Rafter counting chamber and a Nikon TMS-F light microscope (40x). In addition, 2 ml samples of each replicate were taken, filtered, pooled and stored at 4°C in Eppendorf for subsequent nutrient analysis. Nitrate and phosphorus concentrations were determined with standard colorimetric methods using a QuAAtro Autoanalyzer (Hansen and Koroleff, 1999).

2.3 Experiment 2: Initial densities & temperature

To investigate whether initial densities and temperature predetermine the outcome of competition between dinoflagellates, and provide support to the first experiment, another batch of L1 media was prepared. Now, nitrate levels were modified to obtain N:P ratios of 8 or 14 (the annual mean of the North Sea; Brion et al., 2004). Other L1 constituents (phosphate, vitamins, trace elements) were added at the regular dose. Each of the three dinoflagellates (*A. minutum*, *P. reticulatum* and *P. micans*) were added at either 0, 10 or 100 cells.ml^{-1} to 50 ml cultures, which were placed in climate rooms at either 20 or 24°C. Three replicates were made of each of the 108 resulting treatments (27 initial community compositions, 2 nutrient regimes, 2 temperatures). Cultures were left to grow for 68 days with a 12h photoperiod of 20±10 µmol m^{-2} s^{-1}. Cells were counted twice a week, as described in section 2.2. The initial nitrate and phosphorus concentrations (day 0) were verified by standard colorimetric methods using spectrophotometric test kits (Merck Millipore, Darmstadt, Germany) and an Aquamate spectrophotometer (Thermo Scientific, San Jose, USA). Additional nutrient analyses were performed near the middle (day 14) and end (day 28) of the growth by sampling and filtering 21 ml per treatment, split evenly across the three replicates.

2.4 Data analyses and community modelling

Growth rates (µ; d^{-1}) and carrying capacities (K; µm^3.ml^{-1}) were obtained by fitting the cell count data (N(t)), converted to biovolume (µm^3.ml^{-1}), with exponential growth models (Eq. 3.1) or logistic growth models (Eq. 3.2) – based on whether the carrying capacity of the culture was reached - using the 'nls package' in R (Baty et al., 2015).

$$(3.1)\ \mathrm{N(t)} = \mathrm{N_0} \cdot (1+\mu)^{\mathrm{t}}$$

$$(3.2)\ \mathrm{N(t)} = \frac{\mathrm{N_0 \cdot K \cdot e^{\mu t}}}{\mathrm{K + N_0 \cdot (e^{\mu t} - 1)}}$$

MacArthur's resource competition model for non-interacting resources was used to predict community dynamics in mixed cultures (MacArthur, 1970). In this model, n species interact by depleting common resources (Eq. 3.3-3.5).

$$(3.3)\ \frac{1}{N_i}\frac{dN_i}{dt} = (C_{N,i} \cdot U_{N,i} \cdot W_{N,i} + C_{P,i} \cdot U_{P,i} \cdot W_{P,i} - m_i)$$

$$(3.4)\ \frac{\mathrm{dC_N}}{\mathrm{dt}} = -\mathrm{C_N} \sum_{\mathrm{i=1}}^{\mathrm{n}} \mathrm{u_{N,i}} \cdot \mathrm{N_i}$$

$$(3.5)\ \frac{\mathrm{dC_P}}{\mathrm{dt}} = -\mathrm{C_P} \sum_{\mathrm{i=1}}^{\mathrm{n}} \mathrm{u_{P,i}} \cdot \mathrm{N_i}$$

Nitrogen (C_N; mg.l^{-1}) and phosphorus (C_P; mg.l^{-1}) concentrations are depleted by species i through nutrient uptake rates $U_{N,i}$ and $U_{P,i}$ (l.µm^{-3}.d^{-1}). Algal growth is achieved when the biomass production, performed at nutrient conversion efficiencies $W_{N,i}$, and $W_{P,i}$ (µm^3.mg^{-1}), exceeds the maintenance requirement (m; d^{-1}). Here, we assumed that growth was nitrogen limited (i.e. $W_P = 0$) as the changes in phosphorus were much smaller than the depletion of nitrogen (Fig. B1-B6). The monoculture data of each species was used to estimate the remaining parameters. Due to the long lag phase of cultures at 20°C (Fig. B1 & B2), the first 10 days were removed during the data treatment of experiment 1.

The mean average percentage error (MAPE) was used as an objective function for parameter estimation to ensure an equal fit of species densities when densities differed by several orders of magnitude. First, optimal parameters estimates were obtained using a simulated annealing algorithm. Next, credibility intervals were found using Markov chain Monte-Carlo simulations. To ensure fast parameter convergence, the parameter space was limited to a 50% deviation of the optimal parameter estimates. Convergence of the three parallel Markov chains was assessed with the Gelman-Rubin convergence criterion (Gelman and Rubin, 1992). Density predictions for monocultures and mixed cultures were obtained using 1000 Monte-Carlo simulation runs. Every run, parameters were randomly drawn from the posterior probability distributions. The global performance of the model was assessed by comparing the observed species densities to median predicted species densities. All calculations were done in R using the packages deSolve (Soetaert et al., 2010), abind (Plate and Heiberger, 2011), and GenSA (Xiang et al., 2013).

3. Results

3.1 Monoculture growth

The magnitude of macronutrient concentrations, obtained by adding 1:10 fractions of the regular L1 nitrate and phosphate levels and called concentration factors (CF), affected the growth rates and carrying capacities of all three species. Growth rates were highest at CF10 which, according to the parameters of our MacArthur model, coincided with an increase in nutrient consumption rates, rather than changes in resource efficiency or maintenance requirements (Table 3.1; Table B1). As a result, the carrying capacities at CF10 were roughly a tenfold smaller than those at CF100. No exponential growth was, however, observed below CF10. Nutrient stoichiometry (i.e. N:P ratio) had little effect on the growth rate of each species (Table B1). Carrying capacities, on the other hand, increased with N:P ratios (Fig. B1 & B3).

Table 3.1: Performance of *A. minutum*, *P. reticulatum* and *P. micans* at various N:P ratios and temperatures across two large-scale, multifactorial batch culture experiments.

	μ (d^{-1})	K (10^8 µm^3.ml^{-1})	U_N (10^{-7} l.µm^{-3}.d^{-1})	U_P (10^{-7} l.µm^{-3}.d^{-1})	W_N (10^8 µm^3.mg^{-1})	m (10^{-4} d^{-1})
A. minutum						
Exp.1: CF10	0.32±0.01	1.36±0.02	23.5	19.8	1.74	0.06
Exp.1: CF100	0.24±0.01	15.1±0.85	2.25	0.09	1.50	0.05
Exp.2: 20°C	0.12±0.01	3.93±0.50	2.56	7.00	0.76	87.9
Exp.2: 24°C	0.47±0.02	5.72±0.28	15.0	5.04	0.72	0.01
P. reticulatum						
Exp.1: CF10	0.20±0.01	0.74±0.03	27.1	19.9	0.90	0.05
Exp.1: CF100	0.16±0.01	9.43±0.90	2.24	0.13	1.02	0.05
Exp.2: 20°C	0.07±0.01	0.49±0.09	1.86	4.60	0.93	505
Exp.2: 24°C	0.25±0.01	6.34±0.46	3.76	8.14	1.10	9.00
P. micans						
Exp.1: CF10	0.38±0.01	0.58±0.11	71.7	15.3	0.70	0.04
Exp.1: CF100	0.28±0.01	4.23±0.11	7.50	0.12	0.53	0.05
Exp.2: 20°C	0.12±0.01	3.40±0.25	4.40	21.7	0.55	392
Exp.2: 24°C	0.59±0.07	2.98±0.22	17.3	37.3	0.43	3.13

*Dinoflagellates were grown in two experiments under similar conditions: 20°C, initial density of 100 cells.ml^{-1} and a N:P ratio of 16 (exp.1) vs. 14 (exp.2). In addition, a ten-fold decrease in absolute nutrient concentrations (CF10 vs. CF100; exp.1) and an increase in temperature (20°C vs 25°C; exp.2) were included. Growth rate (µ±SD) and carrying capacity (K±SD) were estimated from exponential (20°C; exp.1) or logistic equations. The uptake of nitrogen U_N and phosphorus U_P, nitrogen conversion efficiency W_N and maintenance requirement m were based on MacArthur's consumer-resource equations (ref. 2.4).

As different N:P ratios were obtained by adjusting the nitrate concentration, the link between N:P ratios and carrying capacities supports the underlying assumption that our cultures are predominantly nitrogen-limited (see section 2.4). Growth rates and nitrogen consumption rates increased with temperature (Table 3.1; Table B2). 20°C growth rates were slightly lower in the first experiment, which could be related to the light conditions (see methods). During the second experiment, no growth was observed in the *P. reticulatum* cultures with an initial density of 10 cells.ml^{-1} (Fig B3). The parameter distributions of the monocultures with 100 cells.ml^{-1} were therefore used to predict the community dynamics of this species.

3.2 Community dynamics

MacArthur's resource competition model, tailored to nitrogen, explained 89% of the variation in density data of the first experiment, and 72% (20°C) and 90% (24°C) of the variation in the second experiment (Fig. 3.2). *P. micans* had the highest growth rate and nitrogen consumption rate under all abiotic conditions (Table 3.1; Table B2), and dominated 54 out of the 64 cultures to which it was added. *A. minutum* had the highest nutrient conversion efficiency and, hence, the highest carrying capacities of all three species (Table 3.1). *A. minutum* outcompeted *P. micans* when started at a numerical advantage (i.e. 100 vs. 10 cells.ml^{-1}; Fig B4&5 1:2; Fig B6 1:1:2 & 1:2:2). *P. reticulatum* is the weakest competitor, but it is nonetheless able to produce higher densities when given higher initial start densities than its competitors.

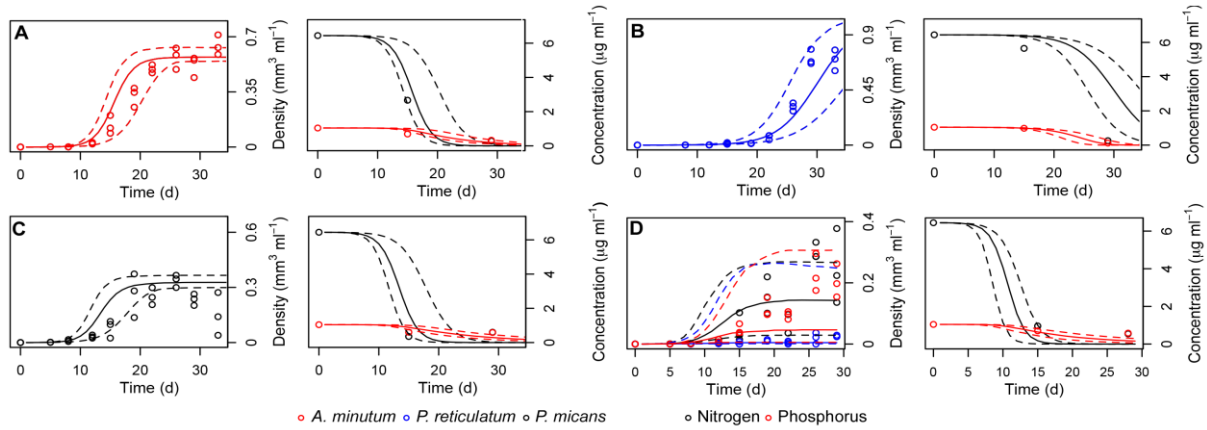


Figure 3.1: Example of the application of our MacArthur's consumer-resource model. Parameterisation was optimized on monocultures of *A. minutum* (A), *P. reticulatum* (B) and *P. micans* (C) and used to predict multispecies competition (D). Full lines represent the mean prediction, dashed lines the 95% confidence interval. All species were added at 100 cells.ml^{-1} under a N/P ratio of 14 and 24°C (experiment 2). Complete set of graphs in Figures B1-B6.

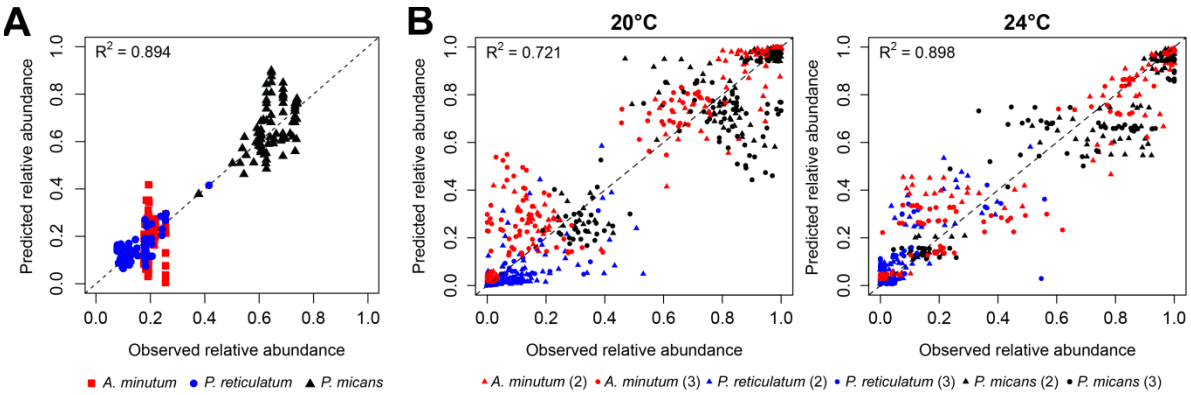


Figure 3.2: Evaluation of MacArthur's consumer-resource model applied to the mixed batch cultures of the first (A) and second experiment (B) of this study. Each species is represented by a unique colour. In (B), triangles represent mixtures of two species while dots represent cultures with all three species.

4. Discussion

Interspecific competition between HAB and non-HAB species is understudied (Wells et al., 2015). Even though we identified several processes that may determine HAB development (e.g. grazer resistance, nutrient competition, allelopathy), we still need to understand the relative importance of these elements during all stages of a bloom cycle. While many studies have investigated the physiological responses of individual HAB species to environmental conditions, few have added environmental variability when looking at interactions between two or more species. Here, we show that temperature and nutrient availability affect communities through direct effects on the nutrient competition, which is the key determinant of bloom development in mixed cultures of dinoflagellates. We also show that small competitive differences can be overcome through changes in the the relative initial densities.

4.1 Nutrient availability, nutrient stoichiometry & temperature

Anthropogenic nitrogen and phosphorus inputs are linked to the global increase in frequency and severity of marine HABs (Anderson et al., 2012; Hallegraeff, 1993). Cultural eutrophication is found in nearly all long-term coastal monitoring records (Brush, 2008; Clarke et al., 2006; Cloern, 2001). The excess availability of nutrients affects the interspecific competition of phytoplankton, enabling the proliferation of one or more species (Anderson et al., 2002; Davidson et al., 2014; Granéli et al., 2008b; Heisler et al., 2008; Hodgkiss and Ho, 1997; Paerl et al., 2014). There is, however, doubt that eutrophication favours HABs per se, as the nutrient requirements of harmful algae are no different from those of non-HAB species (Wells et al., 2015).

Here, the availability of nitrate and phosphate – varied as a percentage of regular L1 medium concentrations (i.e. concentration factors or CFs) – had a clear effect on the performance of each species, changing the growth rates and carrying capacities through the nutrient uptake rates and nitrogen conversion efficiencies (Table 3.1), which was expected as N-limitation is known to upregulate nitrogen transporters (Sciandra, 1991; Zhuang et al., 2015). Regardless, CFs did not alter the competition between our dinoflagellates, as each dinoflagellate responded in a similar fashion. Likewise, the N:P ratio did not influence species dominance, as it had no influence on the growth rate of all species despite small changes in nitrogen uptake rate (U_N) and nitrogen conversion efficiency (W_N; Table B1).

Temperature determines the growth rate as well as key physiological processes (e.g. motility, germination, photosynthesis, nutrient uptake) of algae (Eppley, 1972) and is, hence, a key determinant of HAB development. Here, higher temperatures were associated with higher growth rates and improved nitrogen consumption rates (Table 3.1, Table B2), though some parameter estimates were biologically impossible (e.g. Table B2 *P. micans* NP 14 20°C: W_N 4.72 vs. $0.55.10^8$ $\mu m^3.mg^{-1}$) due to the limited resolution of the nutrient data. Overall, though, temperature did not change the outcome of our communities, as all species had similar responses to this change. In situ, however, climate change is expected to broaden the windows of opportunity for HABs (Smayda and Smayda, 2015; Wells et al., 2015), as most algae live outside their optimal temperature niche (Karentz and Smayda, 1984, 1998).

4.2 Stochasticity, allelopathy & community dynamics

Apart from nutrient availability, temperature and other environmental conditions, phytoplankton communities are structured by species interactions and stochasticity (Armstrong, 1979; Eppley, 1972; Huisman and Weissing, 1994; Legrand et al., 2003; Margalef, 1978; Richerson et al., 1970; Smayda, 2008; Tilman, 1977). As HA often compete poorly for nutrients, toxin-mediated interactions such as grazer deterrence and allelopathy are often seen as the key to HAB development. Yet, while allelopathy is commonly believed to give a competitive advantage during bloom development (Cembella, 2003; Legrand et al., 2003), this may require inhibitory effects which are disproportional to the likelihood of cell-cell interactions and the concentrations of toxins at low densities (Jonsson et al., 2009). To date, however, there is a substantial lack of experimental evidence for exclude either hypothesis.

All of the dinoflagellates used here (*A. minutum*, *P. micans* and *P. reticulatum*) have been shown to produce allelochemicals which reduce the fitness of other algae (Arzul et al., 1999; Fistarol et al., 2004; Ji et al., 2011; Sala-Pérez et al., 2016). Despite of this, we managed to accurately predict the dynamics of each community using only the nutrient consumption rates, conversion efficiencies and maintenance requirements of monocultures (Fig 3.2). While the inclusion of allelopathic interaction terms may mathematically improve the model even further, there is little biological sense to do this exercise. During the initial stages of the mixed cultures, the growth of each species is near identical to their performance in monoculture for all nutrient (CF & N:P ratio) and temperature scenarios. While initial densities had little effect on the parameter estimates (Tables B1 & B2), they could change species dominance (e.g. mixtures of *A. minutum* and *P. micans*), which demonstrates the importance of a sufficient holo- or meroplanktonic inoculum to seed HABs (Granéli and Turner, 2006). Still, allelochemicals are common in HA (Granéli et al., 2008a; Legrand et al., 2003). Based on our results, the prevailing species is first decided by nutrient competition, which does allow slow species to outcompete faster species when given a head start, before densities are reached where allelopathic interactions start to play a vital role. Allelopathy may, however, be used to maintain established blooms and prevent the long-term competitive exclusion of HA within a given phytoplankton community (Chakraborty et al., 2015; Granéli et al., 2008a; Jonsson et al., 2009; Lewis, 1986; Roy, 2009; Roy and Chattopadhyay, 2007; Smayda, 1997; Solé et al., 2005).

4.3 Remarks & recommendations

Due to the static nature of controlled laboratory conditions and the lack of grazing (mixotrophy and zooplankton) in this experiment, it is clear that competitive outcome of our experiments do not necessarily represent the dynamics of similar assemblages in the wild. It is, for instance, known that *P. micans* is readily consumed by copepods (Gill and Harris, 1987; Guisande et al., 2002) which can increase their uptake rates after exposure to grazer deterrents released by both *A. minutum* and *P. reticulatum* (Barreiro et al., 2006; Guisande et al., 2002; Huntley et al., 1987). Mixotrophy is also a crucial mechanism to attain higher growth rates under nutrient limited conditions (Burkholder et al., 2008). Moreover, it is known that most HAB species prefer $NH4^+$ and urea (Glibert et al., 2008; Kudela et al., 2008a; Lomas and Glibert, 1999, 2000; Zielinski et al., 2011). While nitrate is most common in marine ecosystems, and the uptake rates of NO_3^-, NH_4^+ and urea rarely differ by more than a factor 3 in laboratory cultures (Chang et al., 1995; Fan et al., 2003; Kudela et al., 2008b; Smayda, 1997), the inclusion of nutrient variation into the design is still recommended.

This study uses a simple model, which requires a minimum of density and nutrient data to accurately predict the competition in exponentially growing mixed cultures of dinoflagellates. However, the utility of the model is limited to the initial growth phase. Due to its underlying assumptions, this model is ill suited to investigate quiescence and transient growth dynamics. Here, the model is already prone to underestimate densities when nutrient concentrations were rapidly declining. Dinoflagellates are known to “luxury consume”, storing nutrients in intracellularly for times of deficiency (Cembella et al., 1984; Dortch et al., 1984). Growth is, in fact, based on the internal concentration of nutrients (Droop, 1974). Yet, as the model coupled growth to media concentrations, the densities cannot increase in the absence of external nutrients. Future work may resolve this, by coupling growth to internal nutrient concentrations that are replenished through the uptake of external nutrients (John and Flynn, 2000). This, however, requires data on the internal nutrient concentrations of each species, which need to be measured. Future experiments can also extend the experiments passed the stationary phase. To the best of our knowledge, no studies have recorded allelopathic interactions throughout the entire bloom cycle.

5. Conclusions

Despite decades of experimental research, we were still unable to unravel the key mechanisms behind interspecific competition in mixed cultures of dinoflagellates. Crucially, this hampers our understanding of the importance of resource competition, allelopathy and mixotrophy, which undermines our ability to forecast the risk of HABs in changing environments like the North Sea. By analysing the growth of mixed batch cultures, this study demonstrates that nutrient competition and not allelopathy is the driving force behind the interspecific competition between dinoflagellates. In addition, this study demonstrates how the relative densities of competing algae and, hence, seed beds can initiate blooms, and shows how abiotic variability e.g. temperature, nutrient stoichiometry and nutrient availability can affect HABs through changes in resource uptake rates and resource efficiencies. This study provides further support to the opinions of Davidson et al. (2012) and Flynn (2010), i.e. that summer nutrient concentrations should be favoured over nutrient ratios, as the latter provides little to no information about the risk of HABs. To validate this study, we recommend using monitoring data to predict HAB development with nutrient consumption and grazing pressure alone. To this end, the work of Sourisseau et al. (2017) - that predicts the interannual variability of HABs in a French estuary using only nutrient competition and abiotic forcing – may provide a solid basis for future work.

4

The absorption, distribution, metabolization and excretion of two major groups of marine toxins by adult blue mussels

Abstract

Lipophilic marine biotoxins (LMBT) are produced by several cosmopolitan algae, e.g. *Dinophysis* spp., *Prorocentrum* spp., *Alexandrium* spp. and *Protoceratium* spp., which may become more abundant due to global change. The most common vector of lipophilic marine biotoxins (LMBT) to humans is seafood. Shellfish are particularly important, as they can quickly accumulate LMBT and create esterified metabolites. Yet, despite risks to human health, little is known about the absorption, distribution, metabolization and excretion (ADME) of LMBT in shellfish. With the emergent mussel farming projects inside the Belgian EEZ, this information is needed to reduce the risk of shellfish poisoning in consumers and prevent the destruction of the new produce. For this reason, this study investigates the ADME kinetics of two groups of LMBT - okadaic acid analogues (OA) and spirolides (SPXs) - in *Mytilus edulis*. To this end, adult blue mussels were exposed to two of their respective producers, the harmful dinoflagellates *Alexandrium ostenfeldii* and *Prorocentrum lima*, in either a single or combined two-week laboratory exposure. At the same time, mussels were exposed to the natural concentrations of toxic phytoplankton in Ostend harbour (Belgium). During both experiments, the toxin profiles of the mussel tissues were recorded by ultra-high performance liquid chromatography coupled to high-resolution Orbitrap mass spectrometry (UHPLC-HR-Orbitrap MS). Overall, both experiments found a rapid accumulation of OA analogues and SPXs in the visceral tissues of *M. edulis*. Biotransformation of multiple toxins, in particular of the OA analogues, occurred in less than 3 days. Within 15 days of exposure, some fatty esters such as 14:1 DTX-2, 14:0 OA and 16:2 OA were found at concentrations up to 80 $\mu g/kg^{-1}$, exceeding the EU regulatory limit for OA analogues by a large margin. This, hence, demonstrates the potential risk that seafood exposed to mixtures of LMBT poses to human health and urges research into the toxicological effects of LMBT mixtures.

1. Introduction

Harmful algal blooms have increased in frequency and intensity on a global scale. Blooms of potentially toxic dinoflagellates such as *Dinophysis* spp., *Alexandrium* spp. and *Prorocentrum* spp. have become more prevalent due to regional climate change (Anderson et al., 2012; Hallegraeff, 2010). During certain “windows of opportunity”, i.e. specific and often unknown combinations of ideal biotic and abiotic conditions, these species bloom, which entails enormous economic costs in the aquaculture, tourism and, crucially, public health sectors (Hoagland et al., 2002; Shumway, 1990). A significant fraction of this impact is caused by the toxins that these cosmopolitan microalgae produce. Lipophilic marine biotoxins (LMBT), e.g. azaspiracid (AZA), yessotoxins (YTX), spirolides (SPX), okadaic acid (OA) and dinophysistoxins (DTX), readily accumulate in marine biota and create a risk for severe human poisoning (FAO, 2004; Lawrence et al., 2011). To prevent human poisoning incidences, most European countries (Portugal, Ireland, UK, Denmark, France, the Netherlands, etc.) set up costly, extensive routine monitoring programs which screen edible shellfish, phytoplankton and other biota for LMBT.

Two toxin groups of particular interest are OA analogues and SPXs. OA is related to diarrhetic shellfish poisoning (DSP), the most common type of seafood poisoning (Reguera et al., 2014). It is produced by *Dinophysis* spp and *Prorocentrum* spp., two well-spread genera of dinoflagellates. Its mode of action is well-known. It is a potent phosphatase inhibitor which causes inflammation of the intestinal tract and diarrhoea. For this reason, the amount of OA in seafood is checked against legal limits for safe consumption by institutions such as the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA). SPXs, on the other hand, are produced by *Alexandrium ostenfeldii*, i.e. another cosmopolitan species (Cembella et al., 2000). Despite recent reports of high concentrations in shellfish, little is known about SPXs. While SPXs are highly neurotoxic in mice, where they are able to cross the protective blood-brain barrier, no human illness has ever been associated with spirolid ingestion (Alonso et al., 2013; Sleno et al., 2004). Still, the lack of a known effect on humans can result from the poor recognition and underreporting of moderately adverse health conditions such as gastric distress and tachycardia (Daneshian et al., 2013). There is mounting evidence that SPXs inhibit muscarinic acetylcholine receptors and activate transmembrane calcium channels which disrupt human neuroblastoma cells in vitro (Kantiani et al., 2010; Munday et al., 2011; Wandscheer et al., 2010). The absence of regulatory limits for safe consumption should, hence, be seen as a severe risk for acute and chronic intoxications.

The lack of knowledge on the occurrence and effects of toxin mixtures poses another risk towards the health of human consumers. As the mouse bioassay was replaced by liquid chromatography coupled to mass spectrometry (LC-MS) as the principle monitoring tool for LMBT in European shellfish (Commission Regulation No 15/2011), unknown toxins and synergistic mixture toxicity below regulatory levels are effectively undetectable. Still, the use of powerful analytical tools such as LC-MS has greatly improved our detection of LMBT and LMBT metabolites in all kinds of matrices. Through this approach, more than 50 OA analogues have been found in natural phytoplankton communities (Díaz et al., 2013; Vale and Sampayo, 2002), cultures of *Prorocentrum lima* and *Dinophysis* spp. (Bravo et al., 2001; Nascimento et al., 2005; Nielsen et al., 2013; Suzuki et al., 2009; Vale et al., 2009) and higher-order organisms (Orellana et al., 2017; Trainer et al., 2013; Vale and Sampayo, 2002). Similarly, over 16 SPX esters have been described from toxic strains of *A. ostenfeldii* (Almandoz et al., 2014; Cembella et al., 2000; Kremp et al., 2014; Medhioub et al., 2011; Salgado et al., 2015; Tillmann et al., 2014), and the tissues of other marine life (García-Altares et al., 2014; Medhioub et al., 2012; Orellana et al., 2017; Rundberget et al., 2011; Silva et al., 2013). Now, the next step in the detection and quantification of LMBT and LMBT metabolites, is the use of high-resolution mass spectrometry (HRMS) to explore the absorption, distribution, metabolization and excretion (ADME) of marine toxins in seafood upon HAB exposure.

Despite the substantial amount of available literature on the occurrence and effects of LMBT in shellfish, surprisingly little is still known on ADME processes of marine toxins in common shellfish species. Usually, this knowledge gap results from the inability to reliably identify or (accurately) quantify the amount of absorbed and metabolized LMBT. High-resolution mass spectrometry (HRMS) is a promising new instrument to explore the ADME kinetics of marine toxins in seafood. This technique has already been used successfully on shellfish and algae (Domènech et al., 2014; García-Altares et al., 2014; Gerssen et al., 2011; Orellana et al., 2014, 2015). Now, we use this promising technique to study ADME processes of lipophilic marine toxins in a keystone species. To the best of our knowledge, this is the first study to expose blue mussels *M. edulis* to *A. ostenfeldii* and *P. lima* in a mixed exposure experiment to study the ADME of OA and SPXs through state-of-the-art LC-HRMS analyses. In addition, this study demonstrates through fieldwork that similar processes occur when mussels are exposed to the natural phytoplankton of the Belgian Part of the North Sea (BPNS), an understudied area in the North Sea region.

2. Material and Methods

2.1 Chemicals and standards

A multitoxin standard was made from several certified reference materials, i.e. OA (CRM-OA-c 14.3 ± 1.5 µg ml^{-1}), DTX-1 (CRM-DTX-1 15.1 ± 1.1 µg ml^{-1}), PTX-2 (CRM-PTX-2 8.6 ± 0.3 µg mL-1), AZA-1 (CRM-AZA-1 1.24 ± 0.07 µg ml^{-1}), 13-SPX C (CRM-SPX-1 7.0 ± 0.4 µg ml^{-1}), and YTX (CRM-YTX 5.6 ± 0.3 µg ml^{-1}), as described in detail by Orellana et al. (2015). All certified reference materials were obtained from the National Research Council (Institute for Marine Bioscience, Halifax, Canada). Analytical grade solvents (for extractions) and LC-MS grade solvents for UHPLC-MS applications were obtained from VWR International (Merck, Darmstadt, Germany). Ultrapure water was made in-house by means of a Milli-Q water purification system (VWR International, West Chester, Pennsylvania, USA). Millex-GV syringe filters (PVDF 0.22 µm) were obtained from Millipore (Merck, Darmstadt, Germany) and glass beads of 0.5 mm were purchased from Thistle Scientific Ltd. (Glasgow, UK).

2.2 Algal cultures

Alexandrium ostenfeldii (CCAP 1119/45), *Prorocentrum micans* (CCAP 1136/20) and *P. lima* (CCAP 1136/9) were obtained from the Culture Collection of Algae and Protozoa (Scottish Marine Institute, Oban, UK). In addition to these dinoflagellates, in-house strains of the nutritional algae *Tetraselmis suecica* and *Isochrysis galbana* were kindly provided by the Laboratory of Aquaculture and Artemia Reference center (Ghent University, Belgium). All of these algae were grown in L1 medium, prepared from autoclaved and filtered seawater (30 ± 2 PSU; pH 8.0 ± 0.5) from the Belgian part of the North Sea (BPNS) in accordance with Guillard and Hargraves (1993). *Prorocentrum* spp. and *A. ostenfeldii* were grown in 500 ml Erlenmeyer flasks, while *T. suecica* and *I. galbana* were cultured in bags of 5 litre. Both the Erlenmeyer flasks and the bags were placed in the same room, which had a constant temperature of 20±1.0 °C and a 12:12 h irradiance of 100 µmol m^{-2} s^{-1}. The growth of stock cultures was monitored through frequent cell counts, which were performed frequently using a Sedgewick-Rafter counting cell and an inverted microscope (40x). Cultures were harvested semi-continuously during the late-log or early-stationary phase for feeding *M. edulis* and for performing toxin analysis.

2.3 Mussel culture and depuration

Two months before carrying out the experiments, wild adult mussels (*M. edulis*) were picked from breakwaters along the BPNS, cleaned by hand and placed in a recirculating, filtered aquarium (15°C, 32 PSU) in the laboratory. During this time, mussels could depurate any toxins that may have accumulated during their time in the sea. To keep them healthy, they were fed daily ad libitum with a commercial algal paste (Shellfish Diet 1800®, Varicon Aqua Solution, UK). The artificial seawater of the tank (Instant Ocean^{TM}, Belcopet, Belgium) was replaced on a weekly basis.

2.4 Experimental design

2.4.1 Artificial exposure

After the depuration period, 280 mussels were randomly distributed into nine 30l glass aquaria, i.e. around 30 animals per aquarium. Mussels were constantly fed with either *A. ostenfeldii*, *P. lima* or both simultaneously during 15 days. Each of the three treatments was carried out in triplicate during this experiment. To ensure a realistic scenario, i.e. there are nutritious non-HAB algae present during natural algal blooms, two non-toxin producers (*T. suecica* and *I. galbana*) were continuously added to the aquaria as well. A concentration of 2 x 10^2 cells ml^{-1} was added to the mussels by a regimen of 8 automated-feedings per day, using fully automated peristaltic pumps (Ismatec SA, Switzerland). As a control treatment, two aquaria were added in which the mussels were only fed with *T. suecica* and *I. galbana*. Samples of mussels were collected on days 3, 5, 10 and 15 of the exposure to the experimental treatments. At each time, 7 or 8 mussels were removed from the aquaria. Additionally, faeces and pseudofaeces (mix) and water samples were collected during harvest days.

2.4.2 Field study

A North Sea field study was conducted in Ostend harbour and the adjacent sluice dock called the “Spuikom” (51.226328, 2.931137) during July 2015. 160 depurated mussels were placed into 8 cages, i.e. 20 mussels per cage, at different stations around the harbour. Similar to the lab study, five mussels were collected from each cage on day 3, 5, 10 and 15 for toxin analysis.

2.5 Toxin extraction

2.5.1 Pre-treatment

Samples of the stock cultures of the dinoflagellates were treated as described by Orellana et al. (2015). Samples were poured into PVC tubes with a 10 µm nylon net filter at the bottom, and washed with ultrapure water to remove salts. The filters were subsequently placed into a centrifuge tube and backwashed with 3 mL of methanol. A similar procedure was used for the (pseudo)faeces, but the mesh size was adapted to 80 µm. Mussel meat samples were treated as described by Orellana et al. (2014), but the method was adapted to separate visceral and non-visceral tissues. In short, the in and outside of the shells was rinsed with ultrapure water to remove any debris. Next, mussels were removed from the shell and left to drain on a 100 µm sieve. The visceral (hepatopancreas) and non-visceral tissues (gills, mantle, gonads etc.) were then carefully dissected and weighed.

2.5.2 Water and culture media

Solid phase extraction (SPE) protocols were adopted from De Rijcke et al. (2015) (ref. chapter 6) to determine concentrations of toxins in watery phases. This method was used on both the culture media of *P. lima* and *A. ostenfeldii*, as well as the water samples taken from the experiment to determine concentrations of toxins eliminated from mussels. Samples of 2 mL were placed preconditioned Strata-X polymeric reversed phase cartridges (100 mg / 3 mL; Phenomenex, Utrecht, the Netherlands). Each column was washed with 8 mL of ultrapure water and subsequently eluted with 2 ml of 70% acetonitrile. Extracts were then reduced to 1 ml under a gentle stream of nitrogen gas at 40°C, transferred to LC-MS vials and stored at −20°C until analysis.

2.5.3 Algae and faeces

As done by Orellana et al. (2015), intracellular toxins were extracted from algae by a repeated liquid-liquid phase extraction and simultaneous glass bead disruption. The method was adapted to faecal samples by adding a two minute homogenization step in a ultra turrax™ homogenizer (IKA, Staufen, Germany) prior to the start of the phytoplankton protocol. The resulting internal toxin extracts were dried at 40°C under a gentle stream of nitrogen at 40°C. The residues (1 ml) were transferred into vials and stored at −20°C prior to analysis.

2.5.4 Mussel tissues

To extract toxins from the tissues of mussels, we adopted the extraction steps from Orellana et al. (2014). In short, 1 g of visceral or non-visceral tissue was placed in a centrifuge tube. Next, toxins were extracted with 3 ml of methanol by vortexing and centrifuging (12,000×g) for 3 min and 10 min, respectively. After the supernatant was removed, the entire extraction process was repeated two more times. The total extracted volume of 9 ml was again dried under a gentle stream of nitrogen at a temperature of 40°C until a residue of 1 mL could be transferred into a vial.

2.6 UHPLC-HRMS

Liquid chromatography used a Thermo Fisher Scientific Accela UHPLC pump, coupled to an Accela autosampler and degasser and fitted with a Nucleodur C18 Gravity column (1.8 µm, 50x2 mm, Macherey-Nagel, Düren, Germany) for compoud segregation. Mass spectrometry occurred inside an Exactive^{TM} benchtop Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), equipped with a heated electrospray ionization probe (HESI-II). The instrument was operated in switching polarity mode. All instrument settings were adopted from Orellana et al. (2015).

The operational performance of the UHPLC-HRMS instrument was verified using a CRM mixture of OA, DTX-1, 13-SPX C, PTX-2, AZA-1, YTX and PTX-2 (section 2.1). Prior to the analyses of samples, these toxins were first identified by the mass and retention time of their respective certified standard solutions, and their identity was verified with the 13C/12C isotopic ion ratio (as recommended by CD 2002/657/EC). Peaks of toxins in experimental samples were compared to the listed CRM standard. After the identification of parent compound peaks, metabolites were identified using the Thermo ToxFinder software package and the work of Gerssen et al. (2011) and Torgersen et al. (2008). Based on the strong resemblance between newly detected metabolites and parent ions, and the excellent selectivity and separation the Orbitrap MS provides, metabolites were (semi)quantified using the HRMS response ratio.

3. Results and discussion

3.1 Algal cultures

The UHPLC-HRMS analyses revealed that OA, DTX-1, OA-D6, OA-D8 and OA-T9 were present in both the intra- (n=10) and extracellular extracts (n=40) of *P. lima* (Table 4.1). All of these compounds were present in reasonably similar amounts, but the highest concentrations belonged to the parent toxins OA (9.5 ± 1.5 pg cell^{-1}) and DTX-1 (7.1 ± 2.3 pg cell^{-1}). OA-D8 could only be detected in the intracellular extract, not in the medium outside the cells. Early-stationary phase cultures of *A. ostenfeldii* contained 5 compounds. These were 13-desmethyl SPX C, SPX D, SPX F, SPX I and 27-hydroxy-13-desmethyl SPX C. The latter was only found in the culture media. The predominant toxins were 13-desmethyl SPX C and SPX D, with mean estimated concentrations of 5.6 ± 2.2 pg cell^{-1} and 3.2 ± 1.8 pg cell^{-1}, respectively.

Table 4.1 Intra- and extracellular compounds found in *P. lima* and *A. ostenfeldii* cultures (n=10) during the early-stationary phase. ND = not detected.

Tentative identity*	Elemental composition	Measured mass (*m/z*)	Mean intracellular concentration (pg cell^{-1}) ± SD	Mean extracellular concentration (µg l^{-1}) ± SD
P. lima				
OA	C44H68O13	803.46088	9.5 ± 5.0	1.4 ± 1.1
DTX-1	C45H70O13	817.47650	7.1 ± 3.3	1.5 ± 0.9
OA-D6	C50H76O14	923.51605	3.2 ± 2.2	0.90 ± 0.7
OA-D8	C52H80O14	951,54443	4.2 ± 2.0	ND
OA-T9	C53H82O15	957.55944	6.1 ± 3.1	1.3 ± 1.1
A. ostenfeldii				
SPX D	C43H66NO7	708.48394	3.2 ± 1.8	0.11 ± 0.1
SPX F	C42H66NO7	697.48917	2.7 ± 1.4	0.19 ± 0.1
SPX I	C40H62NO6	653.46326	3.0 ± 1.6	0.18 ± 0.1
13-SPX C	C42H62NO7	693.45898	5.6 ± 2.2	0.21 ± 0.1
27-OH-13-SPX C	C42H61NO8	708.44592	ND	0.16 ± 0.1

* OA = Okadaic acid, DTX-1 = Dinophysistoxin-1, OA-D6 = 5-hydroxy-2-methylene-pent-3-enyl okadaate, OA-D8 = 7-hydroxy-2-methyl-hepta-2,4-dienyl okadaate, OA-T9 = 5,7-dihydroxy-2,4-dimethylene-heptyl okadaate, 13-SPX C = 13-desmethyl spirolide C, 27-OH-13-SPX C = 27-hydroxy-13-desmethyl SPX C.

Table 4.2 Mean concentrations of SPX-related compounds in the tissues (n=14), faeces (n=10) and media (n=10) of mussels exposed to *A. ostenfeldii* or *A. ostenfeldii* and *P. lima*. Concentrations between brackets were found in the non-visceral tissues. Toxins with an asterisk were found in the cultures of *A. ostenfeldii* (ref. Table 4.1). Detailed identification steps are presented in supplementary tables C2 to C10.

		A. ostenfeldii				**Mixed exposure**			
		Day 3	**Day 5**	**Day 10**	**Day 15**	**Day 3**	**Day 5**	**Day 10**	**Day 15**
(Non)-visceral tissues (µg.kg^{-1})	SPX B	-	0.63	-	-	-	2.52	-	-
	SPX C	-	-	-	1.14	-	-	-	-
	SPX D*	0.55	0.96	1.26 (0.56)	3.03 (1.45)	3.43	1.25	0.69 (0.39)	0.89 (0.65)
	SPX E	-	0.62	1.65 (0.75)	1.34 (1.21)	-	0.95	-	-
	SPX F*	-	-	-	1.56 (1.15)	-	-	-	1.56
	SPX H	-	-	1.15	-	3.43	-	-	1.03
	SPX I*	-	0.66	1.87 (0.93)	1.09 (1.07)	-	1.14	0.34	1.08
	13-SPX C*	0.7	-	-	1.89 (1.15)	-	0.58	-	2.19 (1.48)
	27-OH-13-SPX C*	0.69	-	0.31	1.88 (1.64)	0.8	-	0.26	5.2 (2.86)
	27-O-13,19-SPX C	-	-	0.78	1.93	1.99	-	0.47	0.73
(Pseudo)faeces (µg.kg^{-1})	SPX B	-	-	-	0.41	-	0.1	-	-
	SPX C	-	-	-	0.16	-	0.15	1.16	1.83
	SPX D*	1.25	1.45	1.72	0.62	1.61	1.37	1.84	1.77
	SPX E	-	-	-	0.11	-	-	-	-
	SPX F*	-	0.15	0.1	-	-	0.24	0.23	0.79
	SPX H	-	0.26	0.18	-	-	0.36	0.16	0.93
	SPX I*	-	-	-	-	-	0.28	0.47	-
	13-SPX C*	0.11	0.22	0.19	0.24	1.1	1.25	1.16	1.83
	27-OH-13-SPX C*	-	-	0.11	0.13	-	-	0.15	-
	27-O-13,19-SPX C	-	1.03	0.25	0.13	-	-	0.14	1.73
Water (µg.l^{-1})	SPX D*	-	1.89	1.96	1.88	1.06	1.04	1.36	2.37
	SPX E	-	-	-	0.11	-	-	-	0.97
	13-SPX C*	-	0.91	1.22	1.76	1.13	1.56	1.25	3.58
	27-OH-13-SPX C*	-	-	0.69	0.6	-	-	3.45	3.76
	27-O-13,19-SPX C	-	-	0.84	0.71	-	-	1.77	3.84

3.2 Mussel physiology

The mussels used in both experiments had an average length of 4.0 ± 0.5 cm and a mean whole body wet weight of 2.5 ± 0.5 g per individual. Prior to the experiments, depurated mussels (n=20) contained no toxins and appeared to be in good health. Approximately one hour after the start of the experiment, filtration and the production of faeces was observed in all treatments. During the course of the experiment, only one dead mussel was reported. After 15 days, no toxins were detected in the tissues of mussels which only fed on *I. galbana* and *T. suecica* in the control aquaria.

3.3 Exposure to *A. ostenfeldii*

From the start, the mussels accumulated SPX-related compounds in their digestive glands (Table 4.2). At first, these compounds were identical to those found in *A. ostenfeldii* (i.e. SPX D, 13-SPX C & 27-OH-13-SPX C; 0.55 to 0.7 $\mu g.kg^{-1}$). Note, however, that 27-OH-13-SPX C was also formed as a metabolite (Figure 4.1). SPX D and 13-SPX C were found in the (pseudo)faeces at 1.25 and 0.11 $\mu g.kg^{-1}$, respectively. While this may indicate that the mussels partially rejected toxic cells, it should be noted that *A. ostenfeldii* produces these toxins in rather similar quantities (3.2 and 5.6 $pg.cell^{-1}$, respectively). As the faeces contained relatively little 13-SPX C, one of the toxins is involved in a preferential uptake or active excretion process that, based on the evolution of these toxins in the faeces and visceral tissues, continued throughout the experiment. No toxins were detected in the water column at this time.

The first true metabolites, i.e. compounds which were not found in *A. ostenfeldii*, were detected when SPX B (0.63 $\mu g.kg^{-1}$) and SPX E (0.62 $\mu g.kg^{-1}$) appeared in the visceral tissues on day 5. At that time, metabolites (SPX H and 27-O-13,19-SPX C) first appeared in the faeces. Evidence of translocation was found later, when SPX D, SPX F, SPX I, 13-SPX C and 27-OH-13-SPX were detected in both the visceral and non-visceral tissues of *M. edulis* after 10 days. These compounds are all produced by *A. ostenfeldii*. SPX E was the only metabolite which was transported to the mantle and gonads. This SPX metabolite was also found in the faeces (0.11 $\mu g.kg^{-1}$) and water column (0.11 $\mu g.l^{-1}$). Other faecal metabolites included SPX B (0.41 $\mu g.kg^{-1}$), SPX C (0.16 $\mu g.kg^{-1}$) and 27-O-13,19-SPX C (0.13 $\mu g.kg^{-1}$), of which only the latter was also present in the water column (0.71 $\mu g.l^{-1}$). The highest concentrations in each of the several compartments were, however, always linked to compounds which are directly produced by *A. ostenfeldii* (e.g. SPX D, 13-SPX C and 27-OH-13-SPX C).

Table 4.3 Mean concentrations of OA-related compounds in the tissues (n=14), faeces (n=10, and media (n=10) of mussels exposed to *P. lima* or *P. lima* and *A. ostenfeldii*. Concentrations between brackets were found in the non-visceral tissues. Toxins with an asterisk were found in *P. lima* cultures (ref. Table 4.1).

		P. lima				**Mixed exposure**			
		Day 3	**Day 5**	**Day 10**	**Day 15**	**Day 3**	**Day 5**	**Day 10**	**Day 15**
(Non)-visceral tissues (µg.kg^{-1})	OA*	5.17	9.12 (4.83)	12.05 (8.64)	9.77 (3.55)	5.97 (1.27)	3.88 (2.24)	5.57 (4.25)	7.64 (3.84)
	DTX-1*	8.63	11.81 (6.56)	15.13 (4.45)	13.21 (7.24)	8.04 (6.67)	6.31 (3.93)	10.03 (3.75)	15.61 (7.03)
	OA-C3	3.39	-	-	-	-	-	3.15	-
	OA-D6*	-	-	5.3	-	-	-	-	-
	OA-D8*	-	-	10.85	2.02 (2.81)	-	-	-	2.82 (1.67)
	OA-D10	-	-	-	1.76 (1.61)	-	-	2.57	1.24 (1.08)
	OA-T9*	-	-	-	1.2	-	-	-	-
	14:0 OA	5.35	2.55	9.38 (4.22)	6.72	-	-	9.01	13.59
	14:3 OA	4.47	10.44 (6.39)	11.17 (7.87)	3.26	4.47	5.31	4.54	12.47
	15:0 OA	-	13.4	26.5	67.33	4.4	4.82 (2.02)	7.57	13.94 (30.3)
	16:0 OA	-	11.07	26.69	7.39	3.84	2.79 (1.95)	8.66	22.94 (19.89)
	16:1 OA	-	15.91 (10.67)	28.08	15.77	-	-	8.96 (3.83)	14.26 (11.29)
	16:2 OA	4.76	35.2 (23.24)	126 (59.08)	49.81 (35.39)	8.59 (3.34)	3.65 (2.89)	28.6 (17.25)	- (12.21)
	17:1 OA	-	5.11	2.08	-	-	-	-	5.93
	18:1 OA	-	13.92	3.58	2.3 (2.26)	-	-	-	-
	18:2 OA	-	19.95 (13.57)	14.77 (9.78)	4.77 (3.91)	-	-	4.88	7.62
	18:4 OA	-	16.87	28.08	3.41	-	-	-	36.35 (11.99)
	20:5 OA	3.77	10.25 (7.21)	14.77 (10.29)	4.3 (3.9)	4.4 (1.05)	-	16.21 (9.35)	42.25 (4.15)
	14:1 DTX-2	-	-	-	-	18.26 (10.11)	6.09	82.82	13.82 (6.32)
Faeces (µg.kg^{-1})	OA*	-	0.47	0.79	1.58	1.04	3.41	5.01	1.28
	DTX-1*	-	-	-	0.36	2.03	7.23	9.12	4.37
	OA-C3	-	0.34	0.34	1.23		0.35	1.59	0.89
	OA-D8*	-	-	-	0.55	-	0.23	0.27	0.23
	OA-T9*	6.12	37.23	41.24	22.89	11.04	58.74	9.88	76.73
	14:3 OA	-	-	-	-	-	-	-	0.36
	16:2 OA	-	0.37	0.51	0.49	-	0.36	0.58	0.25
Water (µg.l^{-1})	OA*	-	1.78	3.35	5.15	0.74	1.22	4.22	4.32
	DTX-1*	-	-	1.43	2.83	1.13	-	4.57	3.15
	OA-C3	-	-	-	0.53	-	-	-	1.5
	OA-D6*	-	1.12	1.52	3.22	-	1.29	1.16	2.35
	OA-T9*	3.66	2.76	4.2	5.28	2.15	2.63	4.57	5.82
	16:2 OA	-	-	-	-	-	0.51	-	-

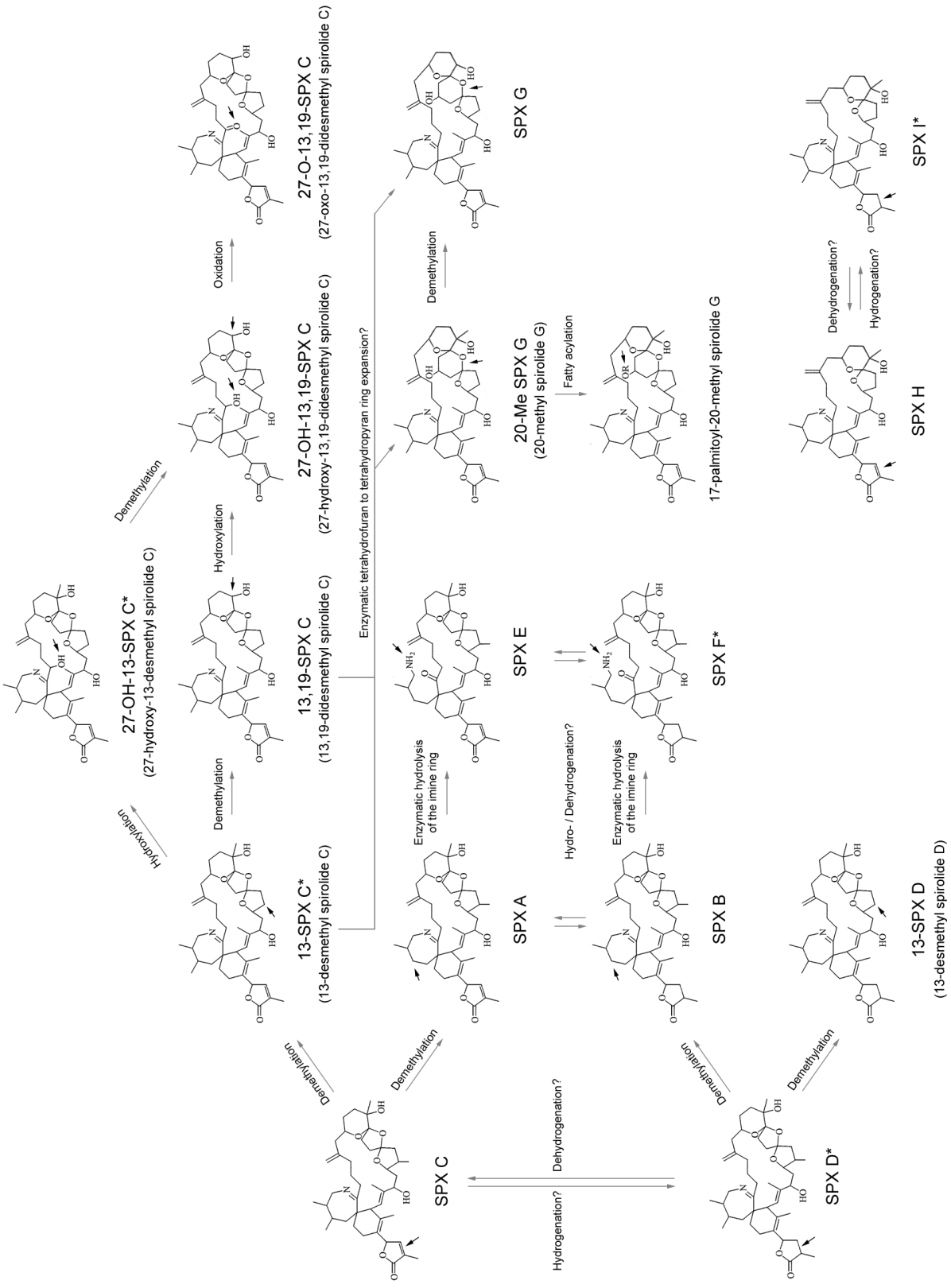


Figure 4.1 Potential biotransformation routes of known spirolides and SPX metabolites. Structures and reactions based on the work of Aasen et al. (2006), Cembella et al. (1999), Ciminiello et al. (2006, 2010), Guéret and Brimble, (2010), Hu et al. (2001), Hui et al. (2012), Molgó et al. (2007) and Roach et al. (2009).

3.4 Exposure to *P. lima*

Throughout the experiment, 26 unique OA compounds were found in *M. edulis* (Full list in Table C2). The parent toxins, OA and DTX-1, accumulated in both mussel tissues, and were (to a lesser extent) also present in the (pseudo)faeces (Table 4.3). By contrast, high amounts of the triol ester OA-T9 – which was produced by *P. lima* in similar quantities as OA and DTX-1 (Table 4.1) – were found in the faeces, but not in the tissues. This either suggests a rapid absorption of OA and DTX-1, or an active excretion of OA-T9. Other esters, such OA-C3, OA-D6, OA-D8 and OA-D10 were intermittently present in the tissues. These compounds are produced by *P. lima* and were probably rapidly metabolized (ref. Figure 4.2) by the mussels.

Biotransformation and translocation were observed throughout the experiment. From day 3, OA was coupled to fatty acids of varying length and degree of saturation (i.e. 14:0 to 20:5). The resulting acyl derivates - most notably 14:3, 15:0, 16:2, 18:2 and 20:5 OA – accumulated strongly in both the visceral and non-visceral tissues, often reaching high concentrations (e.g. 16:2 OA 126 $\mu g.kg^{-1}$). The most abundant fatty ester, 16:2 OA, was also found in the faeces. None of the metabolites were, however, detected in the water column. Both the number of OA-related compounds in the mussels and the concentrations of compounds peaked after 10 days of exposure. By contrast, the toxin diversity and concentrations went up in both the medium and the faeces at day 15. At the same time, the excretion of faecal OA-T9 decreased. These results suggest a behavioural response – such as changes in the filtration rate or particle selection - to stress, but mortality was not observed.

3.5 Mixed exposure to *A. ostenfeldii* and *P. lima*

The initial accumulation of spirolides found in *A. ostenfeldii* (i.e. 27-OH-13-SPX C and especially SPX D) in the mixed exposure was generally higher than that of the single exposure, but 13-SPX C was absent (Table 4.2). In part, more 13-SPX C was excreted as the faecal concentrations were an order of magnitude higher than those found in the single exposure. The dissolved concentrations in the water column were higher as well. At the same time, this compound may have been metabolized towards 27-O-13,19-SPX C. Similarly, SPX I is excreted or transformed to SPX H. Overall, biotransformation appears faster in the mixed exposure. More metabolites were excreted via the faeces (e.g. SPX C, SPX H and 27-O-13,19-SPX C), and fewer compounds were translocated to the mantle and gonads. The few compounds which accumulated faster, i.e. 13-SPX C and 27-OH-13-SPX C, may originate from both the algae as well as metabolic processes.

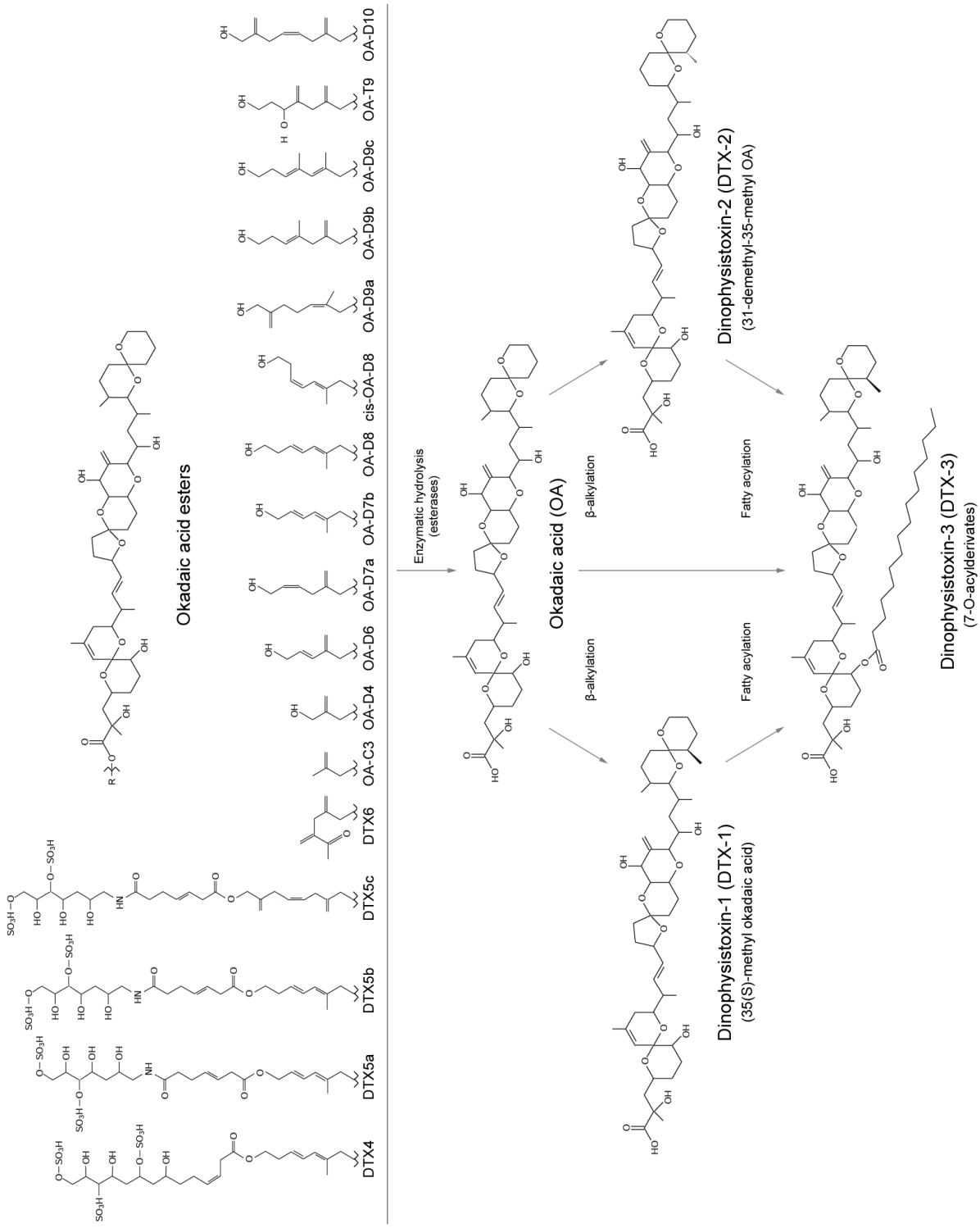


Figure 4.2 The potential transformation of OA structures, based on the combined work of Cruz et al. (2006), Hu et al. (1992), Marr et al. (1992), Needham et al. (1995), Paz et al. (2007), Suárez-Gómez et al. (2001), Torgersen et al. (2008a), Vale (2008), Van Wagoner et al. (2014) and Wright et al. (1996). Molecular names in supplementary materials (Table C1).

Like SPXs, the mussels accumulated more toxins (OA and DTX-1) in their tissues while, at the same time, rejecting more food-based compounds through their faeces (OA, DTX-1, OA-T9; Table 4.3). Faecal deposits became richer in both diversity and quantity of compounds than the single exposure. The dissolved concentrations of compounds were often higher as well. Accumulated concentrations in visceral tissues were generally lower, and metabolites(e.g. 16:2 OA and 20:5 OA) appeared earlier. Again, the parental compounds were less translocated towards non-visceral tissues, but several metabolites were nonetheless detected. Remarkably, the predominant metabolite 16:2 OA of the single exposure was replaced by 14:1 DTX-2. This form of OA was never observed during the single exposure.

3.6 Exposure to the natural conditions of Ostend harbour

No toxins were initially found inside mussels of any station (Table 4.4). By day 5, however, the first compounds were detected in the visceral samples of *M. edulis*. These were all fatty esters of OA and DTX-2. The number and concentrations of OA metabolites increased until the end, reaching a maximum of 32.5 µg kg^{-1} for 14:0 OA in station 3, but the parent toxins were never detected. After 15 days, spirolides were found in the viscera of station 1 and 3. Likewise, day 15 revealed SPX metabolites, i.e. SPX H and 27-oxo-13,19-SPX C, without the detection of other spirolides. Virtually no compounds were detected in the non-visceral samples.

Table 4.4 Compounds found in the (non)-visceral tissues of *M. edulis,* accumulated from Ostend harbour, Belgium. Detailed identification is presented in supplementary table C10.

Day	Tentative identity	Formula	Mean concentration (µg kg^{-1})	Station
5	14:1 DTX-2	C58H92O14	6.0 ± 2.1	1
	14:0 OA	C58H94O14	10.6 ± 4.3	3
	16:2 OA	C60H94O14	4.1 ± 1.3	4
10	14:1 DTX-2	C58H92O14	24.1 ± 8.6	1
	14:0 OA	C58H94O14	26.8 ± 9.1	3
	16:2 OA	C60H94O14	9.8 ± 4.3	4
	17:1 OA	C61H98O14	5.4 ± 1.2	3
	18:2 OA	C62H98O14	8.1 ± 3.9	3
15	14:0 OA	C58H94O14	26.8 ± 10.6	3
	14:1 DTX-2	C58H92O14	76.9 ± 14.2 (10.1 ± 4.3)	1
	15:0 OA	C59H96O14	12.4 ± 4.3	4
	16:2 OA	C60H94O14	26.9 ± 9.7	4
	18:2 OA	C62H98O14	8.0 ± 4.1	3
	SPX H	C40H60NO6	4.2 ± 1.4	1
	27-oxo-13,19-SPX C	C41H58NO8	3.1 ± 1.5	1

4. Discussion

Collaborative work recently revealed that OA- and SPX-related compounds are present in organisms, of different trophic levels, of the Belgian part of the North Sea (Orellana et al., 2017). Here, we present additional evidence that exposure to the natural phytoplankton of the BPNS leads to an accumulation of these two groups of toxins in mussels. As the commercial exploitation of mussels is set to recommence in this region in the near future, and this species is a major vector of toxins to humans, we aimed to elucidate the absorption, distribution, metabolization and excretion of these specific compounds in *M. edulis*. Mussels were shown to rapidly acquire and metabolize both toxins, leading to considerable accumulations of toxic compounds despite the active secretion of toxins and metabolites. In addition, we demonstrated – for the first time – that the simultaneous exposure to multiple toxins can affect metabolic pathways, leading to the creation and accumulation of different structures such as 14:1 DTX-2.

Due to the relative ease of culturing and their extreme resilience to environmental stressors such as salinity, temperature and drought, mussels are one of the most cultured and consumed shellfish species around the world (Dame, 2011; FAO, 2012). Their ability to accumulate various pollutants (e.g. chemicals, plastics and toxins), often without physiological effects , makes them both an ideal bioindicator as well as a potential threat to human health (FAO, 2004; Ostapczuk et al., 1997; Tanabe et al., 2000; Van Cauwenberghe et al., 2015). Regulatory limits were imposed to protect consumers as a result. These are, however, commonly based on acute toxic effects and, hence, do not account for chronic repeated exposure. As most people consume seafood on a regular basis, the presence of unregulated compounds and toxins below regulatory limits highlights the need for further investigation.

Here, the blue mussel *M. edulis* – an common aquaculture species with a huge economic importance - was exposed to two toxin producing dinoflagellates, *P. lima* and *A. ostenfeldii*, to study the ADME of OA- and SPX-related structures. As blooms of both algae are rarely monospecific (e.g. Foden et al., 2005; Hakanen et al., 2012), mussels are able to nourish themselves on multiple phytoplankton species during natural blooms. Feeding on non-toxic phytoplankton improves the depuration rates of LMBT in bivalves (Blanco et al., 1997; Medhioub et al., 2012). For this reason, our mussels were fed with both toxic and non-toxic algae (i.e. *I. galbana* and *T. suecica*). Through this approach, mussels were actively feeding at all times, and virtually no mortality was seen throughout the experiment.

M. edulis accumulated OA-related structures faster than SPXs. To the best of our knowledge, this is the first study to compare the uptake kinetics of these toxin groups. While the difference is, in part, related to the difference in toxin production between these two dinoflagellates (Table 4.1), mussels may have also selected more strongly against *A. ostenfeldii*. Toxin accumulation is an energetically optimized, complex balance between food selection, adsorption and enzymatic transformations (Reguera et al., 2014; Ward and Shumway, 2004). Bivalves can adjust their filtration rates and egestion in response to HABs (Hégaret et al., 2007), but this response is specific for each algae and differs between bivalve species. Several authors have found intact cells of *Alexandrium* spp. and *Prorocentrum* spp. in the (pseudo-)faeces of bivalves, suggesting avoidance through selective feeding (Bauder et al., 2001; Galimany et al., 2008a; Medhioub et al., 2012; Romero-Geraldo et al., 2014). The higher accumulation of toxins in the mixed exposure suggests a shift in the balance between biotransformation and the particle selection and avoidance behaviour.

Toxins accumulated predominantly in the digestive gland. After 15 days, visceral and non-visceral tissues contained a respective 73% and 27% of all OA on average. Similar results were found in bay scallops *Argopecten irradians* exposed to *P. lima* (Bauder et al., 2001), though our mussels accumulated more DSP toxins overall. SPXs, on the other hand, had a 61% (visceral) vs. 39% (non-visceral) distribution, which differs from the 83% vs. 17% found in Pacific oysters *Crassostrea gigas* exposed to *A. ostenfeldii* (Medhioub et al., 2012). *Mytilus* spp. and *Crassostrea* spp. are, however, known to have different avoidance behaviour towards harmful algae (Hégaret et al., 2007). The accumulation of 13-desmethyl spirolide C (13-SPX C) found here (i.e. 0.58–2.19 $\mu g.kg^{-1}$) resembles the ranges found in natural *Mytilus* spp by Rundberget et al. (2011), Silva et al. (2013) and García-Altares et al. (2014): i.e. 1.1-63 $\mu g.kg^{-1}$, 0.5-3.9 $\mu g.kg^{-1}$ and 2-16 $\mu g.kg^{-1}$, respectively.

The production rates of OA, DTX-1 and 13-SPX C were similar to those reported in literature (Bravo et al., 2001; Ciminiello et al., 2006; Medhioub et al., 2011; Nascimento et al., 2005). *P. lima* also produced diol and triol esters, which hydrolyse rapidly inside bivalves (Hu et al., 1992; Miles et al., 2006; Suárez-Gómez et al., 2005; Torgersen et al., 2008a). Little is, however, known about OA esters. Here, we provide the first proof of transient visceral accumulations of diol esters. Moreover, we found high deposits of OA-T9 (5-hydroperoxy-7-hydroxy-2,4-dimethylene-heptyl okadaate) in the faeces, that suggest that this triol ester is poorly absorbed or strongly excreted through mechanisms which are yet to be studied. Likewise, we found high amounts of 27-oxo-13,19-didesmethyl spirolide C in the faeces. This end-of-the-line metabolite may provide a previously unknown, alternative excretion route for SPXs.

The detoxification of xenobiotics (e.g. toxins) often starts with esterification by acyl transferase activity (Janer et al., 2004; Labadie et al., 2007; Rossignoli et al., 2011). The resulting fatty esters may then be oxidized (lipid peroxidation) to form insoluble lipofuscin granules which, together with the xenobiotic, are excreted via the faeces (Galimany et al., 2008a). For DSP toxins, fatty acids are attached to the 7-OH group of either OA and DTX-1 or, in rare cases, the terminal hydroxyl group of diol esters (Torgersen et al., 2008a). SPX esters can be formed by conjugating the 17-OH group of 20-methyl SPX G (Aasen et al., 2006), but this phase I metabolite first needs to be created by several CYP-mediated reactions (Bebianno et al., 2007; Guengerich, 2001; Hui et al., 2012; Ortiz de Montellano and Nelson, 2011). During these steps a tetrahydropyran ring is formed which resembles the terminal spiroketal domain of OA. Other SPX transformations involve demethylation, a process needed to create DTX-2 from OA. The structural similarity between the target sites of both toxins may enable CYPs of the SPX metabolism to interact with OA, and explain why 14:1 DTX-2 is only detected during the mixed exposure.

After 3 days of exposure, acyl-OA esters were already used to excrete OA through the water and faeces, which was also observed by Rossignoli et al. (2011). Most OA esters were made from fatty acids (e.g. 16:0, 20:5) which are common in *Mytilus* spp. (Kluytmans et al., 1985; Leonardos and Lucas, 2000; Torgersen et al., 2008b). Some (e.g. 14:3 and 16:2) fatty acids are, however, rarely found in bivalves. Interestingly, 14:3 is also involved in the detoxification of gymnodimine (de la Iglesia et al., 2013), another complex toxin, and is probably produced from 20:5 (Williard et al., 1998). 16:2, on the other hand, is found in Cholorophyceae and may have been produced by *I. galbana* and *T. suecica* (Dunstan et al., 1992). Similar fatty esters of SPXs may reasonably be expected, but we were unable to confirm their presence.

Bivalves rapidly accumulate several SPXs and DSP toxins, plus their metabolites, (Medhioub et al., 2012; Nielsen et al., 2016; Rossignoli et al., 2011; this study) but mixed or chronic toxicity effects of these compounds are unknown. Both antagonistic and synergistic interactions between OA and SPXs have been demonstrated in vitro (Dragunow et al., 2005; Ferron et al., 2016). As such, the transformation of mixtures will most likely affect the acute toxic effect of some compounds, e.g. SPX E, F and H (Hu et al., 1996; Roach et al., 2009). For other metabolites, like OA and SPX esters, biotransformation just delays the onset of symptoms while the human body reverses the biotransformation (Aasen et al., 2006; Couesnon et al., 2016; Doucet et al., 2007; Guéret and Brimble, 2010; Torgersen et al., 2005; Vale and Sampayo, 2002). While there is still much to be learned about these mixtures, the presence of metabolites should still be treated as a health risk to humans.

Here, we also demonstrated that metabolized toxins are present in *M. edulis* after exposure to the natural phytoplankton of Ostend harbour. These findings support our previous observations on OA- and SPX-related compounds in seafood of the BPNS (Orellana et al, 2017). Despite a few dubious incidences with HAB toxins in the past (cfr. the Belgica mussel story), these are the first studies to find toxin accumulation in shellfish of the BPNS. It is worth noting that we do not really know the effect of these mixtures on the health of human consumers. While most of the toxin concentrations were low, especially in the non-visceral tissues which are the bulk of the body, there is no telling how (positive or negative) the human body would react to these toxins. For all OA metabolites and esters, except DTX-2 which is roughly half as toxic as OA, it is known that they have the same toxicity as OA (Botana et al., 2017). As a result, it is common practice to sum their concentrations and compare them to the safe limit of 80 $\mu g.kg^{-1}$ (whole body). Looking at our concentrations, none of the stations came close to these levels. However, the single and mixed toxicity effects of SPXs are still largely unknown. Although SPXs are readily absorbed and redistributed to the central nervous system, where they inhibit nicotinic acetylcholine receptors, there is little evidence of oral toxicity (Bourne et al, 2010, Munday et al., 2011; Otero et al., 2012). As a result, the limits of safe consumption have not been designed for SPXs to date. Still, these compounds could increase the toxicity of other neurotoxins such as OA (Dragunow et al., 2005; Ferron et al., 2016), enhancing the risk towards consumers.

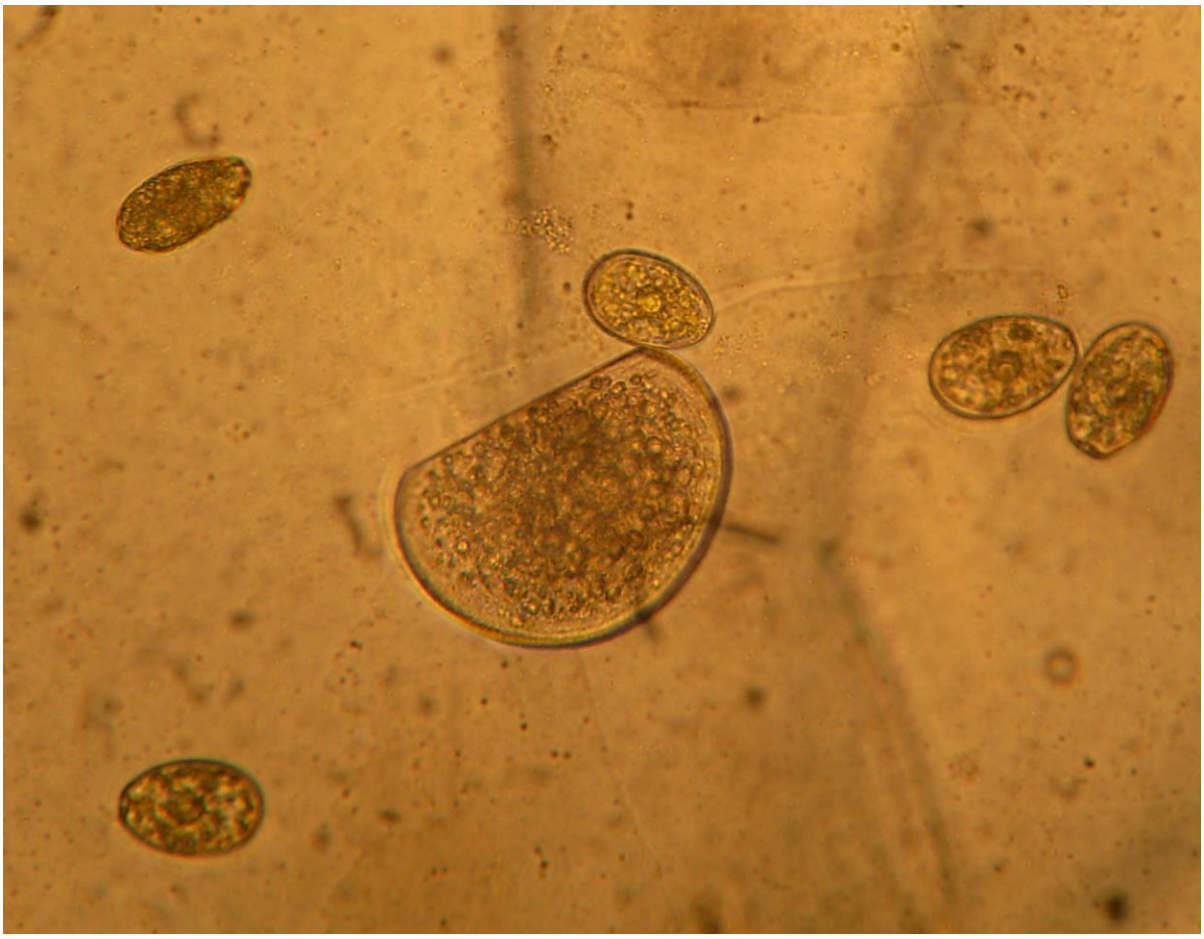
Due to toxin monitoring by the “Federal Agency of the Safety of the Food Chain”, the regional food safety authority, shellfish poisoning is rare in Belgium. As a result, the public and general practitioners are unfamiliar with the risk of shellfish poisoning. While shellfish harvesting is forbidden, there is no enforcement of this law and people might still be tempted to harvest mussels from the Belgian beaches. In addition to the previous news items on the occurrence of PCBs, plastics and bacterial contaminants in the regional mussel populations, our research also shows that wild shellfish harvesting is ill advised. More crucially, though, our results demonstrate the need to closely monitor the new shellfish farming project in the BPNS. Because of the legal designation as the only offshore shellfish area, the phytoplankton of the windmill zone will need to be monitored. Here, though, we demonstrated toxin accumulation in the absence of a bloom. As the BPNS contains a wide variety of toxic dinoflagellates (ref. Table 1.3), complex toxin mixtures can be formed (ref. Orellana et al., 2017) which have unknown acute and chronic toxicity effects. This study, therefore, not only reports on the urgency to evaluate the possible effects of toxin mixtures on key stone species such as blue mussels, but it also highlights the need to evaluate the risk of these (unregulated) compounds to human health.

5. Conclusions

M. edulis accumulates SPX and DSP toxins when exposed to natural and artificial toxic phytoplankton assemblages. Adult mussels assimilate, metabolize, translocate and excrete SPX- and OA-related chemicals when fed with *A. ostenfeldii* and *P. lima*. The simultaneous exposure to both algae increased the rate of nearly all processes. Lipid peroxidation was identified as a major excretion pathway, but other metabolites can provide alternatives for toxin depuration. In addition, we have found some toxins, e.g. SPX D and 5-hydroperoxy-7-hydroxy-2,4-dimethylene-heptyl okadaate, which are directly rejected or rapidly excreted through an unknown mechanism. To improve our understanding of the ADME processes in mussels, we would recommend future research to explore both this mechanism as well as the effect of abiotic variability on the different kinetics found in this study.

5

Bivalves in a changing environment: the effect of two common marine toxins, and their producers, on the survival and immunological resilience of mussel larvae



Abstract

Harmful algal blooms (HABs) and marine diseases are both increasing in severity, frequency, and geographical scale around the world. As a result, bivalve populations are increasingly facing the combined threat of toxic algae and marine pathogens. Individually, both stressors are known to affect the recruitment of ecologically and economically important bivalve species. When combined, HABs and pathogens could further reduce the growth, viability and development of bivalve larvae. Yet, to date, little is known about the effects of HABs on the immunological resilience of bivalves. Here, we investigate whether two most common toxins of the North Sea influence the larval viability, development and innate immune response of blue mussel larvae. Embryos of *Mytilus edulis* were exposed (48h) to the toxic diatom *Pseudo-nitzschia multiseries* or the toxic dinoflagellate *Prorocentrum lima*. In addition to varying cellular densities, the larvae were also exposed to six different concentrations of their respective toxins: i.e. domoic acid (DA) and okadaic acid (OA). After 48 hours, OA was found to have significantly reduced the viability ($p < 0.01$) at concentrations as low as 37.8 $\mu g.l^{-1}$. This toxicity was attributed to its ability to inhibit larval protein phosphatases in vitro ($p < 0.001$). While *P. multiseries*, *P. lima*, and DA did not have such an effect on the viability of the larvae, they increased the phenoloxidase (PO) innate immune activity of the mussel larvae. These results suggest that the innate immune response of even the earliest life stages of bivalves is susceptible to the presence of HABs.

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1. Introduction

Harmful algal blooms (HABs) are globally increasing in frequency, magnitude and scale due to shipping, natural dispersal, eutrophication, climate change, overfishing and aquaculture activities (Anderson et al., 2012). Harmful phytoplankton can cause significant economic and environmental losses through hypoxia, shading, physical disturbance, unpalatability and the production and release of potent marine toxins (Glibert et al., 2005; Granéli and Turner, 2006; Hoagland and Scatasta, 2006). These phycotoxins are readily accumulated by commercially important filter feeding bivalves (e.g. oysters, clams, mussels and scallops; FAO, 2004). As a result, toxic HABs pose a significant threat to both the food safety and the food security of mankind. Shellfish farming provides nearly a quarter of the global aquaculture production, equivalent to 10% of the the world's annual seafood production (FAO, 2012). Still, these numbers are expected to rise as the human population and, hence, the demand for (sea)food continues to grow (Speedy, 2003; UN, 2015).

In addition to their economic value, bivalves are often keystone species of their respective habitats. They bioturbate sediment, create natural hard substrate reefs and play a major role in the bentho-pelagic coupling (Dame, 2011). Crucially, it has been shown that the collapse of bivalve populations can significantly increase the risk of HABs in certain coastal areas (Gobler and Sunda, 2006, 2012). Wild and cultured populations of these ecosystem engineers have a crucial thing in common. Both are dependent on the recruitment of "spat", i.e. the settlement of pelagic veliger larvae (Lucas and Southgate, 2011; Seitz et al., 2001; Smaal, 2002). To start their life-cycle, i.e. before settling down as epi- or endobenthos, bivalves undergo an extended, highly dispersive larval phase. These early life stages are, however, characterized by a high interannual variation in abundance (Dame, 2011). Despite their economic and ecological importance, we do not fully understand which environmental conditions affect the larval viability of bivalves (Pronker et al., 2008).

The earliest life-stages of bivalves are highly sensitive to abiotic stress (e.g. pH), chemical pollution and other environmental disturbances (ASTM, 2004). Pathogens such as *Vibrio* spp., in particular, are known to cause severe mortality among larvae (Paillard et al., 2004). Now, the evidence is mounting that HABs affect larvae as well (Bricelj et al., 2011; Jeong et al., 2004; Padilla et al., 2006; Rolton et al., 2014). Yet, little is known about the potential for mixed adverse effects between both stressors. As both harmful algae and marine pathogens may soon benefit from climate change (Burge et al., 2014; Vezzulli et al., 2016; Wells et al., 2015), their combined toxicity increasingly threatens bivalve populations.

The high availability of organic matter and detritus during algal blooms promotes the growth of associated bacterial communities (Buchan et al., 2014; Cole, 1982). While we do not fully understand the role and diversity of bacteria in HAB dynamics, it is clear that notorious pathogens such as *Vibrio* spp. are present in HAB associated communities (Eiler et al., 2006; Mourino-Perez et al., 2003; Simidu et al., 1971). Yet, little is still known about the mixed effect of HABs and pathogens on bivalve larvae. Despite evidence that HABs affects the number, shape and function of haemocytes in adult bivalves (Hégaret and Wikfors, 2005a, 2005b; Prado-Alvarez et al., 2013), not a single study to date has looked into the effect of HABs on the immunological resilience of bivalve larvae.

To enhance our understanding of the impacts of HABs on bivalve populations, this study investigates whether two common North Sea toxins, and their producers, can affect the viability and innate immune response of blue mussel *Mytilus edulis* larvae. Domoic acid (DA) which is produced by the diatom genus *Pseudo-nitzschia* spp., and okadaic acid (OA) which is found during dinoflagellate blooms of *Dinophysis* spp. or *Prorocentrum* spp. are found around the world, and appear to be increasing globally (Hattenrath-Lehmann et al., 2013; Reguera et al., 2014; Trainer et al., 2012). Both DA and OA are genotoxic (Dizer et al., 2001; González-Romero et al., 2012) and are known to affect the early life stages of several invertebrates (Escoffier et al., 2007; Liu et al., 2007; Patel and Whitaker, 1991; Picard et al., 1989; Tiedeken et al., 2005). Yet, to date, no studies have assessed the impact of these toxins on the recruitment of bivalves. Moreover, not a single study has examined the phenoloxidase (PO) innate immune activity, which is known to respond to several anthropogenic stressors (Bado-Nilles et al., 2010; Ittoop et al., 2009; Luna-Acosta et al., 2011, 2012), and HAB exposure. Here, the common model organism *M. edulis* was used to investigate whether these types of toxic HABs can have an effect on the viability, development and PO activity of bivalve larvae. To this end, blue mussel embryos were exposed for 48h to various concentrations of *Pseudo-nitzschia multiseries*, *Prorocentrum lima* and their respective toxins DA and OA. The results of this study include assessments of (1) the viability of the larvae, (2) the development of the embryos into veliger larvae, (3) the PO activity as an innate immunological response, and (4) the inhibition of larval protein phosphatase (PP) activity by OA.

2. Material and Methods

2.1 Algal cultures and toxins

Cultures of *Pseudo-nitzschia multiseries* (CCAP 1061/32) and *Prorocentrum lima* (CCAP 1136/9) were obtained from the Culture Collection of Algae and Protozoa (CCAP) in Oban, Scotland. A second strain of *P. multiseries* (CLN-47) was kindly provided by the Pacific Northwest Center for Human Health and Ocean Studies (University of Washington, Seattle, USA). f/2 medium (Guillard and Ryther, 1962) and L1 medium (Guillard and Hargraves, 1993), prepared from Instant Ocean^{tm} artificial seawater (Belcopet, Belgium) were used for *P. multiseries* and *P. lima*, respectively. Algae were grown semi-continuously by replacing 80% of the culture volume when needed. Algal densities were counted three times a week with a Sedgewick Rafter counting chamber (SPI supplies, West Chester, USA) to determine the growth phase. Experiments used cultures in the early stationary phase, i.e. when the toxin content is generally highest (Lelong et al., 2012; Nascimento et al., 2005). Toxin exposures used HPLC grade domoic acid (≥ 90%) (Sigma-Aldrich, Steinheim, Germany) and HPLC-grade okadaic acid (≥ 98%) (LC Laboratories, Woburn, USA), dissolved in 100% ethanol and stored as suggested by the National Research Council of Canada: DA stocks (1 µg.l^{-1}) were kept at 4°C while OA stocks (0.3 µg.l^{-1}) were kept at -20°C.

2.2 Spawning procedures

Between March and April, i.e. the natural spawning season of *Mytilus edulis*, adult mussels of approximately 3 to 4 cm were collected from breakwaters on the Belgian North Sea coast. These were then transported on ice, hand cleaned and placed in a recirculating, filtered aquarium at a density of 2 individuals per litre (8°C, 34 PSU). Mussels were kept healthy with daily ad libitum additions of a commercial algal paste (Shellfish Diet 1800 ®, Varicon Aqua Solutions, UK) and weekly replenishments of the artificial seawater (Instant Ocean^{tm}, Belcopet, Belgium). Following the E724-98 ASTM standard guideline for acute toxicity tests with bivalve embryos, spawning was induced by moving mussels between water baths of 18°C and 26°C (ASTM, 2004). Reproducing individuals were separated to isolate sperm and eggs. After checking the quality of these gametes, sperm and eggs were mixed to achieve a controlled fertilization. The resulting embryos were deemed suitable for a larval test when ≥80% of the eggs were fertilized (i.e. displayed a polar body).

2.3 Embryonic development test

Tests were performed in accordance with the ASTM standard E724-98 (2004). Algal cultures were diluted with ASTM artificial seawater (34 PSU) to obtain six densities of *P. multiseries* (140 to 14,000 cells.ml^{-1}) or *P. lima* (15 to 1500 cells.ml^{-1}). The source culture of each algae was subjected to cellular toxin extraction (ref. 2.6). Purified toxins were diluted with ASTM artificial seawater to get six concentrations of domoic acid (25 to 800 µg.l^{-1}) or okadaic acid (10 to 320 µg.l^{-1}). Six 2 ml replicates of each dilution were placed in 24-well cell culture plates. The remainder was stored for toxin analysis. Control treatments included ASTM seawater and a solvent control of 0.001% EtOH which is equivalent to the EtOH content of the highest toxin treatment (i.e. OA 320 µg.l^{-1}). Embryonic tests were initiated by introducing approximately 160 fertilized eggs (i.e. 10 µl of a dense egg suspension) to each well. The culture plates were then placed at 15°C in the dark. After 48h, 50 µl of formaldehyde was added to each well to terminate the assay. Inverse microscopy was used to determine larval viability and development: Veliger larvae with a closed D-shaped prodissoconch were scored as viable, while embryos with severe growth delays and malformations were seen as non-viable. Like Mottier et al. (2013), we distinguished “perfectly developed” larvae that are straight-hinged with well-rounded corners, from “viable imperfect” larvae with concave or convex hinges and more sharply defined corners (Fig. 5.1).

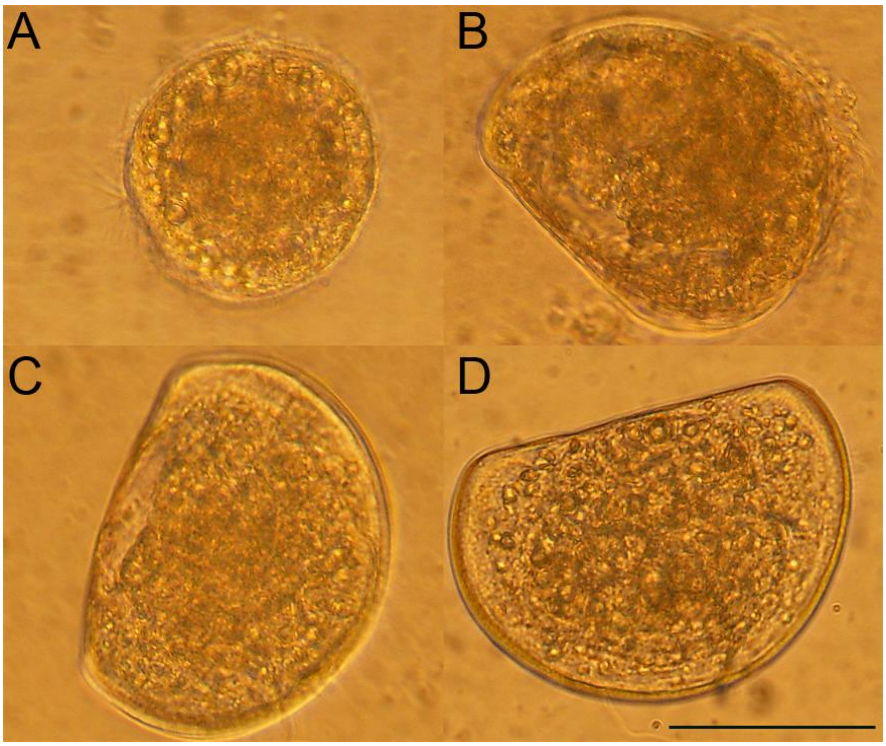


Figure 5.1: Categories for the visual assessment of *M. edulis* larvae: (A) non-developed (B) misdeveloped or growth delayed (C) viable imperfect (D) perfectly developed veliger.

2.4 Phenoloxidase activity

The transformation of L-3,4-dihydroxyphenylalanine (L-DOPA) to dopamine can be used to determine the phenoloxidase (PO) activity of tissues (Baruah et al., 2010). First, sufficient cellular material (> 0.1g) needed to be gained. To this end, five 200 ml replicates with 100,000 embryos each were exposed for 48 hours to environmentally relevant concentrations *P. multiseries* (1400 cells.ml^{-1}) or *P. lima* (150 cells.ml^{-1}) and the highest non-lethal concentrations of their toxins: 800 µg DA.l^{-1} or 150 µg OA.l^{-1}. Exposures were performed at 15°C in the dark. After two days, larvae were collected on a 50 µm sieve, transferred to 2 ml centrifuge tubes, weighed, flash-frozen in liquid nitrogen and stored at -86°C.

Before the PO analysis, a buffer was made (0.43% NaCl, 1.25 mM EDTA, 0.5% Triton^{tm} X, 5 mM CaCl2) in which a 0.5 mM stock solution of L-DOPA was prepared (all chemicals from Sigma-Aldrich, Steinheim, Germany). Then, the mussel larvae were homogenized in buffer (10% w:w) using a pestle and stored overnight at 4°C. Debris was removed with 0.22 µm cellulose-acetate Spin-X® centrifuge tube filters (10,000 g, 20 min) the following day. Triplicate 20 µl aliquots of the resulting extracts were placed in a 96-well tissue culture plate with 200 µl of the L-DOPA solution. Blank buffer samples were added to assess the non-enzymatic dopamine production. The culture plates were dark-incubated at 30°C and the absorbance (λ = 490 nm) was measured three times a day during the next 48 hours using a Thermo Multiskan Ascent 96/384-well spectrophotometer (Thermo Scientific, San Jose, CA, USA).

2.5 Protein Phosphatase activity

The dephosphorylation of p-nitrophenylphosphate (pNPP) to p-nitrophenol is used to measure the protein phosphatase activity of tissues (Takai and Mieskes (1991). As before (see above), 10 times 100,000 embryos were cultured, collected, weighed and stored. Larvae were pulverized by hand with a pestle in 350 µl of a Tris-EDTA buffer (pH 8-8.5) containing 1 µM phenylmethanesulfonylfluoride and 11.7 µM leupeptine. Cellular debris was removed using 0.22 µm cellulose-acetate Spin-X® centrifuge tube filters (10,000 g, 20 min). Triplicate 100 µl aliquots of each sample were placed in a 96-well tissue culture plate and 50 µl of 20 mM pNPP disodium salt hexahydrate (Sigma-Aldrich, Steinheim, Germany) in buffer was added. Five biological replicates were spiked with 8.5 ng OA in 20 µl buffer, resulting in a final ambient concentration of 50 µg OA.l^{-1}. The other five biological replicates were spiked with blank buffer as a control. PP activity was measured at 5-minute intervals for two hours with the Thermo Multiskan Ascent 96/384-well spectrophotometer (λ = 405 nm).

2.6 UHPLC-MS/MS analysis

The protocols of Bravo et al. (2001) and Cerino et al. (2005) were used to extract the intracellular and extracellular toxins of the early stationary phase cultures of *Pseudo-nitzschia multiseries* and *Prorocentrum lima*, respectively. DA samples were purified by solid phase extraction on Strata-X polymeric reversed phase cartridges (33 µm, 100 mg / 3 ml) (Phenomenex, Utrecht, the Netherlands) using the acidic procedure of Wang et al. (2007). Likewise, a new solid phase extraction method was developed to isolate dissolved OA: The same Strata-X (33 µm, 100 mg / 3 ml) polymeric reversed phase cartridges were conditioned and washed with 3 ml of 70% acetonitrile (ACN) and 3 ml ultrapure water before passing 2 ml of OA samples over the column. Salts were then removed with 8 ml of ultrapure water and the absorbed toxins were eluted with 2 ml of 70% ACN.

All of the resulting toxin extracts were analysed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Samples were put in an Accela Autosampler and Degasser, coupled to an Accela UHPLC pump fitted with an Acquity UPLC BEH C18 column (Ethylene Bridged Hybrid - 1.7 µm, 100x2.1 mm; Waters, Milford, MA, USA) for compound separation. Mass spectrometry was done by a TSQ Vantage mass analyser (Thermo Fisher Scientific, San Jose, USA), fitted with a heated electrospray ionisation (HESI-II) interface. The system was operated in the positive ion mode, using the operational parameters of the Community Reference Laboratory for Marine Biotoxins (CRLMB, 2009) and the work of Suzuki et al. (2009). Quantification was based on 8-point calibration curves of certified reference materials (CRM-OA-c and CRM-DA-f) from the National Research Council (Ottawa, Canada).

2.7 Data analysis

First, the larval viability and larval development were calculated for each replicate. Viability was defined as the sum of all viable larvae (perfect L_D and imperfect L_C), divided by the total number of larvae (Eq. 5.1). The development was determined as the fraction of imperfect larvae (Eq. 5.2). Based on the larval viability, the LC_{50} was calculated through the “drc” R package (Ritz and Streibig, 2005). Between-group comparisons were then made using non-parametric Mann-Whitney U (MWU) and Kruskall-Wallis (KW) tests after visually assessing the distribution of each group.

$$(5.1)\ \mathrm{V}_{\%} = \frac{(L_C+L_D)}{(L_A+L_B+L_C+L_D)}$$

$$(5.2)\ \mathrm{D}_{\%} = \frac{L_C}{(L_A+L_B+L_C+L_D)}$$

To determine the enzymatic PO activity, the average optical density of the blank samples – which is caused by spontaneous, non-enzymatic dopamine production - was subtracted from the optical density of each sample for every time point (Eq. 5.3). Next, the increase in optical density over time (ΔOD) was determined by subtracting the initial optical density of samples (OD_0) from all ensuing measurements (Eq. 5.5). Lastly, the optical density was adjusted for mortality (m) as dead larvae are expected to contribute to the weight of the samples, but not the signal of the immune response. Outliers resulting from human error were removed. Treatments were compared by a one-factor ANOVA with unequal sample size. Pairwise group comparisons used the Least Significant Differences test (LSD).

$$(5.3)\ \mathrm{OD}_t = OD_{sample,t} - \langle OD \rangle_{blank,t}$$

$$(5.4)\ \Delta\mathrm{OD} = OD_t - OD_0$$

$$(5.4)\ \Delta\mathrm{OD}_a = \frac{\Delta\mathrm{OD}}{(1-m)}$$

PP activity ($\Delta OD.g^{-1}$) was standardized by calculating the dividing the average increase in optical density (ΔOD; Eq. 5.4) by the wet weight of the sample (Eq. 5.6). This was done for each time-interval, creating two time series of PP activity: one with OA and one without OA. The slopes of the fit lines were compared using the t-test method of Andrade and Estévez-Pérez (2014).

$$(5.5)\ \Delta\mathrm{OD}_g = \frac{OD_t - OD_0}{W}$$

3. Results

3.1 Algal growth and toxin production

The two strains of *Pseudo-nitzschia multiseries* entered the stationary phase at cell densities of 255,000 $cells.ml^{-1}$ (CLN-47) and 261,000 $cells.ml^{-1}$ (CCAP 1061/32). At this point, CLN-47 produced more toxins than CCAP 1061/32: 0.06 $pg.cell^{-1}$ versus 0.02 $pg.cell^{-1}$ intracellular and 81.6 $\mu g.l^{-1}$ (0.32 $pg.cell^{-1}$) vs. 24.0 $\mu g.l^{-1}$ (0.09 $pg.cell^{-1}$) extracellular DA. *Prorocentrum lima* reached a density of 62,000 $cells.ml^{-1}$ during the stationary phase. At this point, 13.3 $\mu g.l^{-1}$ OA (equivalent to 0.2 $pg.cell^{-1}$) was detected in the medium while 1.2 pg intracellular $OA.cell^{-1}$ was detected. Based on these results, the extracellular amount of toxins was calculated for each exposure to algae (Table 5.1). In addition, *P. lima* was found to produce non-quantifiable amounts of dinophysistoxin-1 using the ToxID software (Thermo Scientific, San Jose, USA).

Table 5.1: An overview of the exposure conditions of the embryonic development test. Nominal and measured toxin concentrations (µg.l^{-1}) as well as the cell density and equivalent toxin concentration, calculated from the measured production per cell, are shown.

Treatment	**Concentrations**						
Domoic acid - nominal	25	50	100	200	400	800	µg.l^{-1}
DA - UHPLC-MS verified	18.1	49.3	109.0	214.0	466.4	723.7	µg.l^{-1}
***P. multiseries* CCAP 1061/32**	140	700	1400	7000	14000		cells.ml^{-1}
Eq. extracellular DA	0.01	0.06	0.13	0.64	1.29		µg.l^{-1}
***P. multiseries* CLN-47**	140	700	1400	7000	14000		cells.ml^{-1}
Eq. extracellular DA	0.04	0.22	0.45	2.24	4.48		µg.l^{-1}
Okadaic acid - nominal	10	20	40	80	16	320	µg.l^{-1}
OA - UHPLC-MS verified	10.9	19.5	37.8	79.5	162.5	345.2	µg.l^{-1}
P. lima	15	75	150	750	1500		cells.ml^{-1}
Eq. extracellular OA	0.00	0.02	0.03	0.16	0.32		µg.l^{-1}

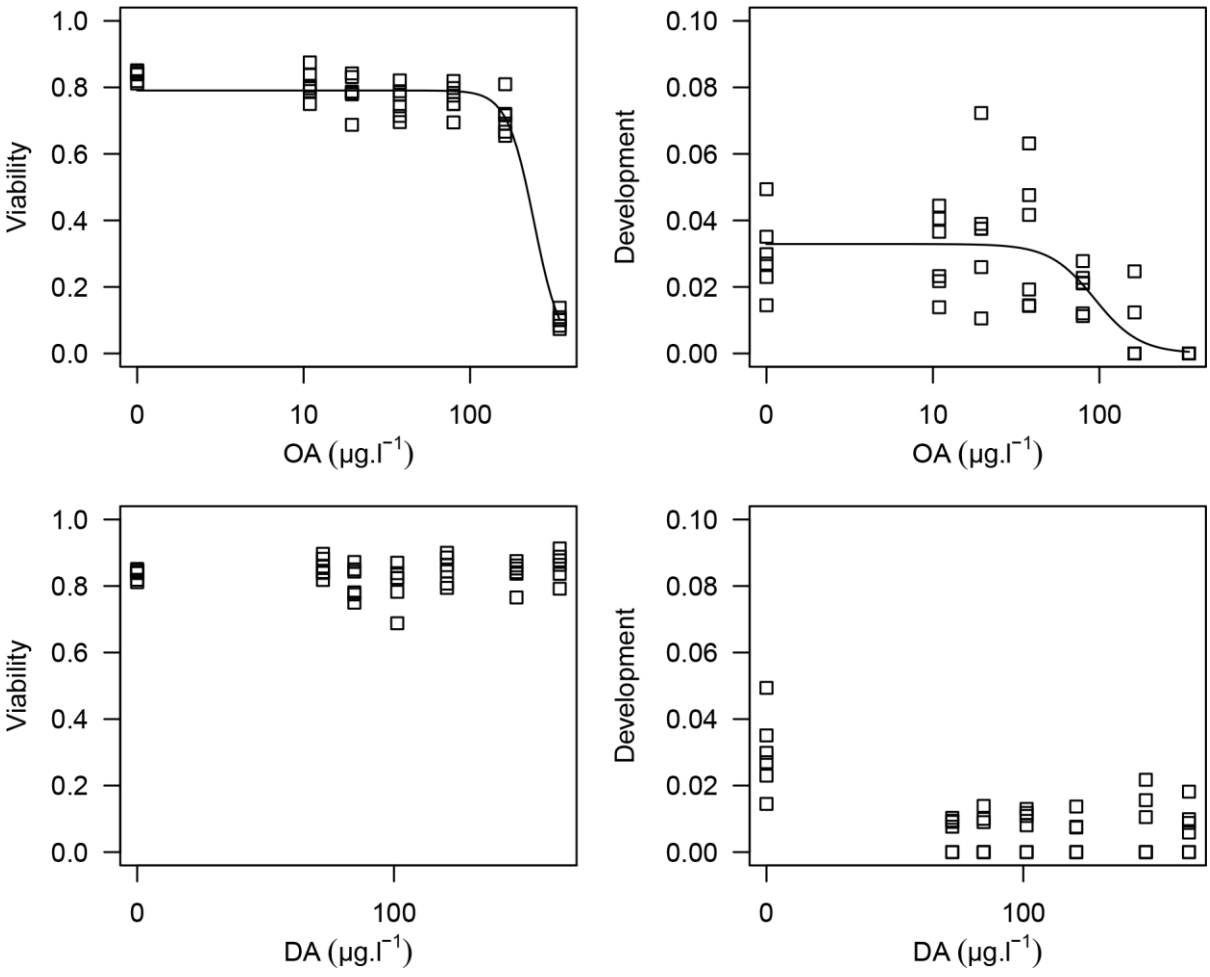


Figure 5.2: Fraction of viable (left) and misdeveloped (right) *M. edulis* larvae, exposed for 48h to okadaic acid (top) or domoic acid (bottom). OA significantly reduced both indicators. Toxin concentrations were determined by UHPLC-MS/MS.

3.2 Embryonic development and viability of D-larvae

None of the *P. multiseries* (up to 14,000 cells.ml^{-1}), *P. lima* (up to 1500 cells.ml^{-1}) or DA treatments (up to 723.6 µg.l^{-1}) had an effect on the viability of *Mytilus edulis*. OA, on the other hand, significantly reduced the viability of *M. edulis* veliger larvae (KW $p < 0.001$; Fig. 5.2) at concentrations greater than 19.5 µg.l^{-1} (MWU $p < 0.01$). 0.001% ethanol (equivalent to the 320 µg.l^{-1} OA treatment) did not affect the larvae. The LC_{50} of OA was 242.4 ± 8.1 µg.l^{-1} for viability and 93.5 ± 19.5 µg.l^{-1} for the larval development. None of the other treatments affected the larval development (Fig. 5.3).

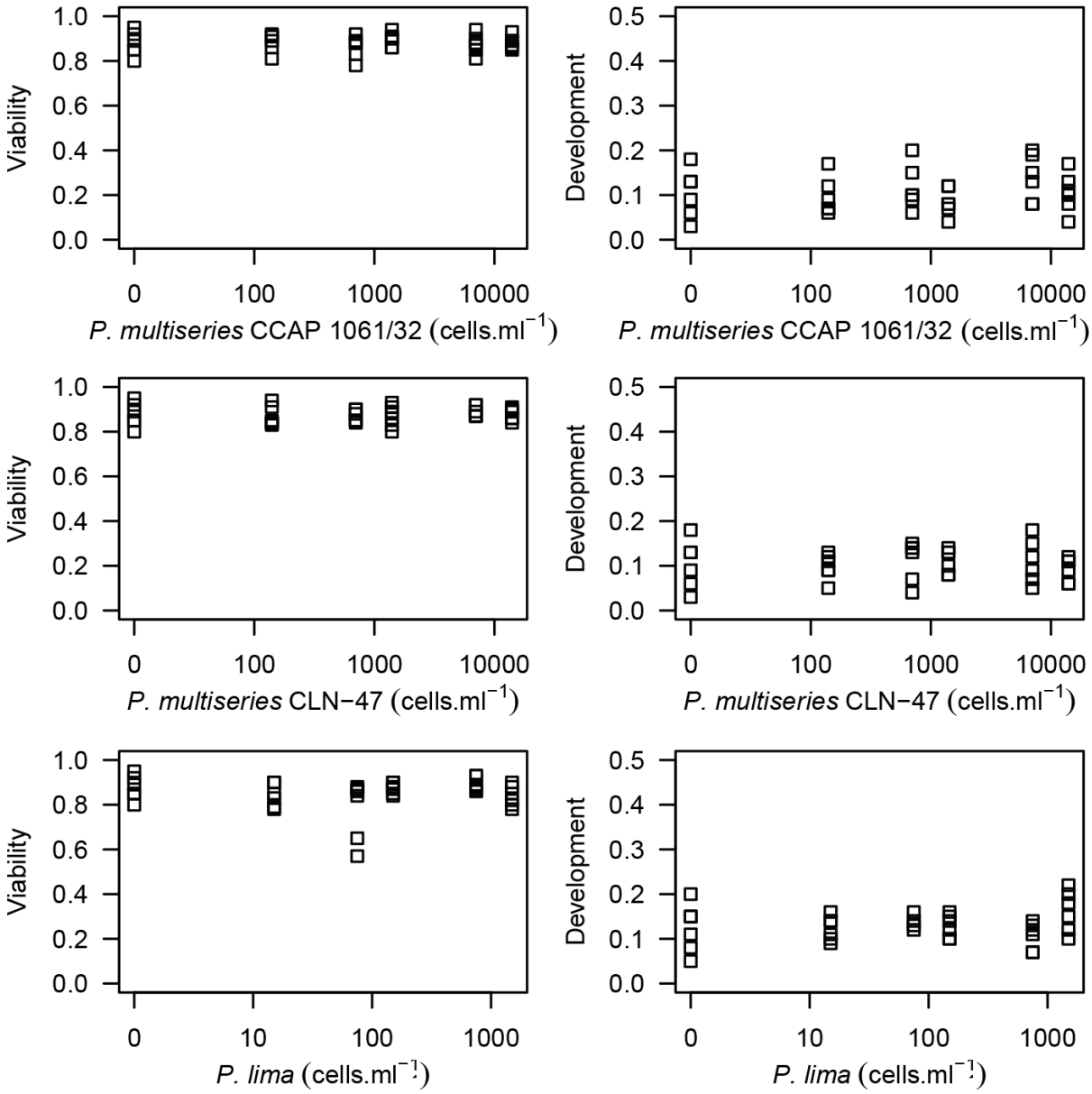


Figure 5.3: Fraction of viable (left) and misdeveloped (right) *M. edulis* larvae, after a 48h exposure to *P. multiseries* (CCAP1061/32; top and CLN-47; middle) or *P. lima* (bottom). None of the treatments had a significant effect.

3.3 Phenoloxidase and protein phosphatase activity

Bloom densities of both strains of *P. multiseries* (cf. above) triggered an increase in larval PO activity during the first 22 h of the enzyme assay (Fig. 5.4: LSD $p < 0.01$). Likewise, a 48h exposure to *P. lima* (150 cells.ml^{-1}) induced a significant increase in larval PO activity during the first hours of the PO assay (LSD $p < 0.05$). In addition, we found a difference in response to the two strains of *P. multiseries* (LSD $p < 0.05$). By contrast, the PO activity i.e. the mortality adjusted optical density (ΔOD_a) of both the DA (623.2 µg.l^{-1}) and the OA (146.9 µg.l^{-1}) treatment were similar to the control. However, we found that the PP activity of larval enzyme extracts could be inhibited by 42% by 50 µg.l^{-1} OA (Fig. 5.5: significantly different slopes $p < 0.001$).

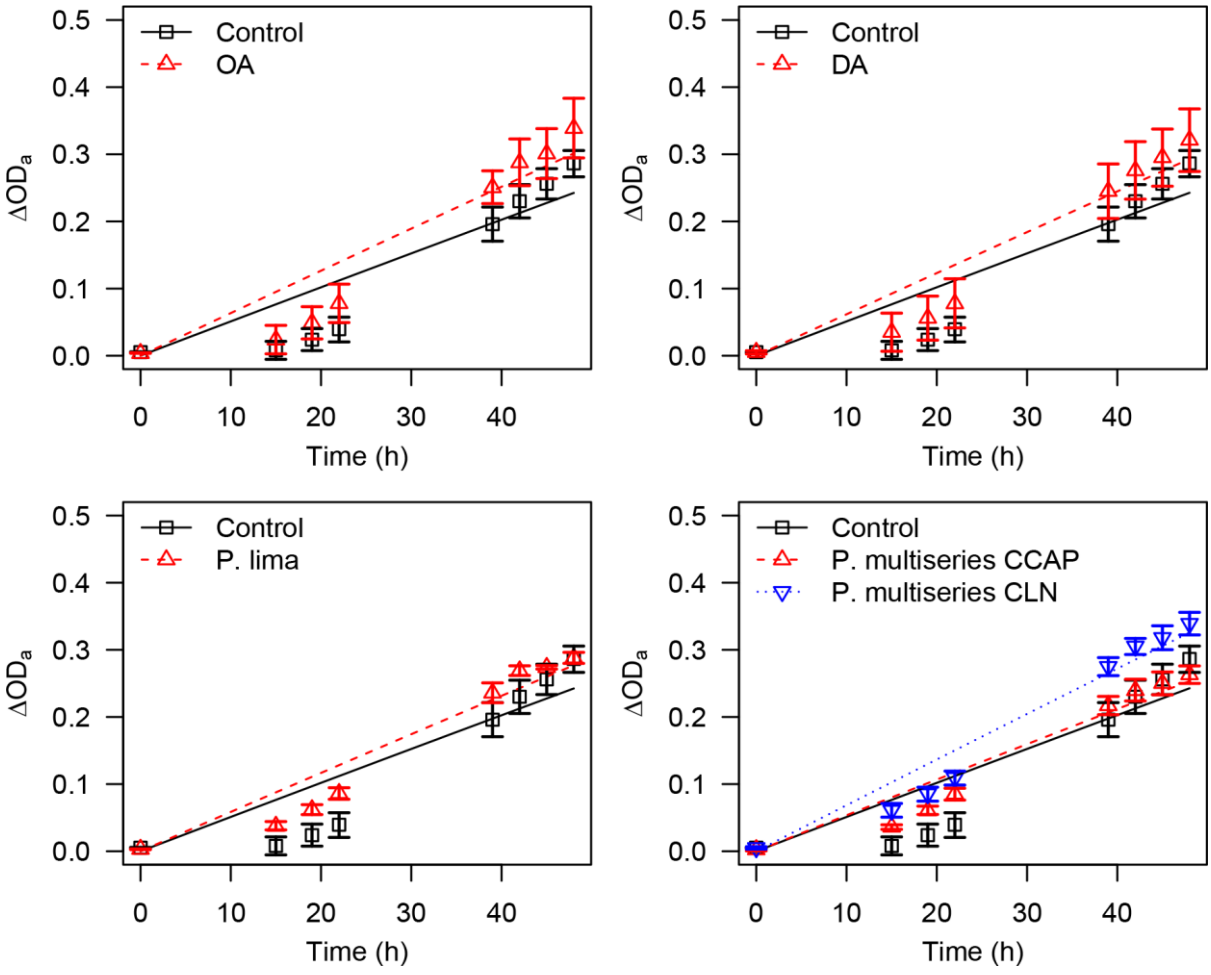


Figure 5.4: Phenoloxidase immune activity of veliger larvae - quantified by changes in the optical density (λ_{abs} = 490nm) of extracts due to the production of dopamine and adjusted for larval mortality ($\Delta OD_a \pm SE$) - after 48h of in vivo exposure to okadaic acid (146.9 µg.l^{-1} OA), domoic acid (623.2 µg.l^{-1} DA), *P. lima* (150 cells.ml^{-1}) and *P. multiseries* (1400 cells.ml^{-1}). The live algae significantly increased the PO activity, which was not observed for either toxin.

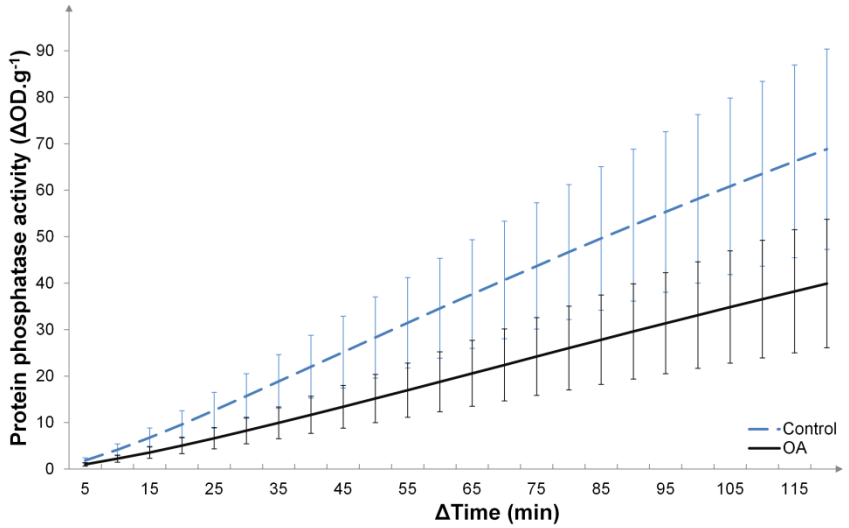


Figure 5.5: Protein phosphatase activity of enzyme extracts of veliger larvae. On average, the control extracts (black) produced 42% more p-nitrophenol than extracts with 50 µg.l^{-1} OA (blue). The in vitro PP activity was expressed as the optical density increase (λ_{abs} = 405nm) over time per unit wet weight (g) of the tissue (mean ΔOD.g^{-1} ±SD).

4. Discussion

This study is the first to explore whether DA and OA, two common marine toxins, pose a significant threat to the recruitment of wild and cultured bivalves. In addition, this study provides the first evidence that harmful algae can induce responses of the phenoloxidase innate immune system. Overall, blooms of *Pseudo-nitzschia* spp. were of low concern as these algae, and their toxin DA, had no distinguishable effect on the development of *M. edulis* larvae. OA producing blooms of *Prorocentrum* spp. and *Dinophysis* spp., by contrast, could potentially affect the recruitment of bivalves, as environmentally relevant concentrations of OA were shown to decrease larval viability through the inhibition of protein phosphatase activity. Both *P. multiseries* and *P. lima* were found to trigger the larval phenoloxidase innate immune response.

Pseudo-nitzschia multiseries is frequently observed at densities >1000 cells.ml^{-1} (Orlova et al., 2008; Parsons et al., 2013; Stonik et al., 2011). Reports of natural DA concentrations are scarce, but range from 3 µg.l^{-1} to 136 µg.l^{-1} (Fawcett et al., 2007; Trainer et al., 2012, 2007). Crucially, DA production may vary between strains and is known to depend on environmental conditions (Mos, 2001; Trainer et al., 2012). Strain-dependency was also observed here. Despite identical culture conditions, the intra- and extracellular DA levels of CLN-47 exceeded those of the CCAP strain by nearly a factor four: 0.02 vs. 0.06 pg.cell^{-1} intracellular DA and 0.09 vs. 0.32 pg.cell^{-1} extracellular DA. The toxin levels of both were, however, within the ranges found by Fuentes and Wikfors (2013): i.e. 0.01-1.5 pg.cell^{-1} and 0.01-2.9 pg.cell^{-1}, respectively.

Experimental exposures to *Pseudo-nitzschia multiseries* (140 to 14,000 cells.ml^{-1}) and DA (up to 723.6 µg.l^{-1}) deliberately exceeded the environmental concentrations by a wide margin. Despite of this, no significant differences in the larval viability and development of *M. edulis* were found. Based on our results, it seems unlikely that blooms of *Pseudo-nitzschia* spp. affect the recruitment of bivalves. Liu et al. (2007), however, show that the viability, growth and development of king scallops larvae (*Pecten maximus*) can be reduced after a chronic exposure (28 days) to 30 µg l^{-1} DA. While the absence of such effects here could be associated with species-dependent sensitivity differences and the vast difference in exposure time, we are not convinced that persistent chronic exposure to DA is environmentally relevant. *Pseudo-nitzschia* blooms can, indeed, last for weeks but their densities exhibit high temporal variability (Liefer et al., 2009). As a result, DA levels are also likely to fluctuate noticeably as DA degrades rapidly in natural water matrices (Bouillon et al., 2006).

Bloom densities of *P. lima* (15 to 1500 cells.ml^{-1}) did not affect the viability and development of veliger larvae, despite an OA production which was similar to those found in literature (Bravo et al., 2001; Koike et al., 1998; Nascimento et al., 2005). However, we estimated that the highest simulated bloom of *P. lima* only produced 0.32 µg.l^{-1} extracellular OA (Table 5.1) while significant acute OA toxicity, i.e. reduced larval viability, was only found at concentrations of 37.8 µg.l^{-1} and above (Fig. 5.2). The lack of a response is, hence, not surprising. While there are currently no known observational records of natural OA concentrations in the range needed for toxicity, we suspect that OA levels could reach the required concentrations for acute toxicity as *Dinophysis* blooms can reach densities between 480 cells.ml^{-1} and 1,300 cells.ml^{-1} (Hattenrath-Lehmann et al., 2013; Heddenhorf, 2013; MacDonald, 1994) and produce between 10 to 100 pg OA.cell^{-1} (Andersen et al., 1996; Nielsen et al., 2013).

As shown here, OA may inhibit protein phosphatases. 50 µg.l^{-1} reduces PP activity in larvae by nearly 50%, which is similar to what was observed in adult mussels (Svensson and Förlin, 1998). Protein phosphatases regulate gene transcription, cytoskeletal structuring, signal transduction, cellular movement, protein stability, the progression of the cell-cycle and apoptosis (Cohen, 2002). These are key processes during the successful division, rearrangement and differentiation of embryonic cells. Picard et al. (1989) already found that microinjections of OA arrest the development of starfish embryos. Subsequent work by Patel and Whitaker (1991) revealed that these injections prevent the reformation of nuclear envelopes during mitosis in sea urchin embryos. A similar mechanism may very well explain the acute OA toxicity observed in *M. edulis* embryos.

Phenoloxidases, which have antibacterial functions, are found in all life stages of *Mytilus edulis* (Coles and Pipe, 1994; Dyrynda et al., 1995; Luna-Acosta et al., 2011). While these immunological enzymes are most commonly found in adult haemocytes (Luna-González et al., 2003), it is known that embryonic cells - which are also able to perform phagocytosis to some extent - have a restricted level of PO activity as well (Dyrynda et al., 1995; Luna-González et al., 2003). Previous studies have shown that (chemical) stressors tend to reduce the PO activity of several marine organisms (Bado-Nilles et al., 2010; Ittoop et al., 2009; Luna-Acosta et al., 2011, 2012). Yet, here we demonstrate that *P. multiseries* (1400 cells.ml^{-1}) and *P. lima* (150 cells.ml^{-1}) increase the PO activity of mussel larvae (Fig 5.3). As these observations could not be related to their respective toxins, this effect could be related to the bacteria which are associated with the algal cultures. By contrast, DA exposure has been shown to increase the number of haemocytes in adult bivalves, while OA does no such thing (Dizer et al., 2001; Jones et al., 1995; Malagoli et al., 2008).

HA may temper as well as aggravate the host-pathogen interactions in bivalves (da Silva et al., 2008; Hégaret et al., 2010; Lassudrie et al., 2015). The implications of an elevated larval PO activity are, however, largely unknown as the immunological role of larval PO is poorly understood. Two scenarios can be envisioned with bivalve larvae: HABs could ‘prime’ the prophenoloxidase cascade which would then increase the larval resilience to bacterial infections. Such an effect was, for instance, observed in heatshock protein Hsp70 fed nauplii larvae of the brine shrimp *Artemia franciscana* (Baruah et al., 2011). Alternatively, HABs may reduce the immunological resistance as the immune system is ‘overwhelmed’ or ‘depleted’. The energy allocation towards defensive responses may also affect other metabolic processes (e.g. growth) in such a manner that these short-term exposure experiments would neglect.

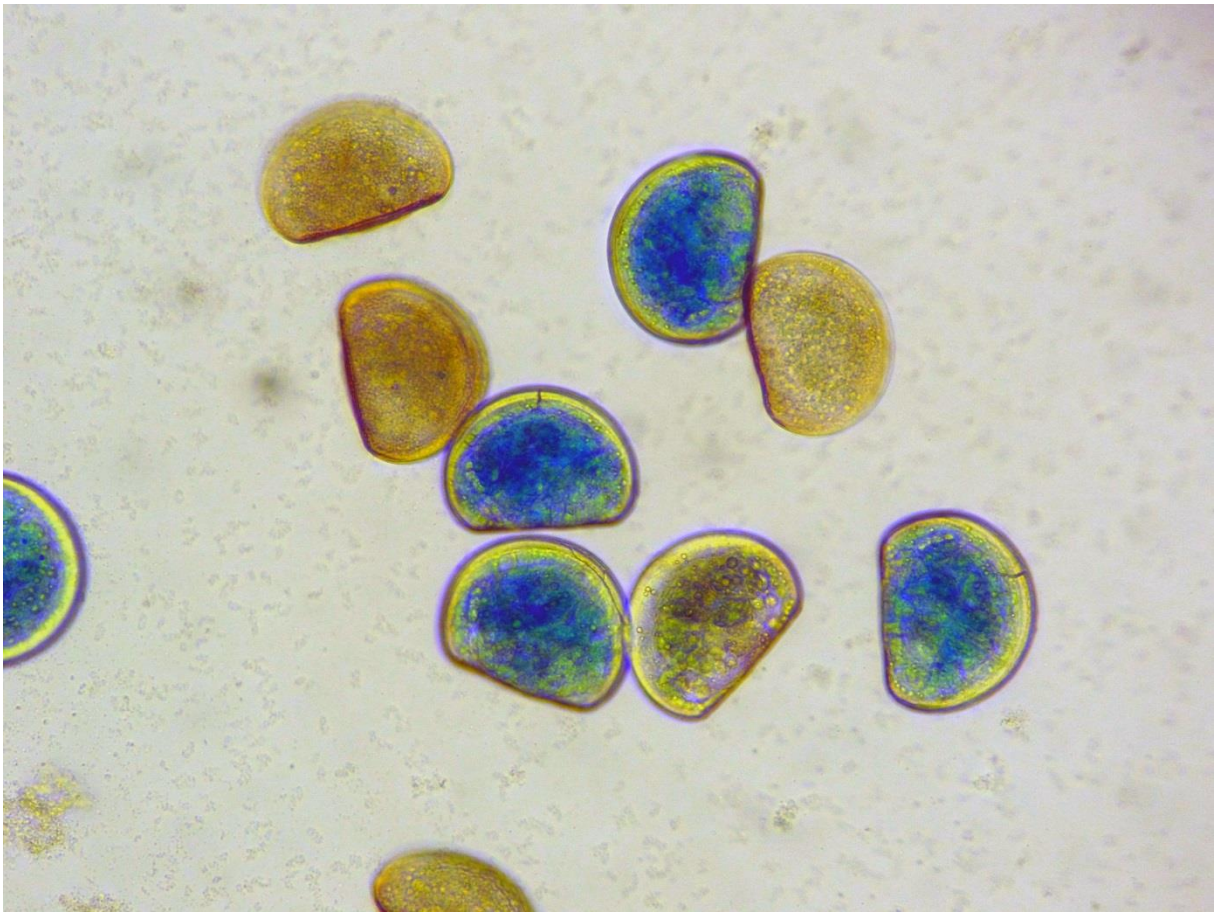
In recent years, pathogens have caused substantial episodic mortality in both wild and cultured bivalve populations (Beaz-Hidalgo et al., 2010; Paillard et al., 2004). As both pathogens and harmful algae may benefit from global change in the future (Anderson et al., 2012; Burge et al., 2014), the combined pressure by both stressors will increasingly affect marine life at all trophic levels. Despite the growing evidence that HABs reduce bivalve recruitment (Bricelj et al., 2011; Jeong et al., 2004; Padilla et al., 2006; Rolton et al., 2014; Wikfors and Smolowitz, 1995), we still know little about the interaction between HABs and the immunological resilience of the earliest, most sensitive, life stages of bivalves. To accurately assess the risk of harmful algae, we should look beyond the short- or long-term effects of HAB exposure and consider including a known pathogen (eg. *Vibrio* spp.) into our experimental designs.

5. Conclusions

Since the original publication of this research chapter, the toxicity of OA has been demonstrated independently in a multitude of cell types of *Mytilus galloprovincialis* (Prego-Faraldo et al., 2015, 2016). Given that OA is perhaps the longest known toxin in the world, with a near cosmopolitan distribution, the recent discovery of until now unknown genotoxic and cytotoxic effects of OA is troubling. It demonstrates the need for continued vigilance and experimental research of this type. As DSP is the most common shellfish poisoning type in the Southern Bight of the North Sea (Figure 1.8), these results could also have implications for the bivalve communities of the BPNS. With the growing spectrum of toxins and harmful species at our disposal, however, it is clear that we are not able to reliably predict the effect of HABs on bivalve stocks. To improve our knowledge on the effect of HABs on bivalves and bivalve recruitment, we will need more robust data on natural dissolved toxin concentrations to design enviromentally ecotoxicological studies of this type. For this reason, and to be able to fully comprehend whether HABs and their associated effects are truly increasing due to climate change, this study urges HAB monitoring programs to include both the density and the toxin production of the current harmful phytoplankton.

6

Bivalves in a changing environment: the potential for subchronic mixed toxicity effects of harmful dinoflagellates and *Vibrio* spp. on developing mussel larvae



Abstract

Harmful algal blooms (HABs) and marine pathogens - like *Vibrio* spp. – are increasingly common in coastal waters due to climate change. These stressors affect the growth, viability and development of bivalve larvae. Little is known, however, about the potential for interactive toxicity between these two concurrent stressors. While some mixed exposures have been performed with adult bivalves, no work has been done on larvae, which are generally more sensitive to environmental stressors. This study examines whether dinoflagellates and bacteria may interactively affect the viability and immunological resilience of blue mussel *Mytilus edulis* larvae. Embryos were exposed to ecologically relevant densities (100, 500, 2,500 & 12,500 cells.ml^{-1}) of dinoflagellates (*Alexandrium minutum*, *Alexandrium ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *P. lima* or *P. micans*), a pathogen (*Vibrio coralliilyticus/neptunius* isolate or *Vibrio splendidus*; 10^5 CFU.ml^{-1}), or both. After five days of exposure, significant ($p < 0.05$) adverse effects on the viability and development of the larvae were found for all dinoflagellates (except *P. cordatum*) and *V. splendidus*. Yet, despite their individual effects, no significant interactions were found between the pathogens and the dinoflagellates. The larval viability and the phenoloxidase innate immune system responded independently to each stressor, which may be related to a differential timing of the effects of HABs and pathogens.

Redrafted from:

De Rijcke, M., Van Acker, E., Nevejan, N., De Schamphelaere, K.A.C., Janssen, C.R., (2016) Toxic dinoflagellates and *Vibrio* spp. act independently in bivalve larvae. *Fish and shellfish Immunology* **57(1)**, 236-242. doi: 10.1016/j.fsi.2016.08.027

1. Introduction

Over the past decades, mankind has inadvertently altered the marine environment through overfishing, eutrophication, invasive species dispersal and global change. These changes have led to an increase in the frequency, scale and magnitude of harmful algal blooms (HABs), which can cause mass mortality amongst bivalves (Anderson et al., 2012; Shumway, 1990). Bivalve populations are key components of coastal communities (Dame, 2011; Gutiérrez et al., 2011). Through starvation, hypoxia, physical damage to vulnerable tissues (e.g. gills) or the accumulation of various marine toxins, HABs may kill bivalves. The associated loss of bioturbation, biogenic reefs and bentho-pelagic coupling upsets the biodiversity of multiple trophic levels. Moreover, these mortality events directly affect our food security as >10% of the global annual seafood production is derived from cultured molluscs (FAO, 2016).

Both wild and cultured bivalve populations rely on the availability of wild larvae (Lucas and Southgate, 2011; Seitz et al., 2001; Smaal, 2002). These early life stages are sensitive to abiotic stress (ASTM Standard E724-98, 2012), bacterial infections (Paillard et al., 2004) and harmful algae (e.g. Bricelj et al., 2011; Jeong et al., 2004; Padilla et al., 2006; Rolton et al., 2014). Recently, it was shown that HABs may also affect the phenoloxidase activity (PO) - which has antibacterial functions in adult bivalves (Hellio et al., 2007; Luna-Acosta et al., 2011; Zhou et al., 2012) – of larvae, potentially increasing their susceptibility towards pathogenic bacteria (Chapter 5). Like HABs, pathogens are long known to cause mass mortality among bivalve stocks (Beaz-Hidalgo et al., 2010; Paillard et al., 2004; Shumway, 1990). In addition, these stressors are known to co-occur since the organic matter and detritus made by HABs promotes the growth of benign and pathogenic bacteria (Cole, 1982; Doucette, 1995; Eiler et al., 2006; Mourino-Perez et al., 2003; Simidu et al., 1971). In fact, pathogens and HABs may increasingly co-occur, since both will benefit from climate change (Anderson et al., 2012; Burge et al., 2014). Yet, despite all of this, little is known about the potential for interactive adverse effects between both stressors.

To investigate the combined effect of HABs and pathogens, this study examined the individual and combined effects of these stressors on bivalve larvae. To this end, embryos of the blue mussel *Mytilus edulis* were exposed to various mixtures of (toxic) dinoflagellates (*Alexandrium minutum*, *Alexandrium ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *P. lima* or *P. micans*) and known pathogens (a *Vibrio coralliilyticus/neptunius*-like isolate or *Vibrio splendidus*). After five days of embryonic exposure, the larval viability, development and PO activity were all determined.

2. Material and Methods

2.1 Algal cultures

Dinoflagellates came from different sources: *Prorocentrum lima* (CCAP1136/9) and *P. micans* (CCAP1136/20) were supplied by the Culture Collection of Algae and Protozoa (Oban, Scotland, UK). *Alexandrium ostenfeldii* (CCMP1773) originated from the National Center for Marine Algae and Microbiota (East Boothbay, Maine, USA). *A. minutum* (SCCAP K-0993), *Karenia mikimotoi* (SCCAP K-0260), *Protoceratium reticulatum* (SCCAP K-1478) and *Prorocentrum cordatum* (SCCAP K-1501) were obtained from the Scandinavian Culture Collection of Algae and Protozoa (Copenhagen, Denmark). All these North Sea species were cultured in L1 medium (Guillard and Hargraves, 1993), prepared from filtered and autoclaved artificial seawater (32 PSU, pH 8; Instant Ocean^{tm}, Belcopet, Belgium). Batch cultures were maintained by replacing 80% of the medium every 2 weeks. Algae were grown at 20°C with a light-dark cycle of 12 hours (300-600 µmol m^{-2} s^{-1}) and culture growth was monitored by frequent cell counts using a Sedgewick Rafter counting chamber (SPI supplies, West Chester, USA) and a Nikon TMS-F light microscope (40x). All experiments were performed with cultures in the early stationary phase.

2.2 Bacterial cultures

Two known pathogens of bivalve larvae, i.e. *Vibrio splendidus* (EU358783) and a *Vibrio coralliilyticus/neptunius*-like isolate (EU358784) – further simply referred to as V*. coralliilyticus* - were isolated at the Glenhaven Aquaculture Center (New Zealand). These strains are known to infect and kill Greenshell mussel (*Perna canalicus*) larvae (Kesarcodi-Watson et al., 2009a). Bacterial stocks were frozen at -80°C in sterile Marine Broth 2216 (Difco, BD, New Jersey, USA) containing 30% glycerol. Before the experimental work, bacteria needed to be reactivated at 25°C by incubating 200 µl of bacterial stock with 10 ml of sterile Marine Broth in 50 ml falcon tubes on a shaker. This culturing procedure was repeated daily. Bacterial densities were counted with a Thermo Aquamate spectrophotometer (Thermo Fisher Scientific, San Jose, USA) operating at 550 nm using the method of Baruah et al. (2011). All procedures were performed in a Holten LaminAir laminar flow cabinet to prevent contamination (Thermo Fisher Scientific, San Jose, USA).

2.3 Viability and development

Fecund mussels were collected from breakwaters near Middelkerke (Belgium) between January and May 2015. These were placed in a recirculating aquarium (8°C, 34 PSU), filled with artificial seawater (Instant Ocean^{tm}, Belcopet, Belgium) and fed ad libitum (± 10 µl.l^{-1}) with Shellfish Diet 1800 (Varicon Aqua Solutions, UK). Spawning was induced, in accordance with the standard E724-98 ASTM guideline (ASTM, 2012), by moving the mussels between water baths of 18°C and 26°C. Sperm and eggs of multiple individuals were isolated, purified and combined in a single fertilization. Gamete quality was visually assessed throughout the subsequent hour. Embryos were used when ≥80% of the eggs had developed a polar body.

2.4 Embryonic development test

Sterile 24-well tissue culture plates were filled with 2 ml of ASTM artificial seawater containing 100, 500, 2,500 or 12,500 cells.ml^{-1} of one of seven dinoflagellate species (*Alexandrium minutum*, *A. ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *P. lima* or *P. micans*). Each series was made in threefold: once as single exposures, once with *Vibrio coralliilyticus* (10^5 CFU.ml^{-1}) and once with *Vibrio splendidus* (10^5 CFU.ml^{-1}). Each treatment was replicated six times. Control treatments included pure ASTM (i.e. 0 cells.ml^{-1} algae and no pathogens) and two single exposures to *V. coralliilyticus* or *V. splendidus* (10^5 CFU.ml^{-1}). As recommended by ASTM (2004), additional replicates of the control (18) and the *Vibrio* control treatments (12) were made to potentially detect diminishing egg quality and quantity between the start, middle and end of the spiking process. Such patterns were not detected. Around 120 fertilized eggs were transferred to each well and the plates were incubated at 18°C with a light-dark cycle of 12h/12h.

Short-term (48h) exposure assays with bivalve embryos typically determine the effect of stressors by the shape of the veliger larvae (ASTM, 2004). If the exposure is prolonged past 48 hours, however, dead but well-formed larvae are indistinguishable from healthy, D-shaped, veliger larvae (personal observations). For this reason, a staining procedure was developed and optimized in preliminary experiments. After four days, 25 µl of 2.4 mM methylene blue (10% EtOH) was added to all treatments. This dye stains actively filtering larvae while dead or moribund larvae are colourless. After five days, the development was halted with 25 µl of 30% formaldehyde. A 40x Oxion inverse light microscope (Euromex, Arnhem, The Netherlands) was used to distinguish and count the healthy (i.e. blue) veliger from larvae with compromised feeding (i.e. non-blue) and underdeveloped larvae (i.e. non-veliger).

2.5 Phenoloxidase activity

Glass jars of 500 ml were filled with 200 ml ASTM seawater solutions containing one of six dinoflagellates (*Alexandrium minutum*, *A. ostenfeldii*, *Karenia mikimotoi*, *Prorocentrum cordatum*, *P. lima* or *P. micans*; 2,500 cells.ml^{-1}). Of each, five single exposures and five replicates with *Vibrio splendidus* (10^5 CFU.mL^{-1}) were prepared. *V. coralliilyticus* was excluded from this test based on earlier results (ref. section 3.1). Control treatments with pure ASTM or *V. splendidus* were also replicated five times. Around 100.000 embryos were added to each vessel before they were incubated at 18°C, with a 12h light cycle. After five days, larvae were collected on a 37 µm sieve, flash-frozen in liquid nitrogen and stored at -86°C. Larval PO activity was determined by the transformation of L-3,4-dihydroxyphenylalanine to dopamine, as explained in chapter 5 (section 3.3). Three aliquots of each biological replicate were analysed. Over the course of 48 hours, the optical density of the samples was determined by a Thermo Multiskan Ascent 96/384-well spectrophotometer operating at 490 nm.

2.6 Data analysis

Due to cell lysis, conventional indices (cfr. chapter 5) were not appropriate here. Larval viability was, therefore, defined as the number of healthy (i.e. blue) veliger larvae divided by the average total number of larvae in the control treatment. Larval development was defined as the total number of veliger larvae (blue and non-blue) divided by the average total number of larvae in the control (Eq. 6.1 & 6.2). The larval viability and larval development were fitted with a three-parameter log-logistic model using the “drc” package of Ritz and Streibig (2005). Each dinoflagellate yielded three dose-responses: one without pathogens, one with *Vibrio coralliilyticus* and one with *Vibrio splendidus*. The estimated slope, EC_{50} and upper limit of these three models were then compared by means of a Z-test.

$$(6.1)\ \mathrm{V}_{\%} = \frac{B}{\langle B+nB+D\rangle_{control}}$$

$$(6.2)\ \mathrm{D}_{\%} = \frac{B+nB}{\langle B+nB+D\rangle_{control}}$$

with V% and D% representing the viability and development rates, respectively. B, nB and m are the number of blue, non-blue and dead larvae of each replicate.

The enzymatic transformation of L-DOPA to dopamine was used to study the phenoloxidase activity. First, the average autoxidation of L-DOPA - determined from samples without enzymes - was subtracted from the optical densities of each sample. Then, the increase in optical density (ΔOD) was calculated for each sample, adjusted for mortality (m) based on the embryonic development test, and fitted to the following linear regression model (Eq. 6.3).

$$(6.3)\ \frac{\mathrm{OD_t} - \mathrm{OD_0}}{1 - \mathrm{m}} = \Delta\mathrm{OD_m} \sim \alpha[\mathrm{D}] + \beta[\mathrm{V}] + \gamma[\mathrm{D}][\mathrm{V}] + \delta\mathrm{t}$$

This basic linear regression model was first used for each dinoflagellate. In short, the mortality adjusted optical density (ΔODm) was expressed as a function of the presence-absence of a dinoflagellate [D], the presence-absence of *V. splendidus* [V], an interaction term between both [D]x[V] and time [t]. The Least Squares F-test was then used to remove non-significant parameters through backward elimination from the model of each dinoflagellate. The validity of the residual model was assessed by the F-statistic, the R-squared and the distribution of residuals.

3. Results

3.1 Development and viability of veliger larvae

Non-exposed *Mytilus edulis* larvae were in good health after 5 days of growth, showing on average 85±12% larval development and 81±12% viability within the control treatment. Exposure to *Vibrio coralliilyticus* improved the larval development slightly (+6%; $p < 0.05$), but did not affect the larval viability ($p > 0.05$). *V. splendidus*, on the other hand, significantly decreased the larval viability ($p < 0.001$) - by 16.8% on average – but did not adversely affect the larval development ($p > 0.05$). All algae, except *Prorocentrum cordatum*, had significant adverse effects on the development ($p < 0.001$) and viability of *M. edulis larvae* ($p < 0.05$; Fig. 6.1). *Alexandrium minutum*, *A. ostenfeldii* and *Protoceratium reticulatum*, in particular, were observed to greatly reduce the number of larvae. Neither *V. coralliilyticus* nor *V. splendidus* affected the response to any of the dinoflagellates. The ECx estimates of the development and viability did not differ significantly ($p > 0.05$) between each of the three density series. The data of all three series (i.e. single exposure to algae and both series with Vibrio) were, therefore, pooled by dinoflagellate species to improve the ECx estimates (Table 5.2).

Table 6.1: Toxins and natural bloom concentrations of the dinoflagellates used here.

Dinoflagellate	Toxins	Bloom concentration	Reference(s)
A. minutum	STX; GTX	10^3-10^4 cells.ml^{-1}	Ranston et al. (2007); Santos et al. (2014)
A. ostenfeldii	SPX or STX;	2.10^2 cells.ml^{-1}	Hakanen et al. (2012)
K. mikimotoi	Gym-A; Gym-B	3.10^3 cells.ml^{-1}	Silke et al. (2005)
P. cordatum	unknown	10^3-10^4 cells.ml^{-1}	Denardou-Queneherve et al. (1999); Tango et al. (2005)
P. lima	OA; DTX	10^2-10^4 cells.g^{-1} DW	Foden et al. (2005)
P. micans	unknown	1.10^4 cells.ml^{-1}	Pybus, (1990)
P. reticulatum	YTX	$3.\ 10^2$ cells.ml^{-1}	Álvarez et al. (2011)

Table 6.2: Estimates of ECx values (±SE) of each dinoflagellate, ranked by EC50, based on the development (DEV) and larval viability (VIA) of *Mytilus edulis* during a 5d sub-chronic embryonic development test. All values expressed as cells.ml^{-1}.

Strain		EC10	EC20	EC50
Protoceratium reticulatum	DEV	330 ± 139	416 ± 82	616 ± 128
SCCAP K-1478	VIA	364 ± 321	431 ± 179	574 ± 224
Alexandrium minutum	DEV	449 ± 74	669 ± 74	1322 ± 109
SCCAP K-0993	VIA	589 ± 133	825 ± 146	1,465 ± 153
Alexandrium ostenfeldii	DEV	2,091 ± 733	3,117 ± 849	6,169 ± 1,105
CCMP1773	VIA	1,103 ± 433	1,817 ± 528	4,265 ± 774
Prorocentrum micans	DEV	1,621 ± 330	2,747 ± 418	6,767 ± 636
CCAP1136/20	VIA	1,627 ± 392	2,655 ± 489	6,132 ± 763
Prorocentrum lima	DEV	2,569 ± 507	3,817 ± 575	7520 ± 657
CCAP1136/9	VIA	2,657 ± 628	3,876 ± 708	7,392 ± 804
Karenia mikimotoi	DEV	9,783 ± 5,416	11,446 ± 2,307	14,969 ± 6,107
SCCAP K-0260	VIA	9,749 ± 8,710	11,115 ± 4,703	13,907 ± 5,351

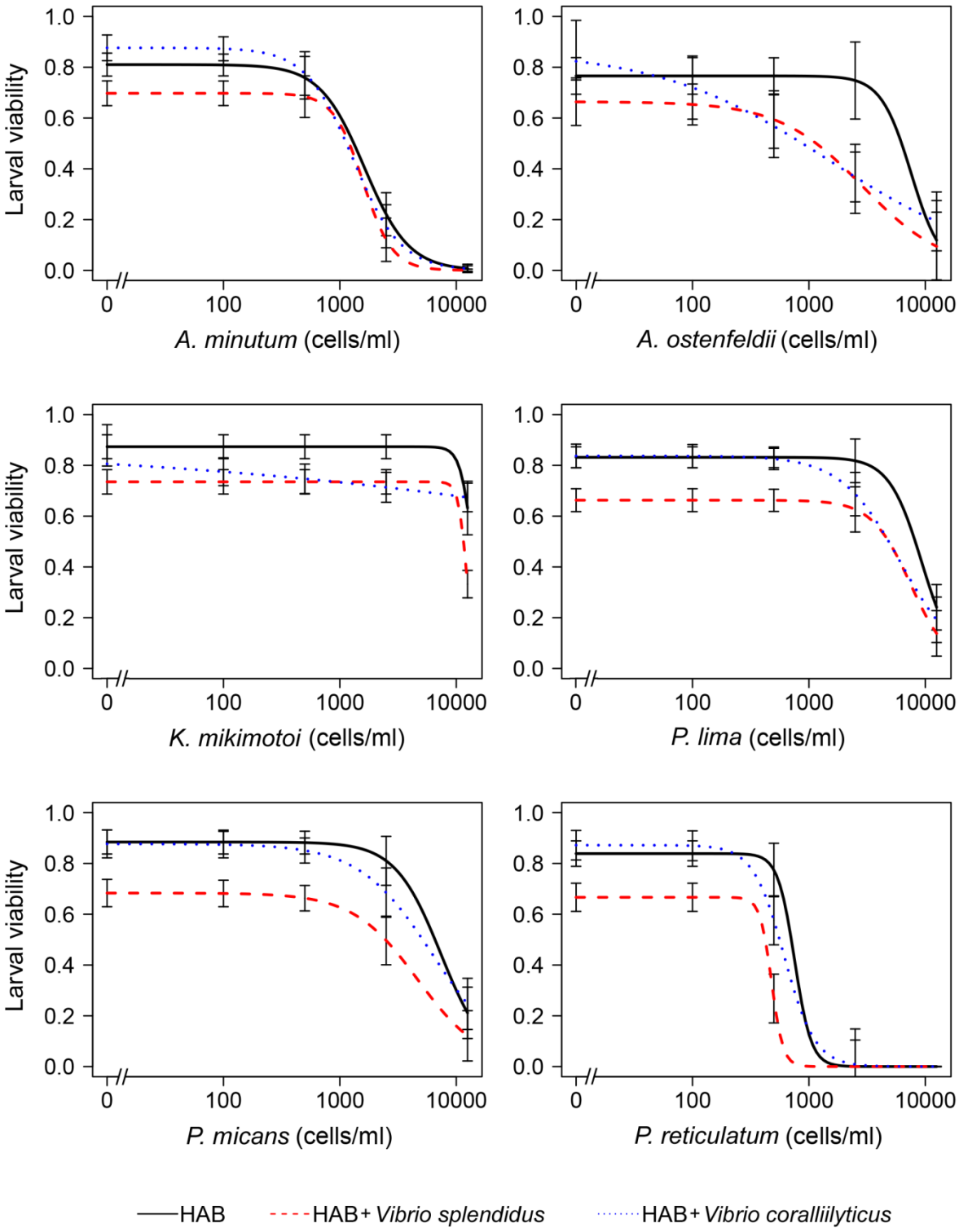


Figure 6.1: Dose-response curves of the larval viability of *Mytilus edulis* after five days of exposure to one of six dinoflagellates, one of two pathogens or both. 0 cells.ml^{-1} represents control treatments with and without the pathogens. Bars are mean±SE. Each dinoflagellate, and *Vibrio splendidus* (10^5 CFU.ml^{-1}), had a significant effect on larval viability ($p < 0.05$).

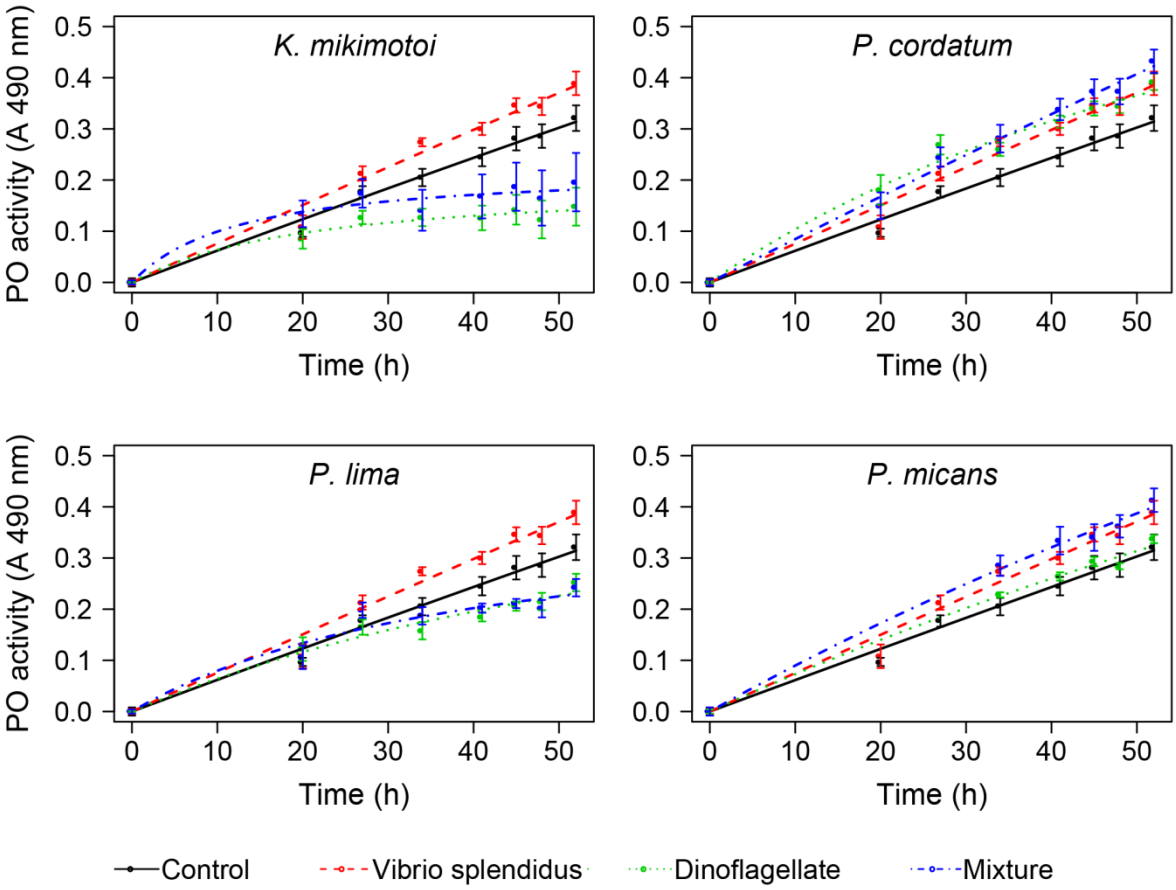


Figure 6.2: The phenoloxidase activity of *M. edulis* larvae after 5 days of exposure to dinoflagellates (2.500 cells.ml^{-1}), *V. splendidus* (10^5 CFU.ml^{-1}), or both. Bars are mean±SE. All algae and *V. splendidus* had a significant effect on the immune response ($p < 0.05$).

3.2 Phenoloxidase activity

The measurement of phenoloxidase activity requires at least 0.1 g of tissue. Due to cell lysis (see section 3.1), this weight requirement was not met by samples of the *A. minutum*, *A. ostenfeldii* and *P. reticulatum* treatments. Two additional samples (one of the *P. micans* with *V. splendidus* treatment and one of the *Karenia mikimotoi* with *V. splendidus* treatment) did not meet the minimum weight either. All of the other remaining samples were processed and measured as described in section 2.4. Dinoflagellates caused species-specific responses (Figure 6.2). The larval PO activity increased significantly in the presence of both *P. cordatum* ($p < 0.001$; R^2 0.83) and *P. micans* ($p < 0.05$; R^2 0.79). By contrast, the larval PO activity was suppressed by *K. mikimotoi* ($p < 0.001$; R^2 0.59) and *P. lima* ($p < 0.001$; R^2 0.69). *V. splendidus* increased the PO activity in all treatments ($p < 0.001$). No significant interactions were observed between the HAB and *V. splendidus* exposures ($p > 0.05$).

4. Discussion

Nearly all bivalve populations exhibit periodical mass mortality. These events are caused by multiple stressors like temperature, salinity, viruses, HABs and pathogens (Barbosa Solomieu et al., 2015; Beaz-Hidalgo et al., 2010; Bricelj et al., 2011; Burdon et al., 2014; Jeffries, 1982; Paillard et al., 2004). Despite of this, we know little about interactive effects between stressors like HABs and pathogenic bacteria (da Silva et al., 2008; De Rijcke et al., 2015; Hégaret et al., 2010). This study is the first to investigate whether pathogens and HABs affect the larval development of bivalves interactively. While novel species-dependent responses to both algae and pathogens were found, no significant combined effects were observed. Overall, the environmentally relevant concentrations of pathogens and dinoflagellates used here did not act synergistically on the development or viability of mussel larvae. Similarly, the phenoloxidase innate immune activity of the mussel larvae was shown to predominantly respond to individual stressors. Despite evidence that these stressors may interact both synergistically and antagonistically in adult bivalves, the results of this study suggest that pathogenic bacteria and harmful algae do not interact during the early development of *Mytilus edulis*.

This is the first report of bivalve mortality, sensu lato, caused by *P. reticulatum*. Despite evidence that yessotoxin causes DNA fragmentation and induces apoptosis (e.g. Pérez-Gómez et al., 2006; Rubiolo et al., 2014), previous work on Pacific oyster *C. gigas* larvae did not find any mortality after exposure to *P. reticulatum* filtrates (Thompson et al., 1994). While it is conceivable that direct cell-cell interactions are needed to obtain toxicity, it should also be mentioned that Thompson et al. (1994) did not measure the yessotoxin production of their strain of *P. reticulatum.* Contrastingly, *Alexandrium* spp. are well known to affect the development of marine organisms (Garcia et al., 2010; Mu and Li, 2013; Yan et al., 2001). This is often attributed to the genetically fixed ability to produce saxitoxins (STX), spirolides (SPX) or goniodomins (Anderson et al., 1990). Here, *A. minutum* and *A. ostenfeldii* both had cytolytic effects on *M. edulis* eggs despite their different suite of toxins: *A. minutum* SCCAP K-0993 has been shown to produce STX - which causes developmental abnormalities – while *A. ostenfeldii* CCMP1173 produces SPX (Bernardi Bif et al., 2013; Cembella et al., 2000; Hansen et al., 2003; Tian et al., 2014). While STX could potentially explain why *A. minutum* has a stronger effect on larvae than *A. ostenfeldii*, it does not clarify why *A. ostenfeldii* was toxic*.* Recently, however, a toxin-independent pathway was found in *Alexandrium* spp. which might be present in both our *Alexandrium* species (Basti et al., 2015b).

Like Stoecker et al. (2008a) before us, this study found no adverse effects of *Prorocentrum cordatum* on bivalve larvae. By contrast, Wikfors and Smolowitz (1995) did find an effect on *Crassostrea virginica* oyster larvae. *P. cordatum* is variably toxic and most strains are non-toxic (Glibert et al., 2012; Heil et al., 2005; Wikfors, 2005). Likewise, *P. micans* is considered to be non-toxic despite shellfish kills in the past (Horstman, 1981; Lee et al., 1989; Pinto and Silva, 1956; Reguera et al., 2014). Yet, here *P. lima*, *P. micans* and *K. mikimotoi* all reduced the larval viability of *M. edulis.* The results of this study, together with the work of Ji et al. (2011), suggest that the toxin production of *P. micans* needs to be reconsidered. Similarly, we urge other researchers to further unravel the toxicity of *K. mikimotoi*, which is superficially known to produce toxic compounds like gymnocins and haemolysins that are poorly understood (Chen et al., 2011; Satake et al., 2002, 2005; Yamasaki et al., 2004). Basti et al. (2015b) reported that *K. mikimotoi* affects the hatching of bivalve eggs at doses of 15.10^3 cells.ml^{-1}. This dosage is similar to the ECx values reported here.

HABs promote the growth of associated bacteria (Cole, 1982; Doucette, 1995). *Vibrio* spp., which can cause mortality among bivalves (Beaz-Hidalgo et al., 2010), has been linked to HABs in the past (Eiler et al., 2006; Mourino-Perez et al., 2003; Simidu et al., 1971). Here, *V. splendidus* (10^5 CFU.ml^{-1}) reduced the larval viability of *M. edulis* while the *V. coralliilyticus/neptunius*-like isolate did not. These results are in accordance with the work of Kesarcodi-Watson et al. (2009b) who originally isolated these specific strains: *V. splendidus* affects *Perna canaliculus* larvae at 10^5 CFU.ml^{-1} while *V. coralliilyticus* causes adverse effects at doses of 10^6 CFU.ml^{-1} and up. Similar effects of *V. splendidus* were reported by Jeffries (1982).

While the response to HABs was species-specific - *P. cordatum* and *P. micans* increased the PO activity, while *K. mikimotoi* and *P. lima* reduced it – *V. splendidus* always induced the PO activity. Phenoloxidase responds to bacterial polysaccharides as it is involved in the opsonization and melanization of particles and, hence, mostly found in haemocytes (Luna-González et al., 2003; Thomas-Guyon et al., 2009). Still, embryos also possess PO (Dyrynda et al., 1995) but virtually no other studies have used PO activity, or larvae for that matter, to study the effect of HABs on bivalves. Because of this, we can only match our results to haemocyte responses. *P. cordatum* increases phagocytosis (Hégaret and Wikfors, 2005a, 2005b), though some studies found a downregulation (Hégaret et al., 2011) or no changes in haemocyte functions at all (Galimany et al., 2008b). The inhibition of PO by *P. lima* could be attributed to okadaic acid that reduces phagocytosis in haemocytes (Prado-Alvarez et al., 2013). *K. mikimotoi*, on the other hand, has no effect on haemocytes (Hégaret et al., 2011) and no studies have investigated the effects of *P. micans*.

None of the dinoflagellates changed the sensitivity of *M. edulis* to either pathogen, despite the demonstrated individual effects of both stressors. Regardless of the up or downwards regulation of PO by the algae, the larvae were still able to respond to pathogenic stress. This result implies that the viability, development and PO activity react independently to both stressors. During preliminary experiments, dinoflagellates caused larval mortality within the first 48 hours of exposure. Bacteria, per contra, were mostly observed to exert their adverse effects at the end of the exposure period (personal observations). Larvae are mostly infected by *Vibrio* spp. through ingestion (Kesarcodi-Watson et al., 2009a). The uptake of small particles (< 10 µm) starts during the second day of development, at which point the trochophore larva becomes encased in a protective prodissoconch (Sprung, 1984). As such, two-day-old veliger larvae may filter pathogens and dissolved toxins, but are no longer vulnerable to direct cell-contact with the much larger dinoflagellates.

The concentrations of algal exudates are noticeably higher within 2 to 3 cell radii distance of a toxic cell, relative to the surrounding medium (Jonsson et al., 2009). Even if environmental concentrations are below inhibitory levels, larval toxicity may occur within these “chemical envelopes” when the local concentrations exceed the no-effect concentrations. The likelihood of encountering such a chemical envelope depends on the algal density, toxin production rate and the mobility of both the larvae and the algae (Jonsson et al., 2009). After 48 hours, the prodissoconch may act as a non-permeable barrier which shields larval cells during the brief instant both organisms collide. By the time the toxin molecules work their way around the shell through diffusion, both organisms will have separated by their combined swimming. At the same time, the dissolved toxin concentrations have likely decreased as labile toxins from the algal source cultures degraded over time (Granéli and Turner, 2006). Moreover, we believe that the larvae may have acclimatized to the presence of toxins through the activation of biotransformation and elimination pathways. All of these reasons can explain the lack of mixed adverse effects in the earliest life-stages. Yet, with the current state of knowledge, we are unable to pinpoint the exact reason for their remarkable tolerance. Lastly, we cannot exclude that combined and interactive effects may appear once the mussels are large enough to simultaneously take up pathogens and larger phytoplankton.

5. Conclusions

Like Chapter 5, this study demonstrates that there are still a lot of unknown effects of HABs on bivalve populations. Specifically, we show that several dinoflagellates of the BPNS are able to reduce the viability of bivalve larvae at environmentally relevant bloom densities. Moreover, we show that HABs are able to induce immunological responses in the early life-stages of our model organism *Mytilus edulis.* Against our expectations, these changes did not increase the susceptibility of mussel larvae towards *Vibrio* spp. infections and, hence, does not inflate the risk of environmental damage by HABs or pathogens. Still, caution is advised as later life-stages may yet encounter mixed toxic effects of HABs and pathogens. To truly understand HABs and their myriad of effects on our coastal systems, more research into these “black swan” interactions is highly recommended.

7

Summary, situation, outlook and recommendations

1. Introduction

The European Marine Strategy Framework Directive (2008/56/EC) encourages the development of new eutrophication indicators that include the nutrient concentrations of the water column (and ratios) as well as effects on the community composition and ecological function of the phytoplankton. Descriptor 5 of the EU MSFD explicitly tasks us to investigate the loss of biodiversity due to human-induced toxic algal blooms. Yet, to date, we know surprisingly little about the presence and effects of toxic algae within the Belgian territorial sea. For this reason, the **main objective of this thesis** was to assess whether the ongoing chances in the environmental conditions of the BPNS have an effect on the risk of toxic HABs. To address this objective, we first had to improve our understanding of HAB ecology through several **research questions**. Here, each of those research questions will be answered with reference to the work presented in this dissertation, as well as the current state of the literature. In addition, **research recommendations** will be provided alongside **policy advice** for the BPNS.

2. Nutrient ratios and nutrient loads

Do shifting N:P ratios affect the competitive traits of dinoflagellates?

Coastal eutrophication changes the relative availability of growth-limiting nutrients, such as nitrogen and phosphorus, in systems all over the world (Martiny et al., 2014). Shifts in the N:P ratio are thought to increase the risk of (toxic) HAB development, but there is no substantial evidence that nutrient stoichiometry plays a key role in the bottom-up control of HABs (Davidson et al., 2012; Flynn, 2010). In **chapters 2 and 3**, we could, indeed, not demonstrate that the N:P ratio alters the competition between potentially harmful dinoflagellates. While variations in the N:P ratio did induce small changes in the nutrient uptake rates, we found no evidence that the growth rate was governed by nutrient stoichiometry. Later, i.e. chapter 3, it was shown that the growth rates are the product of nutrient uptake rates and nutrient conversion efficiencies, and that they are crucial during the initial bloom development stages. A small bloom inoculum may displace a more abundant species, if it is able to outcompete the other species through a higher growth rate. Once a bloom is established, allelopathy might release nutrients from competitors and maintain the bloom at hazardous densities. Toxin concentrations were also not affected by the N:P ratio. This, however, was only tested on a single toxic species, of which we know that the structures of its toxins do not contain nitrogen or phosphorus atoms. Overall, though, it seems unlikely that the N:P ratio has a noticeable direct effect on the risk of dinoflagellate blooms.

This research does, however, not exclude indirect effects of imbalanced nutrients on the food web. As the internal nutrient stoichiometry of phytoplankton often mimics the external nutrient ratio, extreme N:P ratios can reduce the nutritional value of all of the phytoplankton (Glibert et al., 2012). Primary consumers have to allocate energy towards the removal of the excess nutrient, which may affect their fitness and growth (Branco et al., 2010; Elser et al., 2003; Hauss et al., 2012; Schoo et al., 2009) and their value for higher trophic levels (Schoo et al., 2014; Vanni and McIntyre, 2016). The reduced fitness of grazers, combined with the recycling of excess nitrogen as ammonium favours HABs (Bronk et al., 2014; Glibert et al., 2014; Kang et al., 2015). The complex interactions between the bottom-up and top-down control of developing blooms was summarized by Sunda and Shertzer (2014).

In Sunda and Shertzer's model (2014), every dinoflagellate bloom was preceded by one or more blooms of diatoms, whose growth rates were higher than those of the dinoflagellates at high nutrient levels, but were also more grazed by zooplankton. These pre-blooms reduced the available nutrients to low, growth rate-limiting levels and promoted the population growth of zooplankton. Toxic dinoflagellates then proliferated at the expense of the diatoms due to the low grazing mortality rates and their ability to grow at low nutrient levels. At this time, toxic HA can benefit from the ammonium released by zooplankton that graze on nutritionally imbalanced diatoms. As HAB densities increase, a positive feedback emerges. The number of diatoms decreases through the combined action of the nutrient limitation and grazers, while nutrient limited HA become even more toxic. Nutrient recycling diminishes, increasing the nutrient restriction even further and, hence, promoting the production of toxins. Allelopathic interactions are then able to release some nutrients from competitors, prolonging the quiescence phase of the established bloom.

The developing field of ecological stoichiometry is still under considerable debate. A meta-analysis of datasets from the terrestrial, freshwater and marine environment found no effect of resource imbalance on the diversity and productivity of ecosystems (Lewandowska et al., 2016). Resource imbalance may even restrict nutrient recycling and provide stability to autotroph – herbivore systems instead (Striebel et al., 2009). In addition, it is not entirely known how this process relates to broad scale impacts, e.g. pollution, climate change and overfishing, which directly affect the fitness of grazer populations. More research into this emerging field is highly recommended. For experimental approaches, we encourage researchers to expand the range of N:P ratios to extremer cases. In addition, we recommend the inclusion of r-selected algal species, like *Phaeocystis* spp. which is still frequently used as an example of the structuring role of the N:P ratio

Do nutrient load reductions affect the competitive traits of dinoflagellates?

As seen in **chapter 3**, where we found a near perfect linear relation between the carrying capacities of dinoflagellate cultures and the initial nutrient concentrations, the total availability of nitrogen and phosphorus directly affects the intensity of HABs (Anderson et al., 2002; Cloern, 2001). The accumulation of toxins, which enables the deterrence of grazers and the inhibition of competitors, can be avoided by decreasing the potential for biomass accumulation of an ecosystem. For most aquatic systems, this is best achieved by simultaneously reducing the nitrogen and phosphorus inputs (Conley et al., 2009; Paerl et al., 2014). This, however, should be done gradually as the longevity of phosphorus in aquatic systems (and sediments) is noticeably longer than that of nitrogen, creating the risk of upsetting the nutrient balance even further (Burson et al., 2016; Philippart et al., 2007).

All of the fundamental work of this PhD dissertation was done using batch cultures of dinoflagellates. This approach affects our ability to predict the behaviour of dinoflagellates in natural systems. The growth of algae at low nutrient concentrations is difficult to simulate in these types of cultures. Moreover, we are aware that cultures are able to modify their pH which skews the interpretation of interspecific competition. It would be interesting to use a setup like Kayser (1979), i.e. a battery of turbidostats, that can replenish the medium (semi-)continuously. Such a system would allow us to stabilize the pH – also useful for simulating ocean acidification – and perform tests at environmentally relevant nutrient concentrations. Moreover, these types of systems would allow us to further our understanding of mixotrophy. It would be interesting to see whether dinoflagellates prefer to consume nutrients directly (autotrophic growth) or rather rely on their prey (heterotrophic growth) to gather scarce nutrients for them.

Finally, it is worth repeating that we used nitrate as the only nitrogen source in all of the experiments. While the uptake of NO_3^-, NH_4^+ and urea is similar in lab cultures (Chang et al., 1995; Fan et al., 2003; Kudela et al., 2008b; Smayda, 1997), more reduced nitrogen forms may still promote HAB development (Glibert et al., 2014). Donald et al. (2013) have demonstrated how different nitrogen forms can change the community composition of natural phytoplankton assemblages. This type of research is worth pursuing further, as our work and that of Sourisseau et al. (2017) show that resource competition structures phytoplankton communities. To date, we are missing key data on the nutrient preferences and nutrient uptake rates of most HAB species, especially in direct comparison to non-HAB species (Wells et al., 2015). To estimate the likelihood of HABs in the future ocean chemical matrix, it is crucial to understand whether HA will be able to outcompete other species.

3. Climate change

Will global change affect the competitive traits of dinoflagellates?

In our studies, i.e. **chapter 2 and 3**, simulated climate change temperatures led to higher nutrient consumption rates and, hence, higher growth rates of dinoflagellates. The maximum attainable daily growth rate of marine phytoplankton is directly related to temperature (Bissinger et al., 2008; Eppley, 1972). As growth rates were shown to be a key parameter in the development of blooms, it is reasonable to expect that global change will affect the competition between HAB and non-HAB species. Yet, due to a lack of climate experiments with mixed cultures, we know little about the effect of climate change on the long-term composition of phytoplankton communities. An ocean basin-wide time series analysis recently revealed that dinoflagellates and copepods are closely tracking the velocity of climate change, measured as the rate of isotherm movement, and that diatoms are moving more slowly (Chivers et al., 2017). Differences in range shifts of each group appear to result from dissimilarities in niche plasticity which, counterintuitively, could locally enhance the HAB risk. Dinoflagellates are well adapted to high temperatures, their temperature range for optimal growth often exceeds 14°C (Fig 7.1), but diatoms cope even better (Chivers et al., 2017). This affects the timing and duration of spring blooms, creating trophic mismatches between the grazer populations and the abundance of toxic algae, and increasing the summer “window of opportunity” for HAB development (Wells et al., 2015).

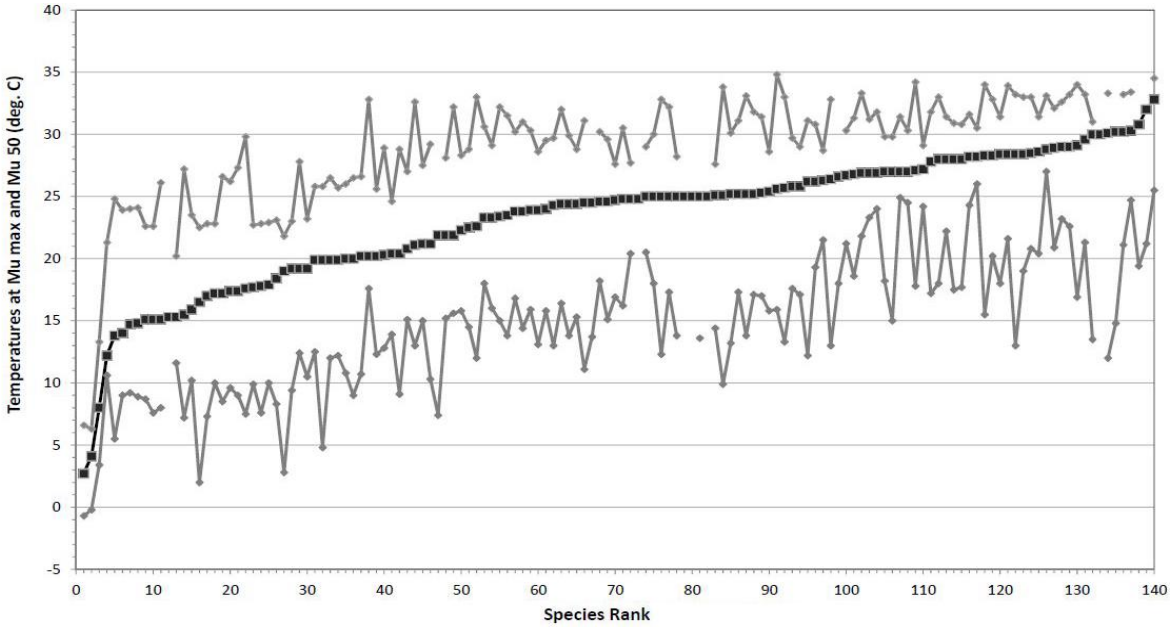


Figure 7.1: Ranking of HAB species by their optimal growth temperature (μ_m; black line). Grey lines are the 50% growth temperatures. Reproduced from Smayda and Smayda (2015).

In addition to the physiological effect of temperature on phytoplanktonic growth, climate change will promote the thermal stratification of coastal waters (IPCC, 2014) which enhances the risk of HABs (Gentien et al., 2005). Regional changes in the precipitation intensity, like the anticipated drier European summers with episodes of flooding (Christensen and Christensen, 2003), will pulse the riverine nutrient inputs and increase the stratification in salt-wedge estuaries even further. Stratification will increase the light penetration, especially in turbulent systems, which could magnify the potential biomass production of HABs (Häder et al., 2007). Changes in the flood and storm frequency will also increasingly help to overcome biogeographical barriers, and transport HA species to areas outside of their current range (Wells et al., 2015).

All of these mechanisms start from the premise that HAB species are able to cope with ocean acidification. The average ocean surface pH is projected to decrease by 0.3 to 0.4 units by the end of this century (Feely et al., 2004). While phytoplankton is generally well adapted to grow at low pH levels (Berge et al., 2010), the acidification will affect the energy requirements of carbon concentrating mechanisms as well as proton driven processes (e.g. mobility, membrane potential) in a species-specific way (Beardall et al., 2009; Beardall and Raven, 2004). On top of the changes in fitness, ocean acidification will change the chemical speciation of nutrients (Shi et al., 2010), change the toxicity of HA (e.g. Hattenrath-Lehmann et al., 2013) and redistribute the top-down control of HABs (Cripps et al., 2014; Waldbusser et al., 2015). Combined, these factors will reshape the competition between HAB and non-HAB species, while global change will affect all other trophic levels as well, reshaping the entire food web (Heuer and Grosell, 2014; Liu et al., 2010; Richardson and Gibbons, 2008).

The uncertainties of climate and ocean science add up. While we have identified a long list of “known unknowns” about the effect of global change on HABs, we expect to find a lot of “black swans” or “unknown unknowns” in the future (Wells et al., 2015). Multifactorial experiments which combine nutrient competition with abiotic variability, preferably temperature, pH and nutrient sources, are urgently needed to improve our predictions of the future occurrence of HABs. At the same time, we need to do more retrospective analyses of long-term phytoplankton or cyst core datasets that reveal to which extent the phytoplankton was already changed by manmade climate change. Fundamental research, as was done here, also needs to have a stronger linkage to climate change research to improve our HAB monitoring and forecasting programs. This can, for instance, be done in a global context through the upcoming “globalHAB” research program of the Intergovernmental Panel on Harmful Algal Blooms of the Intergovernmental Oceanographic Commission of UNESCO.

4. Effects on keystone species

Do toxic HABs affect populations of bivalve species?

In **chapters 4, 5 and 6**, we investigated the various effects of toxic HAB exposure on the blue mussel *Mytilus edulis*. As expected, adult mussels were less sensitive to toxic HABs than their earlier life-stages (ref. *A. ostenfeldii*). As long as the dissolved oxygen concentrations are above the lethal limits, i.e. above the median lethal limit of 1.4 mg $O_2.l^{-1}$ for bivalves (Vaquer-Sunyer and Duarte, 2008), adult bivalves cope well with toxic blooms. By modifying their respiratory and feeding behaviours, bivalves are able to minimize their contact with toxic phytoplankton (Hégaret et al., 2007). Delicate tissues are protected by closing the shell, reducing the movement of water through the animal, reducing the ingestion of particles, sorting the HA cells prior to ingestion or rejecting cells post-ingestion (Bardouil et al., 1996; Brillant and MacDonald, 2002; Galimany et al., 2008a; Hégaret et al., 2007; Matsuyama et al., 1997; Shumway and Cucci, 1987; Ward and Shumway, 2004; Wildish et al., 1998). Infiltration of toxins can activate the lipid peroxidation inside the lysosymes of hemocytes, forming insoluble lipofuchsin granules which are then transported across gastrointestinal epithelia by hemocytes for elimination in the faeces (Estrada et al., 2007; Galimany et al., 2008b). All these mechanisms are employed with species-specific intensities and efficiencies, which may vary with the exposure history of the population (Hégaret et al., 2007).

While we have a decent understanding of the general (de)toxification mechanism, there is still plenty to be learned about the metabolization of HAB toxins by bivalves. In **chapter 4**, we used state-of-the-art analytical techniques to explore how mussels accumulate, metabolize, distribute, and excrete two common toxins. The results were consistent with the lipofuchsin-excretion pathway, and revealed interactions between the metabolic processing of both. To the best of our knowledge, this the first report of the creation of different metabolites by the concurrent exposure to multiple toxic HA. In addition, we also demonstrated that natural phytoplankton assemblages cause the accumulation of multiple toxins and metabolites in bivalves. Similar mixtures of toxins and metabolites were found in several wild animals of the North Sea, including crabs, shrimp and fish (Orellana et al., 2017). As food safety regulations are based on the acute toxicity effect of single compounds, the widespread occurrence of mixtures is a risk to human consumers. Exploring the mixed toxicity effects of realistic toxin mixtures is a highly recommended research priority. Research into the ADME kinetics of multiple toxins, inside different seafood species (incl. bivalves, crustaceans, fish), is equally needed to not only enhance our knowledge of the fate of marine toxins, but also to improve the regulatory limits of safe consumption.

From our results, as well as previous laboratory and field studies, we can conclude that toxic HABs have little effect on adult bivalves. Yet, in **chapters 5 and 6** we found unknown toxic effects on the larval stages of *M. edulis*. As most bivalve populations rely on the natural availability of healthy larvae for the maintenance of their numbers, these adverse interactions could reduce the stock size of bivalves. For the European blue mussel species *M. edulis* and *M. galloprovincialis*, however, the threat of toxic blooms is negligible, as their main spawning period coincides with the spring blooms (Cáceres-Martínez and Figueras, 1998; Seed, 1969). Larvae from secondary spawns in August or September are more likely to encounter toxic HABs, but the fate and importance of these opportunistic secondary spawns is still unknown (Gosling, 2003). Other bivalve species, e.g. the common cockle *Cerastoderma edule*, the soft-shell clam *Mya arenaria* or the invasive Japanese oyster *Crassostrea gigas*, do reproduce during the window of opportunity of dinoflagellate blooms (Philippart et al., 2014). The recruitment of the populations of these species may be threatened by the recent increase in HABs. While *M. edulis* is a good model organism for a variety of reasons, incl. a vast knowledge base, a sequenced genome and its remarkable resilience, comparative studies should be undertaken to relate the sensitivity of mussel larvae towards HABs to the sensitivities of other bivalve larvae.

While no interactions between HABs and pathogens were found during the work of **chapter 6**, there is still a lot to be done on mixed toxicity effects of HA and bacteria. At the start of this PhD, a pilot study revealed that one dinoflagellate (*K. mikimotoi*) increased the susceptibility of adult mussels to pathogenic bacteria, leading to both tissue inflammation and a significant increase in PO activity (De Rijcke et al., 2013). While these results were not pursued further, they were in agreement with available literature on mixed effects of HABs and pathogens in bivalves (Hégaret et al., 2010). By aggravating host-microbial interactions, HA can have a poorly understood, indirect effect on bivalves. Studies into this mechanism would do well to look at the functional properties of the haemolymph system (e.g. Dyrynda et al., 2000) and the expression of vital antibacterial peptides such as defensines, myticins, mytilins and mytimycins (e.g. Mitta et al., 2000) in several species and life-stages of bivalves. Other relevant proteins are nitric oxide synthase, NADPH oxidase, myeloperoxidase and superoxide dismutase for oxidative stress defense, glutathion-S-transferase and CYP450 for detoxification, and dual-specificity phosphatases-2 or macrophage expressed protein-1 as immune markers. All of these markers can be detected at once with new genome-wide approaches, like the Myt-OME microarray. Overall, **Chapters 4 to 6** show just how little we know about the effects of HABs on high-value bivalve species such as *M. edulis*, and the extent of environmental changes caused by HAB events.

5. State of the BPNS

What is the risk of HABs in the BPNS?

To assess the risk of HAB development in the Southern Bight of the North Sea, we need to look at the presence of potentially harmful species, as well as the factors that enable HABs (i.e. the state of overfishing, eutrophication, stratification, grazing etc.). Out of all of the different marine regions that are affected by anthropogenic activities, which is virtually the entire ocean (97.7%), the North Sea may very well be the most severely impacted area in the world (Halpern et al., 2008, 2015). Worryingly, though, we often fail to appreciate its potential, as its former riches have long been forgotten. In part, this is due to the intergenerational ecological obliviousness which is known as the “shifting baseline syndrome” (Pauly, 1995). More unusual, though, is, the fact that we actually know very little about the natural state of the North Sea.

As European settlers explored new parts of the world in the early modern period (i.e. 14-15th century), they frequently found extensive bivalve beds in coastal areas (Ford and Hamer, 2016; Ogburn et al., 2007). In all likelihood, huge oyster reefs once covered the bottom of the North Sea as well (Beck et al., 2011), providing shelter for various species as well as trapping the sediment and filtering out the phytoplankton (Grabowski and Peterson, 2007). As a result, the water was clear enough to support the growth of extensive seagrass beds. Olsen's Piscatorial Atlas of the North Sea, published in 1883, shows the last vestiges of these once abundant oyster reefs in the remotest areas of the North Sea, outside the operating range of traditional coastal fishing boats. At the time of its publication, most of the North Sea seabed had already been trawled for at least 500 years (Roberts, 2007).

Based on zooarchaeological evidence, the European fishing industries expanded dramatically around 1000 AD (Barrett et al., 2004). Shortly after, the “wondyrechaun” – i.e. a primitive beam trawl – is first mentioned in written records. The device was so effective that a group of fishermen petitioned the British Parliament for a ban in 1376, stating that “the device runs so heavily over the ground, that it destroys the flowers of the land below water” (Roberts, 2007). Though bans were eventually implemented, fishing boats continued to trawl until the ban was revoked in 1863. Around this time, new ship designs emerged that could tow substantially larger nets, further increasing the effectiveness and destruction of the trawlers. Around the turn of the 20th century, steam powered trawlers were by far the most important component of the North Sea fishing fleet (Engelhard, 2008). As a result of the continued harvesting of oysters and cod by trawls, oyster reefs became functionally extinct in the North Sea by the 1950s (Airoldi and Beck, 2007).

The disappearance of benthic filter-feeders caused a net decrease in biofiltration, and enhanced the role of planktonic grazers. At the same time, it created a niche for opportunistic polychaetes and brittle stars, which are now still a major component of the benthic biomass (De Groot, 1984; Heip and Craeymeersch, 1995; Reise, 1982). All these changes were set in motion long before the Green Revolution. Interestingly, the Green revolution coincided with an extremely productive period of the North Sea, i.e. the "gadoid outburst" (1960-1980), during which the reproduction, spawning-stock biomass and landings of cod, haddock, saithe and whiting all increased dramatically (Cushing, 1984; Engelhard et al., 2014). These piscivorous species sustain their own populations by removing sprat, herring and mackerel that otherwise target their eggs (Cushing, 1980; Hjermann et al., 2013; Reid et al., 2001a). This feedback loop is part of a stable state or regime that was sustained for several decades. During this time, no changes in the abundance of zooplankton or phytoplankton were observed.

In the 1980s, the gadoid outburst came to an abrupt end. Because of an intricate combination of large-scale weather patterns, e.g. the North Atlantic Oscillation (NAO) and the Atlantic Multidecadal Oscillation (AMO), anthropogenic climate change, and the atmospheric recovery following the eruption of El Chichón (that induced cooling), water temperatures rapidly increased in systems across the globe (Reid et al., 2016). In the North Sea, the warming caused an oceanic incursion onto the continental shelf that altered the nutrient conditions, and the phytoplankton and copepod communities (Alheit et al., 2005; Beaugrand et al., 2002; Reid et al., 2001b). The substitution of the dominant copepod *Calanus finmarchicus* by *C. helgolandicus*, in particular, led to trophic mismatches between the cod larvae and the peak abundance of their prey. Combined with the tremendous fishing pressure, the drop in recruitment success led to the rapid decline of cod, causing a predator-prey reversal between cod and herring which still hampers the recovery of cod today (Fauchald, 2010).

The NAO+ period persisted between 1989 to 2000, evolving from an abrupt shift in the community composition to a long-term adjustment towards higher temperatures (Beaugrand et al., 2014). During the same period, eutrophication was identified as a key issue that affects both terrestrial surface waters and the North Sea (QSR, 1987), leading to legal restrictions (Urban Waste Water Directive and the Nitrates Directive) on nitrate and ammonia levels and voluntary reductions of phosphates in detergents (Blöch, 2001; Köhler, 2006). Yet, despite decreasing nutrients, the herring-dominated regime maintains higher chlorophyll levels than the previous, cod-dominated regime (McQuatters-Gollop et al., 2007; Fig. 7.2). This counterintuitive trend results from improvements to the turbidity of the water, allowing the light-limited phytoplankton to make better use of the available nutrients (Pätsch and Radach, 1997).

Because of all these ongoing environmental changes, the seasonal dynamics and community composition of the phytoplankton is changing. Long-term monitoring data show that the dinoflagellate to diatom ratio has shifted in favour of the dinoflagellates. Permanently mixed North Sea regions that are greatly influenced by river inputs now exhibit strong diatom-based spring blooms, which are followed by prolonged periods of *Phaeocystis* spp. blooms and a peak of dinoflagellates in late summer. In stratified North Sea areas, the spring blooms are succeeded by thermocline based flagellates, upper mixed layer picophytoplankton, and a peak in dinoflagellate abundance around late summer and early autumn (Hernández-Fariñas et al., 2014; Nohe et al., 2016; van Leeuwen et al., 2015). As a result of the increased abundance of dinoflagellates, the number HABs in regions adjacent to the BPNS has increased in the last decades (Figure 1.8; p. 23). Considering the list of harmful dinoflagellates found in the BPNS (Table 1.3; p. 22), the observed shift towards higher abundances of dinoflagellates inside North Sea phytoplankton assemblages also increases the current risk of HABs in the BPNS. Below, we propose a theoretical mechanism for toxic dinoflagellate bloom formation in the BPNS, based on the work of Margalef (1978), Messier (1994), Sunda and Shertzer (2014), and the observations of Lancelot et al. (2005).

The variable availability of silicic acid, which originates from submerged volcanism, continental weathering, decaying terrestrial vegetation and anthropogenic activities (e.g. zeolith in detergents) (Dürr et al., 2011), shapes the phytoplanktonic succession in temperate coastal waters such as the BPNS and, hence, ultimately determines the risk of HAB development (Rousseau et al., 2002). During winter, the temperature and turbulence prevent significant phytoplankton growth, allowing the nitrate, phosphate, and silica concentrations to increase (ref. the void domain IV of Margalef's mandala). When the light intensity and temperature starts to increase during spring, the diatoms develop a modest early spring bloom, which depletes the dissolved silicon availability and causes an exponential growth of the copepod populations (Lancelot et al., 2005). As the silicon depletion increases, the turbulence subsides (thermal stratification) and the light conditions improve, leading to the development of massive *Phaeocystis* spp. blooms that are sustained by silicon-devoid river inputs of nitrogen and phosphorus (Escaravage et al., 1995; Peperzak et al., 1998; Schoemann et al., 2005). During this entire second phase, i.e. domain I of the Mandala, the likelihood of toxic HABs is low. The slow-growing dinoflagellates are not able to effectively compete for nutrients with fast-growing algae such as diatoms and *Phaeocystis* spp. while, at the same time, contending with the size of the grazing pit created by the copepod abundance. Note, however, that this is entirely not the case for the toxic *Pseudo-nitzschia* spp. blooms, which are also known to occur in the BPNS (Andjelkovic et al., 2012).

While the *Phaeocystis* spp. blooms decrease the nutrient availability even further (Gypens et al., 2007; Lancelot et al., 2005; van Leeuwen et al., 2015), they also start to starve the zooplanktonic grazers (Gasparini et al., 2000; Nejstgaard et al., 2007). By the end of the *Phaeocystis* spp. bloom, which is usually around the end of May, Messier's grazing pit has shrunk due to the decline in copepods, and the generated low nutrient levels favor long-lived, nutrient-efficient algae (like dinoflagellates) that may thrive by consuming the bacterio- and nanoplankton that grows on the decaying *Phaeocystis* cells (Rousseau et al., 2000). At this point, the "window of opportunity" for toxic dinoflagellate blooms in the BPNS might be open. These proposed interactions between *Phaeocystis* and HABs have, however, never been studied.

It is vital to remember that HABs are only "optional" during windows of opportunity, and that the role of grazers is not straightforward. The heterotrophic dinoflagellates of the BPNS such as *Gyrodinium spirale*, *Protoperidinium* spp. and *Noctiluca scintillans* (Conrad, 1939; De Pauw, 1975; Louis and Petes, 1979; M'harzi, 1999; Müller, 2004) can, for instance, predate HA cells, but also vector toxins to higher trophic levels (Petitpas et al., 2014). Detritivores might slow down the microbial remineralization of nutrients, preventing diatom growth, but also consume the resting stages of HABs. The polychaetes, nematodes, bivalves and benthic copepods that now dominate the benthic communities of the BPNS might even prevent the development of HABs (Montresor et al., 2003; Persson and Rosenberg, 2003; Tsujino and Uchida, 2004). Little is, however, known about cyst beds in the BPNS. In the past, cyst identification required an expert taxonomist. With the advances in genetic techniques, though, we could reconstruct the current and past presence of dinoflagellates in the BPNS using the genetic material imbedded in sediment cores. This, however, would not yield any new data on the past and present presence of HA that overwinter as a vegetative cell (e.g. *Karenia mikimotoi* or *Prorocentrum cordatum* in the BPNS).

In addition to the unknown effect of benthic detritivores on any present cyst beds, the role of consumers is complicated further by the nutrient stoichiometry. In the past, a lot of unwarranted credit was given to the supposed bottom-up effect of N:P ratios, and its mediating role during HAB development (Davidson et al., 2012; Flynn, 2010). The increase in *Phaeocystis* blooms in the BPNS was, for instance, also unrightfully linked to recent shifts in the N:P ratio (Lancelot et al., 2009; Riegman et al., 1992). Now, we understand that grazing on nutritionally imbalanced algae, that can mimic the external nutrient ratio, can enhance the nutrient recycling during blooms which prolongs the quiescence phase of a HAB top-down. Still, all of these complex species interactions may change in the future.

Considering how even moderate warming is predicted to affect the biodiversity of oceanic regions (Beaugrand et al., 2015), all of the seemingly small interactions that enable or inhibit HAB development are prone to change in ways that are beyond our comprehension (Wells et al., 2015). As the North Sea was identified as a hotspot of climate change (Holt et al., 2014), we should expect to see changes in the food web that will have repercussion for HABs. Yet, at the end of the day, we still know too little to accurately predict how each of the components of the North Sea food web will respond to climate change. Coupled to variations in the growth and distribution of grazers and phytoplankton, changes in the biogenic and chemical remineralization of nutrients will alter the timing of productivity, potentially causing trophic mismatches which may cascade through the food web, and change existing species interactions (e.g. nutrient competition, allelopathy, grazer deterrence) that, ultimately, all influence the current risk of HABs (Le Moigne et al., 2013; Segschneider and Bendtsen, 2013).

Because of the uncertainty related to predictions, HAB experts have expressed the need to closely monitor agreed-upon reference sites for all ecosystem types instead. The Southern North Sea was put forward as one of five interesting sites to monitor the impacts of climate change on HABs in open coast systems (Wells et al., 2015). To facilitate investigations into the HAB-climate interactions, these “sentinel sites” should be equipped with the necessary – preferably automated - means to acquire physical, chemical and biological data on a regular, ideally daily, basis, as well as correspond to satellite ground-truthing sites for the Global Ocean Observing System, thereby facilitating the future development of algorithms for remotely monitoring HAB initiation and progression.

While it is difficult to provide an unambiguous answer to the question of whether or not toxic HABs are common and possibly increasing in the BPNS, it is abundantly clear that HA toxins are already finding their way to the tissues of multiple organisms of different trophic levels of the BPNS (Andjelkovic et al., 2012; Orellana et al., 2017). As such, it would indeed be wise to develop our capacity to study the presence of HABs and toxins in the BPNS. While our work suggests that the on-going shifts in the nutrient stoichiometry have, in fact, not affected the risk of HABs in the BPNS, we are unable to track the indirect effects of nutrient stoichiometry throughout the food web. Likewise, our results suggest that the decreasing nutrient levels of the BPNS should reduce the size and intensity of blooms in the North Sea. Yet, if dinoflagellate blooms are currently light- rather than nutrient-limited, these future decline in nutrients might not reduce the risk of HABs at all, potentially threatening the recovery of bivalve beds and the success of new shellfish culture projects in the BPNS

Box 7.1 Risks towards shellfish farming in the BPNS

In 1765, the brothers Jan and Pieter De Loose kicked off the farming of shellfish in Belgium when they built the first oyster farm in Ostende. Two hundred years later, the Belgian shellfish industry was blooming, exporting millions of "ostendaise" oysters to countries all across Europe. Soon after, though, the industry vanished because of the emergence of infectious oyster diseases and the outbreak of the World Wars (Steevens and Van Moerbeke, 2015). Ever since, no sizeable production of shellfish has occurred within the EEZ. Note, though, that one of the last attempts to restart the shellfish industry in our waters, i.e. the "Belgica mussel" in 2008, ran into problems when the national food safety agency found DSP concentrations in excess of the legal limit inside their mussels.

Recently, two new aquaculture projects have started in the BPNS. The "EDULIS" project that wants to explore the possibility of growing mussels in suspended cultures between the offshore windmills, and the "Value at Sea" project that aims to establish an integrated multi-trophic aquaculture farm in front of Nieuwpoort. Shellfish of either project will need to be checked for toxins by our regional food safety agency, though this instance will only look at the toxins which have a well-known acute toxicity effect in consumers and, hence, have a legal limit (Table 7.1). All the other compounds that could be produced by toxic phytoplankton in the BPNS, like gymnodimins and SPXs, should pass through the inspection, and end up on the plate of human consumers. There is, hence, still a risk of chronic health effects of the monitored toxins, as well as unknown acute (mixed) toxicity effects of the emerging compounds.

Table 7.1: Toxins which may threaten shellfish industries in the BPNS, and their legal limit cfr. the EU Regulation (EC) No 853/2004: Health standards for live bivalves.

Toxins	Producers in the BPNS	EU Legal limit
Domoic acid (DA)	*Pseudo-nitzschia seriata*	20 $mg.kg^{-1}$ whole body
Azaspiracid (AZA)	*Azadinium* spp.*	0.16 $mg.kg^{-1}$ whole body
Okadaic acid (OA) Pectenotoxins (PTX) Dinophysistoxins (DTX)	*Dinophysis acuta* *Dinophysis acuminata* *Dinophysis norvegica* *Dinophysis rotundata* *Prorocentrum lima*	0.16 $mg.kg^{-1}$ whole body
Saxitoxins (STX)	*Alexandrium ostenfeldii*	0.8 $mg.kg^{-1}$ whole body
Yessotoxin (YTX)	*Protoceratium reticulatum*	1 $mg.kg^{-1}$ whole body

* *Azadinium* spp. has not been observed in the BPNS (cfr. Table 1.3), but its toxins have been found in oysters and mussels from the BPNS (Orellana et al., 2017).

6. Policy-making

Harmful algal blooms are a quintessential “wicked problem” for policy-makers. Wicked problems span across the ecological, social, economic, and political systems, and are set aside from traditional planning challenges due to their unique, complex and contentious character (Rittel and Webber, 1973). They are the symptom of other underlying problems and, hence, cannot be resolved independently. The process of solving a wicked problem matches the process of understanding its nature. Due to its multi-faceted, incremental nature, a wicked problem knows no true or false answers. Any implemented solution will, however, generate waves of consequences over an extended period of time. As we lack the opportunity for rigorous experimentation, and we have no way to predict all of the repercussions ahead of time, every attempt to fix wicked problems is consequential. They leave “irrevocable traces” that can outweigh the intended advantages of the solution. As a result, legislators dealing with wicked problems bear a huge responsibility for the consequences of the actions they take.

Each of the three major drivers of the recent success of HABs - i.e. eutrophication, overfishing and climate change – should be seen as wicked problems on their own (Khan and Neis, 2010; Levin et al., 2007; Thornton et al., 2013). All of these issues need a coordinated, international approach to be “resolved”. Note, however, that the “solution” to any wicked problem is a “good enough” state. Policy-makers dealing with wicked problems can constantly do better, but they are constrained by external reasons (e.g. societal costs, time, feasibility). The need to balance ecological versus social and economic interests will determine the desired outcome of an intervention against a wicked problem. Discretionary decision-making may, however, impede the intervention against wicked problems over an extended period of time.

The EU’s Common Fisheries Policy has largely failed to enhance the sustainability of fish stocks and their associated fisheries, because it was designed to function with discretionary decision-making (Khalilian et al., 2010). Likewise, the Paris agreement may fail, as the self-imposed actions by the nations imply an increase in temperature in excess of the agreed upon 2°C above the pre-industrial levels (Rogelj et al., 2016). Both of these issues demonstrate the danger of autoregulation of a shared resource, and should be addressed as they will continue to facilitate HABs in the near future. Eutrophication, however, was successfully tackled by the OSPAR convention, whose strategic goals are now part of the Marine Strategy Framework Directive. The coastal nutrient concentrations of the North Sea are gradually decreasing, though we still find severe eutrophication in certain regions (Burson et al., 2016; Claussen et al., 2009).

The nutrient concentrations of the Southern Bight of the North Sea are influenced by oceanic inputs and rivers such as the Scheldt, Seine, Meuse, Rhine and Thames (Gypens et al., 2007; Lacroix et al., 2004). Although the nutrient fluxes of these rivers are expected to decrease by 2050, largely due to the regional implementation of the Water framework Directive, the majority of these watersheds will probably still have a high potential for harmful algal blooms (Blaas and Kroeze, 2016). Despite the already substantial improvements in the water quality of the Scheldt over the last 30 years (ref. Passy et al., 2013), our regional policies should continue – and are continuing – to take steps towards the Good Environmental Status of our rivers and coastal areas. As the North Sea receives a large amount of nitrogen from atmospheric depositions (ref. Djambazov and Pericleous, 2015), the progress in improved water quality should be intertwined with reduced NOx emissions. As such, the MSFD GES criteria can be linked to the EU strategy on the adaptation to climate change.

As part of the MSFD implementation, we should develop indicators to monitor the evolution of eutrophication within the Belgian EEZ. In addition to chlorophyll a and nutrient monitoring, the MSFD recommends the development of Quality Descriptors that can detect changes in the phytoplankton composition and occurrence of HA (Ferreira et al., 2011). With the development of a new Essential Ocean Variable on phytoplankton (Muller-Karger and Kudela, 2016), similar data will soon be needed to participate in the Global Ocean Observing System (GOOS) as well. Monitoring the diversity, abundance and toxicity of HA in the BPNS is, hence, not only relevant for our blue economy (e.g. food safety, regional productivity) and the assessment of international or EU legislations (i.e. ballast water, climate change and eutrophication), but also for our scientific participation in interdisciplinary international programs. Investing in the development of a comprehensible phytoplankton monitoring program, preferably using autonomous infrastructure, is therefore highly recommended.

By coupling in situ sensors to remote sensing and hydrodynamic forecast models, a HAB warning system can be made. A pilot study (i.e. the ASIMUTH project) created an operational European HAB forecast system that combines the early detection of HABs with Lagrangian transport models (Davidson et al., 2016; Maguire et al., 2016). To date, no ecophysiological modelling has come close in terms of predictive value (Glibert et al., 2010; McGillicuddy, 2010). We should dedicate time and effort to join this EU-wide collaboration. A warning system improves the cost-effectiveness of our seafood safety program, reduces the economic impact on aquaculture, provides an estimate of the effect on the regional productivity, and allow us to deploy emergency measures like aeration, clay flocculation, surfactants, peroxide or algicidal bacteria and fungi in the future (Anderson et al., 2015 and references therein).

The development of a Belgian HAB warning system would require no investment in new equipment, as the required the tools are available, but are still uncoordinated. In short, the Remote Sensing and Ecosystem Modelling team of the Royal Belgian Institute of Natural Sciences (REMSEM) should adopt the optimized discrimination algorithms of Kurekin et al. (2014) to be able to separate blooms of *Phaeocystis* spp. from *Karenia mikimotoi* and other algal blooms in the turbid waters of the North Sea. If a Chl A peak is found, the MERIS and MODIS spectral bands are used to estimate the risk of a HAB. If a potential HAB is identified, its transport can be predicted using the OPTOS hydrodynamic model of Management Unit of the North Sea Mathematical Models (MUMM) of the Royal Belgian Institute of Natural Sciences. If the bloom is predicted to enter vulnerable areas (e.g. the shellfish area between the windmills), samples for species identification can be taken with the RV Simon Stevin. If a HAB species is present, a warning is issued to the farmers and the food safety agency.

Box 7.2 Risks related to the masterplan "Vlaamse Baaien"

To improve the coastal resilience against the challenges posed by climate change, e.g. sea level rise and the "thousand-year storm", the Belgian government is mulling over a long-term masterplan called "Vlaamse Baaien" which could become a staple of the revised marine spatial plan (het Marien Ruimtelijk Plan) of the BPNS in 2020. The transition from a narrow strip of coast, which requires dykes and constant beach suppletions to keep its protection, to a wide coast with dunes, sandbanks and islands promises to combine the coastal defences with the production of sustainable energy, nature restoration, and improve the attractiveness of coastal tourism. If approved, the Flanders Bays project would drastically reshape the Flemish coast by 2100.

The proposal contains 10 specific projects to reduce the vulnerability of the BPNS. Some of these projects - i.e. the extension of harbor walls, the raising of sandbanks, the creation of artificial lagoons on both sides of Zeebruges, and the construction of a multifunctional atoll – will increase the number of sheltered zones inside the BPNS. Because of the reduced hydrodynamic forcing in these new areas, stagnant water bodies along the entire coast will favor the growth of dinoflagellates, which are then seeded into the coastal zone. As shown here, the size of inocula can determine the outcome of competition. As a result, the Flanders Bay project might increase the risk of HABs in the future BPNS, an aspect that is currently overlooked during the design of these activities. Together with other on-going environmental changes to the BPNS, these "Blue growth" initiatives will influence the biodiversity in unknown ways and, hence, strengthen the need for a robust monitoring system of HABs in the future.

7. General conclusion

Since the start of the Industrial Revolution, mankind's activities have increasingly transgressed Earth's self-regulating capacity, accelerating the environmental change which gradually pushes our planets system out of the stable state of the Holocene (Rockström et al., 2009). Overexploitation, eutrophication, ocean acidification, global warming, alien species introductions, chemical pollution and marine debris, are just a few of the challenges that are threatening the diversity and functioning of marine systems around the globe (Halpern et al., 2008). Among many other stressors of the environment, these impacts coincide with a global increase in harmful algal blooms, and an enhanced risk of HAB development in the North Sea. Yet, rather than being pessimistic about the future oceans, let us create a narrative of hope instead.

After decades of stepwise improvements to the water quality of European rivers, the nutrient levels of the North Sea are gradually decreasing. While the North Sea is still caught in its herring-based regime, which allows a greater accumulation of algae, scientists are seeing the initial signs of recovery of the cod populations (ICES, 2016). In addition to improved catch quota, this upwards trend can be related to the rapidly growing EU network of de jure and de facto protected areas (e.g. windmills parks) (Moland et al., 2013). Besides the return of cod, we are also seeing pilot studies which are attempting to restore some of the lost bivalve reefs (e.g. Sas et al., 2016). These restoration projects – which have been hugely successful in the United States (Schulte et al., 2009) – could enhance the filtration potential of our coastal zone, and improve the coastal protection at the same time (Grabowski and Peterson, 2007). Their success may, however, be intertwined with the future of HABs.

Some of the greatest potential for technological innovation and sustainable growth is found in the marine environment (Pauli, 2010). The predicted increase in economic activities inside coastal areas could affect the biodiversity in various ways. Future, large-scale developments like the "Vlaamse Baaien" will have a tremendous effect on our coastal ecosystems, and could enhance the risk of HABs even further. However, these projects also represent opportunities as well as incentives to improve the state of future oceans. They can contribute to the expansion of de facto protected areas that generate returns on both biodiversity and productivity, strengthen the resilience of coastal communities to climate change, and create unique chances for biological experimentation (cfr. reef restoration). Now, more than ever, marine scientists should join forces with legislators and entrepreneurs to envision an economic and ecologic future for the marine environment. The ocean is, after all, the greatest component of Spaceship Earth. The great unifier which affects us all.

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Summaries

Summary

The growing need to feed the world's population has led to significant advances in agricultural practices over the last 50 years. The Haber-Bosch process, in particular, enabled an intensification of the global fertilizer use, resulting in higher crop yields across the world. Yet, due to the inefficient incorporation of fertilizers into agricultural products, these nutrients led to significant environmental pollution and eutrophication. Fuelled by nutrient enriched runoff, rivers upset the biogeochemical balance of the marine environment, leading to a global increase in size, frequency and distribution of harmful algal blooms (HABs). During these events, a phytoplankton species is able to proliferate at the expense of others, causing severe harm to the environment through hypoxia, shading, physical disruption and the release of potent toxins. As a result, HABs are a severe threat to marine biodiversity as well as the safety and security of seafood. Now, with climate change looming over the horizon, scientists fear that HABs could become more prevalent in our future oceans. To date, however, there is a lack of experimental evidence that global change will affect HABs.

Despite the implications for human health and ecosystem health, the link between eutrophication and HAB development is still not fully understood. Yet, while scientists are discussing the fundamental ecological importance of biological features such as toxicity, mixotrophy and allelopathy, we are slowly missing our opportunity to prevent a major escalation of HABs. For this reason, we urgently need to identify and employ model species in cross-validated, long-term multifactorial studies with co-occurring species to rapidly progress our understanding of HABs, and quantify the impact of HABs on the socioeconomic well-being of our species to persuade policy makers. During this PhD research, we tried to achieve both.

Given our lack of knowledge on the occurrence of HABs in our own regional sea, the **main objective of this thesis** was to assess whether the ongoing changes in the environmental state of the Belgian Part of the North Sea (BPNS) have enhanced the risk of HAB development. In addition to regular sampling campaigns, we identified several knowledge gaps in the available literature that we first had to resolve in order to answer this main question. In particular, we needed to determine whether changes in the total or relative nutrient availability, both effects of eutrophication, affect the competitive traits of potentially harmful dinoflagellates. Next, we needed to verify that climate-change driven temperature increases would not affect these traits either. Lastly, we wondered if toxic dinoflagellates could have unknown mixed toxicity effects – by themselves or in together with marine pathogens - on keystone bivalve species like *Mytilus edulis*.

In **chapter 2**, we examined how the relative availability of nutrients – simulated by 10 different additions of nitrate at constant phosphate concentrations - can affect the growth of dinoflagellates at two temperatures. To this end, we measured the density, nutrient concentrations and toxin production of 300 single and mixed cultures of *Prorocentrum lima*, *P. micans*, *Protoceratium reticulatum* and *Scrippsiella trochoidea* – four dinoflagellates commonly found in the BPNS – for well over a month. Overall, the external nutrient stoichiometry had little effect on the growth and toxin production of our species. As a result, the N:P ratio was found to have no structuring role in the competition between dinoflagellates with closely resembling nutrient requirements. For this reason, we urge HAB ecologists to reconsider the use of resource ratios as a reliable measure of eutrophication. The N:P ratio did, however, affect the carrying capacity of *P. micans* and *S. trochoidea*, leading to the suspicion that the competition was nitrogen driven, which was explored in the subsequent chapter. Climate change conditions were mostly found to accelerate the process.

Chapter 3 explores how initial densities may shape the outcome of interspecific competition between dinoflagellates, and whether conditions such as temperature, nutrient availability and nutrient stoichiometry are able to change this outcome. Two large-scale culture experiments were set up using the common dinoflagellate species *Alexandrium minutum*, *Prorocentrum micans* and *Protoceratium reticulatum*. The first experiment applied different macronutrient concentrations (total nutrient availability) and multiple N:P ratios (relative nutrient availability) to verify the results of chapter 2. The second experiment varied the initial community compositions of the cultures by changing the relative species densities at the start, and included abiotic variability as different temperatures and N:P ratios. Again, we monitored the densities and nutrient levels of these 468 cultures for over a month. Then, we used mechanistic modelling based on MacArthur's resource-consumer model to unravel the mechanisms behind the competition, and accurately predict the outcome of competition – under every set of conditions - between these three species. We found that the community dynamics could be predicted using only the nutrient uptake rates, conversion efficiencies and the maintenance requirements of each individual species. All these parameters were estimated from their performance in monocultures. Abiotic variability like temperature, nutrient stoichiometry and nutrient availability affected the parameter estimates, but did not change the underlying mechanism of the competition. Overall, this study demonstrated how initial densities may overturn the outcome of bloom development, giving credit to the importance of cyst beds in the marine environment, and shows that the pre-bloom competition between dinoflagellates is nutrient driven, illustrating the need to need to restrict the nutrient inputs into the North Sea.

In the second half of this dissertation, we looked at the effect of potentially harmful algae on the survival and reproduction of bivalves. To this end, we used the common blue mussel as a model organism, a true North Sea ecosystem engineer on its own. The recent success of HABs is partially associated with the global decline of coastal bivalve reefs. In addition to the loss of natural filtration, the disappearance of bivalve populations has increased the importance of planktonic grazers which tend to avoid toxic algae and, hence, enable HAB development. The future recovery of the bivalve reefs could, however, hinge on their ability to withstand HABs.

In **chapter 4**, we investigated how the exposure to multiple toxic algae affects the feeding of adult mussels. In addition, we looked at the absorption, distribution, metabolization and excretion kinetics of their toxins. More specifically, we exposed adult mussels to the dinoflagellates *Alexandrium ostenfeldii* and *Prorocentrum lima*, in a single and combined two-week laboratory exposure. In parallel, mussels cages were left in the harbour of Ostend to study the accumulation of toxins under natural BPNS conditions. During both exposures, the toxin profiles were regularly recorded by ultra-high performance liquid chromatography coupled to high-resolution Orbitrap mass spectrometry (UHPLC-HR-Orbitrap MS). Both experiments revealed a rapid accumulation of okadaic acid, dinophysis toxins and spirolides in the visceral tissues of *M. edulis*. Worryingly, the simultaneous exposure to both algae increases the rate of the accumulation and metabolization processes, and led to the creation of different metabolized compounds. As such, this study mostly highlighted the need to evaluate the risk of mixtures of (unregulated) compounds to human health, and the improved monitoring of toxic phytoplankton inside the BPNS.

Chapter 5 and 6 examined the effects of toxic HABs on the larvae of *M.* edulis, i.e. the most sensitive life-stage. In chapter 5, we first looked effect of domoic acid and okadaic acid – the most common toxins in European waters – on larval viability, development as well as innate immune responses. By exposing mussel embryos to various concentrations of dissolved toxins and their producers, the toxic diatom *Pseudo-nitzschia multiseries* and the dinoflagellate *Prorocentrum lima*, we found a previously unknown toxic effect of okadaic acid, which was attributed to its ability to inhibit larval protein phosphatase. *P. multiseries*, *P. lima*, and DA, on the other hand, increased the phenoloxidase innate immune activity of the larvae. This discovery was was cause for concern, as it could signify that HABs are affecting the immunological resilience of bivalve larvae. Considering that notorious pathogens, like Vibrio spp., have been found in close association with HABs, such an interaction would greatly enhance the effect of HABs on bivalve recruitment.

Since HABs and pathogens could both become more prevalent in the future, we further explored the interactive toxicity effects between these two stressors. During **chapter 6**, *M. edulis* larvae were exposed to various mixtures of dinoflagellates (*Alexandrium minutum*, *A. ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *P. lima* or *P. micans*) and two notorious bivalve pathogens (a *Vibrio coralliilyticus/neptunius*-like isolate or *Vibrio splendidus*). Again, the viability, development and immune response of the larvae was recorded. Yet, while we found several previously unknown toxicity effects of dinoflagellates, we found no compelling evidence of strong interactions between both stressors. Against all our expectations, none of the immunological responses to HABs increased the susceptibility of mussel larvae towards *Vibrio* spp. infections. Overall, we concluded that the main effects of each stressor is separated in time. Still, we believe that these interactions can occur at a later stage, once bivalves are large enough to consume pathogens and harmful dinoflagellates at the same time. As a result, the natural association between HABs and pathogens remains a concern.

Coupled to a review of the current state of the North Sea in **chapter 7**, this work suggests that the risk of HABs in the region has increased over the last decades. While significant steps have been taken towards the reduction of nutrient loads in our coastal areas, there is a very real possibility that climate change will further enhance the occurrence of HABs in the North Sea. In addition to more research on the nutrient competition between co-occurring phytoplankton, and the potential for harmful effects on key stone bivalve species, we highly recommend the development of a coherent plankton monitoring network in our coastal zone. Such a system would not only allow us to study interactions between phytoplankton and zooplankton populations in situ, and improve our environmental assessment of the effects of HABs in the North Sea, but it could also provide a direct measure of the efficacy of nutrient legislation, a tool to fulfil our obligations towards the Marine Strategy Framework Directive, a system to improve the efficiency of our seafood monitoring strategy, and an early warning system which would benefit future Blue Growth activities.

Samenvatting

Door de toenemende wereldbevolking groeit de wereldwijde vraag naar voedsel, wat leidt tot verbeterde landbouwpraktijken. Dankzij het Haber-Bosch proces is het gebruik van meststoffen sterk toegenomen in de afgelopen 50 jaar, met een hogere landbouwopbrengst tot gevolg. Door de inefficiënte opname van nutriënten door de gewassen, komt een groot deel van deze kunstmeststoffen terecht in het grond- en oppervlaktewater, wat eutrofiëring en milieuvervuiling veroorzaakt. De afgevloeide nutriënten komen via sloten, beken, kanalen en rivieren uiteindelijk in de zeeën en oceanen terecht, waar ze de chemische balans van het water verstoren. Dit proces heeft geleid tot een globale toename in het voorkomen van schadelijke algenbloei. Tijdens een schadelijke algenbloei (SAB) wordt het fytoplankton overwoekerd door cellen die, door middel van zuurstof vermindering (hypoxia), licht reductie, fysieke verstoring of krachtige gifstoffen, schade berokkenen aan het mariene ecosysteem. SAB is, met andere woorden, een bedreiging voor de biodiversiteit en productiviteit van de oceanen. Wetenschappers vrezen nu dat klimaatverandering de impact van SAB zal vergroten. Daar is tot op heden, echter, weinig experimenteel bewijs voor.

Desondanks de gevolgen voor mens en dier, weten we verrassend weinig over de verbanden tussen SAB en eutrofiëring. Terwijl de wetenschappers nog druk aan het discussiëren zijn over het ecologisch belang van bepaalde eigenschappen van SAB, zoals toxiciteit, mixotrofie en allelopatie, verliezen we langzamerhand onze kans om een escalatie van SAB te vermijden. Als we beleidsmakers willen overtuigen van het (toekomstig) belang van schadelijke algen, moeten we dringend onze kennis over de ontwikkelingsprocessen van SAB verbeteren. Daarnaast moeten we een beter zicht krijgen op de socio-economische gevolgen van SAB. In dit proefschrift proberen we dit te bereiken aan de hand van verschillende multifactoriële langetermijnstudies met relevante modelorganismes.

Gezien er vrij weinig gekend is over het voorkomen van schadelijke algen in het Belgisch deel van de Noordzee, was het **doel van dit doctoraat** om na te gaan of de veranderingen in de Noordzee het risico op SAB vergroot hebben. Naast regelmatige bemonsteringen van het zeewater, identificeerden we belangrijke ecologische vragen in de beschikbare literatuur die we dienden te beantwoorden in functie van ons doel. Zo was het nodig om te weten in welke mate de totale en relatieve beschikbaarheid, beide gewijzigd door eutrofiëring, een effect hebben op het competitief gedrag van schadelijke algen in het huidig en toekomstig klimaat van de Noordzee. Daarnaast zijn we nagegaan of schadelijke pantserwieren of dinoflagellaten het voorkomen van ziektes in sleutelsoorten als de mossel *Mytilus edulis* kunnen verhogen.

In **hoofdstuk 2** werd door middel van verschillende toevoegingen van nitraat bij constante fosfaat concentraties nagegaan of de verhouding van stikstof tot fosfor een effect heeft op de groei van dinoflagellaten. Daarvoor werden 300 enkelvoudige en gemengde kweken van vier veelvoorkomende dinoflagellaten - *Prorocentrum lima*, *P. micans*, *Protoceratium reticulatum* en *Scrippsiella trochoidea* – geplaatst bij twee temperaturen (20°C en 24°C) en 10 verhoudingen van stikstof en fosfor. De densiteit, nutriënten concentraties en toxine productie werd gedurende een maand opgevolgd. Algemeen beschouwd, bleek uit dit experiment dat de N:P ratio weinig effect heeft op de groei en de toxine productie van dinoflagellaten. Deze veelbesproken parameter speelt dus geen structurerende rol tijdens de competitie tussen nauwverwante SAB soorten met gelijkaardige nutriëntenbehoeftes. Extra stikstof veroorzaakte veelal wel een verhoging in de densiteit van de algen. Hierdoor ontstond het vermoeden dat de competitie tussen SAB soorten stikstof-gedreven is. De ontwikkeling en competitie in de celculturen verliep meestal sneller bij hogere temperaturen.

Hoofdstuk 3 verdiept zich in het mechanisme van de competitie tussen soorten, en onderzoekt hoe de initiële condities de SAB ontwikkeling kunnen beïnvloeden. Twee grote, multifactoriële kweekexperimenten werden gebruikt om na te gaan wat het effect van nutriënten concentraties, de N:P ratio, temperatuur en de densiteit van verschillende soorten is op de uitkomst van de competitie tussen de dinoflagellaten. Om de resultaten van hoofdstuk 2 te bevestigen, werden in het eerste experiment opnieuw drie Noordzee SAB soorten, nl. *Alexandrium minutum*, *Prorocentrum micans* en *Protoceratium reticulatum*, gekweekt bij verschillende nutriënten concentraties en N:P verhoudingen. In het tweede experiment varieerden we zowel de N:P ratio, als de temperatuur en de initiële samenstelling van de gemengde culturen. Tijdens beide experimenten werd opnieuw gekeken naar het verloop van het aantal cellen en de nutriënten concentraties. Vervolgens werd een bestaand mechanistisch groeimodel (MacArthur's consumer-resource model) gebruikt om de uitkomst van de competitie, onder alle omstandigheden, te voorspellen aan de hand van de nutriëntenopname, omzettingsefficiëntie en onderhoudsbehoeftes van elke soort. Deze laatste werden bepaald aan de hand van monoculturen. Abiotische variantie zoals temperatuur, de N:P verhouding en de totale beschikbaarheid aan nutriënten hadden elk een invloed op deze parameters, maar veranderden niets aan het onderliggende mechanisme van de competitie. Competitie is nutriënten gedreven, waardoor variaties in de initiële densiteit van elke alg kan leiden tot een verschillende dominantie in de finale SAB. Met dit werk werden het belang van cysten en nutriënt concentraties in de Noordzee, en de preventieve maatregelen met betrekking tot beide, nogmaals onderstreept.

In het tweede deel van dit proefschrift werd gezocht naar onbekende directe en indirecte effecten van SAB op de overleving en reproductie van de gewone mossel, een ecologische en economische sleutelsoort van de Noordzee. Het recente succes van SAB in geassocieerd met het verdwijnen van de natuurlijke riffen van mosselen en oesters voor onze kust. Naast het verlies van biofiltratie, verhoogt dit namelijk het belang van planktonische grazers die zich niet voeden op toxische algen. Het herstel van deze riffen hangt samen met het toekomstig succes van SAB in de Noordzee.

In **hoofdstuk 4** werd onderzocht hoe de blootstelling aan meerdere giftige algen het voedingsgedrag van de mossel kan beïnvloeden. Daarbij werd gekeken naar de absorptie, distributie, metabolisatie en excretie van de toxines. Volwassen mosselen werden gedurende twee weken onder labo condities enkel of gelijktijdig blootgesteld aan *Alexandrium ostenfeldii* en *Prorocentrum lima*. Daarnaast werden mosselkooien in de haven van Oostende geplaatst, om de opname van toxines onder natuurlijke omstandigheden te bestuderen. Gedurende beide experimenten werden regelmatig toxine profielen geregistreerd met vloeistofchromatografie met massaspectrometrie. Zowel de natuurlijke blootstelling als de labo experimenten veroorzaakten een snelle toename in toxines (vnl. okadazuur, dinophysistoxines en spiroliden) en de afgeleide metabolieten binnenin de mossel. Tijdens de gelijktijdige blootstelling aan de algen nam de accumulatiesnelheid toe, en werden zelf andere metabolieten gevonden. Mortaliteit werd echter niet waargenomen. Deze studie toont dus aan dat mengsels van ongereguleerde toxines voorkomen aan onze kust. Een verbeterde monitoring van schadelijke algen is dan ook aan te raden.

Hoofdstukken 5 en 6 spitsen zich toe op de impact van giftige algen op de larven van de mossel. In hoofdstuk 5 onderzochten we eerst of de twee meest abundante toxines (domoizuur en okadazuur) en hun producenten, *Pseudo-nitzschia multiseries* en *Prorocentrum lima*, een effect hebben op de ontwikkeling, overleving en immuun activiteit van deze gevoelige levensstadia. Daar werd ontdekt dat okadazuur een onbekende toxiciteit vertoont in mossellarven, die we verbonden aan de inhibitie van fosfatase activiteit. Daarnaast werd waargenomen dat elke andere behandeling een immuunrespons veroorzaakte, waardoor het immuunsysteem van de larve mogelijk onderdrukt wordt. Dit laatste is zorgwekkend omdat mariene pathogenen reeds een ernstige bedreiging vormen voor mosselpopulaties. Vermits beruchte pathogenen als *Vibrio* spp. in nauwe associatie met SAB zijn waargenomen, kunnen deze interacties leiden tot een sterk vergrote impact van SAB op de productiviteit en stabiliteit van het ecosysteem. Daarom werden in het laatste hoofdstuk de mossel larven blootgesteld aan SAB en pathogenen tegelijk.

De klimaatverandering zal het voorkomen van zowel schadelijke algen als nefaste bacteriën sterk beïnvloeden. Hierdoor kunnen in de toekomst steeds vaker mengsels van deze stressoren opduiken in het mariene milieu. Om na te gaan wat de mogelijke gevolgen zijn natuurlijke schelpdierpopulaties, werden in **hoofdstuk 6** mossellarven blootgesteld aan diverse giftige wieren (*Alexandrium minutum*, *A. ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *P. lima* of *P. micans*) en een pathogeen (a *Vibrio coralliilyticus/neptunius*-like isolate of *Vibrio splendidus*). De ontwikkeling, overleving en immuun respons van de mossel larven werd opnieuw geregistreerd. Daaruit bleek dat verscheidene pantserwieren een dusver ongekende impact hebben op de overleving van mossellarven maar, desondanks de individuele effecten van beide pathogenen, er geen sterkte interacties bestaan tussen deze twee stressoren. Ook het primitieve immuunsysteem kon onafhankelijk reageren op zowel de bacteriën als de toxische pantserwieren. Het algemeen besluit was dan ook dat er geen evidentie is voor sterke interacties tussen pathogenen en SAB in de larven van schelpdieren, omdat hun individuele effecten allicht een verschillende timing kennen. Desalniettemin vrezen we dat deze interacties toch een rol kunnen spelen zodra de mossel groot genoeg is om zich gelijktijdig te voeden op toxische algen en bacteriën.

Gekoppeld aan een overzicht van de beschikbare literatuur in **hoofdstuk 7**, toont deze scriptie aan dat het risico van SAB in de Noordzee is toegenomen gedurende de laatste decennia. Hoewel er al grote stappen zijn genomen richting de verbetering van de oppervlaktewaterkwaliteit in België, is er nog steeds een reëel gevaar dat de klimaatverandering het voorkomen van SAB in de Noordzee zal bevorderen. Naast een voortzetting van het onderzoek naar de competitie in natuurlijk gemeenschappen van algen, raden we de verdere ontplooiing van een planktonmonitoring netwerk ten stelligste aan. Een autonoom monitoringsnetwerk laat ons toe om in situ interacties tussen planktonische grazers en fytoplanktonsoorten te bestuderen, verbetert onze inschatting van de frequentie en impact van SAB in onze kustwateren, vervult onze plicht aan Europese wetgeving zoals de Marine Strategy Framework Directive, geeft ons een maat voor de efficiëntie van de nationale nutriëntenwetgeving, en creëert een vroeg waarschuwingssysteem voor de toekomstige industriële activiteiten van de Blauwe economie.

Curriculum vitae

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Employment

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2012-2017 Ghent University (UGent), Ghent, Belgium
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Education

2012 Ghent University (UGent), Ghent, Belgium
M.Sc. in Environmental Management and Sanitation
M.Sc. Dissertation: “Toxicity of two marine algal toxins to blue mussel and brine shrimp larvae”

2011 Ghent University (UGent), Ghent, Belgium
E.M. M.Sc. in Marine Biodiversity and Conservation
M.Sc. Dissertation: “The influence of windmill artificial reefs on the diurnal feeding pattern of cod *Gadus morhua* in the BPNS.”

2011 University of Leuven (KU Leuven), Leuven, Belgium
B.Sc. in Biology minor Biochemistry and Biotechnology
B.Sc. Dissertation: The introduction of artificial markers into the genome of yeast *Saccharomyces cerevisiae*

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Mentored 13 B.Sc. / M.Sc. students throughout 4 years of Ph.D.
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Annexes

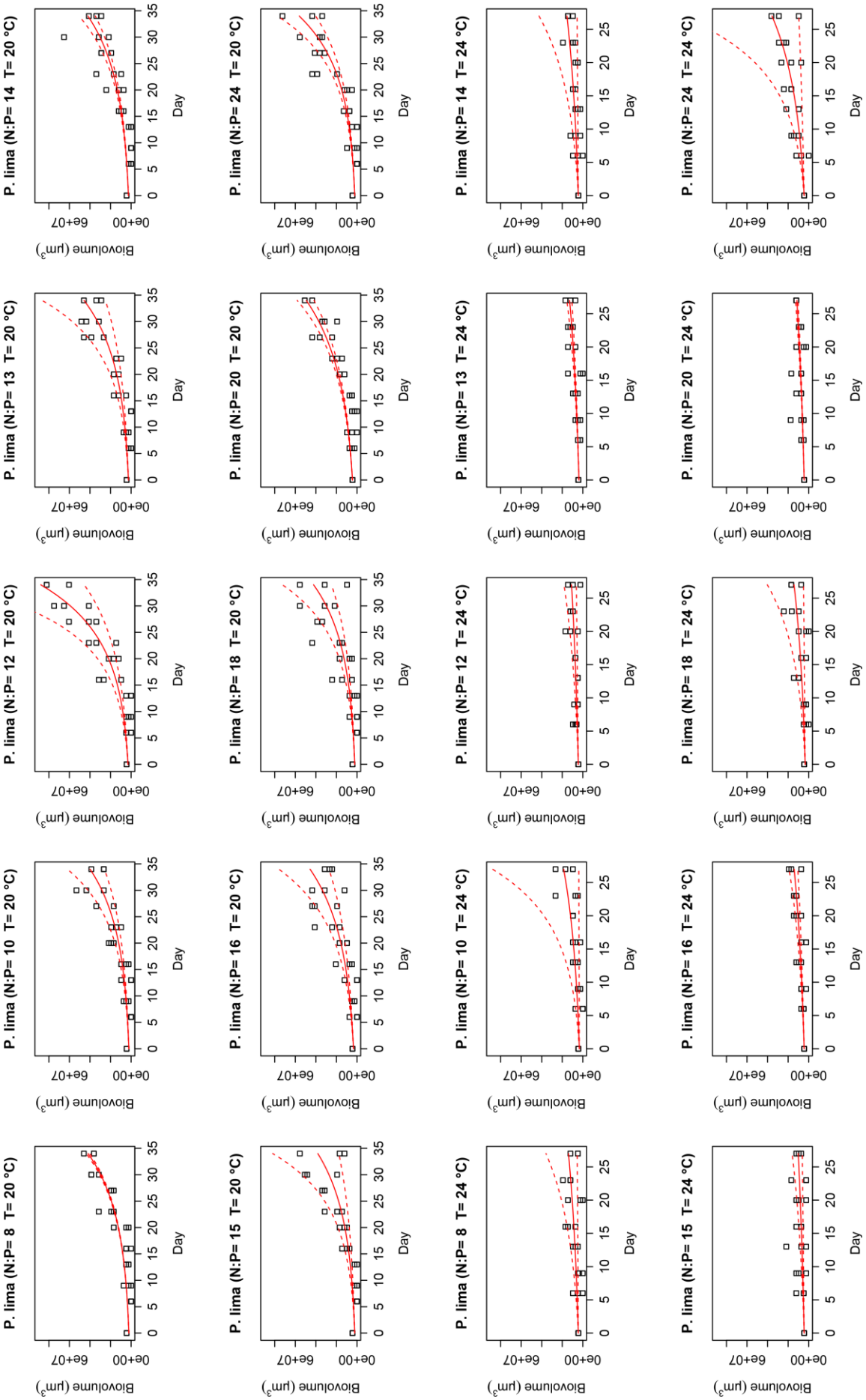

Figure A1: Monoculture growth of *P. lima* under various N:P and temperature scenarios.

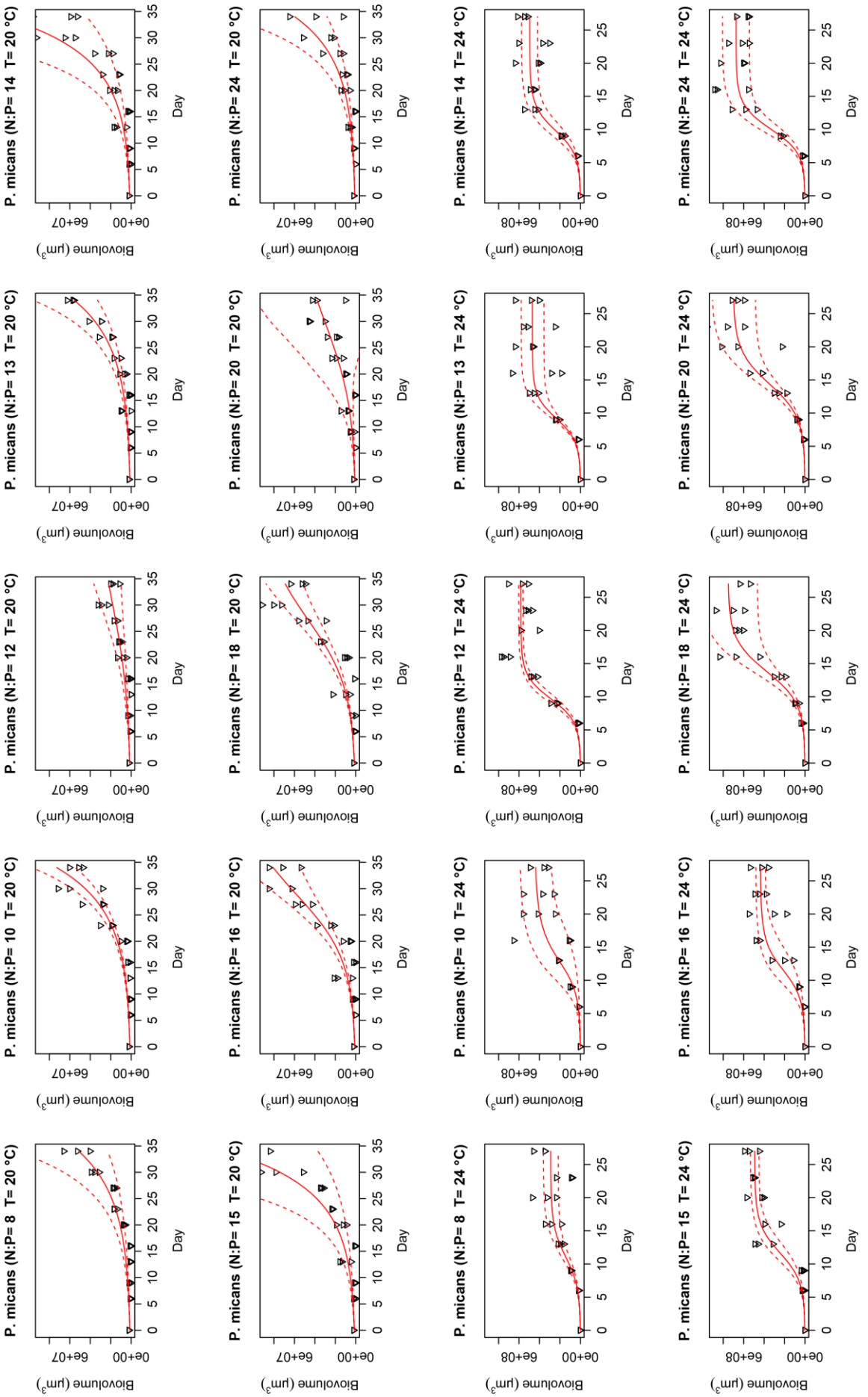

Figure A2: Monoculture growth of *P. micans* under various N:P and temperature scenarios.

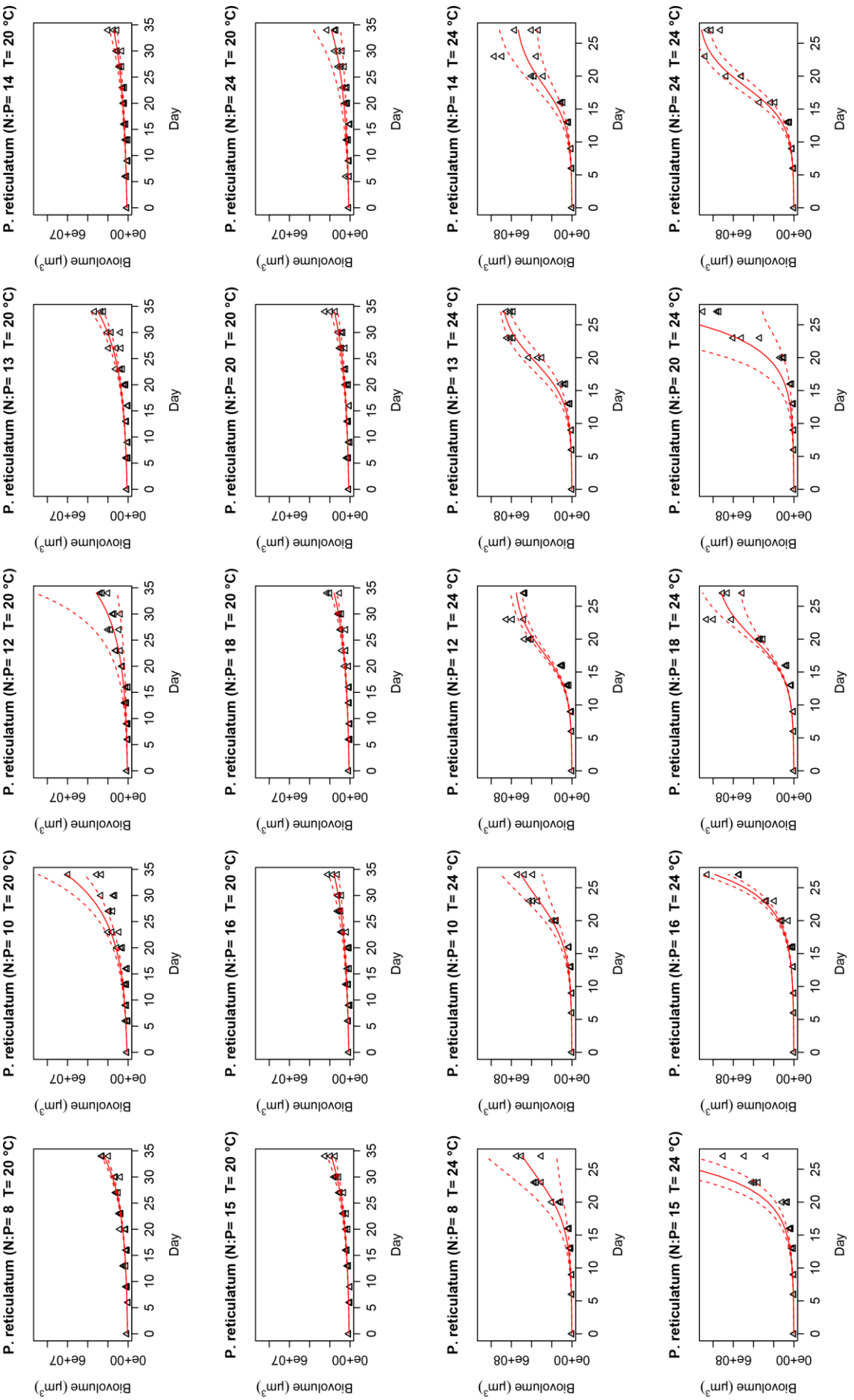

Figure A3: Monoculture growth of *P. reticulatum* under various N:P and temperature scenarios.

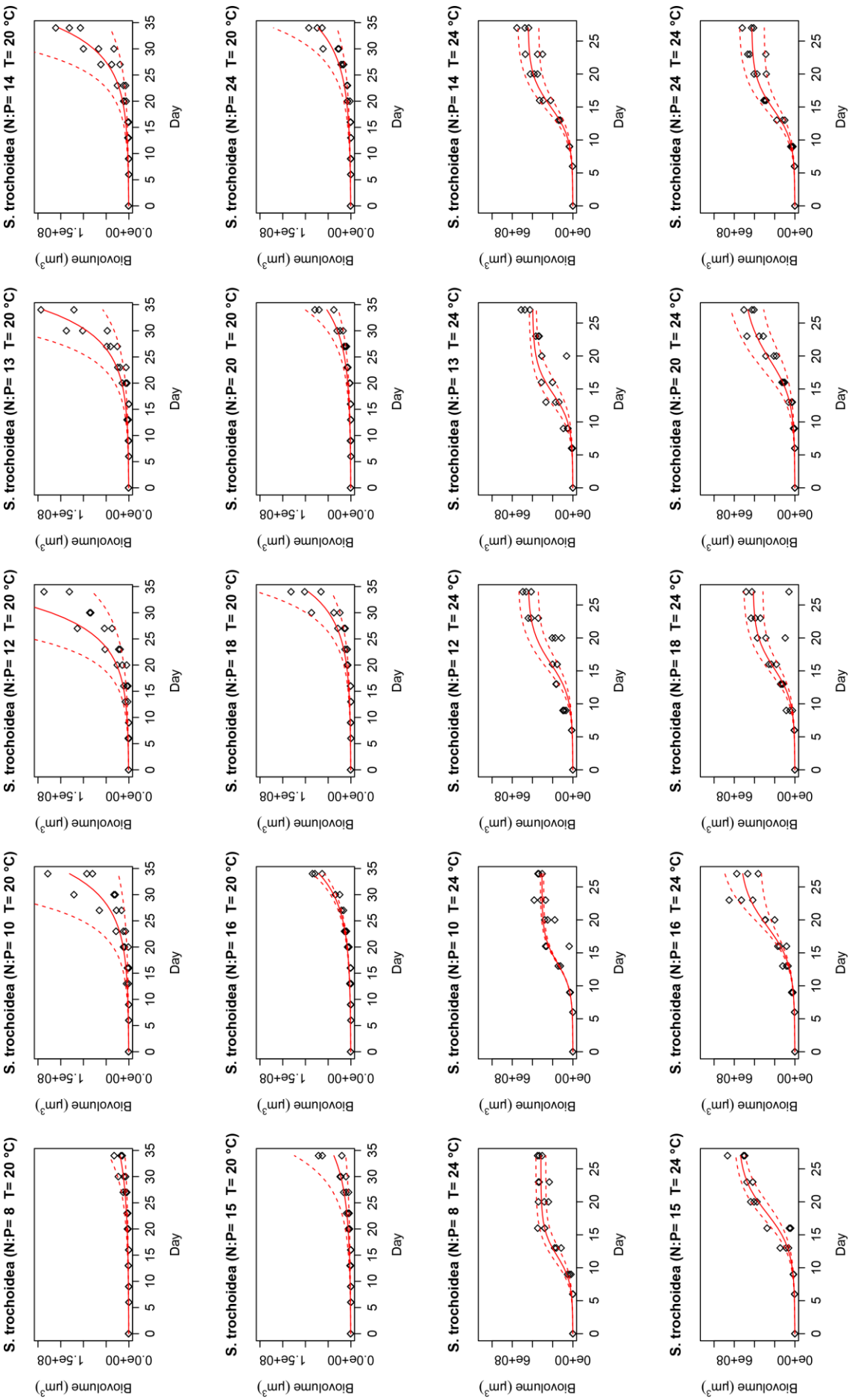

Figure A4: Monoculture growth of *S. trochoidea* under various N:P and temperature scenarios.

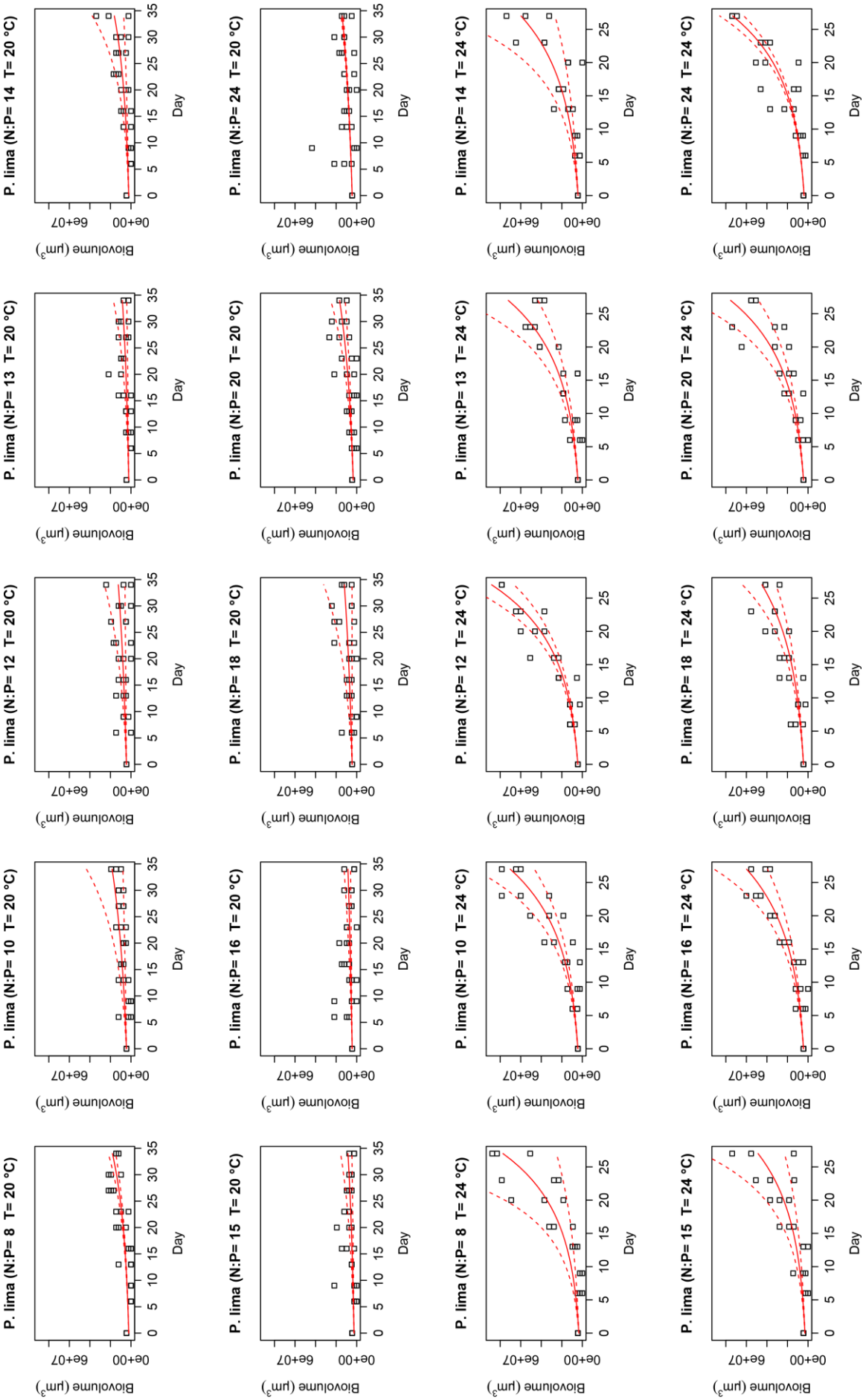

Figure A5: Growth of *P. lima* in mixed batch cultures under various N:P and temperature scenarios.

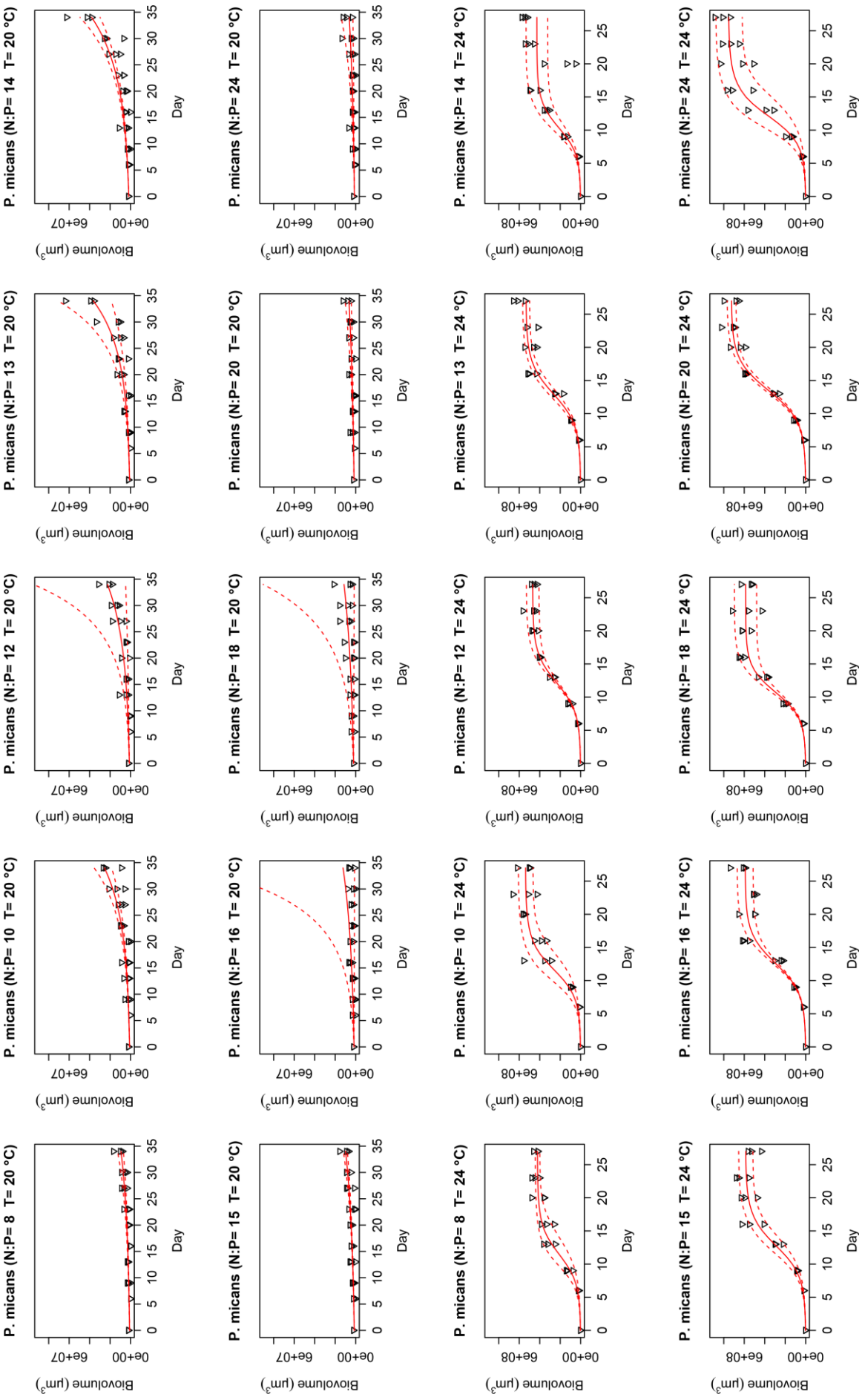

Figure A6: Growth of *P. micans* in mixed batch cultures under various N:P and temperature scenarios.

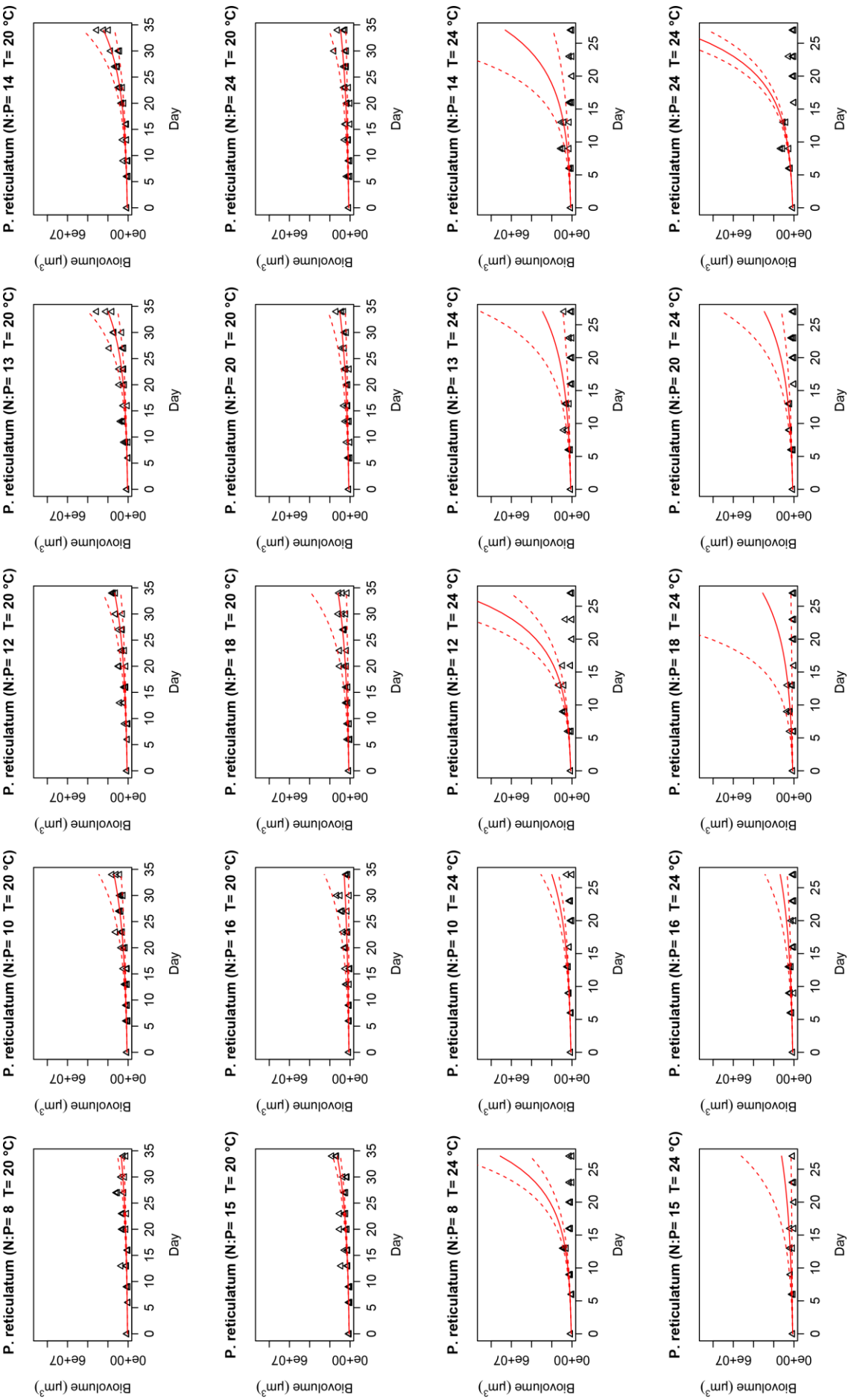

Figure A7: Growth of *P. reticulatum* in mixed batch cultures under various N:P and temperature scenarios.

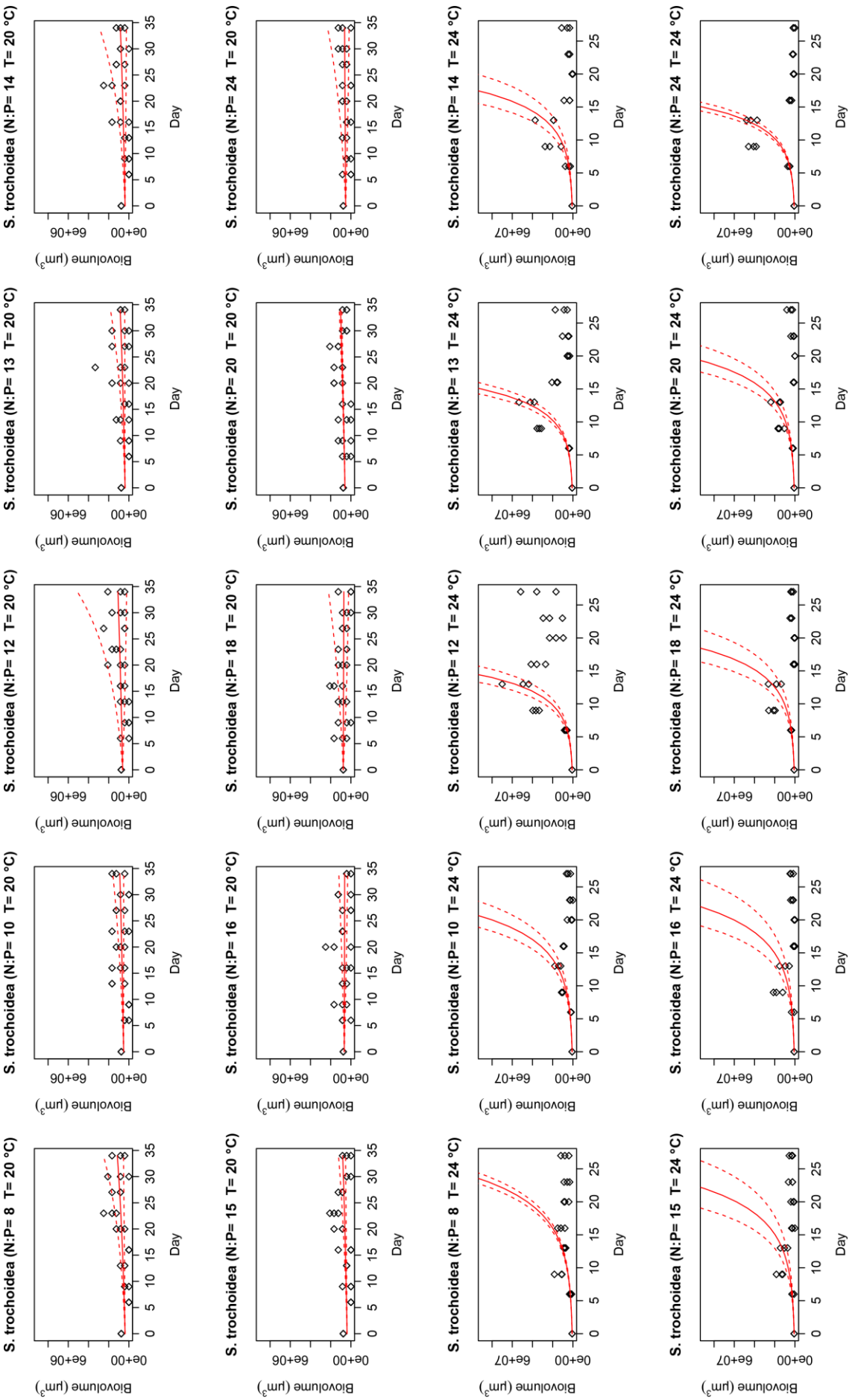

Figure A8: Growth of *S. trochoidea* in mixed batch cultures under various N:P and temperature scenarios.

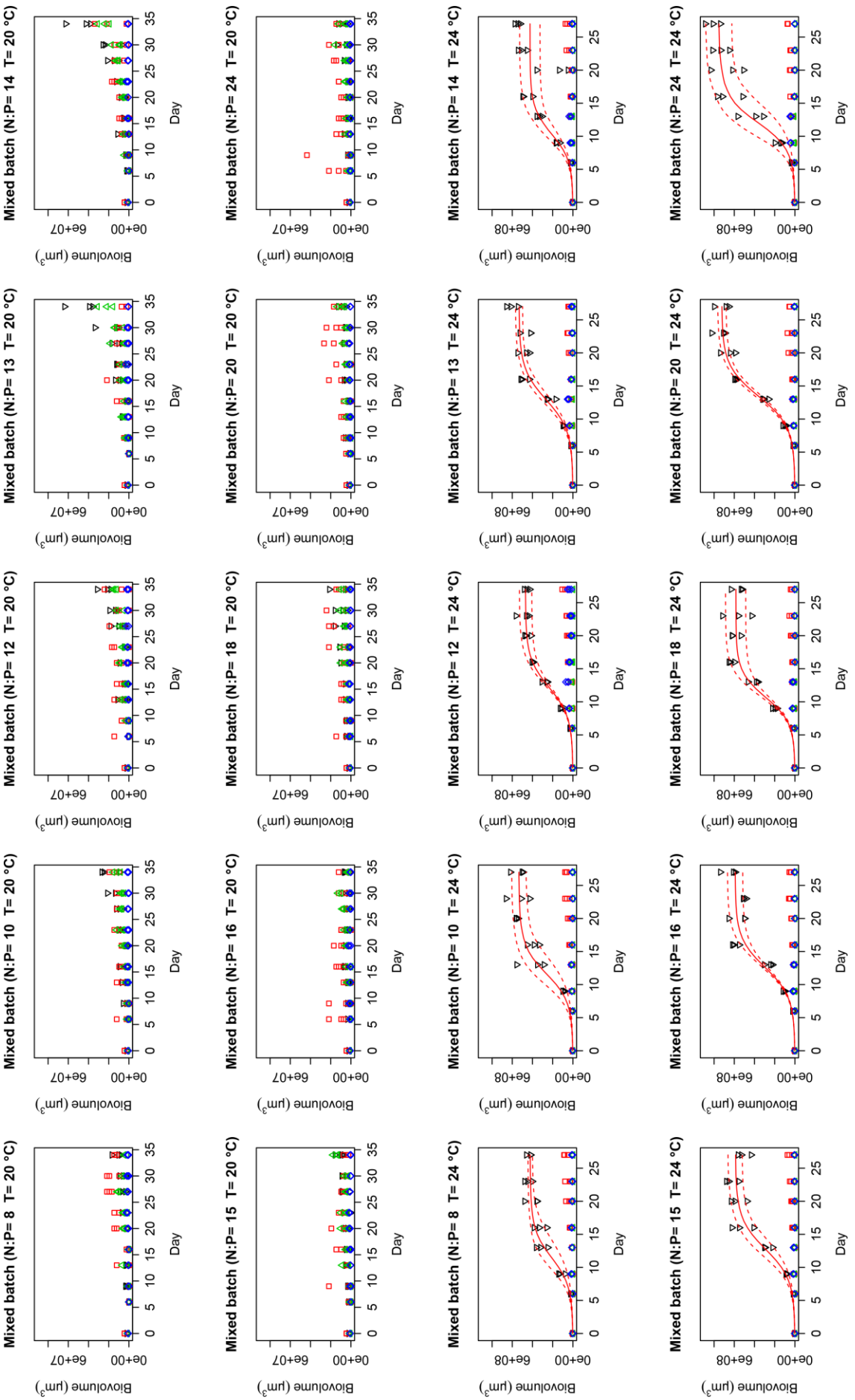

Figure A9: Mixed culture growth of all dinoflagellates, grown together under various N:P and temperature scenarios.

Table B1: Parameter estimates of the first experiment with different nutrient concentrations and N:P ratios

			μ (d^{-1})	K (10^4 cells.ml^{-1})	U_N (10^{-7} l.μm^{-3}.d^{-1})	U_P (10^{-7} l.μm^{-3}.d^{-1})	W_N (10^8 μm^3.mg^{-1})	m (10^{-6} d^{-1})
A. minutum	N:P 8	CF10	0.31±0.01	1.36±0.02	33.0 (31.7-34.6)	44.5 (7.44-91.8)	2.30 (2.25-2.35)	5.39 (0.66-9.59)
		CF100	0.27±0.01	11.7±0.31	3.32 (3.13-3.62)	0.19 (0.03-1.31)	1.92 (1.81-2.01)	5.19 (0.47-9.48)
	N:P 16	CF10	0.32±0.01	1.86±0.03	23.5 (21.5-24.7)	19.8 (8.31-36.8)	1.74 (1.68-1.81)	6.37 (0.49-8.96)
		CF100	0.24±0.01	20.7±1.16	2.25 (1.95-2.66)	0.09 (0.02-0.48)	1.50 (1.34-1.67)	4.88 (0.40-9.41)
	N:P 24	CF10	0.32±0.01	2.18±0.06	21.4 (19.5-22.8)	20.5 (14.7-42.2)	1.35 (1.30-1.43)	5.20 (0.56-9.41)
		CF100	0.25±0.01	26.4±0.09	1.86 (1.61-2.23)	0.61 (0.40-0.86)	1.16 (1.02-1.30)	4.84 (0.40-9.36)
P. reticulatum	N:P 8	CF10	0.21±0.01	0.37±0.01	42.4 (36.9-47.7)	25.0 (19.1-31.1)	1.22 (1.14-1.31)	4.80 (0.56-9.30)
		CF100	0.17±0.01	3.17±0.11	4.38 (4.04-4.90)	1.16 (0.93-1.70)	0.97 (0.90-1.03)	4.35 (0.45-9.45)
	N:P 16	CF10	0.20±0.01	0.51±0.18	27.1 (24.1-31.3)	19.9 (17.7-22.4)	0.90 (0.83-0.97)	4.93 (0.45-9.50)
		CF100	0.16±0.01	6.44±0.61	2.24 (1.97-2.54)	1.28 (0.48-2.03)	1.02 (0.93-1.13)	5.09 (0.60-9.61)
	N:P 24	CF10	0.24±0.01	0.52±0.03	34.7 (31.6-37.9)	38.1 (18.9-87.3)	0.62 (0.59-0.66)	5.27 (0.56-9.32)
		CF100	0.19±0.01	6.57±0.15	2.07 (2.02-2.13)	0.67 (0.05-3.74)	0.76 (0.74-0.77)	5.67 (0.73-9.54)
P. micans	N:P 8	CF10	0.36±0.01	0.33±0.01	88.3 (80.9-94.3)	6.32 (3.61-11.9)	1.00 (0.98-1.04)	5.46 (0.66-9.58)
		CF100	0.28±0.01	2.36±0.03	9.66 (9.31-1.02)	1.32 (0.94-2.68)	0.70 (0.69-0.72)	4.96 (0.54-9.24)
	N:P 16	CF10	0.38±0.01	0.43±0.01	71.7 (64.9-80.8)	15.3 (7.97-50.1)	0.70 (0.68-0.74)	4.32 (0.40-9.46)
		CF100	0.28±0.01	3.18±0.08	7.50 (6.78-8.19)	0.12 (0.02-0.69)	0.53 (0.50-0.56)	4.86 (0.58-9.45)
	N:P 24	CF10	0.36±0.01	0.52±0.01	60.0 (57.1-62.5)	19.4 (12.1-42.1)	0.57 (0.56-0.58)	4.71 (0.46-9.09)
		CF100	0.30±0.01	3.43±0.04	6.76 (6.47-7.03)	0.50 (0.07-13.1)	0.37 (0.37-0.38)	4.86 (0.67-9.37)

Growth rate (μ±SD) and carrying capacity (K±SD) from logistic growth models. Uptake of nitrogen U_N and phosphorus U_P, conversion efficiency of nitrogen W_N and maintenance requirement m – with 95% confidence intervals – based on MacArthur's consumer-resource model (ref. chapter III section 2.4).

Table B2: Parameter estimates of the second experiment with varying initial densities, N:P ratios and temperatures

			Initial density (cells.ml^{-1})	U_N (10^{-7} l.µm^{-3}.d^{-1})	U_P (10^{-7} l.µm^{-3}.d^{-1})	W_N (10^8 µm^3.mg^{-1})	m (d^{-1})
A. minutum	N:P 8	20°C	10	3.09	1.00	1.08	0.004
			100	3.90	2.00	0.86	0.001
		24°C	10	10.9	1.00	1.20	0.001
			100	13.0	0.98	1.05	0.011
	N:P 14	20°C	10	1.97	0.74	1.01	0.009
			100	2.56	7.00	0.76	0.008
		24°C	10	7.80	2.00	1.00	0.001
			100	15.0	5.04	0.72	<0.001
P. reticulatum	N:P 8	20°C	10	NA	NA	NA	NA
			100	1.69	1.08	1.00	0.010
		24°C	10	3.08	5.28	4.98	0.259
			100	7.51	1.67	1.00	0.009
	N:P 14	20°C	10	NA	NA	NA	NA
			100	1.86	4.60	0.93	0.051
		24°C	10	4.73	10.3	1.15	0.001
			100	3.76	8.14	1.10	0.008
P. micans	N:P 8	20°C	10	1.10	1.01	0.52	0.136
			100	7.87	6.89	0.50	0.016
		24°C	10	38.3	5.69	0.38	0.011
			100	22.8	0.97	0.50	0.001
	N:P 14	20°C	10	1.10	1.05	4.72	0.223
			100	4.40	21.7	0.55	0.039
		24°C	10	15.5	2.58	0.50	<0.001
			100	17.3	37.3	0.43	<0.001

Uptake rates of nitrogen U_N and phosphorus U_P, conversion efficiency of nitrogen W_N and the maintenance requirement m based on MacArthur's consumer-resource model. No significant growth was observed for 10 cells.ml^{-1} *P. reticulatum* at 20°C (ref. Fig B3)

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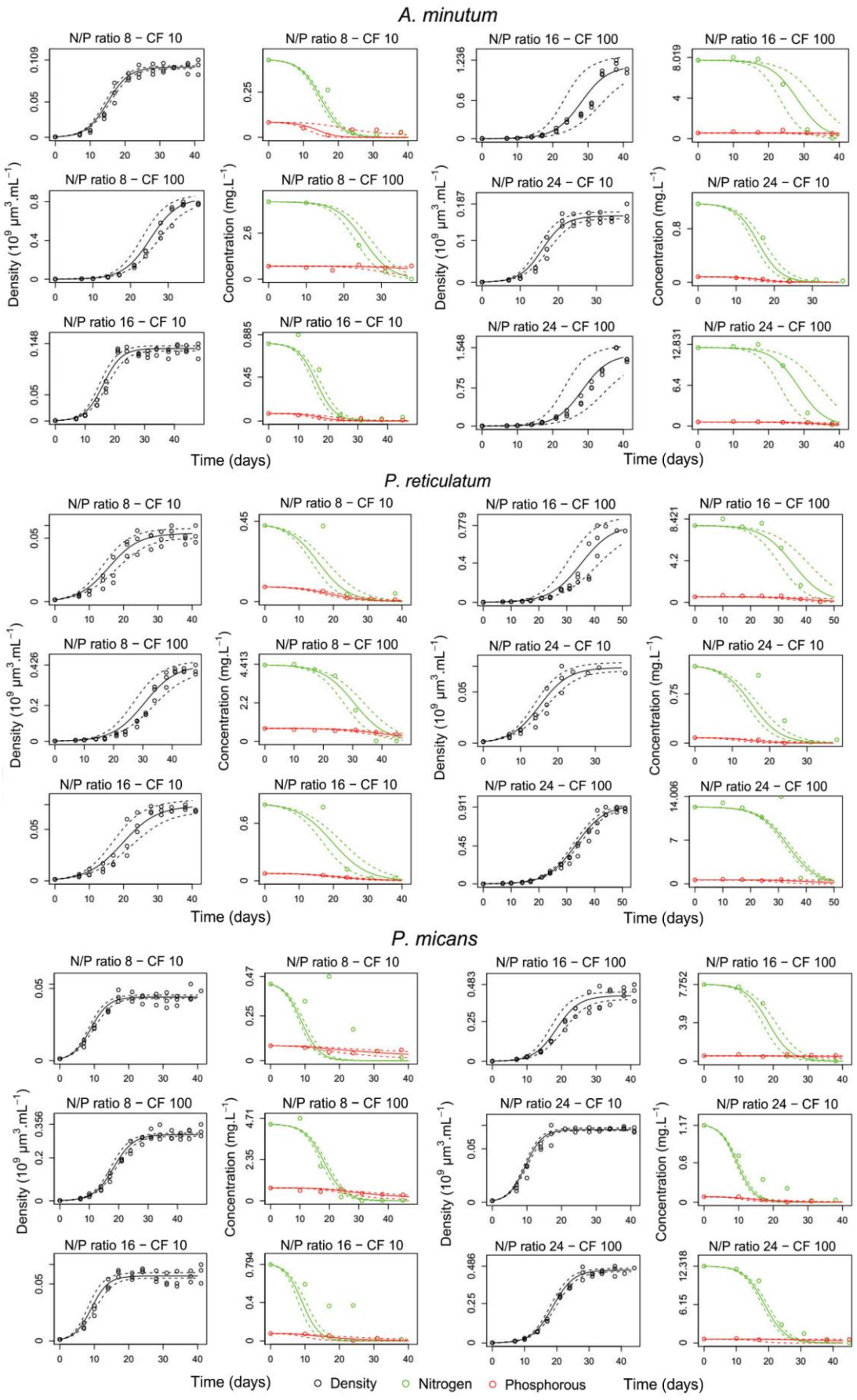

Figure B1: Densities and nutrients of monocultures of all species in the first experiment.

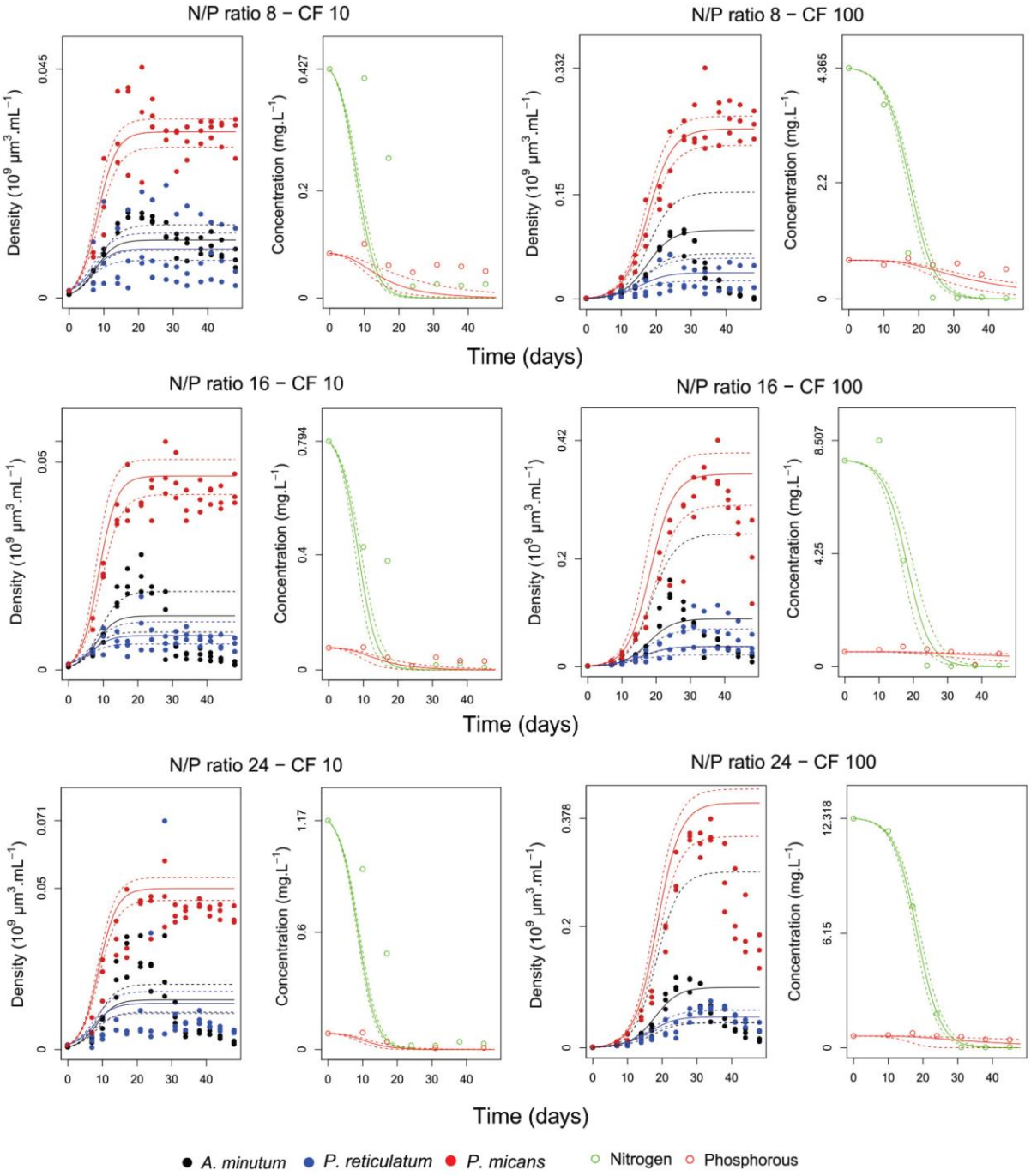

Figure B2: Nutrient and density data of mixed cultures of all three species, grown with different N:P ratios and macronutrient concentrations (i.e. % dilutions of L1 medium called concentration factors), fitted with predictions of MacArthur's resource competition model. Full lines represent the mean prediction, dashed lines are 95% confidence intervals.

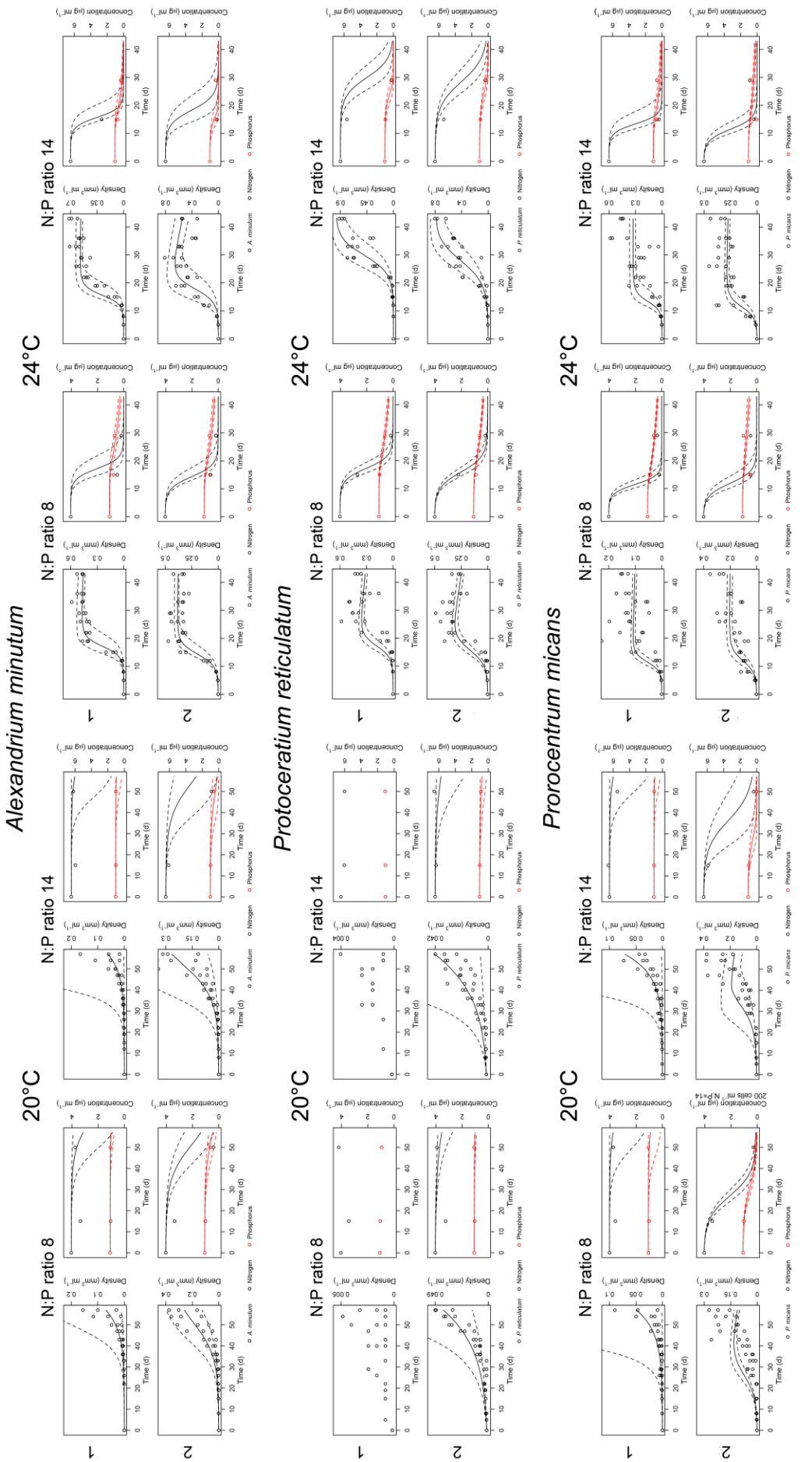

Figure B3: Densities and nutrients of monocultures of all species in the second experiment. 1 represents cultures with an initial density of 10 cells.ml^{-1}, 2 signifies cultures with an initial density of 100 cells.ml^{-1}. Note that *P. reticulatum* did not grow with 10 cells.ml^{-1} at 20°C.

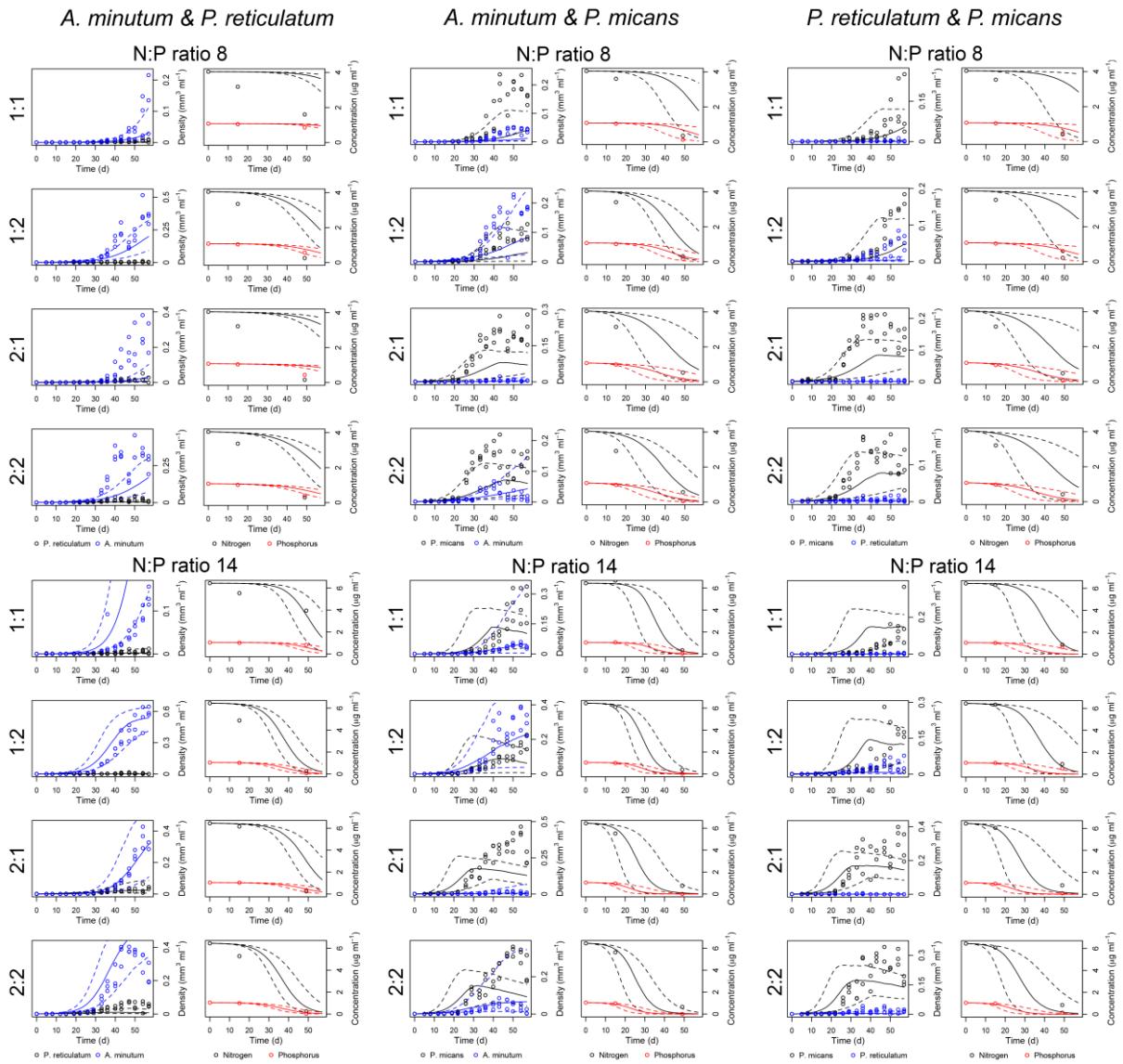

Figure B4: Nutrient and density data of binary mixtures of three dinoflagellate species, grown at 20°C and two different N:P ratios (8 or 14). The ratio on the y-axis represent the respective initial densities of each species (1 = 10 cells.ml^{-1}; 2 = 100 cells.ml^{-1}). Full lines represent the mean predictions of MacArthur's resource competition model, dashed lines are 95% confidence intervals.

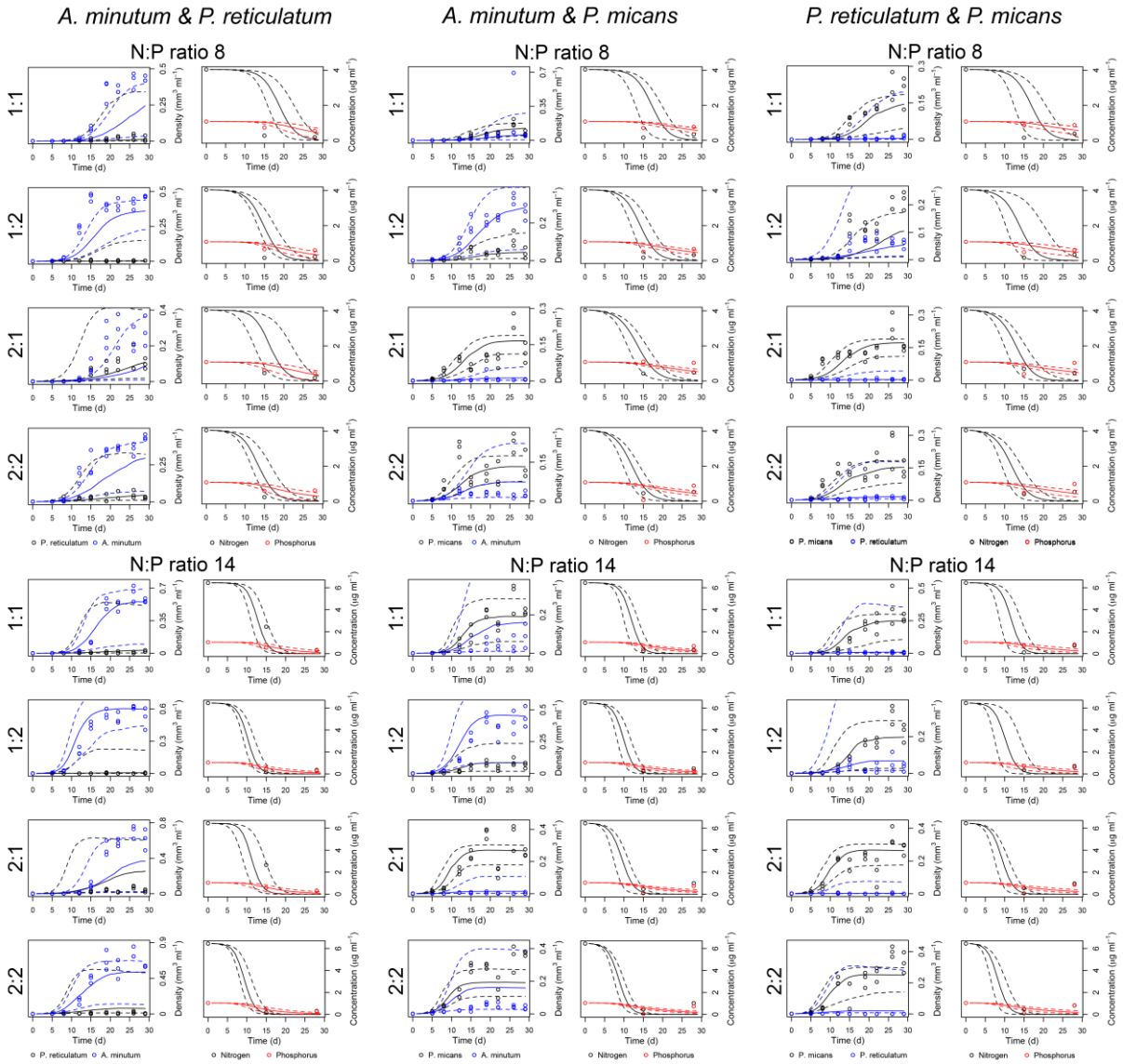

Figure B5: Nutrient and density data of binary mixtures of three dinoflagellate species, grown at 24°C and two different N:P ratios (8 or 14). The ratio on the y-axis represent the respective initial densities of each species (1 = 10 cells.ml^{-1}; 2 = 100 cells.ml^{-1}). Full lines represent the mean predictions of MacArthur's resource competition model, dashed lines are 95% confidence intervals.

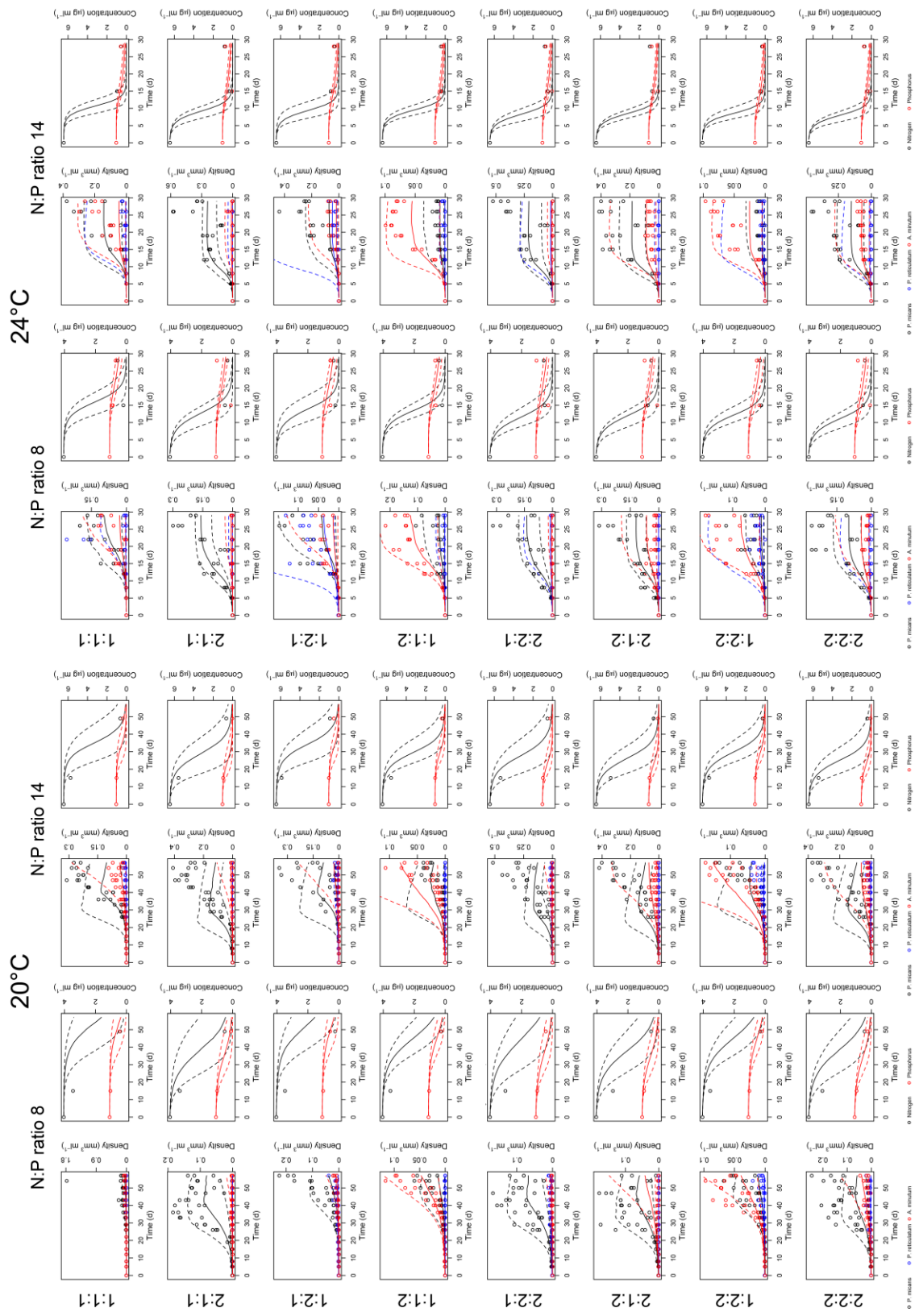

Figure B6: Nutrient and density data of the tertiary mixtures of three dinoflagellate species, grown at either 20°C or 24°C ,and two different N:P ratios (8 or 14). The y-axis shows the respective initial densities of each species (1 = 10 cells.ml^{-1}; 2 = 100 cells.ml^{-1}). Full lines represent the mean predictions of MacArthur's resource competition model, dashed lines are 95% confidence intervals.

Table C1: The abbreviations and official nomenclature for chemicals found in chapter 4. The abbreviated names for OA precursors are based on the number of carbon atoms and the number of double bonds in the cleavable chain.

Abbreviation	Chemical nomenclature
OA	Okadaic acid
OA-C1	Methyl okadaate
OA-C3	Norokadanone
OA-D4	2-hydroxymethyl-allyl okadaate
OA-D6	5-hydroxy-2-methylene-pent-3-enyl okadaate
OA-D7a	6-hydroxy-2-methylene-hexa-4-enyl okadaate
OA-D7b	6-hydroxy-2-methyl-hexa-2,4-dienyl okadaate
OA-D8	7-hydroxy-2-methyl-hepta-2,4-dienyl okadaate
OA-D9a	7-hydroxy-2-methyl-6-methylene-hept-2-enyl okadaate
OA-D9b	7-hydroxy-4-methyl-2-methylene-hept-4-enyl okadaate
OA-D9c	7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate
OA-T9	5-hydroperoxy-7-hydroxy-2,4-dimethylene-heptyl okadaate
OA-D10	7-hydroxymethyl-2- methylene-octa-4,7-dienyl okadaate
DTX-1	Dinophysistoxin-1 or 35(S)-methyl OA
DTX-2	Dinophysistoxin-2 or 31-demethyl-35-methyl OA
DTX-3	Various fatty esters (i.e. 7-O-Acyl derivates) of OA, DTX-1 and DTX-2
13-SPX C	13-desmethyl spirolide C
27-O-13,19-SPX C	27-oxo-13,19-didesmethyl spirolide C
27-OH-13-SPX C	27-hydroxy-13-desmethyl spirolide C

Table C2.1 OA related compounds found in the extracts of mussels exposed to *P. lima* (*n*=55). The results were obtained through the ToxID software, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.kg^{-1}$)	Tissue
3 DAYS										
$C_{44}H_{68}O_{13}$	1.95	803.46234	4.50	-	47.59	46.43	9.46	OA	5.17	visceral
$C_{45}H_{70}O_{13}$	2.09	817.47717	3.46	-	48.67	48.39	10.28	DTX-1	8.63	visceral
$C_{58}H_{94}O_{14}$	3.55	1037.65662	2.92	+	62.73	56.55	13.18	14:0 OA	5.35	visceral
$C_{58}H_{88}O_{14}$	3.15	1009.62524	0.55	+	62.73	59.22	10.51	14:3 OA	4.47	visceral
$C_{60}H_{94}O_{14}$	3.28	1061.6570	3.20	+	64.89	57.44	13.45	16:2 OA	4.76	visceral
$C_{64}H_{96}O_{14}$	3.32	1111.67358	3.91	+	69.22	64.02	15.20	20:5 OA	3.77	visceral
$C_{43}H_{66}O_{11}$	3.09	781.45148	2.23	+	46.51	40.31	16.20	OA-C3	3.39	visceral
$C_{48}H_{74}O_{14}$	4.87	892.53790	-4.23	+	51.92	50.23	16.14	OA-D4	2.50	visceral
5 DAYS										
$C_{44}H_{68}O_{13}$	1.96	803.46198	4.05	-	47.59	44.32	13.26	OA	9.12	visceral
$C_{44}H_{68}O_{13}$	1.97	803.46007	1.68	-	47.59	45.86	15.65	OA	4.83	Non-visceral
$C_{45}H_{70}O_{13}$	2.11	817.47687	3.05	-	48.67	47.22	9.45	DTX-1	11.81	visceral
$C_{45}H_{70}O_{13}$	2.13	817.47488	0.66	-	48.67	47.68	14.69	DTX-1	6.56	Non-visceral
$C_{58}H_{94}O_{14}$	2.82	1037.65659	3.20	+	62.73	58.61	10.12	14:0 OA	2.55	visceral
$C_{58}H_{88}O_{14}$	3.14	1009.62508	0.64	+	62.73	56.74	9.99	14:3 OA	10.44	visceral
$C_{58}H_{88}O_{14}$	3.15	1009.62529	0.99	+	62.73	56.75	12.10	14:3 OA	6.39	Non-visceral
$C_{59}H_{96}O_{14}$	3.61	1029.68616	-1.08	+	63.81	62.37	11.44	15:0 OA	13.40	visceral
$C_{60}H_{98}O_{14}$	3.73	1065.68616	-0.4	+	64.89	60.77	14.12	16:0 OA	11.07	visceral
$C_{60}H_{96}O_{14}$	3.55	1063.67163	2.25	+	64.89	61.63	13.26	16:1 OA	15.91	visceral
$C_{60}H_{96}O_{14}$	3.56	1063.67073	1.41	+	64.89	65.46	14.29	16:1 OA	10.67	Non-visceral
$C_{60}H_{94}O_{14}$	3.70	1061.65265	-0.87	+	64.89	59.48	9.41	16:2 OA	35.20	visceral
$C_{60}H_{94}O_{14}$	3.73	1061.65122	-2.22	+	64.89	66.78	14.29	16:2 OA	23.24	Non-visceral
$C_{61}H_{98}O_{14}$	3.93	1055.70166	-1.2	+	65.98	57.78	10.20	17:1 OA	5.11	visceral
$C_{62}H_{100}O_{14}$	3.96	1069.7200	1.29	+	67.06	66.57	14.49	18:1 OA	13.92	visceral

Table C2.2 OA related compounds found in the extracts of mussels exposed to *P. lima* (*n*=55).

$C_{62}H_{98}O_{14}$	3.61	1067.70056	-2.2	+	67.06	61.24	13.12	18:2 OA	19.95	visceral
$C_{62}H_{98}O_{14}$	3.61	1067.70114	-1.67	+	67.06	66.40	15.82	18:2 OA	13.57	Non-visceral
$C_{62}H_{94}O_{14}$	3.57	1085.65515	1.44	+	67.06	59.31	10.75	18:4 OA	16.87	visceral
$C_{64}H_{96}O_{14}$	3.27	1111.67175	2.26	+	69.22	62.09	14.11	20:5 OA	10.25	visceral
$C_{64}H_{96}O_{14}$	3.28	1111.66955	0.28	+	69.22	68.27	14.13	20:5 OA	7.21	Non-visceral
$C_{66}H_{98}O_{14}$	3.29	1137.68005	-4.24	+	71.38	68.46	11.92	22:6 OA	13.71	Non-visceral
10 DAYS										
$C_{44}H_{68}O_{13}$	1.96	803.46275	5.01	-	47.59	44.32	13.26	OA	12.05	visceral
$C_{44}H_{68}O_{13}$	1.98	803.46007	1.68	-	47.59	47.05	14.93	OA	8.64	Non-visceral
$C_{45}H_{70}O_{13}$	2.11	817.47544	1.34	-	48.67	47.22	15.45	DTX-1	15.13	visceral
$C_{45}H_{70}O_{13}$	2.11	817.47401	-0.40	-	48.67	45.67	11.45	DTX-1	4.45	Non-visceral
$C_{58}H_{94}O_{14}$	3.61	1037.65381	0.22	+	62.73	57.52	15.21	14:0 OA	9.38	visceral
$C_{58}H_{94}O_{14}$	3.61	1037.65206	-1.46	+	62.73	59.46	15.21	14:0 OA	4.22	Non-visceral
$C_{58}H_{88}O_{14}$	3.13	1009.62531	0.62	+	62.73	60.13	12.60	14:3 OA	11.17	visceral
$C_{58}H_{88}O_{14}$	3.13	1009.62624	1.93	+	62.73	59.06	12.60	14:3 OA	7.87	Non-visceral
$C_{59}H_{96}O_{14}$	3.68	1029.68481	-2.39	+	63.81	59.75	14.06	15:0 OA	26.50	visceral
$C_{60}H_{98}O_{14}$	3.77	1065.68616	-0.4	+	64.89	61.43	13.46	16:0 OA	26.69	visceral
$C_{60}H_{96}O_{14}$	3.59	1063.67090	1.57	+	64,89	60.94	13.95	16:1 OA	28.08	visceral
$C_{60}H_{94}O_{14}$	3.71	1061.65471	1.06	+	64.89	66.23	9.34	16:2 OA	126.00	visceral
$C_{60}H_{94}O_{14}$	3.71	1061.65369	0.10	+	64.89	61.48	10.34	16:2 OA	59.08	Non-visceral
$C_{61}H_{98}O_{14}$	3.92	1055.70113	-1.10	+	65.98	56.24	9.74	17:1 OA	2.08	visceral
$C_{62}H_{100}O_{14}$	3.97	1069.71965	1.28	+	67.06	60.95	11.11	18:1 OA	3.58	visceral
$C_{62}H_{98}O_{14}$	3.65	1067.70286	-1.9	+	67.06	59.81	10.25	18:2 OA	14.77	visceral
$C_{62}H_{98}O_{14}$	3.66	1067.70680	3.62	+	67.06	62.49	8.26	18:2 OA	9.78	Non-visceral
$C_{62}H_{96}O_{14}$	3.79	1065.68433	-2.79	+	67.06	61.22	15.84	18:3 OA	20.70	visceral
$C_{62}H_{94}O_{14}$	3.68	1063.67090	-0.68	+	67.06	64,44	12.62	18:4 OA	28.08	visceral
$C_{64}H_{96}O_{14}$	3.77	1111.67161	1.50	+	69.22	58.68	10.54	20:5 OA	14.77	visceral
$C_{64}H_{96}O_{14}$	3.77	1111.67383	2.14	+	69.22	63.85	11.76	20:5 OA	10.29	Non-visceral
$C_{61}H_{100}O_{14}$	4.04	1057.7179	-0.64	+	65.98	60.12	15.86	16:0 DTX1	7.37	visceral
$C_{47}H_{73}O_{14}$	2.94	860.49249	-0,3	-	50.83	48.77	12.23	27-O-acetyl OA	2.08	visceral

Table C2.3 OA related compounds found in the extracts of mussels exposed to *P. lima* (*n*=55).

$C_{54}H_{82}O_{14}$	2.68	953.56256	-0.65	-	58.40	50.11	13.29	OA-D6	5.30	visceral
$C_{52}H_{80}O_{14}$	2.66	927.54761	0.08	-	56.24	48.27	13.66	OA-D8	10.85	visceral
$C_{53}H_{82}O_{14}$	2.74	943.57788	0.15	+	57.32	50.39	9.12	OA-D9	10.94	visceral
15 DAYS										
$C_{44}H_{68}O_{13}$	1.94	803.46136	3.20	-	47.59	41.88	15.36	OA	9.77	Viceral
$C_{44}H_{68}O_{13}$	1.95	803.46115	3.13	-	47.59	42.32	11.82	OA	3.55	Non-visceral
$C_{45}H_{70}O_{13}$	2.11	817.47601	2.01	-	48.67	47.76	12.45	DTX-1	13.21	Viceral
$C_{45}H_{70}O_{13}$	2.12	817.47607	2.07	-	48.67	45.29	13.13	DTX-1	7.24	non-visceral
$C_{61}H_{100}O_{14}$	4.04	1057.71960	0.96	+	65.98	61.85	14.13	16:0 DTX1	3.70	Viceral
$C_{61}H_{100}O_{14}$	3.93	1057.71936	0.73	+	65.98	59.46	12.21	16:0 DTX1	3.11	Non-visceral
$C_{58}H_{94}O_{14}$	3.61	1037.65161	-1.89	+	62.73	58.52	14.90	14:0 OA	6.72	Viceral
$C_{58}H_{88}O_{14}$	3.14	1009.62317	-1.49	+	62.73	59.03	14.22	14:3 OA	3.26	Viceral
$C_{59}H_{96}O_{14}$	2.34	1027.67725	4.39	+	63.81	56.88	9.36	15:0 OA	67.33	Viceral
$C_{60}H_{98}O_{14}$	3.76	1065.68127	-3.38	+	64.89	62.78	15.63	16:0 OA	7.39	Viceral
$C_{60}H_{96}O_{14}$	3.58	1063.6709	1.57	+	64,89	61.45	10.35	16:1 OA	15.77	Viceral
$C_{60}H_{94}O_{14}$	3.71	1061.65335	-0.21	+	64.89	59.53	13.21	16:2 OA	49.81	Viceral
$C_{60}H_{94}O_{14}$	3.69	1061.65289	-0.64	+	64.89	60.06	14.13	16:2 OA	35.39	Non-visceral
$C_{62}H_{100}O_{14}$	3.95	1069.71814	0.41	+	67.06	61.95	15.85	18:1 OA	2.30	Viceral
$C_{62}H_{100}O_{14}$	3.95	1069.71655	-1.89	+	67.06	60.77	14.95	18:1 OA	2.26	Non-visceral
$C_{62}H_{98}O_{14}$	3.72	1065.68774	-0.60	+	67.06	59.81	12.04	18:2 OA	4.77	Viceral
$C_{62}H_{98}O_{14}$	3.71	1067.70074	-2.05	+	67.06	60.81	13.16	18:2 OA	3.91	Non-visceral
$C_{62}H_{94}O_{14}$	3.67	1063.67090	-0.68	+	67.06	65,34	14.44	18:4 OA	3.41	Viceral
$C_{64}H_{96}O_{14}$	3.75	1111.66968	0.40	+	69.22	66.72	14.78	20:5 OA	4.30	Viceral
$C_{64}H_{96}O_{14}$	3.75	1111.66822	-0.90	+	69.22	68.72	11.56	20:5 OA	3.90	Non-visceral
$C_{53}H_{82}O_{15}$	2.12	959.57227	-0.39	+	57.32	50.08	14.7	OA-T9	1.20	Viceral
$C_{52}H_{80}O_{14}$	4.73	951.54004	-4.19	+	56.24	50.29	9.33	OA-D8	2.02	Viceral
$C_{52}H_{80}O_{14}$	2.76	929.56104	-1.11	+	56.24	52.77	9.21	OA-D8	2.81	Non-visceral
$C_{54}H_{82}O_{14}$	2.78	953.56195	-1.28	-	58.40	62.85	12.66	OA-D10	1.76	Viceral
$C_{54}H_{82}O_{14}$	2.76	955.57642	-1.37	+	58.40	51.13	15.66	OA-D10	1.61	Non-visceral

Table C3.1 SPX related compounds found in the extracts of mussels exposed to *A. ostenfeldii* (*n*=57). The results were obtained through the ToxID software, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.kg^{-1}$)	Tissue
3 DAYS										
$C_{42}H_{61}NO_8$	4.09	708.44513	-2.62	+	45.43	42.07	15.86	27-OH-13-SPX C	0.69	Visceral
$C_{42}H_{62}NO_7$	3.40	693.45862	-1.84	+	45.43	44.18	13.31	13-SPX C	0.70	Visceral
$C_{43}H_{66}NO_7$	3.30	708.48621	3.39	+	46.51	48.91	9.02	Spirolide D	0.55	Visceral
5 DAYS										
$C_{42}H_{64}NO_7$	2.35	716.45184	-3.02	+	45.43	44.77	12.33	Spirolide B	0.63	Visceral
$C_{43}H_{66}NO_7$	3.30	708.48621	3.39	+	46.51	48.91	15.02	Spirolide D	0.96	Visceral
$C_{42}H_{64}NO_8$	5.82	728.49719	0.23	+	45.43	46.69	15.31	Spirolide E	0.62	Visceral
$C_{40}H_{62}NO_6$	3.42	651.44781	-4.03	-	43.26	41.55	14.28	Spirolide I	0.66	Visceral
10 DAYS										
$C_{43}H_{66}NO_7$	3.30	708.48639	4.24	+	46.51	48.91	14.73	Spirolide D	1.26	Visceral
$C_{43}H_{66}NO_7$	3.30	708.48621	3.39	+	46.51	46.91	10.02	Spirolide D	0.56	Non-visceral
$C_{40}H_{60}NO_6$	4.83	649.43158	-4.94	-	43.26	42.22	16.08	Spirolide H	1.15	Visceral
$C_{42}H_{61}NO_8$	4.09	708.44513	-2.62	+	45.43	42.07	15.86	27-OH-13-SPX C	0.31	Visceral
$C_{41}H_{58}NO_8$	4.15	693.42114	-3.43	+	44.34	44.02	14.32	27-O-13,19-SPX C	0.78	Visceral
$C_{42}H_{64}NO_8$	5.82	728.49719	0.23	+	45.43	46.69	15.31	Spirolide E	1.65	Visceral
$C_{42}H_{64}NO_8$	5.81	728.49806	1.42	+	45.43	44.45	10.39	Spirolide E	0.75	Non-visceral
$C_{40}H_{62}NO_6$	3.42	651.44781	-4.03	-	43.26	42.55	9.28	Spirolide I	1.87	Visceral
$C_{40}H_{62}NO_6$	3.41	651.44845	-3.05	-	43.26	41.38	15.46	Spirolide I	0.93	Non-visceral

Table C3.2 SPX related compounds found in the extracts of mussels exposed to *A. ostenfeldii* (*n*=57).

15 DAYS										
$C_{42}H_{62}NO_7$	3.42	693.45862	-1.84	+	45.43	44.18	15.31	13-SPX C	1.89	Visceral
$C_{42}H_{62}NO_7$	3.42	693.45975	-0.21	+	45.43	45.13	16.74	13-SPX C	1.15	Non-visceral
$C_{43}H_{64}NO_7$	5.25	707.47571	0.22	+	46.51	46.13	16.38	Spirolide C	1.14	Visceral
$C_{43}H_{66}NO_7$	3.30	708.48621	3.39	+	46.51	48.91	14.02	Spirolide D	3.03	Visceral
$C_{43}H_{66}NO_7$	3.32	708.48547	2.94	+	46.51	46.53	13.28	Spirolide D	1.45	Non-visceral
$C_{42}H_{64}NO_8$	5.82	728.49719	0.23	+	45.43	46.69	14.31	Spirolide E	1.34	Visceral
$C_{42}H_{64}NO_8$	5.81	728.49789	1.19	+	45.43	44.36	16.55	Spirolide E	1.21	Non-visceral
$C_{42}H_{66}NO_7$	3.40	695.47388	-3.98	-	45.43	46.56	11.99	Spirolide F	1.56	Visceral
$C_{42}H_{66}NO_7$	3.41	695.47432	-3.35	-	45.43	47.13	15.12	Spirolide F	1.15	Non-visceral
$C_{40}H_{62}NO_6$	3.42	651.44781	-4.03	-	43.26	41.55	16.28	Spirolide I	1.09	Visceral
$C_{40}H_{62}NO_6$	3.43	651.44885	-2.44	-	43.26	43.78	16.77	Spirolide I	1.07	Non-visceral
$C_{42}H_{62}NO_8$	4.35	707.43707	-4.52	-	45.43	44.21	12.61	27-OH-13-SPX C	1.88	Visceral
$C_{42}H_{62}NO_8$	4.35	708.44695	-0,56	-	45.43	44.95	14.61	27-OH-13-SPX C	1.64	Non-visceral
$C_{41}H_{58}NO_8$	1.99	710.45301	4.12	+	44.34	41.49	13.04	27-O-13,19-SPX C	1.93	Visceral

Table C4.1 LMBT found in the extracts of mussels exposed to both *A. ostenfeldii* and *P. lima* (*n*=58). The results were obtained through the ToxID software, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.kg^{-1}$)	Tissue
3 DAYS										
$C_{44}H_{68}O_{13}$	1.95	803.46111	2.97	-	47.59	45.75	14.07	OA	5.97	Visceral
$C_{44}H_{68}O_{13}$	1.93	803.46013	1.75	-	47.59	43.76	15.13	OA	1.27	Non-visceral
$C_{45}H_{70}O_{13}$	2.09	817.47651	2.65	-	48.67	44.39	13.57	DTX-1	8.04	Visceral
$C_{45}H_{70}O_{13}$	2.08	817.47679	2.96	-	48.67	45.12	11.25	DTX-1	6.67	Non-visceral
$C_{58}H_{92}O_{14}$	3.65	1013.65295	-2.98	+	62.73	64.38	13.75	14:1 DTX-2	18.26	Visceral
$C_{58}H_{92}O_{14}$	3.66	1013.65123	-4.68	+	62.73	60.43	11.84	14:1 DTX-2	10.11	Non-visceral
$C_{58}H_{88}O_{14}$	3.15	1009.62632	2.01	+	62.73	63.05	12.43	14:3 OA	4.47	Visceral
$C_{59}H_{96}O_{14}$	3.61	1029.68583	-1.4	+	63.81	62.37	11.44	15:0 OA	4.40	Visceral
$C_{60}H_{98}O_{14}$	3.73	1065.68416	-0,67	+	64.89	61.78	16.12	16:0 OA	3.84	Visceral
$C_{60}H_{94}O_{14}$	3.28	1061.65561	1.91	+	64.89	59.23	14.45	16:2 OA	8.59	Visceral
$C_{60}H_{94}O_{14}$	3.29	1061.65720	3.40	+	64.89	61.92	9.54	16:2 OA	3.34	Non-visceral
$C_{64}H_{96}O_{14}$	3.32	1111.67248	2.92	+	69.22	64.15	15.20	20:5 OA	4.40	Visceral
$C_{64}H_{96}O_{14}$	3.32	1111.67345	3.79	+	69.22	66.57	14.26	20:5 OA	1.05	Non-visceral
$C_{43}H_{66}NO_7$	3.30	708.48591	3.57	+	46.51	48.93	13.02	Spirolide D	3.43	Visceral
$C_{40}H_{60}NO_6$	4.83	649.43394	1.3	-	43.26	41.93	12.78	Spirolide H	0.63	Visceral
$C_{42}H_{61}NO_8$	4.09	708.44791	1.29	+	45.43	43.28	11.86	27-OH-13-SPX C	0.80	Visceral
$C_{41}H_{58}NO_8$	4.15	693.42114	-3.43	+	44.34	41.83	15.32	27-O-13,19-SPX C	1.99	Visceral
5 DAYS										
$C_{44}H_{68}O_{13}$	1.95	803.46201	4.09	-	47.59	45.13	12.18	OA	3.88	Visceral
$C_{44}H_{68}O_{13}$	1.94	803.46198	4.05	-	47.59	43.24	12.46	OA	2.24	Non-visceral
$C_{45}H_{70}O_{13}$	2.09	817.47643	2.55	-	48.67	48.39	16.28	DTX-1	6.31	Visceral
$C_{45}H_{70}O_{13}$	2.07	817.47507	0.89	-	48.67	45.73	13.21	DTX-1	3.93	Non-visceral
$C_{58}H_{92}O_{14}$	3.65	1013.65295	-2.98	+	62.73	64.48	12.37	14:1 DTX-2	6.09	Visceral

Table C4.2 LMBT found in the extracts of mussels exposed to both *A. ostenfeldii* and *P. lima* (*n*=58).

$C_{58}H_{88}O_{14}$	3.15	1009.62524	0.55	+	62.73	60.73	14.72	14:3 OA	5.31	Visceral
$C_{59}H_{96}O_{14}$	3.61	1029.68616	-1.08	+	63.81	59.37	14.44	15:0 OA	4.82	Visceral
$C_{59}H_{96}O_{14}$	3.60	1029.68805	0.74	+	63.81	58.85	14.99	15:0 OA	2.02	Non-visceral
$C_{60}H_{98}O_{14}$	3.73	1065.68616	-0.4	+	64.89	60.77	14.12	16:0 OA	2.79	Visceral
$C_{60}H_{98}O_{14}$	3.71	1065.68688	1.87	+	64.89	59.43	16.54	16:0 OA	1.95	Non-visceral
$C_{60}H_{94}O_{14}$	3.28	1061.65674	2.97	+	64.89	57.44	13.45	16:2 OA	3.65	Visceral
$C_{60}H_{94}O_{14}$	3.29	1061.65512	1.45	+	64.89	59.96	12.01	16:2 OA	2.89	Non-visceral
$C_{42}H_{62}NO_7$	3.41	693.45862	-1.84	+	45.43	44.18	16.31	13-SPX C	0.58	Visceral
$C_{42}H_{64}NO_7$	2.35	716.45184	-3.02	+	45.43	44.77	15.33	Spirolide B	2.52	Visceral
$C_{43}H_{66}NO_7$	3.30	708.48621	3.39	+	46.51	48.91	11.02	Spirolide D	1.25	Visceral
$C_{43}H_{66}NO_7$	3.32	708.48557	3.09	+	46.51	42.57	10.53	Spirolide D	1.25	Non-visceral
$C_{42}H_{64}NO_8$	5.82	728.49719	0.23	+	45.43	46.69	11.14	Spirolide E	0.95	Visceral
$C_{40}H_{62}NO_6$	3.42	651.44781	-4.03	-	43.26	41.55	15.28	Spirolide I	1.14	Visceral
10 DAYS										
$C_{44}H_{68}O_{13}$	1.95	803.46088	2.68	-	47.59	44.97	15.46	OA	5.57	Visceral
$C_{44}H_{68}O_{13}$	1.97	803.45906	0.42	-	47.59	46.44	13.18	OA	4.25	Non-visceral
$C_{45}H_{70}O_{13}$	2.09	817.47509	0.91	-	48.67	46.19	11.28	DTX-1	10.03	Visceral
$C_{45}H_{70}O_{13}$	2.05	817.47664	2.81	-	48.67	46.22	10.88	DTX-1	3.75	Non-visceral
$C_{61}H_{100}O_{14}$	3.93	1057.71936	0,73	+	65.98	59.46	12.21	16:0 DTX1	3.02	Visceral
$C_{61}H_{100}O_{14}$	3.91	1057.71903	0.42	+	65.98	60.89	13.15	16:0 DTX1	1.02	Non-visceral
$C_{43}H_{66}O_{11}$	4.46	781.45282	1.35	+	46.51	44.50	12.01	OA-C3	3.15	Visceral
$C_{58}H_{94}O_{14}$	3.61	1037.65161	-1.89	+	62.73	58.52	14.90	14:0 OA	9.01	Visceral
$C_{58}H_{92}O_{14}$	3.59	1013.65356	-2.30	+	62.73	66.13	13.66	14:1 DTX-2	82.82	Visceral
$C_{58}H_{88}O_{14}$	3.15	1009.62524	0.55	+	62.73	59.22	15.51	14:3 OA	4.54	Visceral
$C_{59}H_{96}O_{14}$	3.61	1029.68616	-1.08	+	63.81	62.37	9.44	15:0 OA	7.57	Visceral
$C_{60}H_{98}O_{14}$	3.73	1065.68616	-0.4	+	64.89	60.77	14.12	16:0 OA	8.66	Visceral
$C_{60}H_{96}O_{14}$	3.55	1063.67163	2.25	+	64.89	61.63	16.26	16:1 OA	8.96	Visceral

Table C4.3 LMBT found in the extracts of mussels exposed to both *A. ostenfeldii* and *P. lima* (*n*=58).

$C_{60}H_{96}O_{14}$	3.54	1063.67207	2.67	+	64.89	59.77	13.22	16:1 OA	3.83	Non-visceral
$C_{60}H_{94}O_{14}$	3.26	1061.65228	-1.22	+	64.89	59.53	13.21	16:2 OA	28.60	Visceral
$C_{60}H_{94}O_{14}$	3.27	1061.65315	-0.40	+	64.89	59.53	10.21	16:2 OA	17.25	Non-visceral
$C_{62}H_{98}O_{14}$	3.72	1067.70286	-1.9	+	67.06	59.81	13.04	18:2 OA	4.88	Visceral
$C_{64}H_{96}O_{14}$	3.75	1111.66968	0.40	+	69.22	60.72	14.78	20:5 OA	16.21	Visceral
$C_{64}H_{96}O_{14}$	3.74	1111.66908	-0.13	+	69.22	62.57	10.68	20:5 OA	9.35	Non-visceral
$C_{54}H_{82}O_{14}$	2.78	953.56195	-1.28	-	58.40	62.85	14.66	OA-D10	2.57	Visceral
$C_{43}H_{66}NO_7$	3.30	708.48621	3.39	+	46.51	48.91	11.02	Spirolide D	0.69	Visceral
$C_{43}H_{66}NO_7$	3.30	708.48621	3.39	+	46.51	40.45	16.85	Spirolide D	0.39	Non-visceral
$C_{42}H_{61}NO_8$	4.09	708.44513	-2.62	+	45.43	42.07	15.86	27-OH-13-SPX C	0.26	Visceral
$C_{41}H_{58}NO_8$	4.15	693.42114	-3.43	+	44.34	44.02	16.32	27-O-13,19-SPX C	0.47	Visceral
$C_{40}H_{62}NO_6$	3.42	651.44781	-4.03	-	43.26	41.55	11.28	Spirolide I	0.34	Visceral
15 DAYS										
$C_{44}H_{68}O_{13}$	1.95	803.45921	0.61	-	47.59	41.43	11.46	OA	7.64	Visceral
$C_{44}H_{68}O_{13}$	1.93	803.46159	3.57	-	47.59	40.14	13.34	OA	3.84	Non-visceral
$C_{45}H_{70}O_{13}$	2.09	817.47662	2.78	-	48.67	50.39	15.28	DTX-1	15.61	Visceral
$C_{45}H_{70}O_{13}$	2.09	817.47408	-0.31	-	48.67	43.21	12.07	DTX-1	7.03	Non-visceral
$C_{61}H_{100}O_{14}$	3.93	1057.71688	-1.60	+	65.98	59.46	16.21	16:0 DTX1	6.45	Visceral
$C_{58}H_{94}O_{14}$	3.61	1037.65829	4.53	+	62.73	58.52	14.90	14:0 OA	13.59	Visceral
$C_{58}H_{94}O_{14}$	3.60	1037.65226	-1.27	+	62.73	59.11	15.01	14:0 OA	7.20	Non-visceral
$C_{58}H_{92}O_{14}$	3.59	1013.65582	-0.15	+	62.73	66.13	13.66	14:1 DTX-2	13.82	Visceral
$C_{58}H_{92}O_{14}$	3.57	1013.65621	-0.22	+	62.73	64.13	14.68	14:1 DTX-2	6.32	Non-visceral
$C_{58}H_{88}O_{14}$	3.15	1009.62672	2.40	+	62.73	59.22	13.51	14:3 OA	12.47	Visceral
$C_{59}H_{96}O_{14}$	3.60	1029.68708	-0.19	+	63.81	62.37	15.01	15:0 OA	13.94	Visceral
$C_{59}H_{96}O_{14}$	3.61	1029.68715	-1.12	+	63.81	64.72	14.44	15:0 OA	30.30	Non-visceral
$C_{60}H_{98}O_{14}$	3.73	1065.68776	2.70	+	64.89	60.77	14.12	16:0 OA	22.94	Visceral
$C_{60}H_{98}O_{14}$	3.71	1065.68693	1.92	+	64.89	62.77	15.96	16:0 OA	19.89	Non-visceral

Table C4.4 LMBT found in the extracts of mussels exposed to both *A. ostenfeldii* and *P. lima* (*n*=58).

$C_{60}H_{96}O_{14}$	3.55	1063.67105	1.71	+	64.89	61.63	13.26	16:1 OA	14.26	Visceral
$C_{60}H_{96}O_{14}$	3.55	1063.67103	1.69	+	64.89	60.23	14.08	16:1 OA	11.29	Non-visceral
$C_{60}H_{94}O_{14}$	3.71	1039.66895	-2.50	+	64.89	59.53	10.21	16:2 OA	12.21	Non-visceral
$C_{61}H_{98}O_{14}$	3.92	1055.70113	-1.1	+	65.98	56.24	9.74	17:1 OA	5.93	Visceral
$C_{62}H_{98}O_{14}$	3.72	1065.68774	-0.60	+	67.06	59.81	10.04	18:2 OA	7.62	Visceral
$C_{62}H_{94}O_{14}$	3.68	1063.67090	-0.68	+	67.06	64,44	15.35	18:4 OA	36.35	Visceral
$C_{62}H_{94}O_{14}$	3.65	1063.66792	-3.48	+	67.06	62.42	13.12	18:4 OA	11.99	Non-visceral
$C_{51}H_{76}O_{14}$	4.70	913.52863	-2.35	+	55.16	49.29	15.77	DTX-6	2.87	Visceral
$C_{64}H_{96}O_{14}$	3.75	1111.67010	0.78	+	69.22	60.72	14.78	20:5 OA	42.25	Visceral
$C_{64}H_{96}O_{14}$	3.76	1111.66936	0.11	+	69.22	63.72	13.82	20:5 OA	4.15	Non-visceral
$C_{66}H_{98}O_{14}$	3.33	1137.68042	-3.92	+	71.38	74.36	10.94	22:06 OA	15.07	Visceral
$C_{48}H_{79}O_{14}$	3.67	898.50439	-0.56	+	51.92	49.22	11.55	27-O-Ac DTX 1 Me	4.58	Visceral
$C_{52}H_{80}O_{14}$	2.76	929.56104	-1.11	+	56.24	52.77	15.07	OA-D8	2.82	Visceral
$C_{52}H_{80}O_{14}$	2.77	929.56117	-1.09	+	56.24	53.77	14.21	OA-D8	1.67	Non-visceral
$C_{54}H_{82}O_{14}$	2.78	953.56246	-0.8	-	58.40	62.85	13.21	OA-D10	1.24	Visceral
$C_{54}H_{82}O_{14}$	2.79	953.56227	-0.95	-	58.40	59.35	14.66	OA-D10	1.08	Non-visceral
$C_{42}H_{62}NO_{7}$	3.41	693.45764	-3.25	+	45.43	44.67	15.08	13-SPX C	2.19	Visceral
$C_{42}H_{62}NO_{7}$	3.42	693.45963	-0.38	+	45.43	43.18	16.31	13-SPX C	1.48	Non-visceral
$C_{43}H_{66}NO_{7}$	3.30	708.48554	3.04	+	46.51	48.91	13.34	Spirolide D	0.89	Visceral
$C_{43}H_{66}NO_{7}$	3.31	708.48532	2.73	+	46.51	47.85	15.36	Spirolide D	0.65	Non-visceral
$C_{42}H_{66}NO_{7}$	3.40	695.47518	-2.11	-	45.43	46.56	14.12	Spirolide F	1.56	Visceral
$C_{40}H_{60}NO_{6}$	4.83	649.43854	5.77	-	43.26	42.22	15.08	Spirolide H	1.03	Visceral
$C_{40}H_{62}NO_{6}$	3.42	651.44821	-3.42	-	43.26	41.55	14.28	Spirolide I	1.08	Visceral
$C_{42}H_{61}NO_{8}$	4.09	708.44634	-0.91	+	45.43	42.07	16.86	27-OH-13-SPX C	5.20	Visceral
$C_{42}H_{61}NO_{8}$	4.08	708.44684	-0.21	+	45.43	44.82	15.43	27-OH-13-SPX C	2.86	Non-visceral
$C_{41}H_{58}NO_{8}$	4.15	693.42114	-3.43	+	44.34	44.02	16.32	27-O-13,19-SPX C	0.73	Visceral

Table C5 OA related compounds found in the faeces and pseudofaeces of mussels after exposure to *P. lima* (n=10). The results were obtained through the ToxID software, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

3 DAYS									
Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.kg^{-1}$)
$C_{53}H_{82}O_{15}$	5.57	981.55869	4.24	+	57.32	59.87	12.55	OA-T9	6.12
5 DAYS									
$C_{44}H_{68}O_{13}$	2.04	803.460820	2.61	-	47.59	45.85	16.75	OA	0.47
$C_{60}H_{94}O_{14}$	3.30	1061.65745	3.64	-	64.89	66.81	12.01	16:2 OA	0.37
$C_{43}H_{66}O_{11}$	4.43	781.453610	5,34	+	46.51	48.54	15.13	OA-C3	0.34
$C_{53}H_{82}O_{15}$	5.58	981.55878	4.26	+	57.32	59.36	14.12	OA-T9	37.23
10 DAYS									
$C_{44}H_{68}O_{13}$	2.04	803.46078	2.56	-	47.59	44.31	11.37	OA	0.79
$C_{60}H_{94}O_{14}$	3.29	1061.65775	3.92	-	64.89	60.64	14.34	16:2 OA	0.51
$C_{43}H_{66}O_{11}$	4.44	781.44867	1.35	+	46.51	44.50	12.01	OA-C3	0.34
$C_{53}H_{82}O_{15}$	5.58	981.55750	2.96	+	57.32	59.33	12.03	OA-T9	41.24
15 DAYS									
$C_{44}H_{68}O_{13}$	2.04	803.46228	4.43	-	47.59	45.65	14.13	OA	1.58
$C_{45}H_{70}O_{13}$	2.07	841.47180	1.11	+	48.67	51.39	13.31	DTX-1	0.36
$C_{60}H_{94}O_{14}$	3.31	1061.65661	2.85	-	64.89	63.75	11.23	16:2 OA	0.49
$C_{43}H_{66}O_{11}$	4.46	781.45282	1.35	+	46.51	44.50	12.01	OA-C3	1.23
$C_{52}H_{80}O_{14}$	3.45	951.54547	1.51	+	56.24	57.48	12.02	OA-D8	0.55
$C_{53}H_{82}O_{15}$	5.58	981.55847	3.95	+	57.32	58.33	12.03	OA-T9	22.89

Table C6 SPX related compounds found in the faeces and pseudofaeces of mussels after exposure to *A. ostenfeldii* (n=10). The results were obtained through ToxID, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

3 DAYS									
Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.kg^{-1}$)
$C_{42}H_{62}NO_7$	3.40	693.45852	-1.83	+	45.43	44.29	11.33	13-SPX C	0.11
$C_{43}H_{65}NO_7$	3.30	709.49940	1.21	+	46.51	45.16	11.83	Spirolide D	1.25
5 DAYS									
$C_{42}H_{62}NO_7$	3.42	693.45862	-1.84	+	45.43	44.18	11.31	13-SPX C	0.22
$C_{43}H_{65}NO_7$	3.29	708.48031	1.26	+	46.51	44.67	12.01	Spirolide D	1.45
$C_{42}H_{66}NO_7$	5.14	695.47636	-4.34	-	45.43	46.14	11.28	Spirolide F	0.15
$C_{40}H_{60}NO_6$	4.88	649.43256	-3.43	-	43.26	41.72	12, 56	Spirolide H	0.26
$C_{41}H_{58}NO_8$	2.01	710.45325	4.47	+	44.34	43.56	11. 31	27-O-13,19-SPX C	1.03
10 DAYS									
$C_{42}H_{62}NO_7$	3.41	693.45880	-1.58	+	45.43	43.91	12.08	13-SPX C	0.19
$C_{43}H_{65}NO_7$	3.30	708.48017	-4.53	+	46.51	45.59	10.12	Spirolide D	1.72
$C_{42}H_{66}NO_7$	5.14	695.47382	-4.06	-	45.43	43.93	12.64	Spirolide F	0.10
$C_{40}H_{60}NO_6$	3.42	651.44849	-1.3	+	43.26	42.29	11.15	Spirolide H	0.18
$C_{42}H_{61}NO_8$	4.09	708.44513	-2.62	+	45.43	42.07	12.86	27-OH-13-SPX C	0.11
$C_{41}H_{58}NO_8$	4.31	715.40631	1.18	+	44.34	42.04	12.38	27-O-13,19-SPX C	0.25
15 DAYS									
$C_{42}H_{62}NO_7$	3.40	693.45654	-4.84	+	45.43	44.19	10.32	13-SPX C	0.24
$C_{42}H_{64}NO_7$	5.56	717.45477	-3.80	+	45.43	42.95	13.35	Spirolide B	0.41
$C_{43}H_{64}NO_7$	4.94	707.47382	-2.44	+	46.51	43.73	12.22	Spirolide C	0.16
$C_{43}H_{65}NO_7$	3.30	708.48475	1.93	+	46.51	47.26	11.71	Spirolide D	0.62
$C_{42}H_{64}NO_8$	5.82	728.49719	0.23	+	45.43	46.69	11.31	Spirolide E	0.11
$C_{42}H_{61}NO_8$	4.11	708.44598	-1.42	+	45.43	44.31	12.18	27-OH-13-SPX C	0.13
$C_{41}H_{58}NO_8$	4.32	715.40802	3.57	+	44.34	44.39	10.01	27-O-13,19-SPX C	0.13

Table C7.1 LMBT found in the faeces and pseudofaeces of mussels after exposure to *A. ostenfeldii* and *P. lima* (n=10). The results were obtained through ToxID, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.kg^{-1}$)
3 DAYS									
$C_{44}H_{68}O_{13}$	1.90	803.46195	4.02	-	47.59	46.25	11.33	OA	1.04
$C_{45}H_{70}O_{13}$	2.04	817.47813	4.59	-	48.67	47.53	11.14	DTX-1	2.03
$C_{53}H_{82}O_{15}$	5.06	981.55711	2.56	+	57.32	57.87	10.55	OA-T9	11.04
$C_{42}H_{62}NO_7$	3.41	693.45775	-3.1	+	45.43	43.59	12.11	13-SPX C	1.10
$C_{43}H_{65}NO_7$	3.33	708.48513	2.47	+	46.51	46.11	10.42	Spirolide D	1.61
5 DAYS									
$C_{44}H_{68}O_{13}$	1.89	803.46228	4.43	-	47.59	46.48	11.12	OA	3.41
$C_{45}H_{70}O_{13}$	2.06	817.47729	3.57	-	48.67	48.03	10.59	DTX-1	7.23
$C_{60}H_{94}O_{14}$	5.88	1037.65906	1.91	-	64.89	62.64	14.34	16:2 OA	0.36
$C_{43}H_{66}O_{11}$	5.79	759.46436	-4.51	+	46.51	42.99	13.44	OA-C3	0.35
$C_{52}H_{80}O_{14}$	5.15	927.55194	4.75	-	56.24	55.49	11.22	OA-D8	0.23
$C_{53}H_{82}O_{15}$	5.07	981.55798	3.45	+	57.32	59.12	12.23	OA-T9	58.74
$C_{42}H_{62}NO_7$	3.42	693.45807	-2.63	+	45.43	43.59	12.11	13-SPX C	1.25
$C_{42}H_{64}NO_7$	2.35	716.45184	-3.02	+	45.43	44.77	11.33	Spirolide B	0.10
$C_{43}H_{64}NO_7$	5.26	707.47260	-4.16	+	46.51	43.11	12.41	Spirolide C	0.15
$C_{43}H_{65}NO_7$	3.31	708.48639	4.24	+	46.51	46.11	10.42	Spirolide D	1.37
$C_{42}H_{66}NO_7$	5.14	695.47394	-4.34	-	45.43	46.33	11.12	Spirolide F	0.24
$C_{40}H_{60}NO_6$	4.83	649.43158	-4.94	-	43.26	42.22	12.08	Spirolide H	0.36
$C_{40}H_{62}NO_6$	3.42	651.44781	-4.03	-	43.26	41.55	12.28	Spirolide I	0.28
10 DAYS									
$C_{42}H_{62}NO_7$	3.41	693.45880	-1.58	+	45.43	43.91	12.08	13-SPX C	0.19
$C_{43}H_{65}NO_7$	3.30	708.48017	-4.53	+	46.51	45.59	10.12	Spirolide D	1.72

Table C7.2 LMBT found in the faeces and pseudofaeces of mussels after exposure to *A. ostenfeldii* and *P. lima* (n=10).

$C_{60}H_{94}O_{14}$	5.88	1037.65857	1.43	-	64.89	62.13	12.34	16:2 OA	0.58
$C_{61}H_{100}O_{14}$	5.91	1074.74084	-3.99	+	65.98	66.77	11.21	16:0 DTX1	1.70
$C_{43}H_{66}NO_{11}$	4.41	781.45184	2.70	+	46.51	46.48	10.40	OA-C3	1.59
$C_{53}H_{82}O_{14}$	4.68	941.55823	-5.25	-	57.32	56.88	11.55	OA-D9	0.90
$C_{52}H_{80}O_{14}$	3.44	951.54437	0.35	+	56.24	56.88	10.64	OA-D8	0.27
$C_{53}H_{82}O_{15}$	5.07	957.56036	2.37	-	57.32	58.87	11.56	OA-T9	9.88
$C_{42}H_{62}NO_{7}$	3.40	693.45868	-1.75	+	45.43	44.10	11.33	13-SPX C	1.16
$C_{43}H_{64}NO_{7}$	5.25	707.47571	0.22	+	46.51	46.13	10.38	Spirolide C	1.16
$C_{43}H_{66}NO_{7}$	3.30	709.49060	-0.84	+	46.51	45.08	11.42	Spirolide D	1,84
$C_{42}H_{66}NO_{7}$	3.40	695.47388	-3.98	-	45.43	46.56	11.12	Spirolide F	0,23
$C_{40}H_{60}NO_{6}$	4.85	649.43231	-3.81	-	43.26	42.33	11.09	Spirolide H	0.16
$C_{40}H_{62}NO_{6}$	3.42	651.44910	-2.05	-	43.26	42.15	10.11	Spirolide I	0.47
$C_{41}H_{58}NO_{8}$	4.15	693.42114	-3.43	+	44.34	44.02	10.32	27-O-13,19-SPX C	0.14
$C_{42}H_{62}NO_{8}$	4.35	707.43707	-4.52	-	45.43	44.95	10.61	27-OH-13-SPX C	0.15
15 DAYS									
$C_{44}H_{68}O_{13}$	1.89	803.46173	3.74	-	47.59	46.66	11.17	OA	1.28
$C_{45}H_{70}O_{13}$	2.06	817.47778	4.17	-	48.67	47.77	11.51	DTX-1	4.37
$C_{61}H_{100}O_{14}$	5.87	1074.74133	-3.53	+	65.98	62,03	13,91	16:0 DTX1	1.46
$C_{58}H_{88}O_{14}$	5.07	1007.61395	3.79	-	62.73	63.89	11.16	14:3 OA	0.36
$C_{60}H_{94}O_{14}$	5.89	1037.65942	2.25	-	64.89	61.84	13.05	16:2 OA	0.25
$C_{43}H_{66}NO_{11}$	4.46	781.44958	-1.91	+	46.51	46.28	10.30	OA-C3	0.89
$C_{53}H_{82}O_{15}$	5.08	981.55603	1.46	+	57.32	57.56	10.24	OA-T9	76.73
$C_{52}H_{80}O_{14}$	3.44	951.54498	0.99	+	56.24	55.13	11.11	OA-D8	0.23
$C_{42}H_{62}NO_{7}$	3.40	693.45721	-3.87	+	45.43	44.79	11.36	13-SPX C	1.83
$C_{43}H_{64}NO_{7}$	5.25	707.47241	-4.40	+	46.51	44.82	12.31	Spirolide C	0.79
$C_{43}H_{66}NO_{7}$	3.30	708.48621	3.39	+	46.51	48.91	12.02	Spirolide D	1.77
$C_{42}H_{66}NO_{7}$	3.38	695.47424	-3.46	-	45.43	45.78	10.35	Spirolide F	0.79
$C_{40}H_{60}NO_{6}$	3.40	651.44836	1.50	+	43.26	42.76	11,50	Spirolide H	0.93
$C_{41}H_{58}NO_{8}$	1.99	710.453	4.12	+	44.34	41.49	13.04	27-O-13,19-SPX C	1.73

Table C8 OA related compounds in the seawater of mussels exposed to *P. lima* (n=10). The results were obtained through the ToxID software program, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

3 DAYS									
Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.l^{-1}$)
$C_{44}H_{68}O_{13}$	1.89	803.46013	1.75	-	47.59	49.36	13.75	OA	1.64
$C_{53}H_{82}O_{15}$	5.08	981.55653	1.97	+	57.32	59.33	12.03	OA-T9	3.66
5 DAYS									
$C_{44}H_{68}O_{13}$	1.89	803.45745	-1.58	-	47.59	49.36	13.75	OA	1.78
$C_{53}H_{82}O_{15}$	5.08	981.55551	0.93	+	57.32	59.33	12.03	OA-T9	2.76
$C_{50}H_{76}O_{14}$	2.95	923.51542	2.89	+	54.08	52.98	12.68	OA-D6	1.12
10 DAYS									
$C_{44}H_{68}O_{13}$	1.89	803.45745	-1.58	-	47.59	49.36	13.75	OA	3.35
$C_{45}H_{70}O_{13}$	2.14	817.47267	-2.04	-	48.67	49.58	12.31	DTX-1	1.43
$C_{53}H_{82}O_{15}$	5.08	981.55551	0.93	+	57.32	59.33	12.03	OA-T9	4.20
$C_{50}H_{76}O_{14}$	2.95	923.51542	2.89	+	54.08	52.98	12.68	OA-D6	1.52
15 DAYS									
$C_{44}H_{68}O_{13}$	2.04	803.46115	3.02	-	47.59	46.85	16.75	OA	5.15
$C_{45}H_{70}O_{13}$	2.14	817.47633	2.43	-	48.67	49.58	12.31	DTX-1	2.83
$C_{53}H_{82}O_{15}$	5.08	981.55492	0.33	+	57.32	59.33	12.03	OA-T9	5.28
$C_{43}H_{66}O_{11}$	4.46	781.45236	3.36	+	46.51	44.50	12.01	OA-C3	0.53
$C_{50}H_{76}O_{14}$	2.95	923.51503	2.46	+	54.08	52.98	12.68	OA-D6	3.22

Table C9 SPX related compounds in the seawater of mussels exposed to *A. ostenfeldii* (n=10). The results were obtained through the ToxID software program, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

3 DAYS									
Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.l^{-1}$)
NA	-	-	-	-	-	-	-	-	-
5 DAYS									
$C_{42}H_{62}NO_7$	3.42	693.45862	-1.84	+	45.43	44.18	11.31	13-SPX C	0.91
$C_{43}H_{66}NO_7$	3.29	708.48671	4.70	+	46.51	44.67	12.01	SPX D	1.89
10 DAYS									
$C_{42}H_{62}NO_7$	3.42	693.45880	-1.58	+	45.43	43.91	12.08	13-SPX C	1.22
$C_{43}H_{66}NO_7$	3.30	708.48075	-3.71	+	46.51	45.09	11.12	SPX D	1.96
$C_{42}H_{61}NO_8$	4.09	708.44513	-2.62	+	45.43	42.07	12.86	27-OH-13-SPX C	0.69
$C_{41}H_{58}NO_8$	4.31	715.40631	1.18	+	44.34	42.04	12.38	27-O-13,19-SPX C	0.84
15 DAYS									
$C_{42}H_{62}NO_7$	3.41	693.45654	-4.84	+	45.43	44.19	11.32	13-SPX C	1.76
$C_{43}H_{66}NO_7$	3.30	708.48551	3.00	+	46.51	47.26	11.71	SPX D	1.88
$C_{42}H_{64}NO_8$	5.82	728.49719	0.23	+	45.43	46.69	11.31	SPX E	0.11
$C_{42}H_{61}NO_8$	4.11	708.44598	-1.42	+	45.43	44.31	12.18	27-OH-13-SPX C	0.60
$C_{41}H_{58}NO_8$	4.32	715.40802	3.57	+	44.34	44.39	10.01	27-O-13,19-SPX C	0.71

Table C10.1 LMBT in the seawater of mussels exposed to both *A. ostenfeldii* and *P. lima* (n=10). The results were obtained through the ToxID software program, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	Variation (SD)	Tentative identity	Mean estimate ($\mu g.l^{-1}$)
3 DAYS									
$C_{44}H_{68}O_{13}$	1.89	803.46006	1.66	-	47.59	48.54	14.27	OA	0.74
$C_{45}H_{70}O_{13}$	2.04	817.47721	3.51	-	48.67	45.83	10.11	DTX-1	1.13
$C_{53}H_{82}O_{15}$	5.08	981.55612	1.55	+	57.32	53.33	12.39	OA-T9	2.15
$C_{42}H_{62}NO_7$	3.41	693.46128	1.99	+	45.43	44.17	11.31	13-SPX C	1.13
$C_{43}H_{65}NO_7$	3.29	708.48108	-3.24	+	46.51	43.42	8.35	SPX D	1.06
5 DAYS									
$C_{44}H_{68}O_{13}$	1.89	803.45993	1.50	-	47.59	45.36	11.5	OA	1.22
$C_{53}H_{82}O_{15}$	5.08	981.55421	-0.38	+	57.32	54.71	10.83	OA-T9	2.63
$C_{50}H_{76}O_{14}$	2.95	923.51472	2.15	+	54.08	50.24	7.53	OA-D6	1.29
$C_{60}H_{94}O_{14}$	3.29	1061.65616	2.43	-	64.89	63.64	12.31	16:2 OA	0.51
$C_{42}H_{62}NO_7$	3.42	693.45745	-3.53	+	45.43	42.18	8.53	13-SPX C	1.56
$C_{43}H_{66}NO_7$	3.29	708.48124	-3.02	+	46.51	44.31	10.01	SPX D	1.04
10 DAYS									
$C_{44}H_{68}O_{13}$	2.04	803.45802	-0.87	-	47.59	42.37	13.03	OA	4.22
$C_{45}H_{70}O_{13}$	2.14	817.47407	-0.33	-	48.67	45.45	6.53	DTX-1	4.57
$C_{53}H_{82}O_{15}$	5.08	981.55403	-0.57	+	57.32	54.33	11.57	OA-T9	4.20
$C_{50}H_{76}O_{14}$	2.95	923.51442	1.80	+	54.08	50.23	9.62	OA-D6	1.16
$C_{42}H_{62}NO_7$	3.41	693.45620	-5.33	+	45.43	42.47	14.62	13-SPX C	1.25
$C_{43}H_{66}NO_7$	3.30	708.48471	1.87	+	46.51	44.42	15.11	Spirolide D	1.36
$C_{42}H_{61}NO_8$	4.09	708.44732	0.46	+	45.43	42.07	8.48	27-OH-13-SPX C	3.45
$C_{41}H_{58}NO_8$	4.31	715.40523	-0.32	+	44.34	41.55	11. 08	27-O-13,19-SPX C	1.77

Table C10.2 LMBT in the seawater of mussels exposed to both *A. ostenfeldii* and *P. lima* (n=10).

15 DAYS									
$C_{44}H_{68}O_{13}$	2.04	803.46126	3.16	-	47.59	45.85	15.75	OA	4.32
$C_{45}H_{70}O_{13}$	2.14	817.47531	1.18	-	48.67	49.58	12.31	DTX-1	3.15
$C_{53}H_{82}O_{15}$	5.08	981.55548	0.90	+	57.32	59.33	12.03	OA-T9	5.82
$C_{43}H_{66}O_{11}$	4.46	781.45153	2.30	+	46.51	44.50	12.01	OA-C3	1.50
$C_{50}H_{76}O_{14}$	2.95	923.51429	1.66	+	54.08	52.98	12.68	OA-D6	2.35
$C_{42}H_{62}NO_7$	3.40	693.45740	-3.60	+	45.43	43.12	14.16	13-SPX C	3.58
$C_{43}H_{66}NO_7$	3.30	708.48241	-1.36	+	46.51	42.11	7.38	SPX D	2.37
$C_{42}H_{64}NO_8$	5.80	728.49686	-0.21	+	45.43	42.16	8.11	SPX E	0.97
$C_{42}H_{61}NO_8$	4.12	708.44683	-0.22	+	45.43	42.13	11.36	27-OH-13-SPX C	3.76
$C_{41}H_{58}NO_8$	4.31	715.40772	3.15	+	44.34	41.69	13. 56	27-O-13,19-SPX C	3.84

Table C11.1 LMBT detected in field exposed mussels (n=160). The results were obtained through the ToxID software program, using a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The identity of the compounds was confirmed using the $^{13}C/^{12}C$ isotopic ion ratio, according to the criteria described in CD 2002/657/EC (2002).

Station	Elemental composition	RT (min)	Measured mass (*m/z*)	Error (%)	Ion mode	Theoretical isotope ratio	Observed isotope ratio	SD	Tentative identity	Mean estimate ($\mu g.kg^{-1}$)	Tissue
3 DAYS											
All	NA	-	-	-	-	-	-	-	-	-	-
5 DAYS											
1	$C_{58}H_{92}O_{14}$	3.65	1013.65390	-1.99	+	62.73	63.22	13.74	14:1 DTX-2	5.98	Visceral
2	$C_{58}H_{94}O_{14}$	3.61	1037.65107	-2.41	+	62.73	58.52	4.90	14:0 OA	1.34	Visceral
3	$C_{61}H_{100}O_{14}$	3.93	1057.71902	0.41	+	65.98	64.46	15.63	16:0 DTX1	1.56	Visceral
	$C_{58}H_{94}O_{14}$	3.61	1037.65097	-2.51	+	62.73	61.52	14.81	14:0 OA	10.56	Visceral
4	$C_{60}H_{94}O_{14}$	3.29	1061.65547	1.78	+	64.89	65.21	13.45	16:2 OA	4.08	Visceral
	$C_{43}H_{66}NO_7$	3.30	708.48409	0.87	+	46.51	47.91	12.63	Spirolide D	1.15	Visceral
10 DAYS											
1	$C_{45}H_{70}O_{13}$	2.09	817.47544	1.29	-	48.67	47.65	11.13	DTX-1	1.82	Visceral
	$C_{58}H_{92}O_{14}$	3.65	1013.65154	-4.38	+	62.73	63.48	13.21	14:1 DTX-2	24.10	Visceral
	$C_{58}H_{94}O_{14}$	3.61	1037.65084	-2.64	+	62.73	62.25	14.34	14:0 OA	3.36	Visceral
	$C_{47}H_{70}O_{15}$	3.55	873.46294	-1.43	+	50.83	51.23	12.66	PTX-2	1.06	Visceral
2	$C_{58}H_{94}O_{14}$	3.61	1037.65085	-2.63	+	62.73	58.52	4.90	14:0 OA	4.86	Visceral
	$C_{58}H_{88}O_{14}$	3.15	1009.62321	-1.06	+	62.73	59.22	3.51	14:3 OA	2.51	Visceral
	$C_{60}H_{94}O_{14}$	3.28	1061.65588	2.16	+	64.89	57.44	7.45	16:2 OA	1.71	Visceral
	$C_{47}H_{70}O_{15}$	3.55	873.46200	-2.5	+	50.83	56.83	6.08	PTX-2	1.62	Visceral
3	$C_{61}H_{100}O_{14}$	3.93	1057.71822	-0.34	+	65.98	59.46	6.21	16:0 DTX1	1.17	Visceral
	$C_{58}H_{94}O_{14}$	3.61	1037.65113	-2.36	+	62.73	58.52	4.90	14:0 OA	26.78	Visceral
	$C_{58}H_{88}O_{14}$	3.14	1009.62263	-1.64	+	62.73	59.03	4.22	14:3 OA	5.92	Visceral
	$C_{61}H_{98}O_{14}$	3.92	1055.70166	-1.2	+	65.98	56.24	9.74	17:1 OA	5.41	Visceral
	$C_{62}H_{98}O_{14}$	3.72	1067.70298	-1.7	+	67.06	59.81	7.04	18:2 OA	8.02	Visceral
	$C_{43}H_{66}NO_7$	3.30	708.48581	3.42	+	46.51	48.91	2.02	Spirolide D	2.06	Visceral

Table C11.2 LMBT detected in field exposed mussels (n=160).

4	$C_{59}H_{96}O_{14}$	3.61	1029.68771	0.41	+	63.81	62.37	14.28	15:0 OA	3.87	Visceral
	$C_{60}H_{96}O_{14}$	3.55	1063.67190	2.51	+	64.89	63.63	13.56	16:1 OA	4.22	Visceral
	$C_{60}H_{94}O_{14}$	3.28	1061.65682	3.05	+	64.89	62.11	14.09	16:2 OA	9.82	Visceral
	$C_{62}H_{100}O_{14}$	3.95	1069.71987	1.24	+	67.06	68.53	11.88	18:1 OA	1.85	Visceral
	$C_{62}H_{98}O_{14}$	3.72	1067.70164	-1,20	+	67.06	66.55	12.38	18:2 OA	4.28	Visceral
	$C_{64}H_{96}O_{14}$	3.75	1111.66971	0.43	+	69.22	68.23	14.28	20:5 OA	2.60	Visceral
	$C_{43}H_{66}NO_7$	3.30	708.48511	2.44	+	46.51	47.46	12.02	Spirolide D	1.32	Visceral
	$C_{40}H_{60}NO_6$	4.83	649.43358	-1,86	-	43.26	42.22	12.08	Spirolide H	1.09	Visceral
	$C_{42}H_{61}NO_8$	4.09	708.44893	2.73	+	45.43	44.08	15.86	27-OH-13-SPX C	1.54	Visceral
	15 DAYS										
1	$C_{45}H_{70}O_{13}$	2.09	817.47782	4.25	-	48.67	48.90	10.28	DTX-1	2.57	Visceral
	$C_{58}H_{92}O_{14}$	3.65	1013.65158	-4.34	+	62.73	63.91	13.66	14:1 DTX-2	76.91	Visceral
	$C_{58}H_{92}O_{14}$	3.64	1013.65380	0.21	+	62.73	61.87	15.29	14:1 DTX-2	10.07	Non-Visceral
	$C_{58}H_{94}O_{14}$	3.61	1037.65272	-0.82	+	62.73	63.52	14.90	14:0 OA	10.76	Visceral
	$C_{58}H_{88}O_{14}$	3.15	1009.62448	0.18	+	62.73	61.22	13.22	14:3 OA	3.54	Visceral
	$C_{59}H_{96}O_{14}$	3.61	1029.68532	-1.90	+	63.81	62.37	11.73	15:0 OA	11.39	Visceral
	$C_{60}H_{98}O_{14}$	3.73	1065.68210	-2,60	+	64.89	63.26	14.88	16:0 OA	4.78	Visceral
	$C_{60}H_{94}O_{14}$	3.28	1061.65485	1.19	+	64.89	57.44	10.64	16:2 OA	9.65	Visceral
	$C_{62}H_{100}O_{14}$	3.95	1069.71951	1.26	+	67.06	66.56	14.95	18:1 OA	2.86	Visceral
	$C_{62}H_{94}O_{14}$	3.68	1063.6783	6.27	+	67.06	65,83	11.62	18:4 OA	4.37	Visceral
	$C_{47}H_{70}O_{15}$	3.55	873.46429	0.11	-	50.83	51.65	14.85	PTX-2	2.41	Visceral
	$C_{42}H_{64}NO_7$	2.35	716.45214	-2.92	+	45.43	45.97	11.10	Spirolide B	1.40	Visceral
	$C_{43}H_{66}NO_7$	3.30	708.48517	2.52	+	46.51	45.12	15.36	Spirolide D	1.89	Visceral
	$C_{42}H_{66}NO_7$	3.40	695.47457	-2.99	-	45.43	44.26	13.74	Spirolide F	1.96	Visceral
	$C_{40}H_{60}NO_6$	4.83	651.44836	1.50	-	43.26	43.71	10.32	Spirolide H	4.22	Visceral
	$C_{41}H_{58}NO_8$	4.15	693.42114	-3.43	+	44.34	42.68	15.32	27-O-13,19-SPX C	3.05	Visceral

Table C11.3 LMBT detected in field exposed mussels (n=160).

	15 DAYS										
2	$C_{58}H_{94}O_{14}$	3.61	1037.65193	-1.59	+	62.73	58.52	4.90	14:0 OA	10.76	Visceral
	$C_{58}H_{88}O_{14}$	3.15	1009.62516	0.86	+	62.73	59.22	3.51	14:3 OA	3.83	Visceral
	$C_{60}H_{94}O_{14}$	3.28	1061.65561	1.91	+	64.89	57.44	7.45	16:2 OA	3.11	Visceral
	$C_{52}H_{80}O_{14}$	2.76	929.56282	0.79	+	56.24	52.77	9.21	OA-D8	3.40	Visceral
	$C_{47}H_{70}O_{15}$	3.55	873.46531	1.28	+	50.83	56.83	6.08	PTX-2	1.88	Visceral
	$C_{40}H_{60}NO_6$	4.83	649.43396	-1.27	-	43.26	42.22	2.08	Spirolide H	1.21	Visceral
	$C_{43}H_{66}NO_7$	3.30	708.48611	3.85	+	46.51	48.91	2.02	Spirolide D	1.14	Visceral
3	$C_{61}H_{100}O_{14}$	3.93	1057.71836	-0.20	+	65.98	64.15	13.16	16:0 DTX1	1.17	Visceral
	$C_{58}H_{94}O_{14}$	3.61	1037.65088	-2.60	+	62.73	61.89	13.81	14:0 OA	26.78	Visceral
	$C_{58}H_{94}O_{14}$	3.60	1037.65124	-2.25	+	62.73	60.84	11.53	14:0 OA	5.37	Non-visceral
	$C_{58}H_{88}O_{14}$	3.14	1009.62271	-1.56	+	62.73	60.02	14.74	14:3 OA	5.92	Visceral
	$C_{61}H_{98}O_{14}$	3.92	1055.70121	-1.05	+	65.98	65.27	9.74	17:1 OA	5.41	Visceral
	$C_{62}H_{98}O_{14}$	3.72	1067.70251	-1.67	+	67.06	66.46	11.04	18:2 OA	8.02	Visceral
	$C_{52}H_{80}O_{14}$	2.76	929.561870	-0.22	+	56.24	54.18	9.21	OA-D8	2.48	Visceral
	$C_{47}H_{70}O_{15}$	3.55	873.46501	0.93	+	50.83	52.57	16.14	PTX-2	4.53	Visceral
	$C_{47}H_{72}O_{15}$	4.52	894.52166	0.93	+	50.83	51.27	12.23	PTXsa 1	2.34	Visceral
	$C_{47}H_{69}NO_{14}$	4.77	872.48012	1.19	+	50.83	50.25	11.12	AZA 16/17	3.31	Visceral
	$C_{53}H_{82}O_{15}$	5.08	981.55751	2.9	+	57.32	58.18	12.83	OA-T9	2.73	Visceral
	$C_{42}H_{64}NO_7$	2.35	716.45103	0.36	+	45.43	44.28	11.57	Spirolide B	1.39	Visceral
	$C_{43}H_{66}NO_7$	3.30	708.48601	3.71	+	46.51	48.14	15.82	Spirolide D	2.06	Visceral
	$C_{42}H_{64}NO_8$	5.82	728.49691	-0.15	+	45.43	46.06	11.51	Spirolide E	1.87	Visceral
	$C_{40}H_{60}NO_6$	4.83	649.43337	-2.18	-	43.26	42.10	12.08	Spirolide H	2.85	Visceral
	$C_{40}H_{62}NO_6$	3.42	651.44721	-4.95	-	43.26	41.54	12.47	Spirolide I	1.08	Visceral
	$C_{42}H_{61}NO_8$	4.09	708.44417	-3.98	+	45.43	43.97	11.39	27-OH-13-SPX C	1.95	Visceral
	$C_{41}H_{58}NO_8$	1.99	710.45321	4.41	+	44.34	43.16	13.14	27-O-13,19-SPX C	3.75	Visceral

Table C11.4 LMBT detected in field exposed mussels (n=160).

	15 DAYS										
4	$C_{58}H_{88}O_{14}$	3.14	1009.62498	0.68	+	62.73	61.47	14.22	14:3 OA	3.69	Visceral
	$C_{59}H_{96}O_{14}$	3.61	1029.68796	0.66	+	63.81	62.37	11.44	15:0 OA	12.40	Visceral
	$C_{60}H_{96}O_{14}$	3.55	1063.67103	1.69	+	64.89	63.76	13.26	16:1 OA	11.37	Visceral
	$C_{60}H_{94}O_{14}$	3.28	1061.65653	2.77	+	64.89	62.44	15.45	16:2 OA	26.92	Visceral
	$C_{60}H_{94}O_{14}$	3.29	1061.65582	2.10	+	64.89	62.76	12.21	16:2 OA	4.78	Non-visceral
	$C_{62}H_{100}O_{14}$	3.95	1069.71971	1.13	+	67.06	64.77	14.95	18:1 OA	5.21	Visceral
	$C_{62}H_{98}O_{14}$	3.72	1067.70313	2.27	+	67.06	64.81	16.04	18:2 OA	5.01	Visceral
	$C_{62}H_{94}O_{14}$	3.68	1063.67130	-0.31	+	67.06	65,44	12.62	18:4 OA	3.12	Visceral
	$C_{64}H_{96}O_{14}$	3.75	1111.66971	0.43	+	69.22	65.72	14.78	20:5 OA	4.15	Visceral
	$C_{64}H_{96}O_{14}$	3.75	1111.66971	0.43	+	69.22	65.72	14.78	20:5 OA	4.15	Visceral
	$C_{52}H_{80}O_{14}$	2.76	929.56171	-0.39	+	56.24	53.77	15.21	OA-D8	2.95	Visceral
	$C_{54}H_{82}O_{14}$	2.78	953.56522	2.31	-	58.40	60.85	14.66	OA-D10	2.54	Visceral
	$C_{47}H_{70}O_{15}$	3.55	873.46501	0.93	+	50.83	53.83	16.08	PTX-2	2.18	Visceral
	$C_{47}H_{69}NO_{14}$	4.76	872.48007	1.11	+	50.83	49.74	10.85	AZA 16/17	2.81	Visceral
	$C_{42}H_{64}NO_{7}$	2.35	716.45227	2.09	+	45,43	44,77	11.33	Spirolide B	1.76	Visceral
	$C_{43}H_{66}NO_{7}$	3.30	708.48442	1.46	+	46.51	48.91	12.02	Spirolide D	1.58	Visceral
	$C_{40}H_{60}NO_{6}$	4.83	649.43533	0.83	-	43.26	42.22	12.08	Spirolide H	1.23	Visceral
	$C_{42}H_{61}NO_{8}$	4.09	708.44782	1.17	+	45.43	42.07	12.86	27-OH-13-SPX C	2.22	Visceral
	$C_{41}H_{58}NO_{8}$	1.99	710.45301	4.13	+	44.34	41.49	13.04	27-O-13,19-SPX C	1.19	Visceral