EXTREME EVENTS IN THE MARINE ENVIRONMENT:

The role of species-specific traits and adaptive strategies in harmful dinoflagellate bloom formation

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ICBM

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1. GENERAL INTRODUCTION

1.1 Harmful Algal Blooms

Phytoplankton blooms represent a natural seasonal phenomenon in marine ecosystems around the world and provide the basis of the food web for various aquatic life forms (Assmy and Smetacek, 2009). However, some blooms of particular algal species are classified as extreme events, exhibiting an unpredictable temporal and spatial distribution (Smayda and Reynolds, 2003). Such blooms may negatively affect components of aquatic ecosystems, human health, and socioeconomic interests (Anderson *et al.*, 2012b) and are thus often referred to as Harmful Algal Blooms (HABs). Various coastal ecosystems worldwide are affected by recurrent HABs, i.e. the formation and degradation of high algal biomass that can cause low-oxygen conditions in embayments and harbours and lead to fish kills or deaths of other marine fauna (Hallegraeff, 2003). However, HABs are not necessarily associated with high biomass. Moderate or low algal cell abundances of HAB species (~ a few hundred to thousands of cells/L as opposed to millions of cells/L presenting high biomass blooms) can also have adverse effects due to the production of a wide spectrum of potent toxins (Davidson *et al.*, 2011; Glibert *et al.*, 2005; Kudela *et al.*, 2015). These toxins can accumulate within marine food webs (Sunda *et al.*, 2006) and contaminate seafood, thus posing a significant public health threat (Anderson *et al.*, 2012a; Smayda, 1997a). The annual economic losses caused by these HABs are assumed to be approximately 95 million US$ in the USA and more than 850 million US$ in Europe (Bernard *et al.*, 2014).

Box 1:

Harmful algal blooms (HABs)

Proliferation of planktonic algae that have a negative effect on the ecosystem, implying major environmental and human health impacts and potentially causing severe economic losses to aquaculture, fisheries and tourism operations (Hallegraeff, 2003). In this thesis the term HABs will be used in its most general sense. It will refer to blooms of toxic and non-toxic algae that discolour the water, as well as to blooms which are not dense enough to change the water colour, but which are harmful due to the algal toxins they produce.

Microalgal species causing HABs cover a broad range of phylogenetic groups. More than 100 taxa are listed in the IOC-

UNESCO Taxonomic Reference List of Harmful Microalgae including dinoflagellates, diatoms, raphidophytes and cyanobacteria (Moestrup *et al.*, 2009 onwards). There is a general scientific consensus that HABs have globally increased in frequency, magnitude and geographic extent over the past few decades (Anderson, 1989; Anderson *et al.*, 2012b; Burkholder, 1998; Glibert *et al.*, 2005; Glibert and Burkholder, 2006; Hallegraeff, 1993). Some of this apparent increase may be a result of increased scientific awareness combined with increased monitoring of HABs in certain areas rather than an actual increase in bloom occurrence. However, in many well documented cases, coastal eutrophication and the translocation of non-indigenous species via ship ballast water or shellfish stocks to regions where they were previously unknown can be linked to the occurrence of blooms (e.g. Anderson *et al.*, 2008, 2002; Hallegraeff, 1998; Smayda, 2007). For example, the toxic dinoflagellate species *Gymnodinium catenatum*, *Alexandrium catenella* and *Alexandrium minutum* were apparently introduced to Australia only within the last 10-20 years (Hallegraeff, 1998), while no such blooms were documented there before this time. It has also been hypothesized that climate change induced alterations in temperature, stratification, light, ocean acidification, precipitation-induced nutrient inputs, alteration of ocean currents, and the intensification or weakening of nutrient upwelling may increase future harmful algal blooms in frequency and severity (Anderson *et al.*, 2012b; Hallegraeff, 2010; Wells *et al.*, 2015). A range of changes can be expected probably benefiting HAB species, such as earlier timing of peak production of some phytoplankton, potentially resulting in secondary effects for marine food webs, notably when zooplankton and fish grazers are differentially impacted (match-mismatch) by climate. Additionally, some species of harmful algae (e.g., toxic dinoflagellates benefiting from land runoff and/or water column stratification) may become more prevalent, while others may diminish in areas currently impacted (Hallegraeff, 2010).

1.2. Harmful Algal Blooms in the California Coastal Upwelling Region

The coast of Southern California belongs to one of the world's coastal upwelling regions, as part of the eastern boundary current system. The North Pacific Current approaches the US west coast at ~ 45°N, where it splits up into the northern, smaller Alaskan Gyre System and southward into the California Current System (CCS) (Reid *et al.* 1958). Upwelling systems represent an extreme case of natural nutrient inputs driven primarily by the upwelling of cold, nutrient-rich water from the depth due to wind-driven transport (Figure 1-1, upwelling-relaxation-downwelling cycles) and the associated changes in temperature and stratification (Kudela *et al.*, 2010). This builds up a highly productive and biologically rich regime, but also makes the coast of Southern California one of the 'hot-spots' for HAB events that have caused public health concerns and coastal economic impacts for decades (e.g. Garrison S. *et al.*, 1992; Horner and Postel, 1993; Taylor and Horner, 1994; Walz *et al.*, 1994). Common species responsible for HABs along the Californian coast are listed in Table 1-1 and include 17 dinoflagellate species as well as the diatom *Pseudo-nitzschia* spp. and the raphidophyte *Heterosigma akashiwo* (Trainer *et al.*, 2010). These are the causative species for paralytic, amnesic and diarrhetic shellfish poisoning, yessotoxin producers, ichthyotoxic organisms, and high biomass bloom-formers that are often the cause of anoxia when the blooms demise (Kudela *et al.*, 2010).

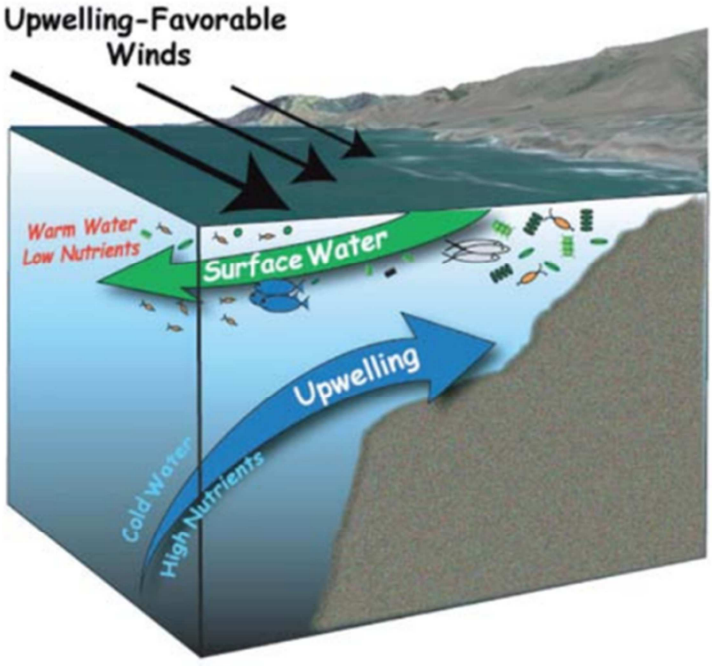

Figure 1-1: Two-dimensional process of coastal upwelling. Cold, nutrient rich water masses are transported to the surface due to divergence in offshore wind-driven transport (Figure from Kudela et al., 2008b)

HAB species	high biomass	moderate biomass	low biomass
	Lingulodinium polyedrum	*Heterosigma akashiwo*[2]	*Ceratium fusus*
	Ceratium furca	*Alexandrium catenella*	*Ceratium lineatum*
	Ceratium dens	*Dinophysis acuta*	*Gymnodinium catenatum*
	Pseudo-nitzschia spp.[1]	*Dinophysis acuminata*	*Prorocentrum minimum*
	Akashiwo sanguineum	*Dinophysis fortii*	
	Noctiluca scintillans	*Protoceratium reticulatum*	
		Prorocentrum micans	
		Cochlodinium fulvescens	
		Scrippsiella trochoideum	

Table 1-1: HAB species observed within the Californian upwelling system. The occurrence is indicated as high-biomass (dark blue), moderate-biomass (blue) or low-biomass (light blue) blooms. Most species listed belong to the group of dinoflagellates, except for one diatom[1] and one raphidophyte[2] (modified after Trainer *et al.*, 2010).

The dinoflagellate *Lingulodinium polyedrum* is one of the high biomass bloom-forming species, which occur frequently along the coast of Southern California (Holmes *et al.*, 1967; Kahru and Mitchell, 1998). Even though *L. polyedrum* are microscopic in size, they can become visible due to their ability to discolour the water and/or bioluminescence (e.g. von Dassow *et al.*, 2005) when mechanically stimulated (Figure 1-2).

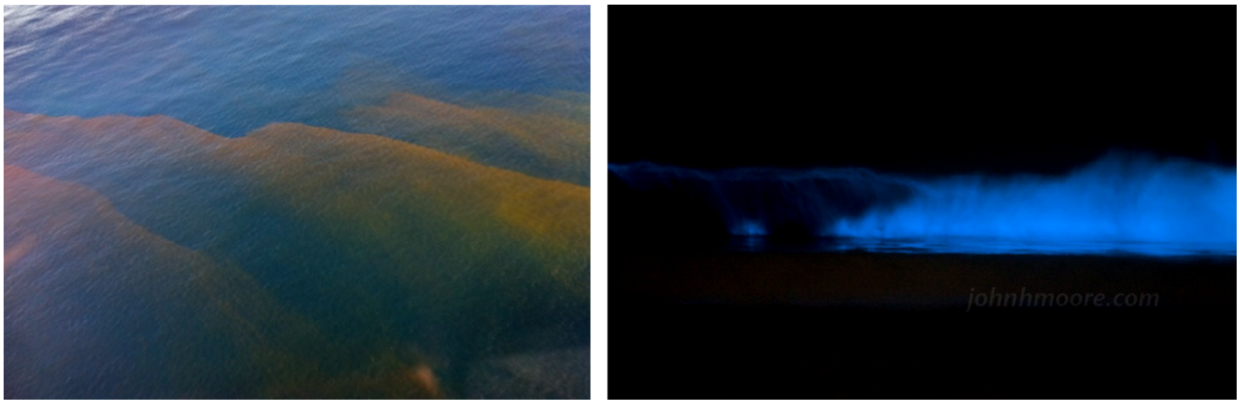

Figure 1-2: Left: *Lingulodinium polyedrum* bloom off the coast of California (Photo E. Kisfaludy). Right: Bioluminescence of a *Lingulodinium polyedrum* bloom in the breaking waves off Torrey Pines State Beach, California (Photo John H. Moore Photography).

While some dinoflagellates form high biomass blooms (indicated in dark blue, Tab. 1-1) along the coast of Southern California of up to 1 Mio cells L^{-1} (Kudela and Cochlan, 2000; SCCOOS, 2013, http://www.sccoos.org/), others (including *A. catenella*) rarely cause visible blooms, but more often toxic events, even at low cell densities (Jester *et al.*, 2009b). The coast of California has a long history of paralytic shellfish poisoning (PSP) events with early cases of PSP reported by Europeans settlers (Meyer *et al.*, 1928; Sommer and Meyer, 1937). Paralytic shellfish toxins (PSTs) with saxitoxin (STX) as the parent compound are a broad group of natural neurotoxic alkaloids, which are highly potent toxins that can paralyse the respiratory system by blocking sodium channels, preventing the propagation of nerve impulses (Gerssen *et al.*, 2010). In recent years an increase in PST activity has been observed at some Southern California sites, most notably in commercial shellfish growing areas in Santa Barbara and San Diego counties (Lewitus *et al.*, 2012). *Alexandrium catenella* is the predominant PSP-toxin producing species in the California Current system and occurs along the entire outer open coast (Taylor and Trainer, 2002).

1.3. Factors Influencing Harmful Dinoflagellate Bloom Dynamics

The main goal of most HAB research is to predict, and possibly prevent or mitigate harmful algal bloom events. This has led to increased scientific and regulatory attention and the development of many new technologies and approaches for research on and management of HABs (Anderson *et al.*, 2012b). For many HAB

species the specific environmental factors driving their bloom dynamics are still poorly understood, thus limiting our capacity to control and potentially prevent such blooms. Substantial research efforts regarding the complex interplay of the species' physiology, ecology and the hydrography of particular area HABs occur are required in order to design and implement effective prevention and control strategies (Anderson, 2007).

The fact that dinoflagellates build the dominant taxonomic group of bloom forming algal species in upwelling regions (Table 1-1) raises the question: what ecological and adaptational strategies and traits make dinoflagellates that successful in upwelling systems?

Factors that control their population dynamics include physical processes (i.e. turbulence, stratification) that influence temperature and nutrient availability, as well as biological interactions, such as competition, grazing, mixotrophy and allelopathy (Smayda, 2000). From a food web perspective, most of these factors can be assigned to bottom-up, sideways and top-down control, while some of them affect more than one trophic level of the food web (e.g. mixotrophy, allelopathy) and thus cannot clearly be assigned.

Bottom-up factors

Bottom-up control is generally defined as resource-driven control, meaning the regulation of growth by an essential growth factor for an organism (Kaiser, 2011). Resources such as light levels and essential nutrient availability are the two main abiotic factors controlling phytoplankton production in the marine environment (Lalli and Parsons, 1997). Many laboratory experiments and field studies on bloom formation of harmful dinoflagellates to date have focused on bottom-up factors that might stimulate the growth and favour the dominance of dinoflagellates (e.g. Dagenais Bellefeuille *et al.*, 2014; Hu *et al.*, 2011; Kudela and Cochlan, 2000; Kudela *et al.*, 2008). As a result of eutrophication, changes in nutrient supply to coastal waters have been suggested to influence the abundance, frequency and toxicity of harmful dinoflagellate blooms through a change in the form or ratio of growth-limiting nutrients (‘nutrient–ratio hypothesis’, Smayda, 1997, 1990; Tilman, 1977; Davidson *et al.*, 2012). For example, declining Si:N ratios may shape community dynamics through the Si-limitation or co-limitation of diatoms, thereby favouring algal groups

that do not require Si, such as dinoflagellates (Smayda, 1990). HAB dinoflagellates in general tend to have lower affinities for dissolved nutrients compared to diatoms, making them inferior competitors for nutrients (e.g. Banse, 1982; Kudela and Cochlan, 2000; Kudela *et al.*, 2008; Smayda, 1997). This general trend, however, does not seem to include upwelling HAB dinoflagellates, which were shown to have a high affinity for nitrate, suggesting that upwelling HABs are adapted for both low and pulsed nitrate supplies (Kudela *et al.*, 2008). The well-studied dinoflagellate *Lingulodinium polyedrum*, for instance, showed nitrate half saturation constants (K_s values) that varied by a factor of 30 (Kudela and Cochlan, 2000b), indicating that this dinoflagellate, which typically occurs in upwelling regions, exhibits a wider range of nutrient affinities (Kudela *et al.*, 2010) in contrast to the overall taxonomic group of dinoflagellates (Smayda, 2000, 1997a).

The different forms of nutrients that are available such as organic/inorganic N and P forms may also play an important role for dinoflagellate bloom formation. While dissolved inorganic nitrogen (DIN) represents the major N input form to coastal systems, there are also significant pools of urea and other dissolved organic N (DON). The ability to use these organic N sources, that mostly result from nutrient regeneration and from terrestrial runoff, in particular, has been demonstrated for many HAB dinoflagellate species (Abadie *et al.*, 2015; Collos *et al.*, 2007; Glibert and Terlizzi, 1999; Glibert *et al.*, 2008, 2006; J. Li *et al.*, 2009).

Box 2:

Mixotrophy *is defined as the combination of phototrophy (use of photosynthesis to obtain inorganic carbon and energy) and heterotrophy (uptake of dissolved organic substrates, and/or feeding on particulate organic carbon) in one organism.*

Allelopathy *is defined as any inhibitory or stimulatory biochemical interaction between and among all types of plants (Molisch, 1937).*

Cembella (2003) has extended the original definition to the production of any non-nutritional compounds by one organism that affect the growth, behaviour, health or population biology of other species.

‘Sideways factors’

Sideways factors refer to resource competition between phytoplankton populations that result from bottom-up control, as well as to direct negative effects of phytoplankton on other organisms which reduces this competition (Kaiser, 2011). Dinoflagellates in particular, possess adaptive strategies to offset potential competitive disadvantages by allelochemically enhanced interspecific competition or feeding on competing phytoplankton (mixotrophy) (Smayda, 1997a). These adaptive strategies distinguish dinoflagellates from diatoms and most other phytoplankton groups and may help to facilitate their competitive success in a plankton community. In the present thesis these adaptions are referred to as sideways factors, additionally controlling the growth of harmful dinoflagellates. However, there is some overlap between sideways factors and bottom-up and top-down factors, as allelopathic anti-predation defence mechanisms may offset top-down control, and as the availability of prey as a resource for mixotrophic dinoflagellates also presents bottom-up control. Mixotrophy refers to the ability of an organism to combine phototrophy and heterotrophy (Burkholder *et al.*, 2008) and has been suggested to be a common nutritional mode in HAB dinoflagellates (e.g. Jeong *et al.*, 2005). Mixotrophic dinoflagellates are able to feed on diverse prey items including bacteria, picoeukaryotes, nanoflagellates, diatoms, other dinoflagellates, and heterotrophic protists due to their diverse feeding mechanisms (Jeong *et al.*, 2010; Jeong *et al.*, 2005a, 2005b; Seong *et al.*, 2006). This enables them to use organic nutrient pools in addition to inorganic nutrients, complement photosynthetic carbon fixation and the uptake of essential nutrients, and function at multiple trophic levels (Cloern and Dufford, 2005; Sanders *et al.*, 1990). The extent to which phototrophy and phagotrophy are employed varies widely among mixotrophs and ranges from ‘ideal mixotrophs’ that equally use both nutritional modes, to primarily phototrophic phagocytic ‘algae’, and predominantly heterotrophic photosynthetic ‘protozoa’ (Stoecker, 1998).

Mixotrophy may provide a competitive advantage, particularly when major nutrients are limited (Thingstad *et al.*, 1996). However, the influence of dissolved inorganic nutrient concentrations and ratios on phagotrophic feeding has only been investigated in the laboratory for a few mixotrophic dinoflagellate species. For some dinoflagellates it was shown that under low nutrient concentrations feeding may

increase their growth rate (e.g. *Prorocentrum donghaiense*, *Karenia mikimotoi*, Zhang *et al.*, 2013) as well as stimulate feeding and increase ingestion rates (e.g. *Ceratium furca and Gyrodinium galatheanum,* Li *et al.*, 2000; Smalley *et al.*, 2012, 2003). However, for others such as *A. catenella* prey availability did not increase dinoflagellate growth and was not dependent on dissolved nutrient concentrations (Zhang *et al.*, 2013). Besides the potential positive nutritional effect of phagotrophic feeding, this strategy can also be beneficial for the dinoflagellates by reducing their competitors under low nutrient conditions. This strategy was described in a mathematical model by Thingstad *et al.* (1996), who demonstrated for a bacteria - mixotroph system that the benefits for the mixotroph were higher due to "eating its competitor" than due to gaining additional nutrients from its prey.

In addition to being mixotrophic, many dinoflagellate species produce secondary metabolites, comprising the very heterogeneous group of intracellular phycotoxins (including paralytic shellfish poisoning toxins, PSTs), and allelochemical compounds of poorly characterized chemical nature that are released into the surrounding water in contrast to intracellular phycotoxins (Ma *et al.*, 2011). Such allelochemicals may negatively affect other organisms in several ways, including phytoplankton competitors and zooplankton consumers, and are thus considered to be one of the key factors determining phytoplankton competition for resources, succession and bloom formation (e.g. Maestrini and Bonin, 1981, and references therein; (Fistarol *et al.*, 2004; Granéli and Hansen, 2006; Hattenrath-Lehmann and Gobler, 2011; Kubanek *et al.*, 2005; Legrand *et al.*, 2003; Rengefors and Legrand, 2001; Yamasaki *et al.*, 2009). Allelopathy in bloom-forming dinoflagellates has been studied extensively in recent years. Allelochemically induced effects on protistan targets include growth inhibition (e.g. Hattenrath-Lehmann and Gobler, 2011; Poulson-Ellestad *et al.*, 2014), encystment (Tillmann *et al.*, 2007), cell lysis (e.g. Ma *et al.*, 2009; Tillmann *et al.*, 2008), and immobilisation of target cells (e.g. Tillmann and John, 2002; Tillmann *et al.*, 2008, 2007). The latter effect might be particularly important in combination with mixotrophy when prey immobilisation is used for subsequent ingestion by mixotrophic dinoflagellates (Blossom *et al.*, 2012).

Top-down factors

Top-down control of phytoplankton growth includes losses due to predation (e.g. Banse, 1994; Lehman, 1991), but can also arise from other biotic factors, like

parasite and viral attacks (Alves-de-Souza *et al.*, 2015; Coats and Park, 2002; Park *et al.*, 2004). Grazing is one of the most important loss factors for phytoplankton and on average more than 80% of the marine phytoplankton production is consumed by herbivores (Calbet and Landry, 2004; Calbet, 2001; Cyr and Pace, 1993). In particular, microzooplankton (< 200µm) grazers (e.g. heterotrophic dinoflagellates and ciliates) can provide a more effective "top-down" control on HAB species compared to mesozooplankton (0.2 - 20 mm size range) because of their faster growth rates (Admiraal and Venekamp, 1986; Strom and Morello, 1998). Many HAB dinoflagellates have been tested as prey in zooplankton grazing experiments (e.g. Colin and Dam, 2002; Frangópulos *et al.*, 2011; Jeong *et al.*, 2002, 2001, 1999; Teegarden, 1999). Part of them were nutritionally accepted by some grazers and rejected by others; some dinoflagellates repressed grazing completely or even caused physiological stress. Thus, the extent to which zooplankton grazing may be a controlling factor for dinoflagellate population dynamics at any given time or place crucially depends on species-specific feeding relationships, i.e. the identity and match between dinoflagellate (prey) and zooplankton (consumer) (Smayda, 2000).

Furthermore, it has been proposed that trophic cascading effects can indirectly contribute to harmful dinoflagellate blooms, when, for instance, decreased copepod numbers release microzooplankton, which feeds on smaller nanophytoplankton, from grazing control, thus increasing top-down control on nanophytoplankton (Granéli and Turner, 2002; Lehrter *et al.*, 1999) and releasing large dinoflagellates from competition with smaller phytoplankton (Stoecker *et al.*, 2008). However, toxicity of dinoflagellate prey or their allelopathic exudates, and poor food quality can also cause an uncoupling between dinoflagellate prey and their grazers and create "windows of low grazing pressure" that may facilitate blooms (Buskey, 2008; Irigoien *et al.*, 2005; Mitra and Flynn, 2006; Stoecker *et al.*, 2008; Sunda *et al.*, 2006).

1.4. The outline of this thesis

This doctoral thesis was part of an integrated interdisciplinary research effort dealing with the investigation, analysis and predictive modelling of extreme events (ExEvs). Extreme events are considered to be rare events characterized by a large impact on a particular system. In this project harmful algal blooms (HAB) were studied as such phenomena in excitable systems, which appear recurrently in rather large, irregularly

spaced time intervals. Modelling these blooms in order to potentially predict HAB dynamics requires an in-depth understanding of environmental factors, species-specific traits and adaptive strategies that determine when and under which conditions certain species are able to dominate the phytoplankton community and form blooms. These interactions are complex and highly variable among different HAB species. In my thesis, I focused on trophic interactions of the two potentially harmful dinoflagellates *Lingulodinium polyedrum* and *Alexandrium catenella* originating from the Southern California Bight (SCB). I tested their competitive ability and potential grazing control by zooplankton consumers, taking species-specific adaptive strategies such as mixotrophy and their ability to produce allelopathic compounds into account. For that purpose, I conducted different microcosm experiments in the laboratory under varied environmental conditions, using different prey/competitor and grazer species. I investigated mixotrophic versus purely phototrophic growth of both dinoflagellates under different inorganic nutrient conditions, the potential allelopathic activity of *A. catenella* on phytoplankton competitors and on metazoan grazers, and finally the bloom dynamics of *L. polyedrum* in a more complex natural plankton community, regarding bottom-up control (manipulating the amount of dissolved nutrients), and top-down control (manipulating zooplankton grazing).

Effects of nutrient concentrations, phagotrophic feeding and allelopathy on bloom dynamics of potentially harmful dinoflagellates

Both mixotrophy and allelopathy may substantially alter food web dynamics, and are likely to increase a species' competitive success in a phytoplankton community. However, there is still a substantial lack of studies investigating the interactions between these factors and their relevance for dinoflagellate growth and cellular nutrient composition, especially in response to a changing nutrient regime. This is particularly important when considering climate driven changes that may affect nutrient flux to surface waters through alteration of vertical mixing and runoff (Wells *et al.*, 2015). In **chapter I**, my aim was to disentangle the effects of dissolved inorganic nutrients, phagotrophic feeding and allelopathy on the performance of the two dinoflagellate species *Lingulodinium polyedrum* and *Alexandrium catenella*, and in turn their effect on potential prey/competitors. I conducted various microcosm experiments to investigate mixotrophic versus purely phototrophic growth of both

dinoflagellates under different inorganic nutrient conditions (different dissolved nitrogen (N) and phosphorus (P) concentrations and ratios), using the picoplankton prey organism *Ostreococcus* sp. Additional tests for lytic activity were conducted to differentiate between the feeding impact on the prey population and potential allelopathic effects. The particulate C:N:P ratios of both dinoflagellates were compared to ingestion rates under different dissolved inorganic nutrient concentrations in order to determine the effects of feeding on the dinoflagellates cellular nutrient content and thus nutritional status.

Allelopathic effects of *A. catenella* on competitors and grazers

In **chapter II**, the potential allelopathic effects of *A. catenella* are described. In addition to the toxic effects of *A. catenella* on metazoan grazers, excreted allelochemicals can cause negative effects on grazers even if toxic cells are avoided or non-toxic phytoplankton prey is available.
I tested the *A. catenella* strain for its potential allelopathic activity on phytoplankton competitors and on the common metazoan rotifer grazer *Brachionus plicatilis*. In order to differentiate between adverse toxic and allelopathic effects on metazoan grazers, I characterized the toxin profile of *A. catenella* and then compared its effects on *B. plicatilis* with the effects of a similarly toxic, but non-lytic strain of *A. tamarense*. Furthermore, I conducted mixed culture experiments with *A. catenella*, a phytoplankton competitor (*Tetraselmis* sp.) and the grazer *B. plicatilis,* to determine the relative effects of allelopathic compounds on different trophic levels and possible cascading effects of allelochemicals through the planktonic food web.

Bottom-up and top-down control of *L. polyedrum* in a natural plankton community

Chapter III is concerned with the question of whether the population dynamics of the dinoflagellate *Lingulodinium polyedrum* in coastal waters of Southern California are ‘bottom-up’- controlled i.e. driven by the amount of dissolved nutrients, or rather top-down regulated by zooplankton grazing. Both control factors are assumed to be important for the formation of high biomass dinoflagellate blooms in the SCB region, which is linked to large metropolitan areas and thus an increased influx of anthropogenic N to coastal waters (Ferreira *et al.*, 2011) in combination with nutrient

input via upwelling. Changes in nutrient regimes may substantially change plankton community structure, microzooplankton grazing and dinoflagellate abundance (Stoecker *et al.*, 2008). I approached this question in three laboratory experiments conducted at the Caron Laboratory at the University of Southern California, Los Angeles, using a natural plankton community off the coast of Los Angeles. In the first experiment, I manipulated concentrations of dissolved nutrients by adding phosphate, nitrogen or both to the plankton community containing a natural *L. polyedrum* population, and investigated its growth under different nutrient conditions. In a second approach, I investigated the effects of potential competitors/prey and consumers on the growth and competitive success of *L. polyedrum* in a natural plankton community. For that purpose, *L. polyedrum* was spiked into two different size fractions of a natural plankton community; i.e., < 20 µm (including competitors/prey, but excluding consumers) and < 110 µm (including consumers). In an additional treatment, the heterotrophic dinoflagellate consumer *Noctiluca scintillans* was added to both size fractions. In a subsequent laboratory experiment, I used laboratory cultures to investigate the grazing effect of *N. scintillans* on a gradient of different cell concentrations of *L. polyedrum* in order to find a potential threshold concentration above which *N. scintillans* is no longer able to control the population growth of *L. polyedrum*.

In the final chapter (**General discussion**), I summarize the results presented in this thesis and synthesize direct and indirect interactions of the studied dinoflagellates at different trophic levels of the plankton community. These interactions are then set into the context of existing conceptual frameworks to show our advancement in the understanding of dinoflagellate bloom dynamics, thus enhancing our predictive capabilities.

Chapter I

2. CHAPTER I: Effects of nutrient concentrations, phagotrophic feeding and allelopathy on bloom dynamics of potentially harmful dinoflagellates

2.1. Abstract

Bloom dynamics of potentially harmful dinoflagellates are influenced by a variety of abiotic factors such as the concentrations of dissolved nutrients as well as biotic factors such as the presence of competitors and consumers. Many dinoflagellates have evolved particular strategies to escape competition by either phagotrophic feeding on other phytoplankton (mixotrophy) or by producing harmful secondary metabolites (allelopathy). In the present study microcosm experiments were conducted to investigate the impact of different nutrient conditions and the availability of prey on growth and feeding characteristics of the bloom forming dinoflagellates *Lingulodinium polyedrum* and *Alexandrium catenella*. To differentiate between the feeding impact on the prey community and potential allelopathic effects, additional tests for lytic activity were conducted with both dinoflagellates. Both species were able to ingest a variety of different prey organisms, but had a particularly strong negative impact on the pico-phytoplankton *Ostreococcus* sp. irrespective of nutrient limitation. However, there was only little benefit for the dinoflagellates from growing with prey in terms of biomass production. Their cellular nutrient contents indicated initial metabolic 'costs' for switching nutritional modes from photosynthesis to phagotrophic feeding, before having a benefit from ingested prey. In the experiments testing for allelopathy, *A. catenella* showed strong lytic activity, while *L. polyedrum* did not. This indicates that *L. polyedrum* may have used phagotrophic feeding to reduce competitors, while for *A. catenella* allelochemicals may play a more important role for escaping competition and becoming dominant in the phytoplankton community. This study emphasizes, that competitive strategies such as mixotrophy and allelopathy need to be considered in the context of nutrient dynamics in order to understand dinoflagellate population dynamics and their impact on planktonic food webs.

2.2. Introduction

Harmful dinoflagellate blooms are a common phenomenon in coastal upwelling regions such as the Californian eastern boundary current system (Kudela *et al.*, 2010; Trainer *et al.*, 2010). Recent evidence suggests that the increased magnitude of such blooms might be linked to anthropogenic nutrient enrichment in coastal waters (Anderson *et al.*, 2002; Glibert *et al.*, 2005); however, coastal upwelling regions have been found to be less affected by eutrophication (Kudela *et al.*, 2008). Nutrient availability along the Californian coast is strongly influenced by the upwelling-relaxation-downwelling cycles and the associated changes in temperature, stratification and seasonal utilization of nutrients by phytoplankton (Goering *et al.*, 1973; Kudela *et al.*, 2010; Sambrotto and Lorenzen, 1986). A variety of different harmful algal species occur along the coast of Southern California, including the diatom *Pseudo-nitzschia* spp., the dinoflagellates *Alexandrium catenella, Lingulodinium polyedrum*, *Dinophysis* spp., and *Ceratium* spp. (Anderson *et al.*, 2008; Horner *et al.*, 1997; Schnetzer *et al.*, 2007). *L. polyedrum* (Stein) Dodge is one of the most frequent high biomass bloom forming dinoflagellates along the Southern California coast (Allen, 1946; Holmes *et al.*, 1967; Kahru and Mitchell, 1998; Kudela and Cochlan, 2000b). Blooms of *L. polyedrum* appear to be associated with high levels of nutrient input to near surface waters via upwelling (Eppley and Harrison, 1975); but blooms can also occur outside the upwelling season, presumably fuelled by high nutrient freshwater runoff (Hayward *et al.*, 1995; Kudela and Cochlan, 2000b; Kudela *et al.*, 2008). These blooms, of up to 1 Mio cells L^{-1} (http://www.sccoos.org, 2013) have been associated with fish and shellfish mortality events due to oxygen depletion when high biomass blooms accumulate in enclosed harbors or bays (Horner *et al.*, 1997). *L. polyedrum* produces yessotoxin, a hepato- and and cardiotoxin (Armstrong and Kudela, 2006; Paz *et al.*, 2004); however, no human health issues or marine mammal deaths associated with yessotoxins have been reported in this area yet (Caron *et al.*, 2010).

In contrast to *L. polyedrum*, the U.S. west coast strain of *A. catenella* rarely forms dense or visible blooms off the cost of Southern California. However, despite their relatively low cell densities (maximum concentration of about 17,000 cells L^{-1}, Jester *et al.*, 2009) they can still cause serious toxic events. *A. catenella* produces saxitoxin (paralytic shellfish poisoning toxins, PST), a very potent neurotoxin that accumulates

in filter feeding shellfish and other organisms or gets transferred through the marine food web, which often leads to the closure of mussel harvesting (Price *et al.*, 1991). Blooms of *A. catenella* are considered to develop in shallow, nearshore waters and to spread regionally (Taylor *et al.*, 1994); these blooms are generally subsurface, often occurring near the nutricline (Taylor and Trainer, 2002). *A. catenella* toxic events off the California coast have also been associated with large scale oceanographic events and occur in particular during relaxation of upwelling and onshore advection. Few datasets from offshore phytoplankton sampling, including maps of sedimentary cysts indicated that cell numbers may increase in offshore waters and then being transported onshore during relaxation-favourable winds (Langlois and Smith, 2001; Price *et al.*, 1991).

Differences in bloom dynamics of harmful dinoflagellates indicate that particular HAB species respond differently to altered environmental conditions, such as resource availability, depending on species-specific competitive ability and nutrient acquisition strategies (Smayda, 1997b). Accordingly, the relationship between inorganic nutrient levels and dinoflagellate blooms appears to be rather complex, entailing different trophic interactions.

In general, dinoflagellates are known to be rather poor competitors for dissolved nutrients, resulting in low growth rates and low nutrient uptake rates compared to other phytoplankton of the same size (Banse, 1982; Collos *et al.*, 2005; Smayda, 1997a). To compensate for this, many dinoflagellates have evolved different adaptive strategies to successfully compete with other phytoplankton (Smayda, 1997a), such as vertical migration to prevail in stratified water (e.g. Eppley and Harrison, 1975), allelopathy, i.e. the production of secondary metabolites harming potential competitors and consumers (e.g. Tillmann and Hansen, 2009; Tillmann *et al.*, 2008), the ability to use dissolved organic matter (DOM) to complement nutrition (e.g. Loureiro *et al.*, 2009) and mixotrophy, i.e. the ability to feed phagotrophically on other organisms (e.g. Burkholder *et al.*, 2008; Stoecker *et al.*, 2006; Stoecker, 1999). Various studies have hypothesised that blooms of some dinoflagellates are closely related to their mixotrophic capability (Burkholder *et al.*, 2008; Glibert *et al.*, 2009; Jeong *et al.*, 2005). The two dinoflagellates *Lingulodinium polyedrum* and *Alexandrium catenella* are also mixotrophic and were shown to ingest a variety of prey organisms including bacteria (Seong *et al.*, 2006), cyanobacteria (Jeong *et al.*, 2005), diatoms (Yoo *et al.*, 2009), haptophytes (*A. catenella*, Zhang *et al.*, 2013) and

even other dinoflagellates (Jeong *et al.* 2005). These studies also showed that mixotrophic dinoflagellates often have a considerable grazing impact on the natural population of their prey.

Mixotrophy may confer a competitive advantage, particularly when major nutrients are limiting (Thingstad *et al.*, 1996). However, only for a few mixotrophic dinoflagellate species, the influence of dissolved inorganic nutrient concentrations and ratios on phagotrophic feeding has been investigated in the laboratory. It was shown that low nutrient concentrations (nitrogen (N) and phosphorus (P)) stimulate feeding in *Gyrodinium galatheanum* and that the ingestion rates of *Ceratium furca*, *K. veneficum* and *Neoceratium furca* decreased after nutrient addition (Li *et al.*, 2000; Smalley *et al.*, 2012, 2003), while ingestion rates of *Fragilidium cf. mexicanum* (Jeong *et al.*, 1999a) were not affected. Zhang *et al.* (2013) studied the growth of 4 mixotrophic dinoflagellate species under different nutrient conditions and found a positive effect of prey addition under nutrient limitation on the growth of *Karenia mikimotoi*, *Prorocentrum micans* and *Prorocentrum donghaiense*. However, prey availability (*Isochrysis galbana*) had no beneficial effect on the growth of an *A. catenella* strain isolated from the East China Sea. For *L. polyedrum* no such data are available, but Jeong *et al.* (2005) showed that the maximum growth rate of *L. polyedrum* increased when prey was available.

Besides the positive nutritional effect of phagotrophic feeding, this strategy can also be beneficial for the dinoflagellates by reducing their competitors under low nutrient conditions. This strategy was described in a mathematical model by Thingstad *et al.* (1996), who demonstrated for a bacteria - mixotroph system that the benefits for the mixotroph were higher due to "eating its competitor" than due to gaining additional nutrients from its prey.

In addition to mixotrophy, allelopathy may also help to escape competition by harming other phytoplankton. The production of allelochemicals has been demonstrated for several strains of *A. catenella* (Arzul *et al.*, 1999; Tillmann *et al.*, 2008) and common effects on target cells include cell lysis and immobilization (Ma *et al.*, 2009; Tillmann *et al.*, 2008, 2007). In this context, paralyzing or trapping prey may be a strategy to facilitate mixotrophic feeding (Sheng *et al.*, 2010), while cell lysis may also result in the uptake of dissolved organic matter (DOM). Earlier studies demonstrated that *A. catenella* is capable of increasing its growth rate when supplied with DOM of terrestrial (Carlsson *et al.*, 1998; Doblin *et al.*, 2001) and autotrophic

origin (Loureiro *et al.*, 2009). Thus the use of allelopathy together with mixotrophic nutrition is likely to increase the species' competitive success in a phytoplankton community.

Even though both mixotrophy and allelopathy may substantially alter food web dynamics, there is still a substantial lack of studies investigating the interactions of those factors and their relevance for dinoflagellates and the remaining food web in response to a changing nutrient regime.

In the present study I aimed at disentangling the effects of dissolved inorganic nutrients, phagotrophic feeding and allelopathy on the performance of the dinoflagellates *Lingulodinium polyedrum* and *Alexandrium catenella* and in turn their effect on potential prey/competitors. Microcosm experiments were conducted to investigate mixotrophic versus purely phototrophic growth of both dinoflagellates under different inorganic nutrient conditions (different dissolved nitrogen (N) and phosphorus (P) concentrations and ratios), using the picoplankton prey organism *Ostreococcus* sp.

I tested the following hypotheses:

1) Both dinoflagellates have a negative impact on the prey population, either through phagotrophic feeding or allelopathy.
2) Mixotrophic growth (phagotrophy and photosynthesis) results in a growth benefit for the dinoflagellates, i.e., higher biomass production compared to purely phototrophic growth.
3) Phagotrophic feeding increases internal cellular nutrient concentrations under limitation of dissolved nutrients, i.e., compared to purely phototrophic algae, mixotrophic dinoflagellates are able to keep their internal nutrient concentrations more constant under nutrient limitation.

2.3. Material & Methods

Strains and culture conditions

The two dinoflagellates *Lingulodinium polyedrum* and *Alexandrium catenella* (toxic group I, based on D1-D2 region of 28S rRNA gene sequencing, Garneau *et al.*, 2011) as well as the potential prey species *Ostreococcus* sp. (Prasinophyceae) were isolated from coastal waters in Southern California near Los Angeles (Caron Laboratory, USC, Los Angeles).

In a recent study, John *et al.* (2014) suggested renaming species belonging to the initially defined *Alexandrium tamarense* species complex (i.e. *Alexandrium tamarense*, *A. catenella*, *A. fundyense*). Based on their analyses (i.e. morphology, ITS/5.8S genetic distances, ITS2 compensatory base changes), the North American ribotype of *A. catenella* used in the present study belongs to group I, which they suggested to be renamed *A. fundyense*. I will, however, continue to use the name *A. catenella* here to be consistent with previous work on this species in the area of Southern California.

All stock cultures were grown non-axenically in enriched f/2 seawater medium (Guillard and Ryther, 1962) without silicate, prepared from 0.2 µm sterile-filtered natural North Sea seawater (pH 8.0). Cultures were maintained in 200 ml culture flasks under controlled conditions at 18°C under cool-white fluorescent light of 60 µmol photons m^{-2} s^{-1} and a 12:12 h light:dark cycle. Medium was renewed weekly by replacing one third of the culture volume to ensure that the cells were maintained in the exponential growth phase. Cultures grown for the experiments were acclimated to different N:P ratios and concentrations (see below).

In order to test the hypotheses, a set of different experiments were conducted: 1) Short-term experiments were conducted to determine dinoflagellate feeding preferences and the feeding / allelopathic effect on prey under different phosphate concentrations; 2) Two long-term experiments were conducted to investigate interactive effects of different nutrient conditions and prey availability on dinoflagellate growth as described below.

Short- term experiments

a) Determination of potential prey species with CMFDA staining

Short-term experiments were conducted to identify potential prey species and to quantify the prey uptake capability of the dinoflagellates. Potential prey species of different sizes and taxonomic groups (Prasinophytes, Raphidophytes and Cryptophytes) were tested that co-occur with *A. catenella* and *L. polyedrum* along the Southern California coast and thus are potential competitors and prey in a natural plankton community. The different prey species were incubated for 1 h with the vital green fluorescent stain 5-chloromethylfluorescein diacetate (CMFDA) according to Li *et al.* (1996). Afterwards, dinoflagellates were incubated with the respective stained prey species in 6-cell-well plates (volume of 6 ml) for 1, 4 and 6 h, in the light (see culture conditions above) and in the dark. The ratio of dinoflagellate to prey cell numbers was between 1:10 and 1:100. In addition, both dinoflagellates were incubated with a concentrated fluorescent polystyrene beads (Fluoresbite YG Microsperes 0.5µm) stock solution for 1 hour. After fixation with 1% glutharaldehyde, samples were filtered on 5 µm Nucleopore Membrane filters and approximately 100 dinoflagellate cells per filter were checked for prey/bead inclusion using an epifluorescence microscope (Zeiss, Axioscope A1) at 1000x magnification using blue light excitation (Zeiss filter set 09). Algal prey uptake was defined as the percentage of dinoflagellate cells containing prey cells divided by incubation time while the bead uptake rates were obtained by dividing the number of ingested beads by the number of counted dinoflagellate cells.

b) Short-term tests for allelopathic ability of *A. catenella* and *L. polyedrum*

Cultures of *A. catenella* (8.9×10^3 cells ml^{-1}) and *L. polyedrum* (9.2×10^3 cells ml^{-1}) were filtered through a 0.45 µm syringe filter. The filtrate was enriched with nutrients, trace metals and vitamins according to f/2 medium (Guillard and Ryther, 1962), so that potential negative effects of allelopathic chemicals on algal competitors could not be confounded with negative effects caused by nutrient limitation. An initial cell concentration of $5x10^4$ *Ostreococcus* sp. cells ml^{-1} was added to the dinoflagellate filtrate and into f/2 medium as control. The experiment was carried out in 50 ml Erlenmeyer flasks in triplicate with an experimental volume of 30 ml and was run for 6 days. Daily samples were taken for the photometrical determination (Thermo

Scientific AquaMate Plus UV-VIS) of *Ostreococcus* cell numbers by calibrating the absorption (A664) to cell numbers.

c) Short-term effects of different phosphorus concentrations on phagotrophic feeding / allelopathic activity of *A. catenella*

Preliminary experiments indicated that *A. catenella* responds more sensitive to P-limitation compared to N-limitation. Therefore, I tested in further short-term experiments the effect of *A. catenella* on a prey population under P-limitation and in a gradient of increasing P concentrations. For the first experiment *A. catenella* was grown in mixed culture with *Ostreococcus* sp. in P-limited medium and in a f/2 medium control. The P-limited medium was prepared according to f/2 medium (Guillard and Ryther, 1962), but without addition of P, i.e. containing only background concentrations of phosphate from filtered North Sea water (0.53 µM P). The second experiment was conducted using a gradient of 5 phosphate concentrations (1, 2, 4, 8 and 16 µM) and N-concentrations according to the f/2 medium. Both experiments were conducted in 100 ml Erlenmeyer flasks containing *A. catenella* at a concentration of 1000 cells ml^{-1} and *Ostreococcus* sp. at $5x10^4$ cells ml^{-1}. Samples for the quantification of *Ostreococcus* sp. cell numbers by flow-cytometry were taken after 1, 2 and 4 h, and the experiments were terminated after 6 h. In order to compare the results with similar studies, the prey loss rate after each time interval (h^{-1}) was converted to carbon content in ng ml^{-1} (see below).

Photosynthetic and mixotrophic dinoflagellate growth (with and without prey) under different N and P concentrations and ratios

Two long-term experiments (12 – 28 days, depending on duration of exponential growth phase) were conducted with the two dinoflagellates *L. polyedrum* and *A. catenella* to investigate the effects of dissolved nutrients on their growth and grazing characteristics. The first experiment was conducted testing different N:P ratios and concentrations in a semi-continuous culture system; the second experiment was conducted using a gradient of increasing N and P concentrations in a batch culture system (see below). In both experiments *Ostreococcus* sp. was used as prey.

Experiment 2 A: Photosynthetic versus mixotrophic dinoflagellate growth under different N:P ratios

In the first experimental approach, *L. polyedrum* and *A. catenella* were inoculated with and without prey (*Ostreococcus* sp.) under four different nutrient conditions, which were nitrogen limited (-N), phosphorus limited (-P), nitrogen and phosphorus limited (-NP) and nitrogen and phosphorus replete (+NP). In addition, an *Ostreococcus* monoculture was set up as a control. All species combinations were set up in triplicate in all nutrient treatments, resulting in a total of 60 experimental units. Growth medium was prepared from sterile filtered natural North Sea water according to f/2 medium (see above), but with different N:P concentrations and ratios: 160:10 (580 µmol L^{-1} N and 36.2 µmol L^{-1} P, +NP), 160:1 (580 µmol L^{-1} N and 3.62 µmol L^{-1} P, +N-P), 16:10 (58.0 µmol L^{-1} N and 36.2 µmol L^{-1} P, -N+P) and 16:1 (58.0 µmol L^{-1} N and 3.62 µmol L^{-1} P, -N-P). Both dinoflagellates were pre-cultured for 7 days under the different experimental nutrient conditions as described above. Then the experiment was started in 200 ml Erlenmeyer flasks containing an experimental volume of 150 ml of medium. The experiment was conducted in a semi-continuous way with an exchange rate of 10% every second day. Initial cell concentrations were 500 cells ml^{-1} for the dinoflagellates and 5 x 10^4 cells ml^{-1} for *Ostreococcus* sp.. Subsamples of 3 ml were taken every second day and were preserved with Lugol´s iodine solution at 1% final concentration and cells were counted under an inverted microscope (Leica DM IL). The experiment was terminated after 13 days (for *L. polyedrum*) and after 26 days (for *A. catenella*).

Experiment 2 B: Photosynthetic versus mixotrophic dinoflagellate growth in a gradient of increasing N and P concentrations

For the second long-term experiment the experimental set up was changed. By using co-culture experiments to study mixotrophy-based growth of the dinoflagellates under different nutrient conditions, the loss of prey can be caused by factors other than dinoflagellate feeding (i.e. nutrient competition and allelopathic effects). Since *A. catenella* showed allelopathic activity in short-term experiments, my approach for this experiment was to use dinoflagellate filtrate as a growth control for *Ostreococcus* sp. to distinguish between the possible grazing and the allelopathic effect in the mixed culture.

Both dinoflagellates were pre-cultured for 7 days in 1 l Schott flasks (Schott Duran, Germany) containing 800 ml growth medium prepared from sterile filtered natural seawater and containing all nutrients according to f/2 medium, except for nitrate and phosphate. These nutrients were added at 1/10 of the concentrations used for f/2 (f/20, 88.3 µM N, 3.6 µM P, respectively) in order to acclimate the dinoflagellates to intermediate experimental nutrient conditions. After this pre-incubation time both dinoflagellate cultures were set up at concentrations of 500 cells ml^{-1} in 36 Schott flasks for each dinoflagellate, respectively (1 L flasks with a final volume of 500 ml). Four different nutrient conditions were established, corresponding to a gradient of increasing N and P concentrations (N:P = 32:2 (N1), 80:5 (N2), 160:10 (N3), 320:20 (N4) µmol l^{-1}), but with constant N:P ratios (Redfield, N:P = 16, Redfield, 1934). Due to the N background concentrations of 19-20 µM, the N:P ratio was increased to 20 in the lower nutrient treatments. Dinoflagellates were inoculated in the different media in monoculture (no prey added), with prey (mixed treatment with *Ostreococcus* sp.), and in addition an *Ostreococcus* monoculture was set up as control. The experiment was set up in triplicate. At first, all flasks (including the mixed treatment and the *Ostreococcus* control) were inoculated with only the dinoflagellates in monoculture. After an acclimation time of 4 days for the dinoflagellates under the four experimental nutrient conditions, *Ostreococcus* sp. was added into the mixed treatment at a concentration of 2 x 10^5 cells ml^{-1}. The *Ostreococcus* control treatment was set up by removing the dinoflagellates from the *Ostreococcus* control flasks by filtration through GF/F filters. After that, *Ostreococcus* was added to the remaining filtrate at the same concentration as in the mixed treatment. This approach allowed the growth of *Ostreococcus* sp. in the dinoflagellates' filtrate containing potential allelochemicals with the same nutrient concentrations as in the mixed treatment.

Samples for phytoplankton particulate carbon, nitrogen and phosphate, for dissolved inorganic nitrogen and phosphate, and for algal cell numbers were taken every other day during the growth phase of the dinoflagellates until the stationary phase was reached (after 12 days for L*. polyedrum* and after 21 days for *A. catenella*). To determine dinoflagellate cell densities, 3 ml subsamples were preserved with Lugol´s iodine solution at 1% final concentration and counted under an inverted microscope (Leica DM IL). Subsamples for *Ostreococcus* sp. cell counts were preserved with 1% Glutaraldehyde and cell numbers were determined by flow-cytometry using an Accuri C6 flow cytometer (BD, San Jose, CA). Volume verification was conducted using

TruCount beads (BD) following Giebel *et al.*, (2009). Data were processed by BD AccuriC6 software v.1.0.264.21. To convert algal cell numbers into biovolume, species-specific cell volumes were calculated by assuming a geometrical shape of a sphere (Hillebrand *et al.*, 1999). The equivalent spherical diameter (EST) was determined microscopically by measuring n= 30 individual dinoflagellate cells; for *Ostreococcus* sp. an average EST determined from 100 cells measured by flow-cytometry was used. Final biovolume per ml^{-1} was calculated by multiplying single cell volume with corresponding cell counts. Growth rates (μ) were calculated using the formula:

(1) $\mu = (\ln(C_2) - \ln(C_1))/\,(t_2 - t_1)$

where C_1 and C_2 are cell numbers at the beginning (t_1) and the end (t_2) of the exponential growth phase (t in days).

The maximum growth rate (μ_{max}) is the intrinsic growth rate of the two dinoflagellate species and was used to determine how prey concentration affected the growth rate under different nutrient conditions. To determine μ_{max} for the dinoflagellates, growth rates under different nutrient starting concentrations (in mono and mixed culture) were fitted to the Michaelis-Menten equation:

(2) $\mu = \frac{\mu_{max}}{K_\mu + S}$

where μ_{max} is the maximum growth rate (d^{-1}); S is the nutrient concentration (μM N or P); and K_μ is the growth rate sustaining ½ μ_{max}. The negative effect of each dinoflagellate on the prey population in the mixed culture (either due to ingestion or lysis of prey cells) was defined as the loss rate (LR). It was determined by calculating the expected prey cell number (C_{exp}) in mixed culture for different time intervals as follows (modified after Frost, 1972):

(3) $C_{\exp t2} = C_{mixed\,t1} * e^{\mu_{mono}}$

where $C_{mixed\ t1}$ is the prey cell number in mixed culture at t_1 and μ_{mono} is the specific growth rate (μ) in monoculture for t_2 - t_1. The loss rate (LR) for the time interval t_2 - t_1 was then calculated according to equation 4:

(4) $LR_{(t2-t1)} = C_{\exp t2} - C_{mixed\ t2}$

In order to convert the loss rate into total prey carbon content (pg C ml^{-1}) the *Ostreococcus* cellular carbon content was estimated using the equation log carbon (pg $cell^{-1}$) = 0.94 * log biovolume (μm^3) - 0.6 (Eppley *et al.*, 1970).

Samples for dissolved inorganic nutrients were retained in PE bottles, stored at -20°C and were analysed later using the SAN++ Continuous Flow Analyzer (CFA) (Skalar Analytical B.V., Netherlands). For the internal cellular C, N and P analyses, a volume of 20 ml was filtered on pre-combusted and acid washed Whatman GF/F filters. The CN and P filters were dried at 60 °C for at least 48 hours. To analyse CN and P content in the mixed treatments for the dinoflagellates and *Ostreococcus* separately, the mixed samples were filtered through a 20μm nylon mesh to separate the dinoflagellates from *Ostreococcus*. The CN elemental composition of dinoflagellates and prey was measured using a CHN analyzer (Thermo, Flash EA 1112). Particulate phosphate was measured as orthophosphate by molybdate reaction after sulfuric acid digestion (Wetzel and Likens, 2003).

Additionally, the treatments containing *A. catenella* were sampled for PSP toxin analysis at the beginning of the experiment (just before *Ostreococcus* was added), and at the end of the exponential growth phase. For analyses of the different PST analogues, 20 ml subsamples were filtered on Whatman GF/F filters and stored at -20°C until analysis. Prior to analyses, cells were re-suspended in 1.2 ml 0.03 mol L^{-1} acetic acid, centrifuged at maximum speed (16,000 x g, 4°C) and the supernatant was then transferred into FastPrep tubes with 0.9 g lysing matrix D (Thermo Savant, Illkirch, France) to disrupt cell membranes by reciprocal shaking in a Bio101 Fast Prep instrument (Thermo Savant, Illkirch, France) for 45 seconds at maximum speed (6.5 m s^{-1}). Subsequently, cell debris was centrifuged in 15 minutes at 13,000 × g and 4° C (Eppendorf 5415 R, Hamburg, Germany). The supernatants were transferred to a spin-filter (pore size 0.45 μm; Millipore Ultrafree, Eschborn, Germany), filtered 30 seconds at 3000 x g, and then the filtrates were transferred into glass vials and stored at – 20 °C until further analysis by high-performance liquid

chromatography (HPLC) via fluorescence detection (LC-FD) with post-column derivatisation (Krock *et al.*, 2007).

Statistical analysis

All data were analysed using the software R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria, 2014). One way analyses of variances (ANOVA) were performed to test the effect of dinoflagellate filtrate on prey cell numbers compared to f/2 medium control (experiment 1.2) as well as to test the effect of different phosphorus conditions on prey abundance and prey loss rates (experiment 1.3). For the long term experiments (experiment 2.1 and 2.2) the interactive effects between the factors nutrient conditions and prey (for the dinoflagellates) or feeding (for *Ostreococcus*) were tested using the two-factor ANOVA. The response variables tested for dinoflagellates and the prey were maximum cell density (MCD) and growth rates as well as the *Ostreococcus* loss rate. Additionally, the cellular nutrient concentrations and ratios were tested in experiment 2.2. Whenever ANOVA's showed significant effects, the significant differences among treatments were determined using a TukeyHSD post hoc test. All data were examined for normal distribution. Homogeneity of variances was tested using the Bartlett's test. Data that failed to meet these criteria were log transformed or, if the assumptions for parametric tests could still not be fulfilled, Box-Cox transformation was performed as indicated in text or table legends. Data for the dissolved nutrient concentrations were not normally distributed, thus differences in remaining nutrients (N and P) between the two dinoflagellates and between mono- and mixed culture were tested using a non-parametric Kruskal-Wallis test (experiment 2.2). The level of significance was defined at $p < 0.05$. Ingestion rates calculated from experiment 2.2 were plotted against dinoflagellate cellular nutrient contents and correlations were determined using a Spearman rank order correlation. After statistical and analytical examinations corresponding graphs were created with the software Sigma plot (version 11.0, from Systat Software, Inc., San Jose California, USA).

2.4. Results

Short term experiments

Determination of potential prey species, Experiment (a)

Among the phytoplankton prey species tested, the CMFDA staining worked well for *Ostreococcus* sp. (EST= 0.9 µm, n=100). The bright yellow-green stain was clearly visible after 1 h of incubation with CMFDA, but decreased after 3 h. Both dinoflagellates were able to ingest fluorescently labeled *Ostreococcus* sp. cells and also fluorescently labelled beads (EST=0.5 µm) (Figure 2-1). Maximum ingestion rates were estimated after 1 h when incubated with beads (4.3 ± 1.2 beads $cell^{-1}$ h^{-1} for *A. catenella* and 5.8 ± 3.4 beads $cell^{-1}$ h^{-1} for *L. polyedrum*). Stained *Ostreococcus* sp. cells were clearly visible inside the dinoflagellate cells, single prey cells, however, were difficult to distinguish; therefore, only dinoflagellate cells containing stained prey cells were counted. Approximately 20% of the dinoflagellate cells were observed with ingested prey cells after 1 h. Since ingestion rates were highest for *Ostreococcus* sp. compared to all other prey species tested, all further experiments were conducted using *Ostreococcus* sp. as a prey species.

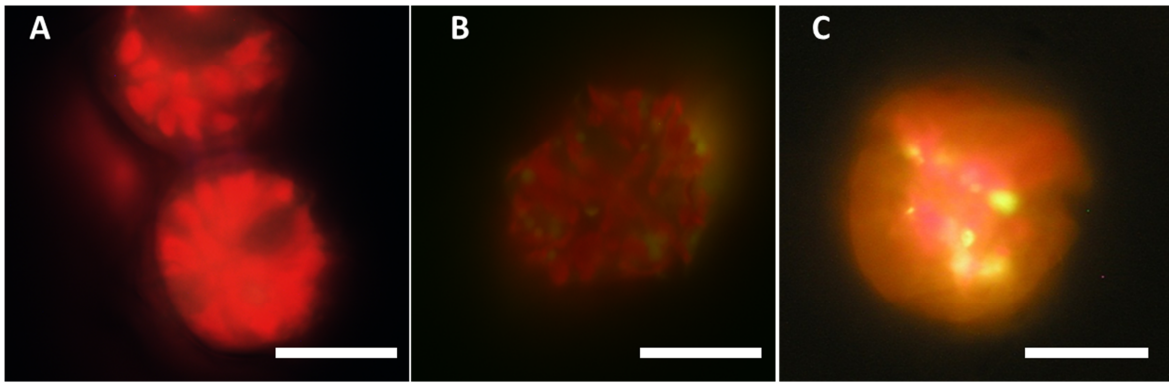

Figure 2-1: Prey uptake by *A. catenella* cells. A: *A. catenella* without ingested prey B: *A. catenella* cell with ingested fluorescently labelled beads C: *A. catenella* cells with ingested fluorescently labelled Ostreococcus cells. Scale bars = 20 µm

Short-term tests for allelopathic ability of *A. catenella* and *L. polyedrum* Experiment (b)

An allelopathic effect of *A. catenella* on *Ostreococcus* sp. cells was visible after 24h. *Ostreococcus* cell density significantly decreased by 70% after incubation in *A. catenella* filtrate compared to f/2 medium ($p < 0.05$, ANOVA). The filtrate of *L. polyedrum,* however, had no negative effect on *Ostreococcus*. The calculated growth rate for *Ostreococcus* in *L. polyedrum* filtrate did not differ from its growth rate in f/2 medium (0.37 d^{-1} in filtrate compared 0.39 d^{-1} in f/2).

Short-term effects of different phosphorus concentrations on phagotrophic feeding / allelopathic activity of *A. catenella*, Experiment (c)

Ostreococcus sp. biovolume significantly decreased when incubated with *A. catenella* for 6 h under P-limitation compared to the f/2 medium control (ANOVA, $p<0.05$, Figure 2-2). The highest loss rate due to *A. catenella* presence was observed after 1 h of incubation in P limited medium and it was higher compared to the loss rate in f/2 medium. After 4 h, the loss rate increased again, but only in P-limited medium and with a lower peak compared to the one after 1 h. (Figure 2-2), while loss rates in f/2 decreased after 2h. In the second short-term experiment in this context, where *A. catenella* was grown together with *Ostreococcus* in the phosphorus gradient, *Ostreococcus* cell numbers decreased with the decreasing P concentrations (data not shown).

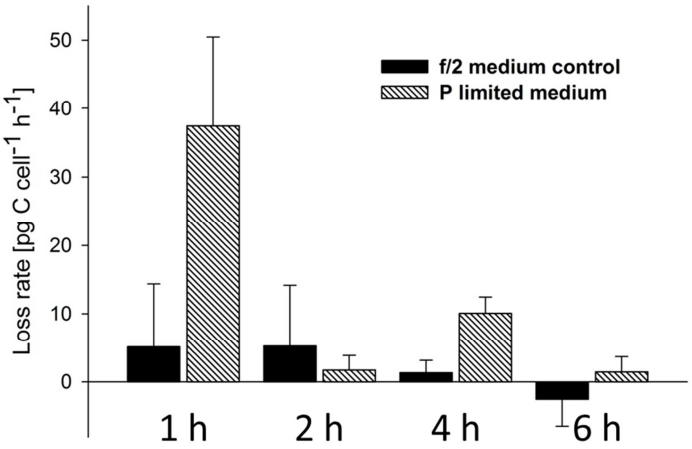

Figure 2-2: Loss rate of *Ostreococcus* (pg C *A. catenella* $cell^{-1}$ h^{-1}) with *A. catenella* over 6 h of incubation in f/2 medium (black bars) compared to P limited medium (patterned bars). Error bars indicate ± SD, n = 3.

Dinoflagellate photosynthetic and mixotrophic growth in different N:P concentrations and ratios

While prey availability had no significant effect on the maximum cell density (MCD) of the two dinoflagellates, nutrient concentrations and ratios significantly affected their MCD at the end of the exponential growth phase (Table 2-1), but in different ways (Figure 2-3). For *L. polyedrum*, the MCD was significantly lower under nutrient depletion (-NP) compared to the +NP treatment (TukeyHSD, $p < 0.05$) and there was a non-significant trend of lower MCD at –P and –N compared to +NP (TukeyHSD, $p < 0.1$ Figure 2-3 A). For *A. catenella*, nutrient limitation effects were much stronger (Figure 2-3 B); MCD was significantly lower at –P, -N and -NP compared to the +NP treatment in both mono and mixed culture (Figure 2-3 B, TukeyHSD, $p < 0.05$).

Table 2-1: Dinoflagellate response in MCD to nutrients (N:P), prey availability and the interaction of both tested with a two- factorial ANOVA (exp. 2.1, nutrient ratios). The table gives degrees of freedom (df) for each factor, its F-ratio and significance level (p).

		L. polyedrum			*A. catenella*		
Response	Factor	df	F	P	df	F	p
MCD	nutrients	3	18.635	*<0.0001*	3	41.97	*<0.0001*
	prey	1	0.041	0.627	1	2.458	0.130
	prey*nutrients	3	0.366	0.406	3	2.061	0.132

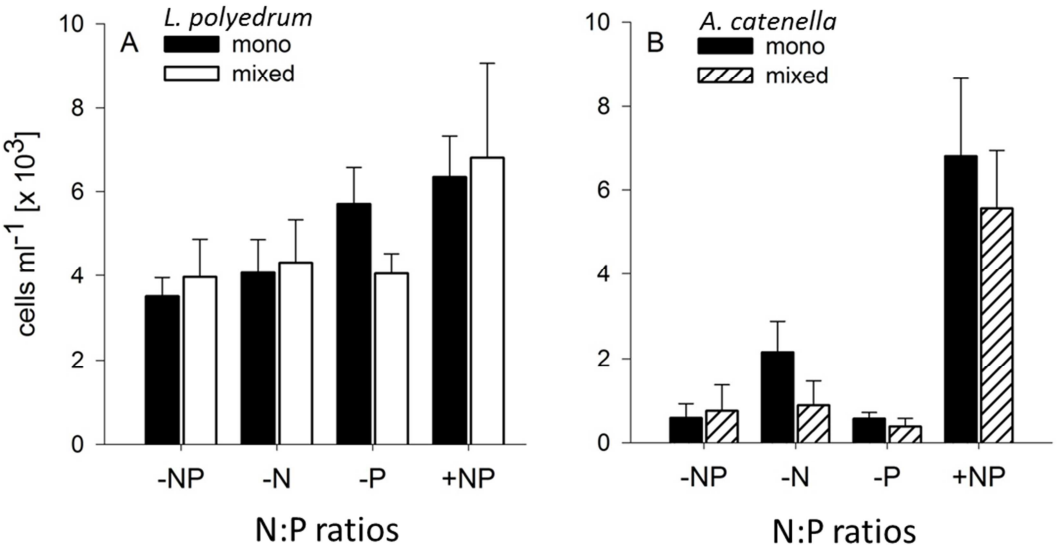

Figure 2-3: Maximum cell density (MCD) of both dinoflagellates growing under different N:P ratios (1. Long term experiment (A: *L. polyedrum* and B: *A. catenella*)). Error bars indicate ± SD, n = 3

L. polyedrum and different N:P treatments significantly affected *Ostreococcus* cell numbers with a significant interaction term (Table 2-2), reflecting that consumer effects differed in different nutrient treatments. *L. polyedrum* had a significantly negative impact on *Ostreococcus* under N limitation (-N) and nutrient depletion (-NP) (TukeyHSD, p< 0.001, Figure 2-4 A, B), but *Ostreococcus* final cell numbers were not reduced by *L. polyedrum* under P limited (-P) and replete nutrient conditions (+NP) compared to the monoculture. On the other hand, *A. catenella* had a significantly negative effect on *Ostreococcus* sp. irrespective of nutrient concentrations and ratios (Table 2-2, Figure 2-4 C, D). *Ostreococcus* cell numbers were reduced by one order of magnitude in all nutrient treatments when grown in mixed culture with *A. catenella* (Figure 2-4**Figure 2-** D). Additionally, *Ostreococcus* cell numbers were negatively affected by nutrient limitation, but these effects were not consistent in mono and mixed culture (significant interaction term (Table 2-2), monoculture: -NP, -P, -N < +NP, TukeyHSD, p< 0.05). In the two experiments (with *L. polyedrum* and *A. catenella*) the *Ostreococcus* monocultures responded differently (in terms of MCD) to different nutrient conditions. These differences were most likely causes by different growth stages of the *Ostreococcus* stock culture used for the experiments.

Table 2-2: Effects of dinoflagellate presence on *Ostreococcus* sp. cell numbers growing under different N:P concentrations and ratios. Data were transformed using Box-Cox transformation. The table gives degrees of freedom (df) for each factor, its F-ratio and significance level (p).

		L. polyedrum			*A. catenella*		
Response	Factor	df	F	P	df	F	p
Ostreococcus	nutrients	3	3.37	<0.05	3	55.09	< 0.0001
cells (ml^{-1})	dinofl. presence	1	23.05	<0.0001	1	437.95	< 0.0001
	nut*dinofl.	3	9.67	<0.0005	3	13.59	< 0.0001

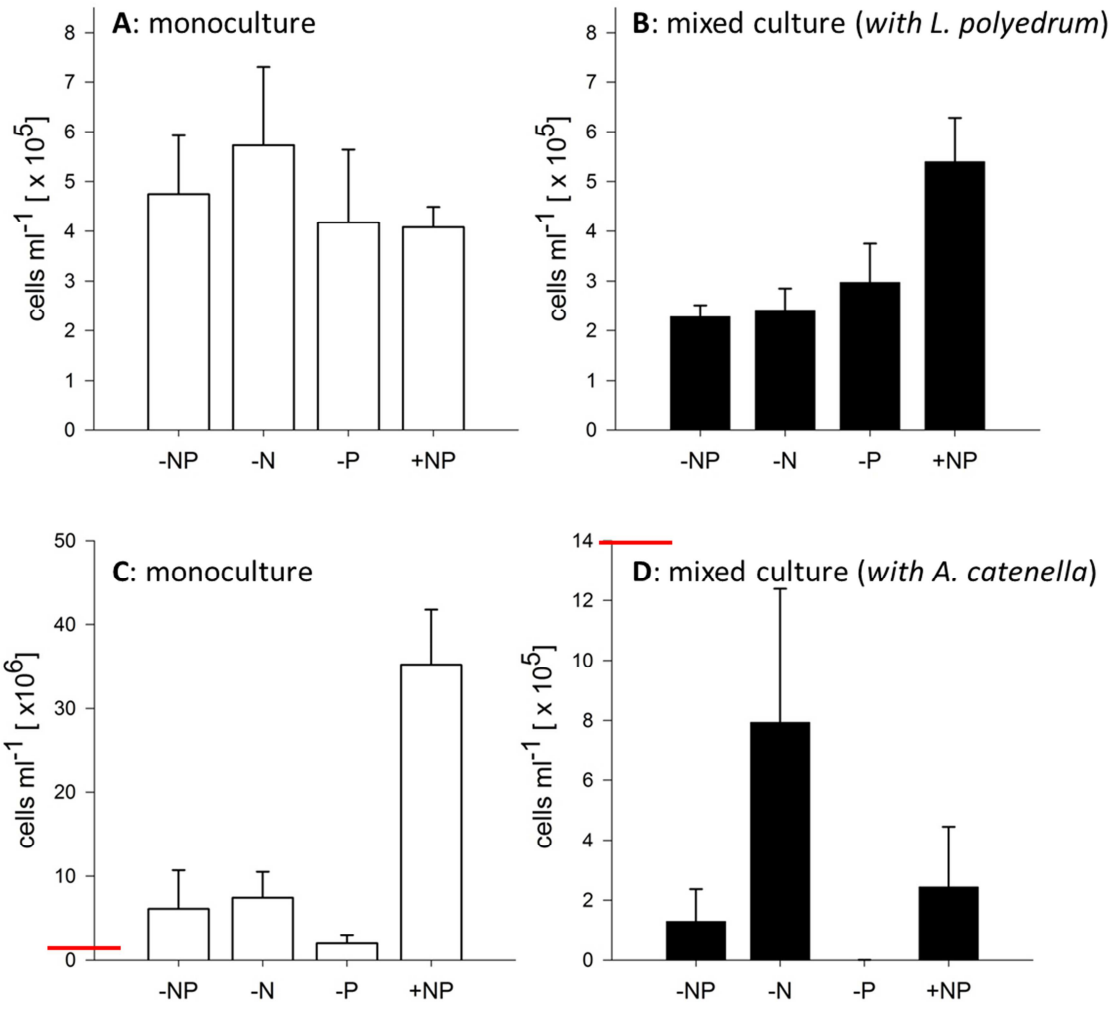

Figure 2-4: *Ostreococcus* cell numbers in different N:P ratios at the end of exponential growth phase A and C in monoculture; B in mixed culture with *L. polyedrum* and D with for *A. catenella*. Values are means and error bars indicate ± SD (n = 3).

Dinoflagellate photosynthetic and mixotrophic growth in a nutrient gradient

L. polyedrum and *A. catenella* growth rates calculated over the length of the exponential growth phase (4-8 days for *L. polyedrum*, 4-10 days for *A. catenella*, depending on the nutrient treatment) exhibited significant nutrient treatment-specific differences ($p < 0.0001$, Table 2-3, Figure 2-5). The addition of prey had no significant effect on the growth rate of *L. polyedrum*, but this effect was marginally non-significant for *A. catenella* ($p = 0.06$, Table 2-3).

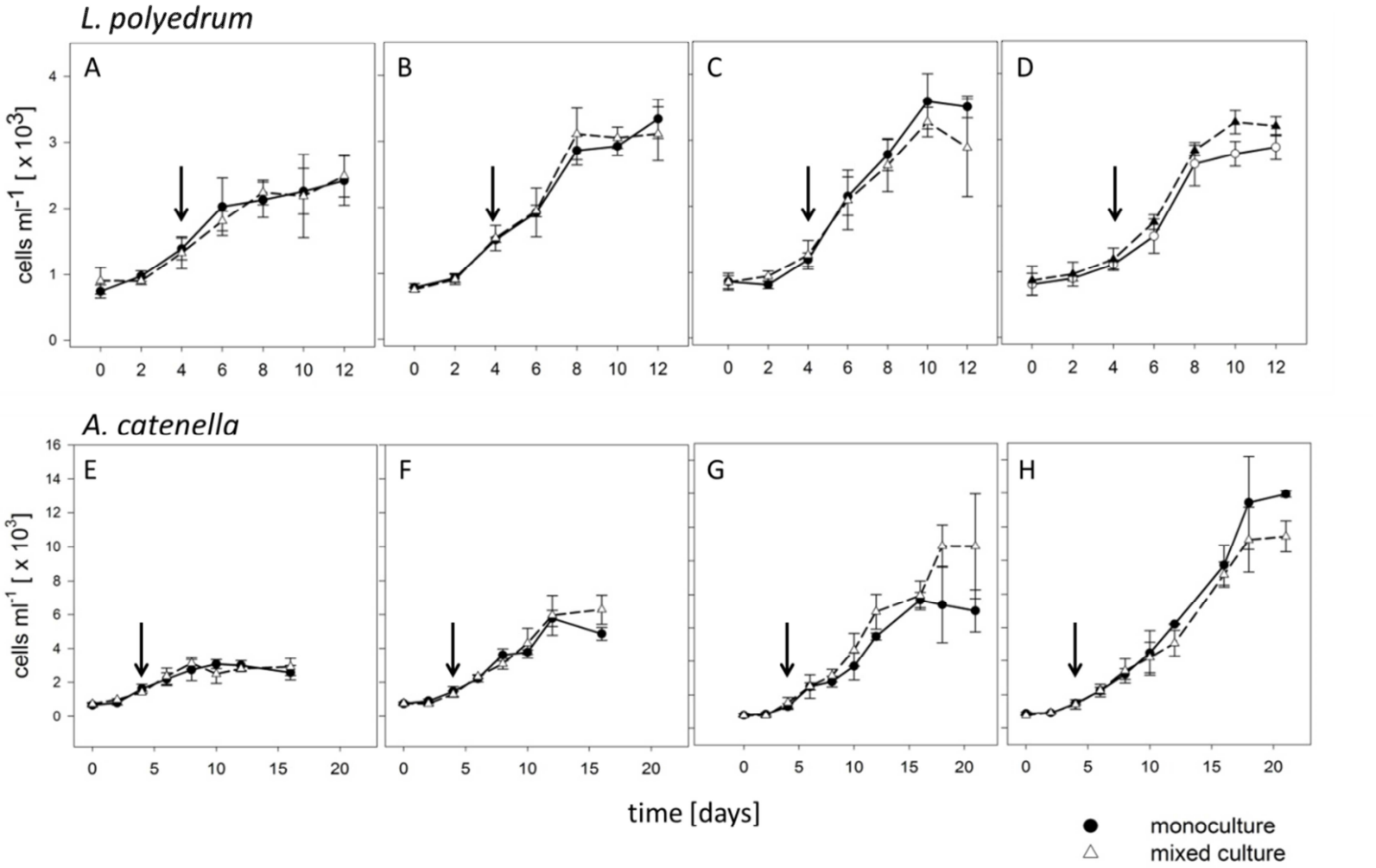

Figure 2-5: Dinoflagellate growth in monoculture and in mixed culture with *Ostreococcus* in a gradient of increasing nutrient (N and P) concentrations. *L. polyedrum* A - D: high (N4) - low (N1) and *A. catenella* E - H: high (N4) - low (N1). Data are presented as means ± 1 SD (n=3) and arrows indicate time points where prey was added.

The significant interaction term for *A. catenella* indicates that the effects of prey addition were not consistent between the nutrient treatments. Prey availability (mixed culture) increased the specific growth rates of *A. catenella* in the N2 treatment compared to the monoculture (Figure 2-6 B). However, no effects of prey presence were observed in the highest (N4), second highest (N3) and lowest (N1) treatments. The addition of *Ostreococcus* sp. slightly promoted the exponential growth of *L. polyedrum* only in the highest nutrient treatment (N4) which resulted in a growth rate of 0.17 ± 0.002 d^{-1} compared to 0.13 ± 0.02 d^{-1} in monoculture (Figure 2-6 A).

Based on these data, the maximum growth rate as a function of increasing nutrient concentrations (Michaelis-Menten fit) was higher for *L. polyedrum* in the mixed culture compared to the monoculture growth, but did not differ for *A. catenella* in mono and mixed culture (Table 2-4). The half saturation constants (k_s), representing the competitive ability of the dinoflagellates under nutrient limitation, were lower for N and higher for P in mixed culture compared to monoculture for *L. polyedrum.* For *A. catenella* the k_s value was 1.2 times lower for both N and P in mixed culture compared to the monoculture (statistically not significant, ANOVA, F=3.3 p > 0.1, Table 2-4).

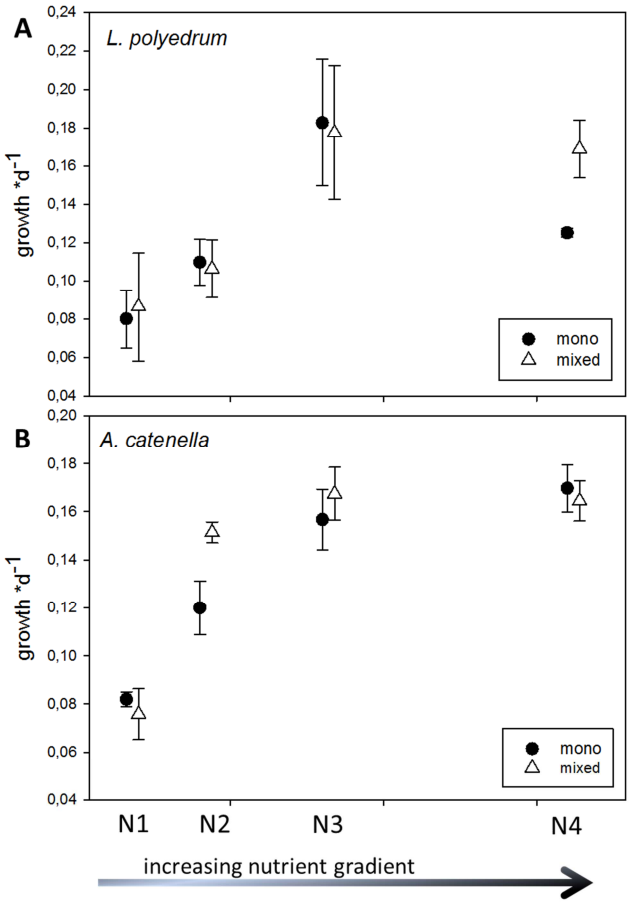

Figure 2-6: Effect of increasing N and P concentrations [µM] on phototrophic (in monoculture) and mixotrophic (in mixed culture) growth rates of (A) *L. polyedrum* and (B) *A. catenella*. Error bars indicate ± SD, n = 3

Only the nutrient gradient, but not the availability of prey (experiment 2.2) had significant effects on *L. polyedrum* MCD at the end of exponential growth (Table 2-3, Figure 2-7A). Maximum cell density (MCD) significantly decreased under low nutrient concentrations (N1) compared to N4 with no differences between mono

and mixed culture (TukeyHSD, $p<0.05$). Only a marginally positive effect of prey availability on *L. polyedrum* cell numbers was visible 6 days after addition of the prey (day 10) in the highest nutrient treatment (Figure 2-7A). However, this elevated MCD level (N4) in the mixed culture of about 10% was not significantly different from the monoculture (TukeyHSD, $p > 0.05$).

A. catenella MCD was also not affected by the availability of prey, but significantly affected by the nutrient gradient, and this effect was much stronger compared to *L. polyedrum* (Figure 2-7, Table 2-3). The MCD significantly decreased with decreasing nutrient concentrations, however, this effect was stronger from nutrient concentration N4 to N3 for *A. catenella* growing alone compared to growing with *Ostreococcus* (significant nutrient * prey interaction, Table 4). Even though there were no significant differences between the MCD of *A. catenella* in mono and mixed culture, there was a non-significant trend ($p <0.1$) of an elevated MCD in the N3 mixed treatment relative to the monoculture (increase of 32%, Figure 2-7 B, Figure 2-5 F).

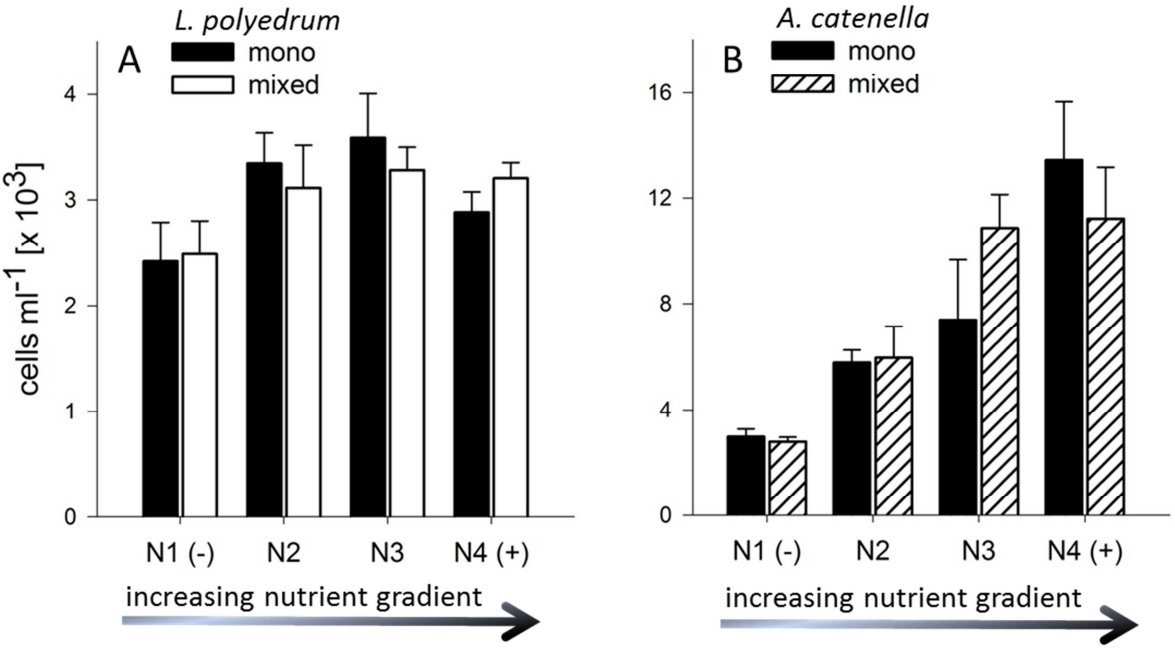

Figure 2-7: Maximum cell density (MCD) of both dinoflagellates growing in an increasing nutrient gradient (2. long term experiment, C and D). Values are means, error bars indicate ± 1 SD, n = 3

Table 2-3: Dinoflagellate response to inorganic nutrients, prey availability and the interaction of both tested with a two- factorial ANOVA (exp. 2.2, nutrient gradient). The table gives the degrees of freedom (df) for each factor, its F-ratio and significance level (p).

		L. polyedrum			*A. catenella*		
Response	Factor	df	F	P	df	F	P
MCD	Nutrients	3	10.6	*<0.001*	3	46.604	*<0.001*
	Prey	1	0.166	0.689	1	0.292	0.596
	prey*nutrients	3	1.109	0.376	3	3.865	*<0.05*
growth rate	Nutrients	3	19.478	*<0.001*	3	110.38	*<0.001*
	Prey	1	0.495	0.492	1	3.967	0.063
	nutrients*prey	3	2.351	0.111	3	5.235	*<0.05*
total N	Nutrients	3	21.79	*<0.001*	3	3.550	*<0.04*
(log)	Prey	1	72.29	*<0.001*	1	162.92	*<0.0001*
	nutrients*prey	3	0.559	0.651	3	3.572	*<0.039*
total C	Nutrients	3	2.215	0.132	3	0.040	0.989
	Prey	1	71.8	*<0.001*	1	56.05	*<0.001*
	nutrients*prey	3	0.63	0.606	3	0.094	0.962
total P	Nutrients	3	6.69	*<0.005*	3	0.455	0.717
(log)	Prey	1	5.619	*<0.05*	1	20.80	*<0.0001*
	nutrients*prey	3	0.63	0.605	3	0.87	0.47
C:N	Nutrients	3	742.786	*<0.001*	3	135.4	*<0.001*
(log)	Prey	1	24.705	*<0.001*	1	53.9	*<0.001*
	nutrients*prey	3	3.829	*<0.05*	3	0.584	0.635
C:P	Nutrients	3	39.2	*<0.001*	3	15.21	*<0.001*
(log)	Prey	1	0.100	0.757	1	1.15	0.303
	nutrients*prey	3	0.235	0.870	3	2.39	0.115
N:P	Nutrients	3	0.054	0.983	3	8.369	*<0.005*
(log)	Prey	1	1.556	0.238	1	0.606	0.452
	nutrients*prey	3	0.478	0.704	3	1.654	0.233

Table 2-4: Maximum growth rate (d^{-1}), half saturation constant (k_s) for PO_4^{3-} and NO_3^- of both dinoflagellates (in [a] mono- and [b] mixed treatment) growing in an increasing nutrient gradient (N1-N4).

	L. polyedrum	*A. catenella*
μ_{max} d^{-1}	0.167±0.02 [a] 0.199±0.01 [b]	0.196± 0.01 [a] 0.197± 0.01 [b]
K_s (N)	30.55 ±16.2 [a] 20.8 ± 5.9 [b]	46.13± 6.5 [a] 37.45± 8.3 [b]
K_s (P)	1.01 ± 1.0 [a] 3.16 ± 1.4 [b]	2.88 ± 0.40 [a] 2.34 ± 0.52 [b]

Ostreococcus sp. cells could no longer be detected in any of the nutrient treatments (experiment 2.2) after 6 days of incubation with *L. polyedrum* and after 4 days of incubation with *A. catenella* (Figure 2-8). Both dinoflagellates as well as different nutrient concentrations had a significantly negative impact on *Ostreococcus* cell numbers at the end of the exponential growth phase (Figure 2-8, Table 2-5).

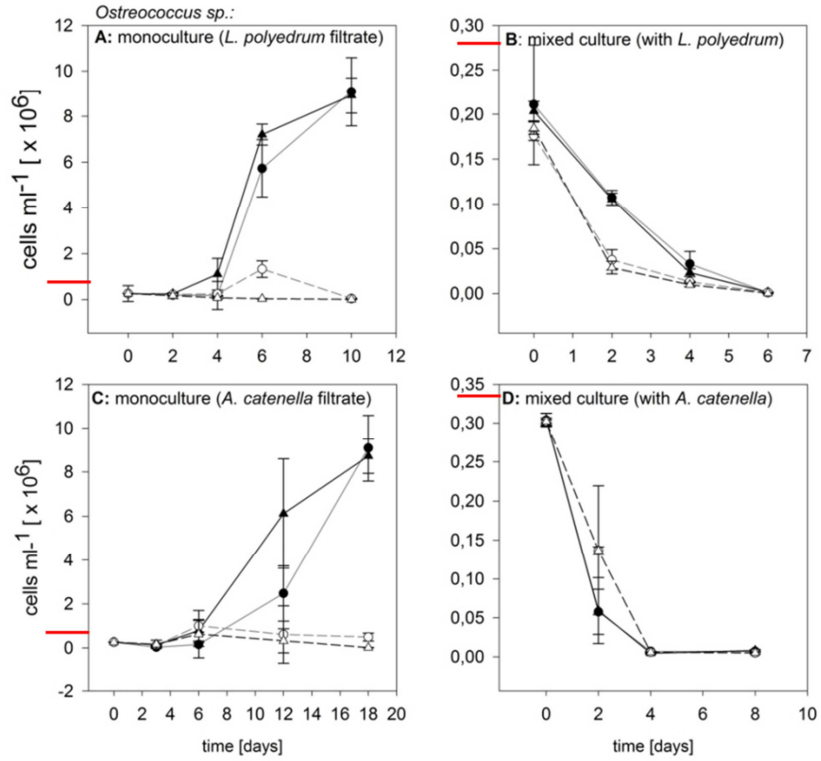

Figure 2-8: *Ostreococcus* sp. growth (A) and (C) in monoculture (dinoflagellate filtrate) and (B) and (D) in mixed culture with the dinoflagellates in different nutrient concentrations (Error bars indicate ± SD, n = 3).

While the influence of low nutrient conditions in the N1 and N2 treatment was stronger than the influence of dinoflagellate presence, the impact of the dinoflagellate consumers was restricted to the higher nutrient levels (N3 – N4), reflecting the significant interaction between nutrients and dinoflagellate presence (Table 2-5). The lag phase before *Ostreococcus* exponential growth started was 2 days in *L. polyedrum* filtrate and 3 days in *A. catenella* filtrate (Figure 2-8). While *Ostreococcus* growth was negative during the first 3 days in *A. catenella* filtrate (growth rates between -0.73±0.18 and -0.21±0.06); *L. polyedrum* filtrate did not have an effect on *Ostreococcus* growth (growth rates between -0.03 ± 0.01 and -0.002 ± 0.006). The *Ostreococcus* loss rate due to dinoflagellate presence per time interval (in pg C dinoflagellate cell^{-1}) was not significantly different across the nutrient gradient in the first time interval for *L. polyedrum*, but was significantly higher in the N1 and N3 treatment compared to N2 and N4 when grown in mixed culture with *A. catenella* (Figure 2-9, Table 2-5). During the second time interval this pattern changed and *Ostreococcus* loss rates were significantly higher with *L. polyedrum* in the two highest nutrient treatments (N4 and N3) compared to N1 and N2 (TukeyHSD, $p< 0.05$, Figure 2-9 A). This result is consistent with the finding that during the second time interval no positive growth due to nutrient limitation was observed in the *Ostreococcus* monoculture in these two treatments (N2 and N1) and thus dinoflagellate feeding had no additional impact. For *A. catenella* feeding significantly increased in the N2 treatment in the second time interval with a tendency of decreasing loss rates with increasing nutrient concentrations (Table 2-5, Figure 2-9 B).

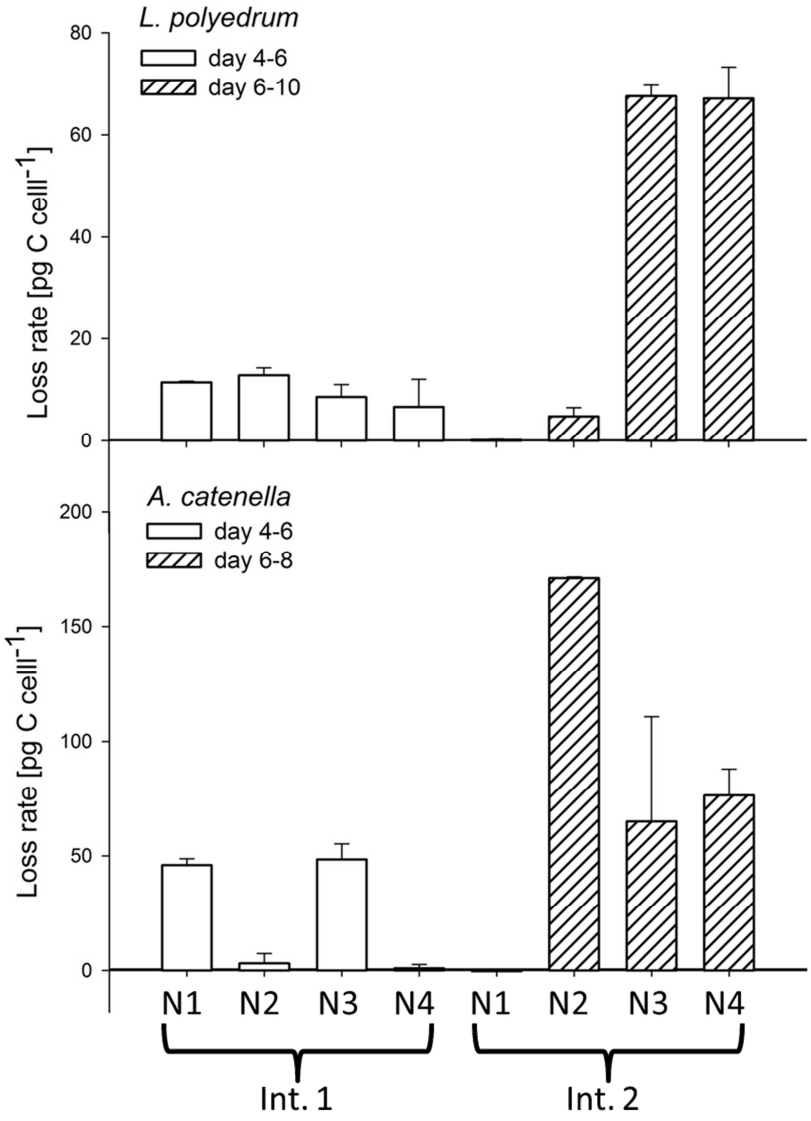

Figure 2-9: Loss rates (pg C cell^{-1}) of *Ostreococcus* sp. in an increasing nutrient gradient A*:* for *L. polyedrum* and B: for *A. catenella*. Interval 1 was calculated for day 4-6 and interval 2 from day 6-10 (*L. polyedrum*) and day 6-8 (*A. catenella*). Data are presented as means ± 1 SD (n=3).

Table 2-5: Effects of dinoflagellate presence (D) on *Ostreococcus* sp. cell numbers and loss rate (pg C dinoflagellate cell^{-1}) in the nutrient gradient (N) tested with a 2-factor ANOVA. Data were transformed using Box-Cox transformation. Loss rate 1 and 2 represent the *Ostreococcus* loss rate in the two time intervals. Loss rate 2 for *L. polyedrum* was tested using the non-parametric Kruskal-Wallis test. The table gives degrees of freedom (df) for each factor, its F-ratio and significance level (p).

			L. polyedrum			*A. catenella*	
Response	Factor	df	F	P	df	F	p
Ostreococcus	N	3	45.704	*<0.0001*	3	64.82	*<0.0001*
cells (ml^{-1})	D	1	93.05	*<0.0001*	1	169.17	*<0.0001*
	N*D	3	3.72	*<0.05*	3	41.07	*<0.0001*
Loss rate 1	N	3	2.918	0.1	3	18.75	*<0.0005*
Loss rate 2	N	3	9.401 (chi-square)	*<0.05*	3	11.54	*<0.05*

Inorganic nutrient concentrations (experiment 2 B)

Due to the pre-culturing of the dinoflagellates under nutrient-limited conditions for 7 days (see Methods) it can be assumed that both dinoflagellates were nutrient limited before the experiment was started. Dissolved nutrient concentrations (N and P) decreased steadily through the course of the experiment throughout all nutrient treatments and species combinations (Figure 2-10). However, the two dinoflagellates showed different uptake patterns for both nutrients N and P, but no clear differences in mono and in mixed culture. The N and P utilization of *L. polyedrum* cultures was highest during the first 4 days of incubation, resulting in significantly lower nutrient concentrations compared to the *A. catenella* approach at the time point when *Ostreococcus* sp. was added to the experiment (Kruskal-Wallis, $p< 0.05$). *L. polyedrum* cells, when grown on less than 320 µM nitrate (treatments N3 - N1), exhausted the N in the medium by late-exponential phase (day 6), whilst those supplied with more than 320 µM N (treatment N1) had an excess of nitrate present even at the beginning of the stationary phase (Figure 2-10). Dissolved inorganic phosphate was depleted (< 1 µM P) by *L. polyedrum* when cells where grown in less than 20 µM P (treatment N3 – N1) by mid-exponential phase (after day 4), but was still present in excess at the end of the experiment in N4. For *A. catenella*, nitrate concentrations decreased only slightly until day 12 and were between 65 µM and 0.2

µM (N4 – N1) at the end of the experiment. Phosphate concentrations in *A. catenella* cultures decreased rapidly at the beginning of exponential growth when supplied at lower concentrations (< 20 µM P, N3-N1 treatments, Figure 2-10), but not when cells were supplied with P in excess of 20 µM (N4). Interestingly, phosphate concentrations were significantly higher in the N4 and N2 mixed treatments compared to the monoculture at the end of exponential growth (Kruskal-Wallis, $p<0.05$).

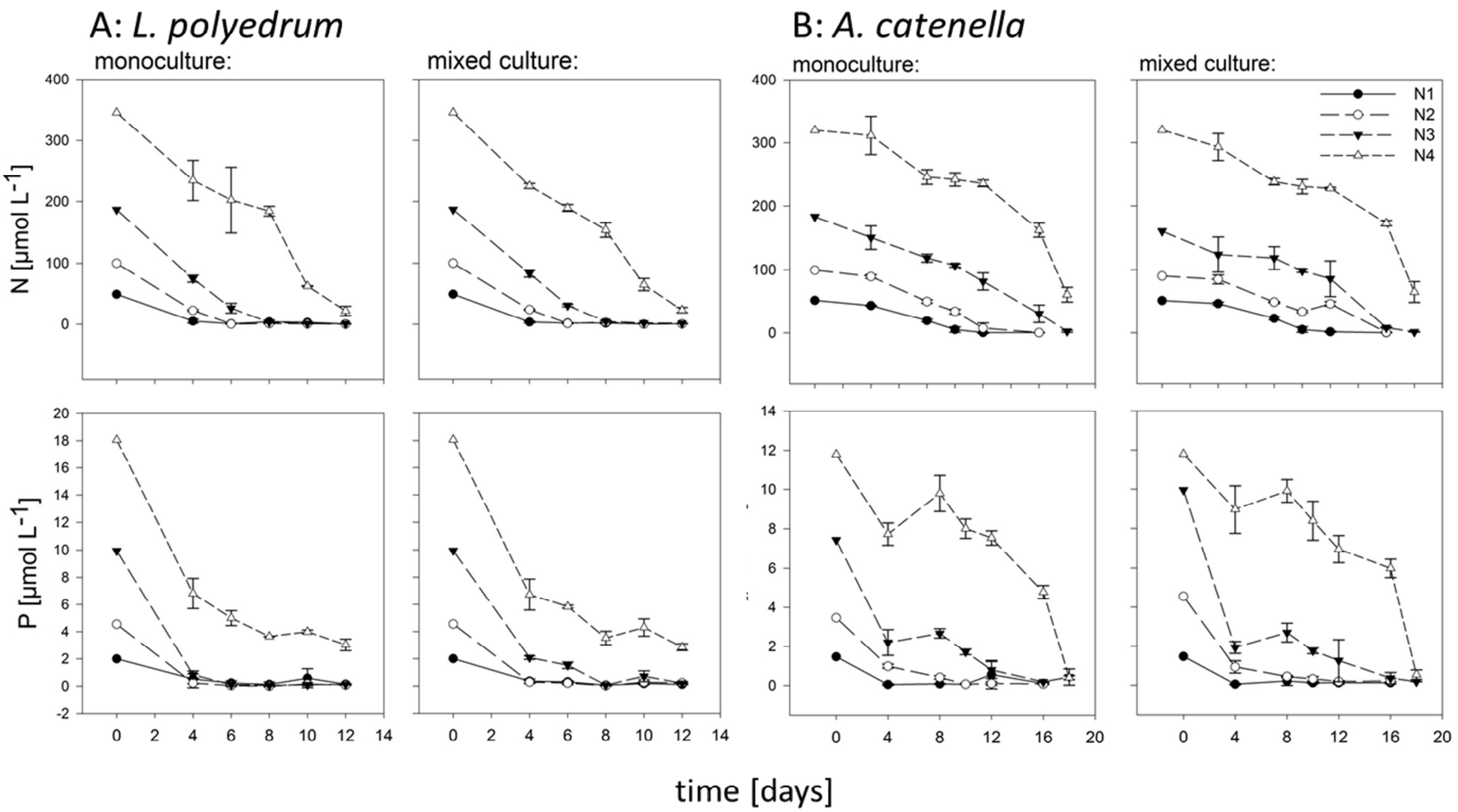

Figure 2-104: Dissolved inorganic N and P concentrations (µmol L^{-1}) over time for both dinoflagellate treatments in mono- and in mixed culture. Data are presented as means ± 1 SD (n=3).

Effects of dissolved inorganic nutrients and prey availability on dinoflagellates' internal cellular nutrient concentrations and ratios

The initial gradient of dissolved nutrient concentrations supplied for all species treatments resulted in the attainment of different nutritional states at the beginning of stationary growth (Figure 2-11). At the end of the exponential growth phase, *L. polyedrum* cellular N and P concentrations were significantly affected by the nutrient gradient and prey abundance ($p<0.001$,Table 2-3, Figure 2-11). Cellular phosphate values for *L. polyedrum* need to be considered with care. They appear to be too low, which is consistent over all replicates and treatments and might be due to analytical problems. Besides, internal phosphate and nitrate concentrations in *L. polyedrum* increased with increasing nutrient concentrations and were lower in mixed culture compared to monoculture (Figure 2-11 B, C). *L. polyedrum* cellular C content was significantly decreased by prey abundance while the nutrient gradient had no effect (Figure 2-11, Table 2-3). *A. catenella* cellular N content significantly increased with increasing nutrient concentrations in monoculture. In mixed culture, N content was consistently significantly lower compared to monoculture, but irrespective of nutrient concentrations (significant interaction of main factors, Table 2-3, Figure 2-11 H). In contrast, the cellular C and P contents were only significantly decreased by prey presence, but hardly affected by dissolved nutrient concentrations (Table 2-3, Figure 2-11 G, I). The molar C:N ratio for both *L. polyedrum* and *A. catenella* significantly decreased with increasing nutrient concentrations in both mono- and mixed culture (Table 2-3, Figure 2-11 D, J). Low nutrient supply also increased the molar C:P ratio at the stationary phase for both dinoflagellates and the N:P ratio for *A. catenella,* while prey addition had no effect (Table 2-3, Figure 2-11 E, K, L). In turn, neither the nutrient gradient nor prey addition had an effect on the cellular N:P ratio of *L. polyedrum* (Figure 2-11F, Table 2-3). Considering different time intervals in the experiment, there was a significant relationship between prey loss rate and the cellular P content as well as the C:P ratio of *L. polyedrum* in the first time interval (day 4-6) after *Ostreococcus* was added (Spearman rank order correlation, Table 2-6). The total P content was negatively and the C:P ratio positively correlated with the prey loss rate. This pattern changed in the second time interval (day 6 - 10).

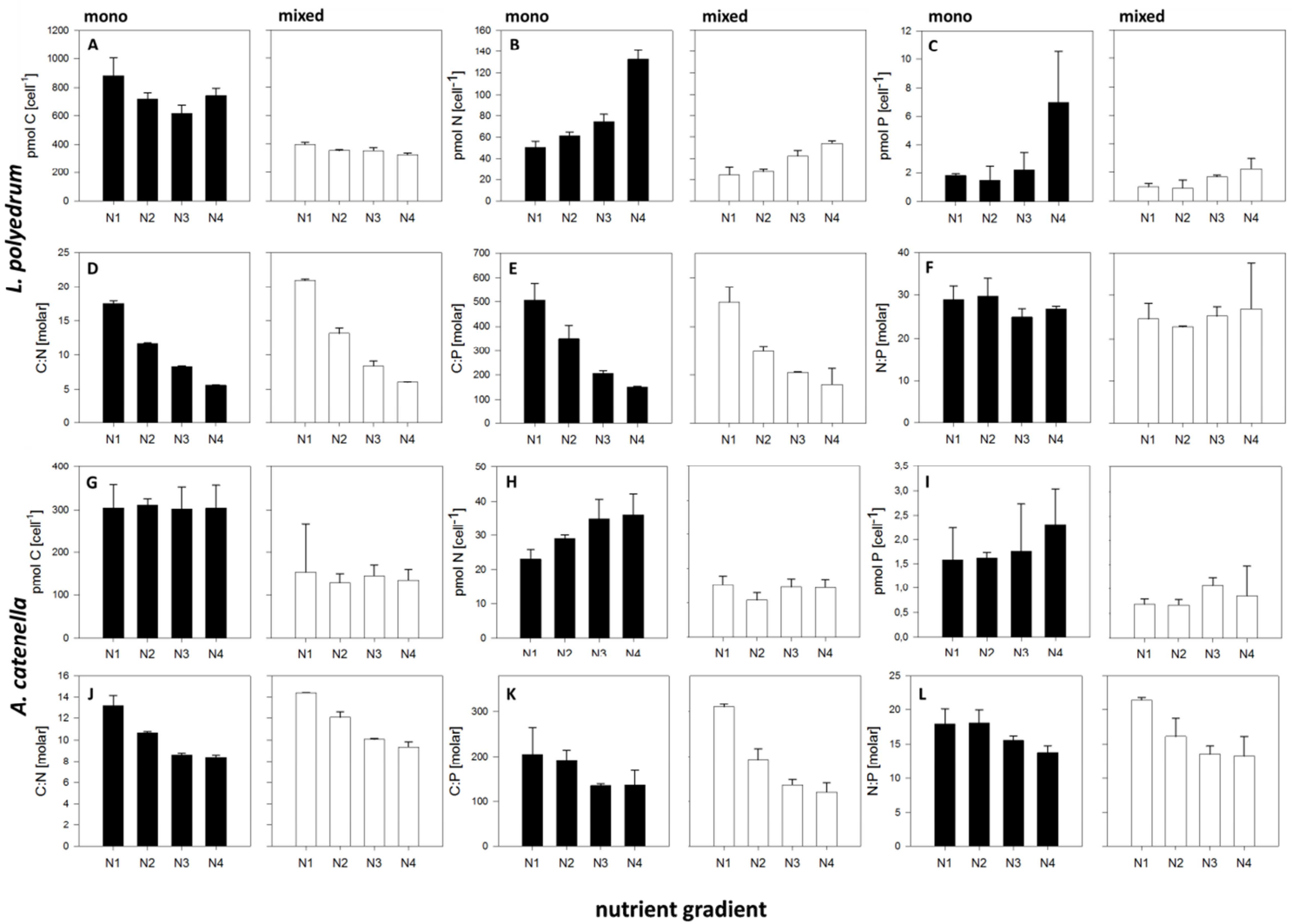

Figure 2-11: Final cellular nutrient contents and ratios for *L. polyedrum* (A-F) and *A. catenella* (G-L) in mono and mixed culture across the increasing nutrient gradient (N1-N4). Data were determined once stationary growth phase was reached and are presented as means ± 1SD (n=3).

At this point, the prey loss rate was positively correlated with the cellular N and P content, but negatively correlated with the cellular C content (Spearman rank order correlation, $p<0.05$). Accordingly, the C:N and C:P ratios were negatively correlated with the prey loss rate (Table 2-6). Similar to *L. polyedrum*, prey loss rates caused by *A. catenella* were significantly negatively correlated with the cellular P content in the first time interval (day 4 - 6), but positively correlated in the second time interval (Table 2-6). The *A. catenella* cellular C and N contents were not significantly correlated with the loss rate.

Table 2-1: Spearman rank order correlation analysis of the prey loss rate (defined as the loss rate of *Ostreococcus* sp. (ng C ml^{-1}) in two time intervals) with cell numbers and cellular nutrient contents and ratios. Coeff. = correlation coefficient. n= 12

	L. polyedrum		*A. catenella*	
Parameter	Int1	Int2	Int1	Int2
Cell numbers				
coeff.	0.294	0.552	0.572	-0.054
p-value	0.354	0.066	0.071	0.881
Total C				
coeff.	0.300	-0.839	-0.591	0.254
p-value	0.343	*0.001*	0.061	0.451
Total N				
coeff.	-0.300	0.783	-0.591	0.164
p-value	0.33	*0.004*	0.061	0.633
Total P				
coeff.	-0.489	0.713	-0.627	0.745
p-value	0.109	*0.012*	*0.044*	*0.012*
C:N				
coeff.	0.483	-0.804	-0.081	-0.218
p-value	0.115	*0.002*	0.81	0.521
C:P				
coeff.	0.692	-0.727	0.381	-0.336
p-value	*0.015*	*0.01*	0.248	0.313
N:P				
coeff.	0.713	0.034	0.427	-0.327
p-value	*0.012*	0.921	0.193	0.327

Toxin content (*A. catenella*)

The PSP toxin profile of *A. catenella* revealed the presence of the following toxins in decreasing order of molar percentage of total toxin content: GTX1/4 (52.9%) and B1 (23.1%), C1/C2 (22.9%). GTX2/3, Neosaxitoxin (NEO) and Saxitoxin (STX) were only present in trace amounts (<1 mol%). However, only C1/C2 and B1 were detectable in the samples at the end of the exponential growth phase. The concentration of GTX1/4 was below the detection limit (<0.5ng μl^{-1}).

The total toxin content (fmol STX-eq $cell^{-1}$) of *A. catenella* was significantly affected by the nutrient gradient but not by prey availability (Table 2-7). At the end of exponential growth the lowest total toxin concentrations were observed under high nutrient concentrations (N4), with 6.9 ± 1.1 fmol STX-eq $cell^{-1}$ (in monoculture). In comparison, total toxin content was almost twice as high in the lowest nutrient treatment (TukeyHSD $p<0.01$, Table 2-7) with no significant differences between mono and mixed culture. This increase resulted mostly from the dramatic increase of one toxin derivative (B1). A separate ANOVA for B1 concentrations showed significantly higher levels in the lowest nutrient treatment N1 compared to N2, N3 and N4 (TukeyHSD, $p< 0.01$ for all comparisons with N1).

Table 2-7: Response of cellular toxin content and composition to inorganic nutrients, prey availability and the interaction of both tested with a two- factorial ANOVA (exp. 2.2, nutrient gradient). The table gives the degrees of freedom (df) for each factor, its F-ratio and significance level (p).

Response	Factor	df	F	p-value
total toxin content	Prey	1	0.039	0.845
(fmol $cell^{-1}$)	Nutrients	3	19.797	*< 0.0001*
	prey*nutrients	3	0.758	0.534
toxin composition	Prey	1	0.099	0.768
	Nutrients	3	20.76	*< 0.0001*
	prey*nutrients	3	1.344	0.295

2.4. Discussion

Effects of dinoflagellates on the prey population (feeding, competition or allelopathic effect)

Both dinoflagellates ingested *Ostreococcus* cells in short-term experiments which supports the findings of previous studies where a variety of different prey sizes and taxonomic groups have been ingested (i.e. cryptophytes, diatoms, dinoflagellates, haptophytes, raphidophytes and the cyanobacterium *Synechococcus*, Jeong *et al.* 2005 a, b, Yoo *et al.*, 2009; Seong *et al.*, 2006). The prey population was significantly negatively affected by dinoflagellate presence, with similar effects under different N:P ratios and with an increasing nutrient gradient (supporting H1). However, these negative effects of both dinoflagellates on prey were driven by different mechanisms. *L. polyedrum* significantly reduced *Ostreococcus* cell numbers under N limitation and under nutrient depletion (-N-P), indicating enhanced phagotrophic feeding under low nutrient conditions. The negative effect of *L. polyedrum* on its prey was significant in all treatments across the nutrient gradient. Several mixotrophic dinoflagellates have been found to ingest prey primarily under nutrient limitation; two separate studies have found feeding to be most strongly induced by P-limitation in *Ceratium furca* (Smalley *et al.* 2003) and *Prorocentrum minimum* (Johnson, 2014). A different feeding pattern was observed for *Karlodinium veneficum* in which feeding occurred under nutrient replete conditions but increased with starvation of N, P or both (Li *et al.* 2000), which is comparable to what I observed for *L. polyedrum*.

A. catenella, on the other hand, had a significantly negative impact on *Ostreococcus* irrespective of N:P ratios and nutrient concentrations. However, under short term conditions the negative effect on *Ostreococcus* was stronger in P-limited medium compared to the f/2 medium. Additionally, *A. catenella* cell free filtrate decreased *Ostreococcus* cell numbers in short-term experiments and in the first 3 days of the second long term experiment, after which the *Ostreococcus* population recovered again, indicating allelochemical degradation or adsorption. These results indicate that *A. catenella* reduced its prey by a combined effect of phagotrophic feeding and the continuous release of allelochemicals causing cell lysis. The production and release of allelochemicals has frequently been reported for different *Alexandrium* species including *A. catenella* (i.e. Arzul *et al.* 1999, Tillmann *et al.* 2008, 2009). Initial characterization of allelochemicals have shown that these substances can adsorb to

all kinds of surfaces and cause lysis of target cells (Ma *et al.*, 2009). Consequently, lytic substances binding to different surfaces such as *Ostreococcus* cells or other organic material may lead to a removal of compounds from the medium. Not much is known about degradation of the compounds or adaptation of target organisms, but since the allelochemicals were not continuously released by *A. catenella* in the filtrate decreasing activity due to adsorption or degradation can be assumed.

L. polyedrum filtrate, however, had no negative effect on *Ostreococcus* sp. and there are no data available regarding allelopathic effects of *L. polyedrum* on other phytoplankton. Therefore, it can be assumed that the loss of prey cells in the mixed culture with *L. polyedrum* was caused by prey ingestion.

Nutrient and prey effects on dinoflagellate growth and maximum cell density

Nutrient effects

Only the lowest nutrient levels significantly decreased the MCD of *L. polyedrum* in both experiments (Exp. 2.1 (ratios) and Exp. 2.2. (gradient)), while its growth rate continuously decreased with decreasing nutrient concentrations. N or P limitation alone (experiment 2.1) had no effect on the MCD and growth rate. After *L. polyedrum* was acclimatised to lower nutrient conditions before the start of the experiment 2.2, a high consumption of inorganic nutrients (both N and P) was observed mainly in the beginning of the experiment across the nutrient gradient, indicating a luxury uptake of nitrogen and phosphorus, i.e. excessive cellular storage not related to growth rate. Dinoflagellates can store large amounts of inorganic and organic N forms (Dagenais-Bellefeuille and Morse, 2013; Flynn *et al.*, 1996; Maguer *et al.*, 2007). The uptake of excess P at increased maximum rates, which is incorporated into the cellular polyphosphate fraction, has also been observed before (e.g. Cembella *et al.*, 1982; Healey, 1973; Smalley *et al.*, 2003). In the present experiments, *L. polyedrum* was able to maintain high MCD irrespective of different nutrient conditions, suggesting that it was able to draw on nutrients mobilized from internal storage pools. This could also be an explanation for the increasing C:N and C:P ratios over time and across the nutrient gradient (measured in experiment 2.2). On the other hand, Dagenais-Bellefeuille *et al.* (2014) found that *L. polyedrum* under N stress immediately stopped cell growth and diminished levels of internal nitrogen, in particular in the form of

protein and chlorophyll. These findings indicated that *L. polyedrum* did not store N in a form that could have sustained growth and cell division under N stress, which is consistent with the decreasing growth rates across the nutrient gradient observed in this experiment. These results indicate that *L. polyedrum* was able to maintain high cell numbers when one nutrient (N or P) was limiting, while co-limitation in the lower nutrient levels had a negative effect. The increasing C:N and C:P ratios over time and across the nutrient gradient further suggest the maintenance of photosynthesis at high levels, even under low nutrient conditions, resulting in accumulated photosynthetic products in the form of starch (Dagenais Bellefeuille *et al.*, 2014).

A. catenella MCD and growth rate was significantly negatively affected by low dissolved nutrient concentrations and N- or P-depletion which can be explained by low nutrient affinities for nitrate and phosphorus as discussed in Collos *et al.* (2009, 2004). Based on results of growth kinetics, Matsuda *et al.* (1999) suggested that *A. catenella* could not become dominant in waters subject to N or P limitation. Collos *et al.* (2004) found that *A. catenella* accumulates large amounts of N before it starts to grow, but this cannot be supported by the present analytical results of dissolved nutrients. In this experiment, *A. catenella* did not exhibit luxury nitrate uptake as was observed for *L. polyedrum.* However, there were indications for higher phosphorus uptake in the first 4 days of the experiment as has been discussed for *A. tamarense* by Van de Waal *et al.*, (2013). They found that phosphorus starved cultures increased their internal phosphorus storage remarkably during the first 2 days when transferred into phosphorus rich medium.

Prey effects

As outlined in previous studies, ingestion of prey organisms can increase the growth of mixotrophic dinoflagellates (e.g. Hansen, 2011; Jeong *et al.*, 2005b; Johnson, 2015; Li *et al.*, 1999; Yoo *et al.*, 2009), possibly through the supplement of cellular C, N or P pools under nutrient-limited conditions. Even under nutrient-replete conditions, the growth of dinoflagellates can be significantly improved by feeding (Burkholder *et al.*, 2008; Stoecker *et al.*, 2006). Therefore, I initially expected the dinoflagellates to be negatively affected in monoculture by nutrient limitation and accordingly to gain a benefit from growing with prey under different nutrient conditions. *L. polyedrum* and *A. catenella* showed different responses to the addition of prey and different nutrient

conditions, but in contrast to previous expectations maximum cell density was not significantly improved for either of the two dinoflagellates in the presence of *Ostreococcus* sp. in any of the nutrient treatments (rejecting H2). Moreover, the MCD of *A. catenella* was significantly decreased by prey abundance under N limitation and under nutrient replete conditions, which can be explained by possible competition for dissolved nutrients with the prey *Ostreococcus*. In the nutrient ratio experiment, *Ostreococcus* was abundant in low cell numbers until the end of the experiment and might have had an impact on dissolved nutrient concentrations. In contrast, in the second experiment *Ostreococcus* was not measurable after 4 days of incubation with *A. catenella*. Thus, there was no competition for dissolved nutrients and *A. catenella* reached similar MCD as in monoculture. Similar results have been reported on *A. catenella* by Zhang *et al.*, (2013), where prey (*Isochrysis galbana*) did not promote *A. catenella* growth, but, as in the present experiments, disappeared quickly after being added to the culture or to cell free filtrate of *A. catenella*. However, Collos *et al.*, (2009) suggested from a field study in the Thau Lagoon (Southern France) that *A. catenella* supplemented limiting N sources for its growth by using particulate N in form of pico-cyanobacteria, indicating that for *A. catenella,* potential benefits through prey may be prey species- and context specific.

Contrary to predictions made by Jeong *et al.* (2005b), *L. polyedrum* MCD and growth rate were not significantly promoted by prey availability in any of the nutrient treatments in this study. There were only negligible indications in both experiments of elevated MCD and growth rates with prey under high nutrient conditions. Jeong *et al.* (2005b) reported higher maximum growth rates for *L. polyedrum* in mixed culture with *Prorocentrum minimum* of 0.254 d^{-1} and with *Scrippsiella trochoidea* of 0.303 d^{-1}. The corresponding growth rates in monoculture were between 0.157 and 0.182 d^{-1}, which are comparable with the growth rates in *L. polyedrum* monocultures of this study. While the maximum growth rates in this study were calculated by fitting the specific growth rates to the nutrient gradient, Jeong *et al.* (2005b) fitted the specific growth rates to an increasing prey concentration. Thus, these data are not perfectly comparable. However, despite these differences my data also suggests an enhanced maximum growth rate in mixed culture at high nutrient concentrations, indicating that the availability of both inorganic and organic resources were advantageous for *L. polyedrum*. Based on these results, it is important to consider whether the available prey carbon content was sufficient to enhance the growth and the MCD of the

dinoflagellates. The initial prey carbon content in this experiment was between 64 and 83.2 ng C ml^{-1} while the maximum growth rate of *L. polyedrum* with prey according to Jeong *et al.* (2005b) was reached at an initial prey C content of between 170 and 210 ng C ml^{-1}. In this context it needs to be considered that the entire prey population disappeared in both mixed cultures (with *L. polyedrum* and with *A. catenella*) after at least 6 days. This might explain why the MCD and specific growth rate for both dinoflagellates was not enhanced by prey abundance in the present experiments.

In addition to the commonly discussed physiological advantage of prey ingestion, phagotrophic feeding can also just be an ecological strategy for eliminating or reducing other phytoplankton competitors (Stoecker *et al.* 2006). Jeong *et al.* (2005b) estimated the grazing impact of a *L. polyedrum* population on co-occurring *Prorocentrum* sp. and *Scrippsiella trochoidea* as a removal of 2.6 and 1.1% of the prey population in 1h, respectively. Another study by Jeong *et al.*, (2005a) suggests that red-tide dinoflagellates potentially have a strong grazing impact on populations of *Synechococcus* which is in the same size range as *Ostreococcus*. This implies that dinoflagellate grazers can have a considerable grazing impact on populations of co-occurring phytoplankton competitors. However, not only the ingestion of competitors is relevant; it is also important to consider the use of allelochemicals (as shown for *A. catenella*), which can be used in combination with phagotrophic feeding to avoid competition and possibly to gain additional organic nutrients by lysing prey cells (Stoecker *et al.* 2006, Jonsson *et al.* 2009). Weissbach *et al.* (2012) provided evidence that the addition of lytic *A. tamarense* filtrate to a plankton community provoked the release of bioavailable dissolved organic material (DOM). Several previous studies have shown that *A. catenella* is able to use dissolved organic material (DOM) (Carlsson *et al.*, 1998; Collos *et al.*, 2007; Legrand and Carlsson, 1998; Loureiro *et al.*, 2009) and shows enhanced growth when DOM is added to laboratory cultures. However, this cannot be supported by the present study.

Nutrient and prey effects on dinoflagellate cellular nutrient concentrations and ratios

The cellular phosphorus content of both dinoflagellates decreased with increasing ingestion rate during the first feeding interval (2 days after the prey was added). This

pattern changed during the second time interval (4-6 days): cellular P content (and N content for *L. polyedrum* only) showed a significant positive response to ingestion rate. This might be an indication that the metabolic costs for switching nutritional modes from photosynthesis to phagotrophy are high at first for the dinoflagellates as a result of maintaining photosynthetic organelles, enzyme systems for the assimilation of inorganic nutrients, and the feeding apparatus (Raven, 1997). However, the positive correlations between cellular N and P content and ingestion rate for *L. polyedrum* during the second time interval (4 days) indicate that feeding had a positive effect on cellular nutrient contents (supporting H3).

In contrast to *L. polyedrum*, it is likely that *A. catenella* reduced the prey population by using a combination of feeding and allelochemicals. Thus, *A. catenella* did not necessarily ingest the whole amount of prey cells that disappeared, but might have ingested organic compounds that resulted from cell lysis. Because the prey cells were lysed *A. catenella* could have taken up only the nutrients that were limited from the organic compound, this could be an explanation as to why only the cellular P content was positively correlated with the ingestion rate. Interestingly, the cellular carbon content of *L. polyedrum* was negatively correlated with the ingestion rate during the second time interval, indicating that the cellular C content decreased with increasing ingestion rate. This is also supported by the decrease in *L. polyedrum* cellular C content immediately after *Ostreococcus* was added into the mixed culture (Exp. 2.2, data not shown) and the lower C content of both dinoflagellates in mixed culture compared to monoculture at the end of exponential growth phase of experiment 2.2. This indicates that photosynthetic carbon fixation was reduced during phagotrophic feeding in *L. polyedrum* and *A. catenella* and that carbon was not supplied in equal amounts by phagotrophy. This phenomenon was studied by Skovgaard *et al.* (2000), who showed that for the mixotrophic dinoflagellate *Fragilidium subglobosum* the photosynthetic apparatus was quantitatively and qualitatively (i.e. reduced chl *a* efficiency) reduced by phagotrophy. Furthermore, Adolf *et al.* (2006) showed that cellular photosynthetic performance of the mixotrophic dinoflagellate *Karlodinium micrum* was 24–52% lower during mixotrophic growth than during autotrophic growth. In the present study, though, photosynthetic rates and chl *a* contents in mixed culture were not directly measured, thus making assumptions about the reduction of photosynthesis difficult.

Furthermore, the dinoflagellate cells in mixed culture also generally contained lower cellular N contents compared to the monocultures at the end of the exponential growth phase. Also the higher C:N ratios in mixed culture compared to the monoculture indicated that cells grown in mixed culture were more affected by N limitation. This may be explained by the higher assimilation efficiency in phototrophically grown cells compared to mixotrophically grown cells as discussed in Hansen *et al.* (2000). Ingested organic substances and photosynthetic products probably differ in the efficiency and pathways by which they are metabolized by a mixotroph. Ingested food must be digested and assimilated before it can be utilized by a cell, while photosynthetic products consist of relatively simple carbon compounds (Raven, 1974). Therefore, it is plausible that these compounds are more readily utilized as a source for respiration and growth than more complex organic compounds obtained through feeding (Hansen *et al.*, 2000). This implies that the metabolic costs of switching from photosynthesis to phagotrophic feeding were too high for feeding to be sufficient to enhance the growth rate under these experimental conditions. Nevertheless, switching between the trophic modes may still be more beneficial than simultaneous phototrophy and phagotrophy (Stoecker, 1998). Due to this ability, it has been suggested that mixotrophs might be favoured in temporarily or spatially heterogeneous environments with respect to resources, while homogenous conditions should favour strict autotrophs and heterotrophs over mixotrophs (e.g. Holen and Boraas, 1995; Stoecker, 1998).

Toxin content (*A. catenella*)

The PSP toxin profile (restricted to C1/C2 and B1 toxins) obtained in this study for the Southern California strain of *A. catenella* was comparable to that found by Tatters *et al.*, (2013). My findings of elevated total toxin content and a changed toxin composition under low nutrient conditions are consistent with previous studies (e.g. Anderson *et al.*, 1990). Toxin content in phytoplankton cells can vary within a wide range as a result of growth phase and external abiotic environmental factors (e.g. temperature, light, nutrient availability; Cembella, 1998; Granéli and Flynn, 2006; Granéli *et al.*, 1998). Under nutrient-balanced conditions toxin production is often low, while increased production arises from different types of nutrient stress (Anderson *et al.*, 1990). Increased cellular PSP content under phosphorus limitation has been frequently observed for different *Alexandrium* species (Guisande *et al.*, 2002; John

and Flynn, 2000; Van de Waal *et al.*, 2013). P-limitation typically results in low cellular C:N ratios as such conditions are likely to favor the synthesis of N-rich secondary metabolites such as PSP toxins (John and Flynn, 2000). However, I observed increasing cellular C:N, C:P and N:P ratios with decreasing nutrient concentrations at the end of the exponential growth phase, indicating that *A. catenella* cells were co-limited by both nutrients. An increase in toxin content under simultaneous N- and P-stress was also observed by John and Flynn (2000) for *A. fundyense* and by Flynn *et al.* (1994) for *A. minutum*. These authors suggest that this increase in toxin content could have been supported by N released within the cell from protein turnover, as it is unlikely that there was sufficient N in the medium to support toxin synthesis. Anderson *et al.* (1990) showed for one strain of *A. fundyense* that under nitrogen limitation the toxin synthesis was not stopped, but the production of C1/C2 and GTX1/5 (B1) toxins was favoured, while phosphorus limitation caused high levels of GTX2/3, which may have been the cause of the increase in B1 toxins in the lowest nutrient treatment in this experiment. These shifts in toxin composition may be an important factor for the evaluation of toxic events caused by *A. catenella* blooms. The specific toxicity of the different PSP analogues has been determined using a mouse bioassay (Oshima *et al.*, 1995; Genenah and Shimizu, 1981) and the results are usually expressed as mouse units (MU: µmole of toxin in which 1 MU is a dose sufficient to kill a ~ 20 g male mouse in 15 min, Oshima (1995)). These data imply that, for example, STX has a much higher toxicity compared to the B1 and C-toxins. Thus, the cellular toxicity of PSP-toxin producing *Alexandrium* species is not only determined by their PSP content, but also by the relative composition of the different PSP analogues.

Ecological implications

Overall, the results of the present study suggest that phagotrophic feeding (*L. polyedrum*) and allelopathy (*A. catenella*), or a combination of both, can be used by mixotrophic dinoflagellates to reduce competition for dissolved inorganic nutrients. The uptake of organic nutrients, both by the ingestion of whole prey cells or by taking up dissolved organic matter resulting from cell lysis, supports the dinoflagellates internal nutrient contents and might help them to survive at high cell numbers under low nutrient conditions. Even though results from the current study cannot directly be

used to estimate an impact on natural pico-phytoplankton populations, it can be assumed that mixotrophic dinoflagellates play an important role as grazers in marine planktonic food webs. Therefore, mixotrophic dinoflagellates can be a link in the flux of material between different trophic levels and can cause a short circuit in the microbial loop (Mitra *et al.*, 2014).

The two dinoflagellates tested here responded differently to low nutrient availability (imbalanced N:P ratios and lower concentrations along a gradient). In particular *L. polyedrum* was able to maintain high cell densities over the entire time of the experiment irrespective of nutrient ratios and absolute concentrations, while *A. catenella* was negatively affected by low nutrient availability. Being able to adapt to low nutrient conditions and thus to survive in high cell densities until the nutrient conditions change to more beneficial growth conditions, e.g. via upwelling events, can be an important factor for bloom initiation and maintenance. However, absolute nutrient concentrations or nutrient imbalances do not seem to be directly related to the growth of the dinoflagellates, as trophic interactions seem to be much more complex. Therefore, predicting bloom formation only based on dissolved nutrient concentrations is hardly possible. Instead, competitive strategies such as mixotrophy and allelopathy need to be considered in the context of nutrient dynamics in order to understand dinoflagellate population dynamics and their impact on planktonic food webs.

Chapter II

3. CHAPTER II: Allelopathy determines competition and grazing control in *Alexandrium catenella*

3.1. Abstract

The production of allelopathic chemicals by the toxic dinoflagellate *Alexandrium catenella* is one suggested mechanism by which this relatively slow growing dinoflagellate out-competes other phytoplankton and reduces grazing pressure. Despite the well documented allelopathic potential of *Alexandrium* spp., its allelopathic potency is very variable among different species and strains. Toxic events caused by *Alexandrium catenella* regularly have a huge impact on coastal ecosystems and the coastal economy in Southern California, but the factors influencing the recurrence of *A. catenella* blooms in this area still remain unclear. In the present study, I investigated potential toxic and allelopathic effects of an *Alexandrium catenella* strain isolated off the coast of Los Angeles, Southern California, on phytoplankton competitors and on the metazooplankton consumer *Brachionus plicatilis*. In order to do so, I conducted comparative experiments with a highly lytic and a non-lytic strain of *Alexandrium tamarense*, testing the effects of cell-free supernatant and whole cell culture. Further, I tested the donor density dependency of these effects. These bioassays showed a steep decline in competitor and consumer numbers, with increasing *A. catenella* concentrations. Compared to the two *A. tamarense* strains *A. catenella* showed an intermediate lytic activity on other phytoplankton as well as on the grazer *B. plicatilis*. *B. plicatilis* was able to feed and grow well on the toxic, but non-lytic strain of *A. tamarense* (Alex5), while its survival rate significantly decreased with increasing *A. catenella* cell concentrations. However, the relatively high *A. catenella* concentrations required to cause a negative effect suggest that such chemically mediated interactions play a greater role for bloom maintenance rather than bloom initiation. These results were also supported by mixed culture experiments including *B. plicatilis* and the non-toxic chlorophyte *Tetraselmis*, in which the negative effect of *A. catenella* was ‘diluted’ by high *Tetraselmis* cell abundance, as *Tetraselmis* was able to counteract the negative effect of *A. catenella* on *B. plicatilis* in mixed culture.

3.2. Introduction

Dinoflagellates are a major cause of harmful algal blooms (HABs) in coastal areas worldwide, with potentially severe consequences for marine ecosystem functioning and services. Among bloom-forming dinoflagellates the genus *Alexandrium* spp. is raising the most public concern due to its ecological, toxicological and economic importance. Some members of this genus have the ability to synthesise and release very potent toxins (Paralytic Shellfish Poisoning toxins = PST) that can accumulate within marine food webs (Sunda *et al.*, 2006) and contaminate seafood, thus posing a significant public health threat (Anderson *et al.*, 2012a; Smayda, 1997a). An ongoing question in understanding the bloom dynamics of these dinoflagellates is how, and under which circumstances, they succeed to become dominant in the plankton community. Generally, species that can effectively compete for growth-limiting nutrients are able to dominate the phytoplankton community. Bloom-forming dinoflagellates, however, are often considered inferior competitors compared to other phytoplankton such as diatoms due to their lower growth and nutrient uptake rates (Banse, 1982; Smayda, 1997). Allelopathy, the release of potentially harmful secondary metabolites (allelochemicals), is considered to be a successful strategy to facilitate bloom formation (Smayda, 1997a). Such allelochemicals negatively affect other organisms in several ways, including phytoplankton competitors and zooplankton consumers, and are thus considered to be one of the key factors determining phytoplankton competition for resources, succession and bloom formation (e.g. Maestrini and Bonin, 1981, and references therein; Rengefors and Legrand, 2001; Legrand *et al.*, 2003; Fistarol *et al.*, 2004b; Kubanek *et al.*, 2005; Graneli and Hansen, 2006; Yamasaki *et al.*, 2009; Hattenrath-Lehmann and Gobler, 2011).

Allelopathy of bloom-forming dinoflagellates has been studied extensively in recent years. Allelochemically induced effects on protistan targets include growth inhibition (e.g. Hattenrath-Lehmann and Gobler, 2011; Poulson-Ellestad *et al.*, 2014), encystment (Tillmann *et al.*, 2007), cell lysis (e.g. Ma *et al.*, 2009; Tillmann *et al.*, 2008), and immobilisation of target cells (e.g. Tillmann and John, 2002; Tillmann *et al.,* 2008, 2007*)*. The latter effect might be particularly important in combination with mixotrophy (phagotrophic feeding by primarily phototrophic species). Prey immobilisation and subsequent ingestion by the mixotrophic dinoflagellate

Alexandrium pseudogonyaulax has recently been reported. *A. pseudogonyaulax* excreted metabolites to immobilize prey species that were subsequently caught in a toxic mucus trap and engulfed through the sulcus area (Blossom *et al.*, 2012).

For *Alexandrium* spp., most of these studies provide evidence that adverse effects on protistan targets are not related to the intracellular PST content (e.g. Tillmann and John, 2002). Instead, these effects are caused by released allelochemical compounds of poorly characterized chemical nature by direct damage to external membranes and loss of cell integrity (Ma *et al.*, 2011; Tillmann and John, 2002). However, studies on allelopathic effects (as opposed to toxic effects) on metazoan micro-grazers are rather rare or have shown inconsistent patterns. Experiments using *Alexandrium* culture filtrates of different species and strains, including *A. minutum* and *A. tamiyavanichii* indicated adverse effects (lethal or sublethal) on copepods (Bagøien *et al.*, 1996; Silva *et al.*, 2013) and on gastropod larvae (Silva *et al.* 2013). On the other hand, no adverse effects of *Alexandrium* spp. filtrate, i.e. allelopathic effects, were found on gastropod larvae (Juhl *et al.*, 2008), and on *B. plicatilis* (Silva *et al.*,2013).

Studies using whole cell culture (including *Alexandrium* spp.) also showed both (1) high ingestion rates of toxic *Alexandrium* with no adverse effects on the grazers (Teegarden and Cembella, 1996) as well as (2) enhanced mortality of the grazer (Bagøien *et al.*, 1996). Wang *et al.* (2005) tested the effects of 10 different *Alexandrium* species and strains on *B. plicatilis* and found that 7 strains, including both PST producing and non-PST producing strains, negatively affected *B. plicatilis,* indicating that other substances than PSTs, i.e. allelochemicals were responsible for these adverse effects. Overall, these studies suggest that adverse effects of *Alexandrium* spp. on metazooplankton consumers may be either caused by PSP toxins or by allelochemicals and that these effects are highly variable, depending on the *Alexandrium* strain/species investigated, as well as on the target species (Tillmann and John, 2002; Turner and Tester, 1997). Furthermore, many experimental studies do not clearly distinguish between toxic and allelopathic adverse effects on metazoan grazers, even though these adverse effects may play very different roles for bloom dynamics of harmful dinoflagellates. In addition to the toxic effect of ingested cells, excreted allelochemicals could also have negative effects on grazers even when toxic cells are avoided or when non-toxic

phytoplankton prey is available. Grazing by microzooplankton is considered to be an important factor in controlling the growth of dinoflagellate populations (Calbet *et al*., 2003; Stoecker *et al*., 2005). Thus, escaping from grazing control by the production and release of allelochemicals may support the ecological success of *Alexandrium* spp. and facilitate its dominance in a plankton assemblage.

Alexandrium catenella blooms and a related increase in PST levels are a common phenomenon in some areas and are expected each year in British Columbia and Washington (Cox *et al*., 2008; Lewitus *et al*., 2012). Dense or visible blooms are rare off the coast of Southern California; however, despite their low densities (maximum concentration of about 17,000 cells L^{-1}, Jester *et al*., 2009) they still can cause serious toxic events. In recent years an increase in PST activity has been suggested at some southern California sites, most notably in commercial shellfish growing areas in Santa Barbara and San Diego counties (Lewitus *et al*., 2012). Despite their huge impact on coastal ecosystems in Southern California the factors influencing the recurrence of *A. catenella* blooms in this area still remain unclear. *A. catenella* cell numbers often are initiated in offshore waters, as these toxic events are correlated with large scale oceanographic events, in particular the upwelling-relaxation cycle, and are then transported onshore during relaxation-favourable winds (Langlois and Smith, 2001; Price, *et al*., 1991).

Toxic/allelopathic effects of *A. catenella* have only been studied in strains isolated from the Mediterranean Sea (Tillmann and John, 2002; Tillmann *et al*., 2007, 2008a), from the East China Sea (Yan *et al*., 2010) and from the Pacific coast of Chile (Arzul *et al*., 1999). These studies showed a variety of different effects including allelopathic effects on a range of autotrophic and heterotrophic protists (Tillmann and John, 2002; Tillmann *et al*., 2007, 2008a) and low inhibitive effects on metazoan grazers (cladocera, Yan *et al*., 2010). Adverse effects of the American west coast strain of *A. catenella* on competing phytoplankton and zooplankton consumers, though, have not yet been described yet.

The present study investigates potential allelopathic effects of a North American strain of *A. catenella* (toxic group I, Garneau *et al*., 2011), isolated from the coast of Southern California. I tested the strain for its potential allelopathic activity on phytoplankton competitors and on the common metazoan rotifer grazer *Brachionus plicatilis*. Furthermore, the aimed was to disentangle whether adverse effects on

metazooplankton are rather due to toxins or allelochemicals. I approached this by comparing the effects of *A. catenella* with the effects of a similarly toxic but non-lytic strain of *A. tamarense*. Additionally, I conducted mixed culture experiments with *A. catenella*, a phytoplankton competitor (*Tetraselmis* sp.) and the grazer *B. plicatilis* to study the relative effects of allelopathic compounds on different trophic levels and possible cascading effects of allelochemicals through the planktonic food web.

3.3. Material & Methods

Algal and rotifer culturing

The dinoflagellate *Alexandrium catenella* was isolated from the coast of Southern California near Los Angeles (Caron Laboratory, USC, Los Angeles, Garneau *et al*., 2011). The two *Alexandrium tamarense* strains (Alex2 and Alex5) used as reference strains were isolated from the east coast of Scotland (North Sea) (Alpermann *et al*., 2009). These two clones were selected based on allelopathic, i.e. lytic capacity quantified by a *Rhodomonas* bioassay (Tillmann *et al*., 2008). Alex5 was found to have no lytic impact on *Rhodomonas salina* and will further be referred to as non-lytic *Alexandrium*. Alex2 had a high lytic capacity and will henceforth be referred to as lytic *Alexandrium*. In a recent study, John *et al*. (2014) suggested a revision of the species name *A. catenella*. Based on their analyses (i.e. morphology, ITS/5.8S genetic distances, ITS2 compensatory base changes), the North American ribotype of *A. catenella* used in the present study, belongs to the toxic group I, which they suggested to rename *A. fundyense*. I will, however, continue to use the name *A. catenella* here to be consistent with previous work on this species in the area of Southern California.

Two different phytoplankton competitor species, *Tetraselmis* sp. and *Rhodomonas salina*, common in temperate waters of the coast of southern California, were obtained from different culture collections (Table 3-1). The cell sizes of target and donor species were determined by measuring the length and width of live cells using an inverted microscope (Leica DM IL, n = 20 to 25 cells), individual biovolumes were estimated using volumetric formulae (Hillebrand *et al*., 1999). All stock cultures were grown non-axenically in enriched f/2 seawater medium (Guillard and Ryther, 1962)

without silicate, prepared from 0.2 µm sterile-filtered North Sea seawater adjusted to a pH of 8.0. Cultures were maintained in 200 ml culture flasks under controlled conditions at 18°C under cool-white fluorescent light of 60 µmol photons m^{-2} s^{-1} and a 12:12 h light:dark cycle (except for the *Rhodomonas salina* culture used in the bioassay, which was grown in K-medium (Keller *et al*., 1987) and cultured at 16°C). Cultures were transferred weekly to fresh medium and were always in exponential growth when used in the experiments. The metazoan rotifer *Brachionus plicatilis* was cultured in filtered seawater and fed with *Tetraselmis* sp. It was transferred once or twice per week to fresh medium containing food organisms. Cultures of *B. plicatilis* for the experiments were grown to high cell concentrations until they became almost deprived of food, as checked by microscopic examination.

Table 3-1: Donor and target species cultures, their approximate cell volume [μm^3] x 10^3 and origin.

donor /target	Species		volume [μm^3] x10^3	origin / collection
donor	*Alexandrium catenella*	Dinophyceae	1.2	Southern California, Caron lab, University of Southern California
donor	Alex2 (*A. tamarense*)	Dinophyceae	1.3	Coast of Scotland (North Sea), Alpermann *et al*. (2009)
donor	Alex5 (*A. tamarense*)	Dinophyceae	1.7	Coast of Scotland (North Sea), Alpermann *et al*. (2009)
target	*Tetraselmis sp.*	Chlorophyceae	0.23	Roscoff culture collection, France
target	*Rhodomonas salina*	Cryptophyceae		Kalmar Algal Collection (KAC 30)
target	*Brachionus plicatilis*	Rotifer		Oliver Thielmann, aquatic retailer, Germany

Determination of PSP toxin profile

A culture of *A. catenella* was harvested during late exponential growth phase when the cell concentration was 12.000 cells ml^{-1}. The culture was centrifuged (3200 x g, 10 min at 18°C) and the cell pellets were processed as described in Krock *et al*. (2007).

Experimental design

Four different sets of experiments were conducted to evaluate the effects of *A. catenella* on algal competitors and metazoan grazers: (1) **short-term experiments,** where adverse effects on phytoplankton competitors were tested in a *Rhodomonas* bioassay and in growth and immobilisation tests (2) **short-term feeding experiments** with *B. plicatilis* (3) **dose-response experiments**, where the growth and grazing of *B. plicatilis* was determined in a range of different *Alexandrium* concentrations (*A. catenella* and non-lytic *A. tamarense*) and (4) **mixed culture experiments***,* where the effects on both competitors and grazers were tested. All experiments were conducted under controlled conditions at 18°C under cool-white fluorescent light of 60 µmol photons m^{-2} s^{-1} and a 12:12 h light:dark cycle.

Experimental set 1: Adverse effects of A. catenella on protistan targets

a) Rhodomonas bioassay

The lytic activity of *A. catenella* compared to the lytic (Alex 2) and the non-lytic strain (Alex 5) of *A. tamarense* was determined using a *Rhodomonas* bioassay as described in Tillmann *et al*. (2008). An exponentially growing culture of *A. catenella* was centrifuged (3200 x g, 10 min at 18°C) and a dilution series was prepared using different amounts of *A. catenella* supernatant and whole cell culture, resulting in 14 concentration levels of supernatant and of whole cell culture (see Table 3-2). *R. salina* was added to all treatments and controls. After an incubation period of 24 h in the dark at 16°C, samples were fixed with Lugol's solution (2% final concentration) and counted with an inverted microscope (Zeiss Axiovert 35). A subsample containing a minimum of 500 cells in the control was counted.

b) Immobilisation

To test whether the *A. catenella* strain used in this study has the ability to produce allelopathic substances that harm protistan targets an immobilization test (based on Fistarol *et al*., 2004) was conducted using the phytoplankton competitor *Tetraselmis* sp.. Exponentially growing cultures of *A. catenella,* non-lytic *A. tamarense* (Alex5) and lytic *A. tamarense* (Alex2) were centrifuged (3200 x g, 10 min at 4°C) and

Tetraselmis sp. was incubated in the supernatant potentially containing allelochemicals. The experiment was conducted in glass petri dishes in triplicates. After one hour of exposure the immotile *Tetraselmis* cells were counted in situ using an inverted microscope (Leica DM IL LED). For further details see Table 3-2.

c) Growth experiment with the target species *Tetraselmis* sp.

To determine the effects of *A. catenella* on the growth of other phytoplankton competitors, *Tetraselmis sp.* was grown in *A. catenella* cell-free supernatant and in mixed culture with *A. catenella* compared to both f/2 medium control and (non-lytic) Alex5 supernatant control. The supernatant (pH 8.2) was enriched with nutrients and vitamins according to the f/2 medium (for details see Table 3-2). The growth rate of *Tetraselmis* in the treatments and controls was calculated according to equation 1 :

(1) $\mu\ (d^{-1}) = \frac{\ln C2 - \ln C1}{(t_2 - t_1)}$

where C2 and C1 are the cell numbers at the time points t_2 and t_1.

Table 3-2: Experimental details for testing adverse effects of *A. catenella* on protistan targets. The table summarizes the treatments, donor and target cell concentration [ml^{-1}], the experimental volume [ml] and the response variable determined at the end of the experiments.

Test	Treatments	donor cells [ml^{-1}]	target cells [ml^{-1}]	Vol. [ml]	Response variable
a) *Rhodomonas* bioassay	*A. catenella*	11-17,300			EC_{50} after 24 h
	Alex 5	8,600	*R. salina*, $12.5x10^3$	4	
	Alex2	*230*			
	medium control	---			
b) Immobilisation	*A. catenella*	8×10^3			% of immotile target cells after 1h
	Alex 5	1×10^4	*Tetraselmis sp.* 9.8×10^3	3	
	Alex2	4.7×10^3			
	medium control	---			
c) *Tetraselmis* growth	*A. catenella* supernatant	*A. catenella*, 800	*Tetraselmis*, 2.8×10^4	30	Tetraselmis growth rate after 6 days
	Alex 5 supernatant mixed with *A. catenella* f/2	Alex5, 1500			
	medium control	---			

Experimental set 2: Adverse effects on the rotifer *B. plicatilis*

a) *B. plicatilis* growth and survival with *A. catenella*

This experimental set tested the potential adverse effects of *A. catenella* on growth and survival of the zooplankton consumer *B. plicatilis*. Cell–free filtrate was obtained by filtration of *A. catenella* cultures through a 0.2 µm syringe filter after preliminary filtration through a 20 µm mesh. The experiment was started with approximately equal biovolume for each algal species (Table 3-3) and was carried out in 6-well cell culture plates (TPP Tissue Culture Plate, area: 8.96 cm^2). Due to destructive sampling all experimental treatments with 3 replicates each were set up twice. After 2 days the first three replicates were fixed with Lugol's iodine solution, while the remaining 3 replicates were fixed after 8 days. Since the enumeration of motile individuals can be challenging, dead and live *B. plicatilis* were first counted under a stereoscope, and after fixation (1% Lugol's solution) samples were counted again using an inverted microscope to determine the total number of individuals. 1 ml sub-samples were preserved to count algal cell densities after 2 and 8 days (for details see Table 3-3).

b) Short term (24 h) effects of *Alexandrium* supernatant on *B. plicatilis*

In order to compare the effects of *A. catenella* on *B. plicatilis* with the effects of the two *A. tamarense* trains (Alex2 and Alex5) *B. plicatilis* was exposed for 24 h to cell-free *Alexandrium* supernatant of all three strains (in glass petri dishes, prepared as described above, Table 3-3) and the survival of *B. plicatilis* was quantified.

c) Growth and survival of *B. plicatilis* in *Alexandrium* supernatant when fed with a non-toxic food

The negative effects of *Alexandrium* supernatant on *B. plicatilis* in the presence of non-toxic prey were tested by growing *B. plicatilis* for 4 days in *A. catenella* and (non-lytic) Alex5 supernatant (prepared as described above, Table 3-3), enriched with nutrients and vitamins according to the f/2 medium and fed with *Tetraselmis*. Live and dead *B. plicatilis* were counted under a stereoscope (5 ml samples) after 24 h and 4 days. Afterwards sub- samples were put back into the flask.

Table 3-3: Experimental details for testing adverse effects of *Alexandrium* on the rotifer *B. plicatilis.* The table summarizes the treatments, donor / prey and target cell concentration [ml^{-1}], the experimental volume [ml] and the response variable determined at the end of the experiments.

Test	Treatments	donor / prey cells [ml^{-1}]	Target cells [ml^{-1}]	Vol. [ml]	Response variable
a) Growth & survival test	*A. catenella* cells	8.7×10^3			*B. plicatilis*
	A. catenella filtrate	---			growth rate and
	Tetraselmis cells	1.8×10^5	10	6	survival after 2
	medium control	---			and 8 days
	Tetraselmis mono	1.8×10^5			
	A. catenella mono	8.7×10^3			
b) Effects of three *Alexandrium* species	*A. catenella* supernatant	$1.5x10^4$			survival after 24 h
	Alex 5 supernatant	$1.4x10^4$	10	3	
	Alex2 supernatant	$1.1x10^4$			
	f/2 medium	--			
c) Growth in supernatant, fed with non-toxic food	*A. catenella* supernatant	$1.6x10^4$	30	30	survival after 1 and 4 days
(*Tetraselmis;* 2.3 × 10^4 cells ml^{-1})	Alex 5 supernatant	$1.7x10^4$	30	30	

d) Density dependent effect of *A. catenella* on *B. plicatilis*

Based on the results of the first two sets of experiments density dependent effects of *A. catenella* on *B. plicatilis* were studied. In this context, the effects of *A. catenella* and the non-lytic *A. tamarense* strain (Alex5) were compared. To avoid a contamination with *Tetraselmis* cells in the experimental flasks *B. plicatilis* was starved in sterile filtered seawater before the experiment for 10 days (until no food cells were observed anymore). Five donor cell concentrations were set up in equal biovolume of *A. catenella* and Alex5 (for details see Table 3-4). The experiment was carried out in triplicate in 100 ml Erlenmeyer flasks with an experimental volume of 30 ml. For each replicate 50 individual *B. plicatilis* were randomly picked from healthy stock cultures and were pipetted into the flasks. The *A. catenella* and Alex5

monoculture controls were run in triplicate in the same cell-concentrations as in the treatments with *B. plicatilis*. The algal cell density and the number of alive and dead rotifers were counted every second day. Additionally, a seawater control treatment where *B. plicatilis* was kept in starvation in 0.2 µm filtered seawater and a non-toxic algal control was set up. For the non-toxic food control *B. plicatilis* was grown in 2 different concentrations of the non-toxic alga *Tetraselmis* chosen to represent prey biovolume of the lower and higher end of the biovolume levels used for the two *Alexandrium* species. *B. plicatilis* concentrations were determined in three 5 ml sub-samples that were taken from each flask and pipetted into a 6-cell-well-dish. After counting live and dead *B. plicatilis* under a stereoscope, sub- samples were put back into the flask. After this procedure a 1 ml sub-sample was taken and preserved using Lugol's iodine solution (1%) for microscopic algal cell counts.

Table 3-4: Experimental details for the dose-response experiment. The table summarizes the prey cell concentration [ml^{-1}], the corresponding biovolume in the 5 different concentration levels, the experimental volume [ml] and the response variable.

Conc.	Prey cell concentration [cells ml^{-1}]			Prey biovolume [μm^3 ml^{-1} x 10^6]	Grazer [ml^{-1}]	Volume [ml]	Response variable
	A. catenella	Alex 5	*Tetraselmis*				
C1	60	40	--	~ 0.67			EC_{50} and ingestion rates of *B. plicatilis* after 48 h (exp. duration 16 days)
C2	470	392	22,000	~ 6			
C3	1400	1090	--	~ 17.5	1	30	
C4	3500	2200	--	~ 37			
C5	7750	5540	195,000	~ 92.7			

The *B. plicatilis* individual numbers after 48 h followed a sigmoidal declining pattern when plotted against log-transformed *A. catenella* cell concentrations. Estimates of EC_{50} (i.e. the *A. catenella* cell concentration yielding a 50% decline in *B. plicatilis* concentration) were determined by fitting the data points to a sigmoidal curve using the non-linear model in R (as described below for the *Rhodomonas* bioassay).

The ingestion rate of *B. plicatilis* in different cell concentrations of the two *Alexandrium* strains and the non-toxic *Tetraselmis* was calculated after 48 h of incubation according to Frost (1972).

Experimental set 3: Mixed culture experiments with *Tetraselmis*, *B. plicatilis* and *A. catenella*

In order to study interactive effects of *A. catenella* on both a competitor and a grazer in a simple food web, *B. plicatilis* was inoculated in a mixture of *A. catenella* and the non-toxic *Tetraselmis sp.* (ATB). The experiment was set up with four control groups: *Tetraselmis sp.* in monoculture (T), *Tetraselmis sp.* together with *B. plicatilis* (TB) *Tetraselmis sp.* in mixed culture with *A. catenella* (AT) and *A. catenella* in monoculture (A). Each treatment was set up in triplicate in 100 ml Erlenmeyer flasks with a total volume of 30 ml. Exponentially growing cultures of *A. catenella* were diluted to an initial cell concentration of 880 cells ml^{-1}. To reach an equal biovolume of 12 x 10^6 μm^3 ml^{-1} the *Tetraselmis* culture was diluted to 28,000 cells ml^{-1}. 30 *B. plicatilis* individuals were added from a healthy stock culture into each experimental flask, resulting in a final concentration of 1 individual ml^{-1}. Samples for cell counts were taken every second day. While *A. catenella* and *B. plicatilis* cell numbers were counted using an inverted microscope, *Tetraselmis* cells were filtered (in mono and mixed culture) through a 20 µm mesh and determined photometrically (Thermo Scientific AquaMate Plus UV-VIS) by calibrating the absorption (A664) to cell numbers.

Statistical analysis

Data were analysed using the software R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria, 2014). One-way analyses of variance (ANOVA) were performed to test for differences in growth rate, percentage of immotile cells and mortality of targets (*Tetraselmis* sp. and *B. plicatilis*) between each treatment and the control (experimental set 1 and 2) as well as for differences in ingestion rate (Imax), µmax and EC_{50} of *B. plicatilis* between each treatment (*A. catenella*, Alex5, *Tetraselmis*, experiment 3 and 4). In the dose response experiments (exp. 1.2 and 3), values of EC_{50}, defined as the *A. catenella* cell concentration causing lysis of 50%

of target cells, were calculated by fitting the data points to the following equation (Tillmann *et al*., 2008) using the non-linear model fit in R.

(2) $N_{final} = \frac{N_{control}}{1+(x/EC_{50})^h}$

N_{final} is the experimental final target cell concentration, $N_{control}$ the final target cell concentration in controls, x the log-transformed cell concentration of *A. catenella* and EC_{50} and h are fit-parameters. Results are expressed as EC_{50} (cells ml^{-1}) including 95% confidence intervals.

Additionally, in experiment 3 (dose response experiment) the interactive effects between the factors grazer presence and cell density (for the dinoflagellates) were tested using the two-factor ANOVA. The response variables were the growth rates of *A. catenella* and Alex5. Whenever ANOVA's showed significant effects, the significant differences among treatments were determined using a TukeyHSD post hoc test. All data were examined for normal distribution. Homogeneity of variances was tested using the Bartlett's test. Data that failed to meet these criteria were log transformed or, if the assumptions for parametric tests could still not be fulfilled, a non-parametric Kruskal-Wallis ANOVA was performed. The level of significance was defined at $p < 0.05$. After statistical and analytical examinations corresponding graphs were created with the software Sigma plot (version 11.0, from Systat Software, Inc., San Jose California, USA).

3.4. Results

Toxin profile *A. catenella*

The PSP toxin profile revealed the presence of the following toxins in decreasing order of molar percentage of total content: GTX1/4 (52.9%) and B1 (23.1%), C1/C2 (22.9%). GTX2/3, Neosaxitoxin (NEO) and Saxitoxin (STX) were only present in trace amounts (<1 mol%) (Table 3-5).

Table 3-5: PSP toxin profile of *A. catenella* compared to the two *A. tamarense* strains (Alex5 and Alex2) as determined by Tillmann *et al*. (2009). Values are mol% of total PST content; nd=not detected

species	C1/C2	GTX1/4	B1	GTX2/3	NEO	STX	Ref.
A. catenella	22.926	52.941	23.127	0.213	0.683	0.109	this study
Alex5	32.0 ± 0.2	6.4 ±0.1	nd	2.2 ± 0.1	31.6 ± 0.6	27.4 ± 0.6	Tillmann *et al*. 2009
Alex2	21.5 ± 1.4	3.6 ± 0.2	nd	3.8 ± 0.4	26.4 ± 0.6	43.1 ± 1.1	Tillmann *et al*. 2009

Short-term effects on protistan targets

a) *Rhodomonas* bioassay

A. catenella caused cell lysis of *R. salina* in a dose dependent manner with an EC_{50} of 566.24 ± 1.04 *A. catenella* cells ml^{-1} (Figure 3-1). Cell–free supernatant was slightly less effective (EC_{50} = 1,124.60 ± 1.03 *A. catenella* cells ml^{-1}). The Alex 5 control did not have any effect and the Alex 2 control caused 100% mortality of *R. salina*.

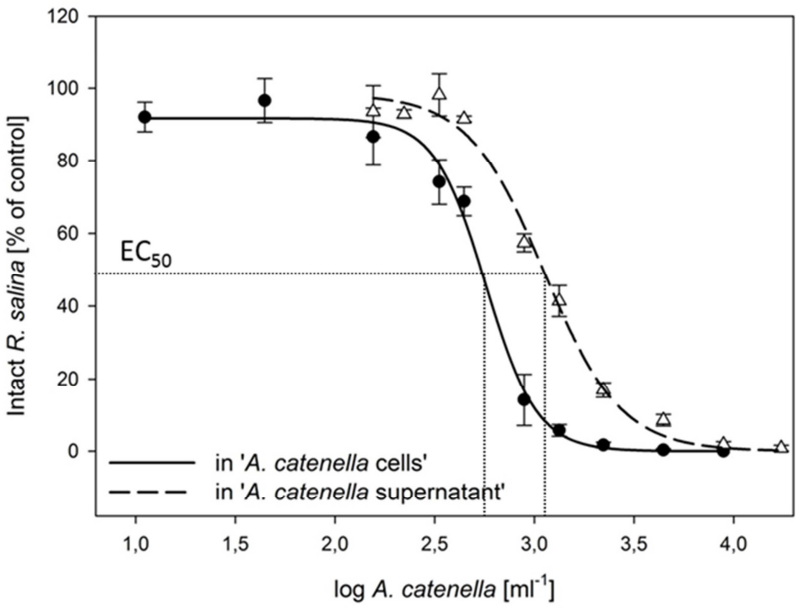

Figure 3-1: Dose–response curves describing the lytic capacity of *A. catenella* cells (solid line) and *A. catenella* supernatant (dashed line) as quantified with the *Rhodomonas* bioassay. Each graph shows the concentration of *Rhodomonas salina* after 24 h incubation (% of control) as a function of log-transformed *A. catenella* concentration of 3 replicate cultures. The EC_{50} values were at 566.2 *A. catenella* cells ml^{-1} and at 1124.6 *A. catenella*

cells ml^{-1} in supernatant. Each data point represents a mean ± SD. Lines represent a non-linear, sigmoidal curve fit.

b) Immobilisation

A. catenella supernatant caused a significant cell immobilization of the chlorophyceae *Tetraselmis sp.* (one-way ANOVA, $F_{3,8}$= 74.18, p< 0.00001). In *A. catenella* supernatant 54% of the *Tetraselmis* sp. cells were immotile, which was significantly higher compared to 24% in the f/2 control (p < 0.01, TukeyHSD) and 13% in Alex5 supernatant (p < 0.001, TukeyHSD). Alex2 supernatant immobilised 95% of the *Tetraselmis* cells. No cell lysis was observed in *A. catenella* or in Alex2 supernatant after 1h of exposure. These results reflect the findings from the bioassay (see above) and show that *A. catenella* has an intermediate lytic activity compared to Alex2 and Alex5.

c) Growth experiment with the target species *Tetraselmis* sp.

A. catenella cell-free supernatant had a significantly negative effect on the *Tetraselmis* sp. growth rate after 24h (Figure 3-2, ANOVA, $F_{2,6}$=7.177, p<0.05), which was significantly lower when grown in *A. catenella* supernatant compared to the non-lytic Alex5 supernatant control (p < 0.05, TukeyHSD). There were no significant differences in growth rate between Alex5 supernatant and the f/2 medium control (p = 0.156, TukeyHSD, Figure 3-2). After 24h the *Tetraselmis* culture recovered and increased its growth rate in all treatments with no significant differences between the treatments anymore (Table 3-6, ANOVA, $F_{2,6}$= 3.37, p= 0.104).

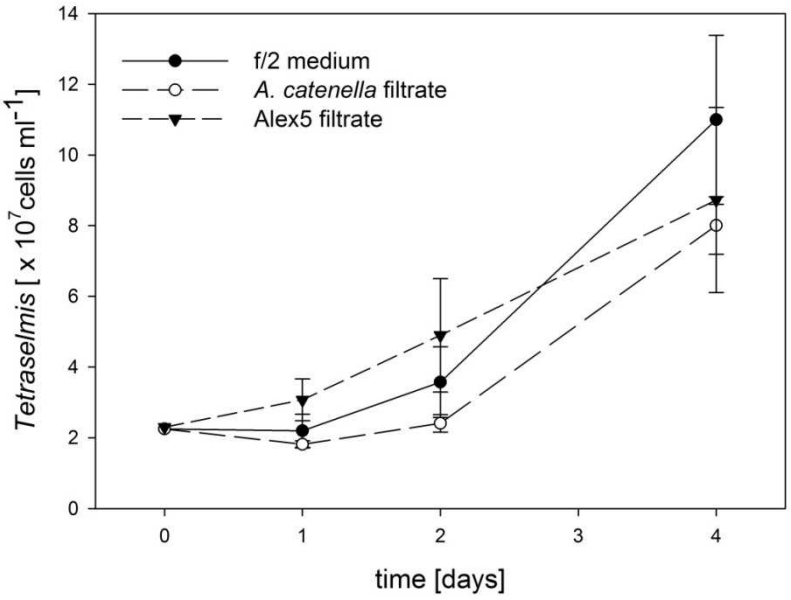

Figure 3-2: *Tetraselmis* growth in *A. catenella* cell-free supernatant compared to the f/2 medium control and Alex5 supernatant control (exp. 1.3). Each data point shows the mean ± SD.

Table 3-6: *Tetraselmis* sp. growth rate when exposed to *A. catenella* and Alex5 cell-free supernatant compared to f/2 medium. The data represent means ± 1 SD.

gr (μ d^{-1})	f/2	*A. catenella* supernatant	Alex5 supernatant
day 0 - 1	0.009 ± 0.128	-0.15 ± 0.07	0.277 ± 0.19
day 1 - 4	0.38 ± 0.02	0.51 ± 0.13	0.34 ± 0.04

Experimental set 2: Adverse effects on the rotifer *B. plicatilis*

In experiment 2.1, *A. catenella* whole cell culture as well as cell-free filtrate had a significantly negative effect on *B. plicatilis* after 8 days of incubation (ANOVA, $F_{3,8}$=44.03, p< 0.0001, Figure 3-3, Figure 3-4 A). The mortality of *B. plicatilis* was significantly higher when grown in mixed culture with *A. catenella* (cells), and in *A. catenella* cell-free filtrate compared to the two control treatments (mixed culture with *Tetraselmis* sp. and seawater, both TukeyHSD, p<0.0005). No significant differences between the mortality in mixed culture with *A. catenella* and in filtrate were observed.

In comparison to the non-lytic *A. tamarense* strain (Alex5), *A. catenella* supernatant significantly increased the mortality of *B. plicatilis* after 24 h of incubation (TukeyHSD, p<0.0001), while there was no difference in *B. plicatilis* mortality between *A. catenella* and the lytic *A. tamarense* strain (Alex2, exp. 2.2, after 24 h exposure, Kruskal-Wallis Test, p< 0.05, data not shown). In experiment 2.3, *A. catenella* supernatant also had a significantly negative effect on *B. plicatilis* survival compared to Alex5 supernatant, even when *Tetraselmis* sp. was provided as a non-toxic food source (ANOVA, $F_{1,3}$= 71.46, p< 0.005, Figure 3-4 B).

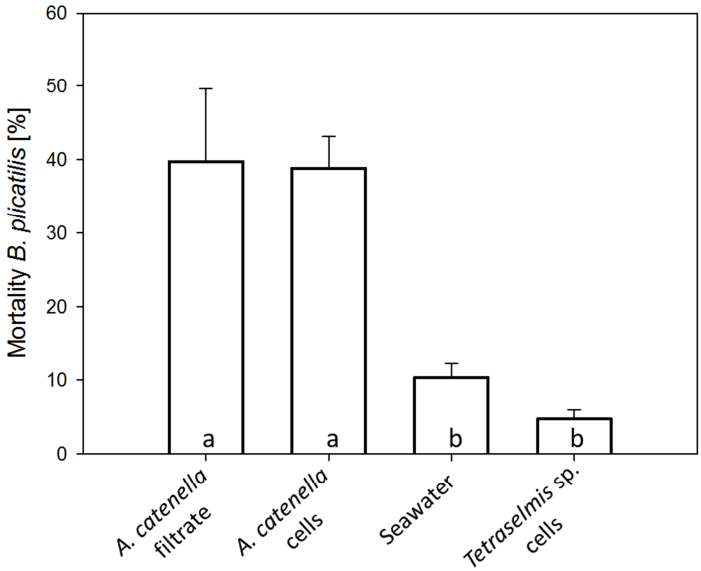

Figure 3-3: *B. plicatilis* mortality (% of total number of individuals) in 4 different treatments including *A. catenella* cell-free filtrate, *A. catenella* cells culture, filtered seawater and *Tetraselmis sp*. cell culture after 8 days of incubation (exp. 2.1). Letters indicate statistically homogenous subsets based on the TukeyHSD post hoc procedure (p < 0.001, ANOVA, df=3, F= 44.03 p < 0.001). Bars show means ± SD.

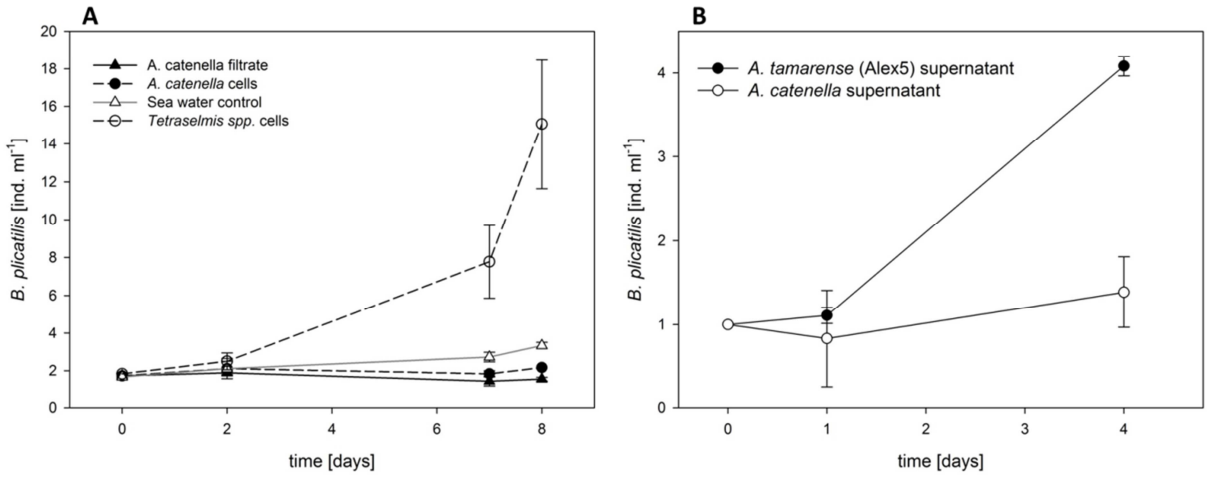

Figure 3-4: Population growth of *B. plicatilis* (**A**) when fed with *A. catenella* or *Tetraselmis* sp. compared to the growth in *A. catenella* cell free filtrate and a seawater control (exp. 2.1), and (**B**) when fed with *Tetraselmis* sp. grown in *A. catenella* and in the non-lytic *A. tamarense* supernatant (exp. 2.3). Each data point represents a mean ± SD.

The dose-response experiment (exp. 2.4) showed that abundances of *B. plicatilis* strongly decreased with increasing *A. catenella* cell concentrations (Figure 3-5 A and B); 100% mortality was observed at about 2,000 *A. catenella* cells ml^{-1}. The calculated EC_{50} concentration (based on the seawater control) was 410 ± 147 cells ml^{-1} after 48 h of incubation. The EC_{50} value calculated based on the Alex5 control was higher (433 ± 167 cells ml^{-1}) but not statistically different (ANOVA, $F_{1,4}$= 0.01, p> 0.05, data log-transformed) from the seawater based EC_{50} value.

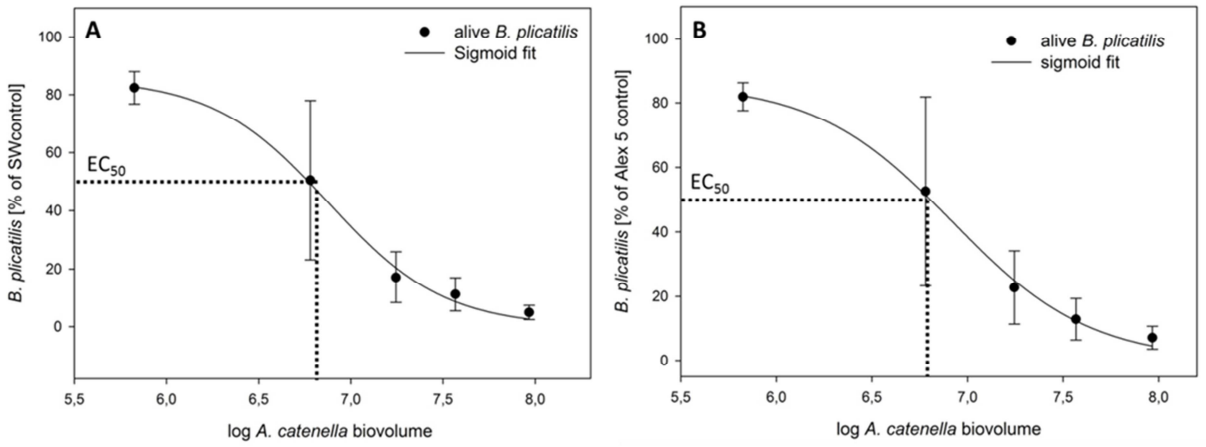

Figure 3-5: Dose-response curves show *B. plicatilis* individuals after 48 h incubation as a function of log-transformed *A. catenella* concentration of 3 replicate cultures. A: % of the seawater control and B: as % of the Alex 5 control (exp. 3). The EC_{50} values were 410 *A. catenella* cells ml^{-1} (SW control) and 433 *A. catenella* cells ml^{-1} (Alex5 control). Each data

point represents a mean ± SD. Lines represent a non-linear, sigmoidal curve fit and indicate the calculated EC_{50}.

However, the ingestion rates of *B. plicatilis* on *A. catenella,* compared to the non-toxic algae *Tetraselmis* sp. and the toxic, but non-lytic *A. tamarense* strain (Alex5) revealed that the rotifer preyed actively on the two toxic dinoflagellates (Table 3-7, Figure 3-6). The ingestion rates of both dinoflagellates were density dependent and increased significantly with increasing dinoflagellate cell concentration (Figure 3-6, non-linear fit, t=2.697, p< 0.05).

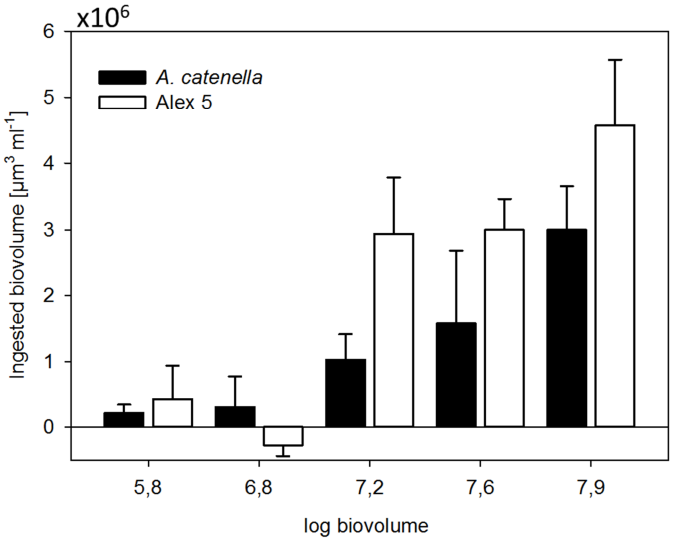

Figure 3-6: Ingestion rate (µm^3 ml^{-1} individual^{-1} d^{-1}) of *B. plicatilis* on *A. catenella* compared to non-lytic *A. tamarense* (Alex 5) in 5 different cell concentrations. Bars indicate mean ingestion rates ± SD.

There were indications of increased ingested biovolume (µm^3 individual^{-1} day^{-1}) of *A. catenella* (3.2 x 10^6) and Alex5 (4.4 x 10^6) compared to *Tetraselmis* sp. (2.8 x 10^6 µm^3) when all three species were provided in the same biovolume at the highest prey concentration levels, but these differences were not statistically significant (ANOVA, $F_{2,5}$=1.527, p > 0.05). *B. plicatilis* was able to maintain positive population growth in all 5 cell concentrations of the non-lytic *A. tamarense* strain (Alex5 control, Table 3-7, Figure 3-7). Its growth rate significantly increased with increasing Alex5 cell concentrations (non-linear fit, t= 7.141, p< 0.0001). However, the population growth of *B. plicatilis* was significantly higher when provided the non-toxic alga *Tetraselmis*

as a food source compared to the highest growth reached with Alex5 (ANOVA, $F_{2,6}$=371.2, p< 0.0001, Figure 3-7).

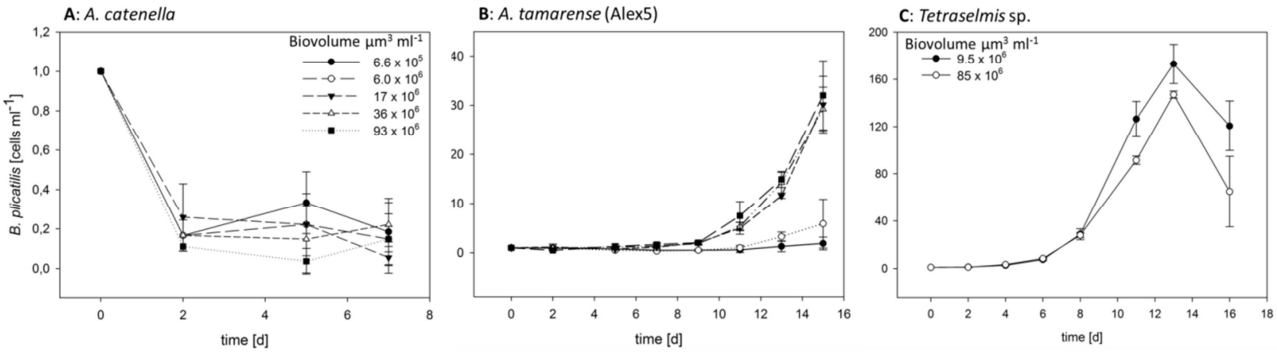

Figure 3-7: *B. plicatilis* population growth in 5 different concentrations [µm^3 ml^{-1}] of (A) *A. catenella* and (B) *A. tamarense* (Alex5) over time compared to the growth in two different concentrations of the non-toxic *Tetraselmis* (C). The 5 different biovolume levels were similar for both *Alexandrium* species, while the two biovolume levels used for *Tetraselmis* represented the lowest and the highest concentration used for *Alexandrium* (exp. 3). Data points represent means ± SD (n = 3). Note the different scaling of the y-axes.

Table 3-7: Maximum Ingestion rates in pg C individual^{-1} d^{-1} calculated for the first 48 h of the experiments and maximum growth rate (day^{-1}) for *B. plicatilis* growing with *A. catenella*, *A. tamarense* (Alex 5) and *Tetraselmis*.

Parameter	*A. catenella*	*A. tamarense* (Alex5)	*Tetraselmis*
I [µm^3 ind^{-1} d^{-1}]	3.2 ± 0.7 × 10^6	4.4 ± 0.9 × 10^6	2.8 ± 1.8 × 10^6
μ_{max} [d]	n.d.	0.30 ± 0.04	0.44 ± 0.02

Even though *B. plicatilis* actively preyed on both dinoflagellates, the growth rate of *A. catenella* was not significantly affected by grazing at any cell concentration (Table 3-8). In contrast, the Alex5 growth rate was significantly lower in the presence of the grazer in the lowest cell concentration (Table 3-8 TukeyHSD, p< 0.05), but was unaffected in the higher cell concentrations (TukeyHSD, p> 0.05).

Table 3-8: Effects of grazer presence and cell concentrations on dinoflagellate growth tested with a two-factorial ANOVA (experiment 3, dose-response). The table gives degree of freedom (df) for each factor, its F-ratio and significance level (p).

Response	Factor	Df	F	p-value
growth (μ d^{-1})	Grazing	1	0.548	0.467
A. catenella	cell conc.	4	3.851	0.017
	grazing*conc.	4	1.260	0.318
growth (μ d^{-1})	Grazing	1	6.624	*<0.05*
Alex5	cell conc.	4	48.763	*<0.00001*
	grazing*conc.	4	2.262	0.098

Experimental set 3: Mixed culture experiments with *Tetraselmis*, *B. plicatilis* and *A. catenella*

In the mixed growth experiments the rotifer fed on both the toxic *A. catenella* and on the non-toxic *Tetraselmis* sp.. There were no significant differences between the rates of *A. catenella* and *Tetraselmis* sp. ingestion in the mixed culture (ANOVA, $F_{2,3}$ = 0.119, p= 0.89), indicating that the rotifer did not select against the toxic dinoflagellate. The *Tetraselmis* growth rate over the first two days of incubation was significantly reduced when both *A. catenella* and *B. plicatilis* were present (Table 3-9, ANOVA, $F_{3,8}$= 6.426, p< 0.01, Figure 3-8). After this initial decrease the *Tetraselmis* population recovered and was able to maintain positive growth with and without the rotifer grazer (0.51 and 0.56 d^{-1}, respectively). The exponential growth rate of *B. plicatilis* was not significantly affected by *A. catenella* presence (comparing BT and ATB, ANOVA, $F_{1,4}$=1.617, p= 0.272).

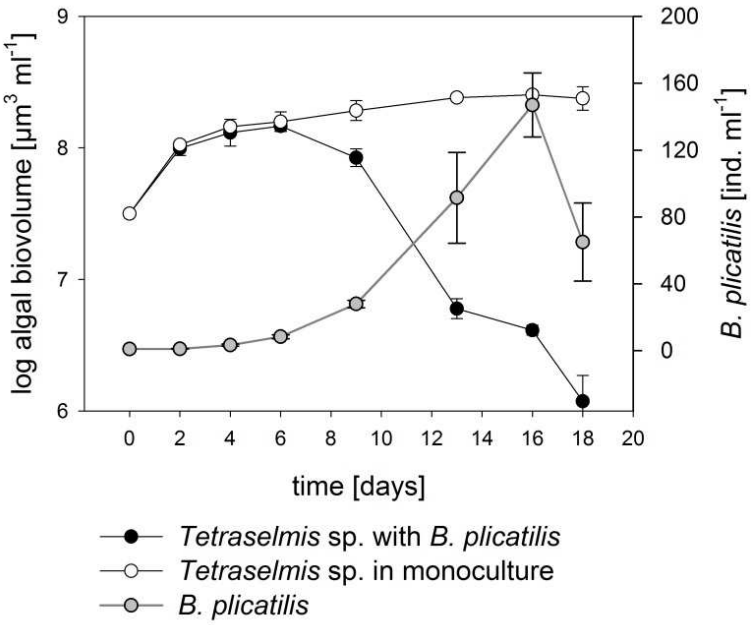

Figure 3-8: *Tetraselmis* sp. growth in monoculture (white symbols) and with the grazer *B. plicatilis* (black symbols) (exp. 3). The grey symbols show the growth of *B. plicatilis* with *Tetraselmis*. Each data point represents a mean ± SD.

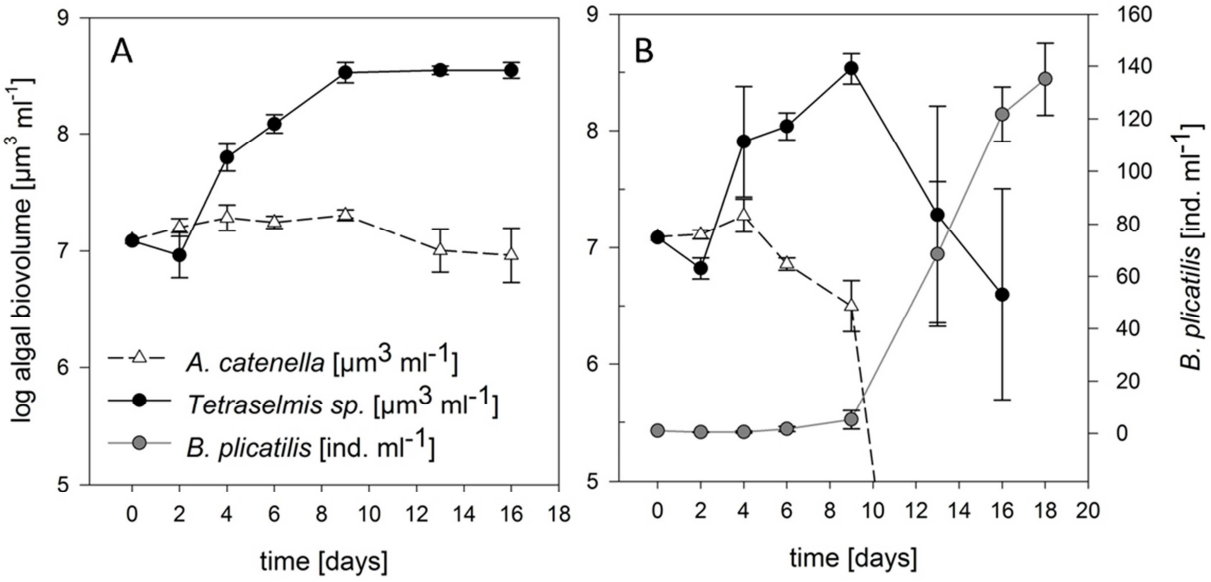

Figure 3-9: Growth curves of *A. catenella* and *Tetraselmis* in mixed cultures (exp. 4). **A**: without grazer (AT) **B**: with the grazer *B. plicatilis* (grey line, ATB). Each data point represents a mean ± SD.

However, *B. plicatilis* showed a lag phase of 6 days when fed with *Tetraselmis* and of 9 days when both algae were provided as food source (Figure 3-8, Figure 3-9 B). The growth rate of *A. catenella* was significantly reduced by *B. plicatilis* presence in the ATB treatment compared to the AT treatment (ANOVA, $F_{1,4} = 49.5$, $p < 0.005$) and by *Tetraselmis* in the AT treatment (compared to the monoculture (A), ANOVA, $F_{1,4} = 29.04$, $p < 0.01$). After 9 days the rotifer reached a sufficiently large population size to have a significant grazing impact on the *Tetraselmis* population (ANOVA, $F_{2,3} = 219.3$, $p < 0.001$).

Table 3-9: Pairwise comparisons (TukeyHSD) of *Tetraselmis* growth rate in monoculture (T), with *A. catenella* (AT), with *B. plicatilis* (TB) and with both (ATB). Significant p-values ($p< 0.05$) are reported in italic.

	T	AT	TB
AT	0.18	---	---
TB	0.98	0.27	---
ATB	*0.02*	0.44	*0.03*

3.5. Discussion

The present study clearly showed that the North American strain of *A. catenella*, isolated from the coast of Southern California, produces PSP toxins as well as extracellular allelopathic compounds that have deleterious effects on phytoplankton competitors and on zooplankton grazers. The negative effects of allelochemicals produced by *A. catenella* included immobilisation and growth inhibition of *Tetraselmis* sp., cell lysis of *Rhodomonas salina* and mortality / growth inhibition of the metazoan grazer *B. plicatilis.* A comparison of these effects with the effects of two well-studied strains of *A. tamarense* (Alex2 (highly lytic) and Alex5 (non-lytic), Tillmann *et al*., 2002) revealed that *A. catenella* had an intermediate negative effect on phytoplankton competitors and on *B. plicatilis* under short-term conditions (24h). The adverse effects on both the protistan competitors and the metazoan grazer were observed in whole cell culture and culture filtrate/supernatant of *Alexandrium* spp.,

indicating that extracellular allelochemicals were the active agents in these experiments and not intracellular PSP toxins.

To my knowledge this is the first study demonstrating allelochemical potency of a North American strain of *A. catenella* isolated from the Southern California coast and to investigate allelopathic effects on competitors and consumers in a food web context.

Allelopathic effects on phytoplankton competitors

The *Rhodomonas* bioassay used in the present study has also been used for the detection of lytic activity in a range of different *Alexandrium* species and strains (e.g. Hakanen *et al*., 2014; Tillmann *et al*., 2009, 2008). Thus, this bioassay was considered to be useful in order to set the observed effect strength into the context of other studies on *Alexandrium* strains. The bioassay showed an intermediate lytic activity compared to Alex2 (~230 cell ml^{-1}, 100% mortality) and Alex5 (~8600 cells ml^{-1}, no effect) for *A. catenella* whole cell culture with an EC_{50} of 566 cells ml^{-1} (cell concentration at which 50% of the target cells were lysed). Based on the same bioassay Tillmann *et al*., (2008) found EC_{50} concentrations of 649 cells ml^{-1} when testing an *A. catenella* strain originating from the Mediterranean Sea. These data are in the same range as my results. In contrast, Tillmann *et al*. (2008) used the same Mediterranean *A. catenella* strain to test lytic effects on the chlorophyte *Dunaliella salina* and found a very low EC_{50}-value of 22 cells ml^{-1}, indicating that different target organisms may show different sensitivities to allelochemical substances and/or that different *Alexandrium* strains produce multiple allelochemicals that target and differentially impact different phytoplankton (Ma *et al*., 2009; Poulson *et al*., 2010; Prince *et al*., 2010). Different sensitivities of target species to allelochemical substances may be due to several factors, including specific growth rates of the target species, growth phase of donor species as well as cell concentrations and ratios of donor and target species (Arzul *et al*., 1999). In contrast to *R. salina,* the chlorophyte *Tetraselmis* sp. was less inhibited in growth and immobilisation tests and was able to recover under these specific conditions (particular donor / target proportions), indicating a lower sensitivity to allelochemicals produced by *A. catenella*. Cell lysis was not observed in the short time frame of the immobilisation experiment (1h). However, the observed immobilisation of *Tetraselmis* cells could

have been a preliminary phase of cell lysis even when part of the cells were able to recover from the inhibiting effects after 3h (data not shown). These results indicate that the weak effects on the *Tetraselmis* population may be explained as a combination of the intermediate potency of the allelochemicals produced by this strain of *A. catenella* (see above) and the relatively high specific growth rates of the target species (~ 0.5 d^{-1}, own observation). The latter reduces the sensitivity of the target population, as new target cells are produced faster than new allelochemical substances, which can adsorb to a variety of surfaces, leading to the removal of compounds from the medium (Ma *et al*., 2009). This is supported by Arzul *et al*. (1999), who showed that an *A. catenella* isolate from the Chilean coast (Pacific Ocean) affected the growth of three different target species in different ways; the effects were dependent on the donor's cell concentration, but also on the target's growth rate. The *A. catenella* cell densities they used in their tests were almost in the same range (~1,800 cells ml^{-1}) as used in the current study and caused a low growth inhibition of the diatom *Chaetoceros gracile*, a strong effect on the dinoflagellate *Gymnodinium mikimotoi* and no inhibition for *Scrippsiella trochoidea*.

Several studies conducted with different isolates of *A. catenella* and different *Alexandrium* species support these observations regarding negative effects of *Alexandrium* spp. on protistan targets (e.g. Fistarol *et al*., 2004; Hakanen *et al*., 2014; Hattenrath-Lehmann and Gobler, 2011; Weissbach *et al*., 2011) and indicate that allelochemicals produced by *A. catenella* can distinctly shape natural phytoplankton communities by affecting different members of the community in different intensities. Hattenrath-Lehmann & Gobler (2011) demonstrated different patterns of allelochemical potency in North American strains of *A. fundyense* (isolated from the American east coast) affecting *R. salina* and natural plankton communities. For example, strains that only weakly reduced densities of *R. salina* in laboratory experiments significantly and strongly decreased densities of autotrophic nanoflagellates, diatoms, and dinoflagellates during field experiments.

Besides the benefit from reducing competition through adverse effects on other phytoplankton, the production of allelochemicals may also be a mechanism involved in prey capture of mixotrophic dinoflagellates as discussed in previous studies (Adolf *et al*., 2006; Blossom *et al*., 2012). *A. catenella* is known to be mixotrophic (e.g.

Jeong *et al*., 2005; Yoo *et al*., 2009, chapter 2 of this thesis) and was also shown to ingest *Tetraselmis sp.* (see chapter 2), indicating that cell immobilisation may be beneficial for *A. catenella* for the capture and ingestion of prey cells. This mechanism has also been observed for the marine dinoflagellate *Karlodinium veneficum*, which used extracellular allelochemicals for immobilising prey cells before ingestion (Sheng *et al*., 2010). Additionally, the lysis of prey cells may indirectly supply the donor species with dissolved organic nutrients (Stoecker *et al*., 2006; Jonsson *et al*., 2009). Weissbach *et al*. (2011), for instance, demonstrated that allelochemicals released by *A. tamarense* made resources available by both increasing the dissolved organic matter and bacteria abundance which were responsible for demineralization of nutrients.

In summary, allelochemicals that negatively affect protistan targets can be beneficial for *A. catenella* by reducing competition for dissolved nutrients as well as by supporting the dinoflagellates' mixotrophic feeding through prey immobilising or by providing organic nutrients due to the lysis of target cells.

Allelopathic / toxic effects on zooplankton consumers

In the second part of this study I investigated harmful effects of *A. catenella* on micro-zooplankton using the rotifer *B. plicatilis* as an assay species, which has been suggested to be a suitable model organism for detecting toxic effects of harmful algae (Yan *et al*., 2009). As discussed before, negative effects of *Alexandrium* spp. on protistan targets such as heterotrophic dinoflagellates are unrelated to the presence of the well-known PSP-neurotoxins (Tillmann and John, 2002), but for metazoan grazers the distinction between toxic and allelopathic effects is not so clear. In contrast to protistan targets, metazoan grazers were shown to be negatively affected by both the ingestion of toxic *Alexandrium* spp. cells (which has been tested mainly for copepods, e.g. Dutz, 1998; Frangópulos *et al*., 2000) and by extracellular allelopathic compounds (Bagøien *et al*., 1996; Silva *et al*., 2013; Yan *et al*., 2009). In the present study *B. plicatilis* mortality significantly increased in *A. catenella* and Alex2 (highly-lytic) cell free supernatant, but was not affected in non-lytic Alex5 supernatant, indicating that extracellular allelochemicals caused the observed deleterious effects. In contrast, Silva *et al*. (2013) tested exudates of a toxic *Alexandrium* sp. on *B. plicatilis* and found no adverse effect. However, they observed

copepod nauplii inactivation during exposure to toxic *A. tamiyavanichii* and *A. minutum* exudates. Likewise, Bagøien *et al*. (1996) found an inactivation effect of *A. minutum* exudates on copepod nauplii and adults (*Euterpina acutions*). On the other hand, Wang *et al*. (2005) used whole cell cultures to test the effects of different *Alexandrium* strains on *B. plicatilis*. They demonstrated that 7 strains (out of 10), including both PST producing and non-PST producing strains, had a negative effect on *B. plicatilis* and also suggested that extracellular allelochemicals produced by *Alexandrium* spp. were the active compounds in their study. Similar results have been obtained with gastropod larvae, where exposure to toxic or non-toxic *Alexandrium minutum* cells resulted in feeding inhibition and ultimate death of the target species (Juhl *et al*., 2008).

The dose response experiments demonstrated that the negative (allelopathic) effect of *A. catenella* on *B. plicatilis* is density dependent. *B. plicatilis* fed on both of the PST producing *Alexandrium* species tested (lytic *A. catenella* and non-lytic *A. tamarense* strain, Alex5). While *B. plicatilis* mortality increased with increasing *A. catenella* densities, the rotifer was able to maintain positive population growth when grazing on Alex5 at all cell concentrations except for the lowest one, where the grazer starved due to a lack of food. Accordingly, the rotifer grazing impact on both *Alexandrium* species was constrained by allelopathic activity, i.e. *B. plicatilis* was able to control the growth of the non-lytic Alex5 but not the growth of *A. catenella*. Both *Alexandrium* species used in the dose response experiment were PST producing strains, but the toxin profiles differed substantially (*A. catenella*: mainly GTX1/4, B1 and C1/C2 toxins; Alex5: C1/C2, NEO and STX (Tillmann *et al*., 2009)). These differences are not unusual as toxin profiles may be highly variable among *Alexandrium* isolates from different geographical origin (Cembella *et al*., 1987). GTX1 is the compound with the highest toxicity in the PST group (Oshima, 1995) and therefore the higher rotifer mortality with *A. catenella* compared to Alex5 could have also been a result of toxicity in addition to the allelopathic effect. Ingestion rates increased with increasing *Alexandrium* cell concentrations for both species, which supports previous studies in which *B. plicatilis* was shown to feed actively on different *Alexandrium* species irrespective of PST content, either with lethal effects or without (Wang *et al*., 2005; Xie *et al*., 2007; Yan *et al*., 2009). However, even though *B. plicatilis* was able to

maintain positive population growth when fed with the toxic, but non-lytic Alex5, its growth rate was significantly lower compared to growth with a non-toxic diet.

Further, long-term ingestion of toxic cells can cause sub-lethal effects in metazoan grazers, resulting, for instance, in reduced egg production (Colin & Dam 2007) and lower hatching success (Frangópulos *et al*., 2000). In the present study, the experimental duration did not allow to test for such potentially sub-lethal effects on reproduction success, which make assumptions of long-term toxic effects on the rotifer *B. plicatilis* difficult. Considering the decreasing trend of surviving *B. plicatilis* with increasing Alex5 cell concentrations in the dose response experiment, there might be a possibility of an additional toxic effects under long-term conditions, which is consistent with the previously described studies on long-term sub-lethal effects on metazoan grazer of ingested PSP-toxins. In summary, the observed effects on *B. plicatilis* in the present study were mainly caused by extracellular allelochemicals, but long term toxic effects also seem to be possible. Microzooplankton grazing is considered to be an important factor in regulating harmful dinoflagellate blooms (Irigoien *et al*., 2005; Stoecker *et al*., 2008). In particular, grazing by heterotrophic dinoflagellates and ciliates is often considered to be most effective because of their fast growth rates and high ingestion rates compared to mesozooplankton grazers such as copepods (e.g. Admiraal and Venekamp 1986,). However, this potential capability to regulate harmful dinoflagellate blooms can be extended also to other non-protistan microzooplankton groups such as rotifers (Mallin *et al*., 1995, Calbet *et al*., 2003). In a field study Calbet *et al*. (2003) found the rotifer *Synchaeta* spp. to be very abundant within a *Alexandrium minutum* bloom and considered the rotifer to actively graze on *A. minutum* under bloom conditions. Since that specific *A. minutum* strain seemed to have no other feeding deterrent effect despite its toxicity (Calbet *et al*., 2003), a bloom of an allelopathic *Alexandrium* spp. strain might have caused a prolongation of the bloom due to missing grazing pressure.

Allelopathy and toxicity of *A. catenella* - Implications for food web dynamics

Mixed culture experiments with *Tetraselmis*, *A. catenella* and *B. plicatilis* showed that providing *Tetraselmis* as a non-toxic food source partly counteracted the negative effect of *A. catenella* in limiting the population growth of *B. plicatilis*. At the chosen inoculum cell proportion of 1:32 (*A. catenella* :*Tetraselmis*, resulting in similar biovolume of 12×10^3 μm^3 ml^{-1}) *A. catenella* had a marginally negative impact on *Tetraselmis* in the first 48 h of the experiment. *Tetraselmis* has higher growth rates and nutrient uptake kinetics compared to *A. catenella* (e.g. k_s of 0.00345 µM for phosphorus uptake for *Tetraselmis*, Laws *et al*., 2011; k_s (P uptake) of 0.7 µM for *A. catenella,* Matsuda *et al*., 1999). Thus, the negative effect of excreted allelochemicals was quickly masked by the high growth of *Tetraselmis* and accordingly *Tetraselmis* became the superior competitor for dissolved nutrients. The results of the single culture experiments revealed that *B. plicatilis* did not select against the toxic dinoflagellate. Concurrently, the cell density of *A. catenella* decreased in proportion to increased *B. plicatilis* grazing and competition with *Tetraselmis*. In combination with decreasing *A. catenella* concentrations the high biomass which was formed by *Tetraselmis* and *B. plicatilis* may have led to a quick removal of compounds from the medium (Ma *et al*., 2009) and thus may have weakened the negative allelopathic effect. Therefore, these results support previous studies demonstrating that the intensity of allelopathic effects is not only dependent on the donor species concentration, but also varies depending on the target density (Fistarol *et al*. 2004, Schmitt & Hansen 2001, Tillmann *et al*. 2007, Hattenrath-Lehmann and Gobler, 2011).

With respect to the allelochemical potential of *A. catenella* on natural plankton communities, it is important to note that typical bloom concentrations of less than 17,000 cells L^{-1} (Jester *et al*., 2009) are low relative to the EC_{50} concentrations estimated for this species in this study. Since the allelopathic effects of *A. catenella* on co-occurring algae and grazers are density dependent, allelopathy is more likely to contribute to bloom maintenance when cell densities are high rather than during bloom initiation when cell densities are low (Jonsson *et al*., 2009).

However, even at low average cell concentrations, spatial variation in cell concentration may be high. Under natural conditions, patches of increased *A. catenella* cell numbers can occur when cells are being transported onshore during relaxation-favourable winds (Langlois and Smith, 2001; Price *et al*., 1991) and thus accumulate in shallow nearshore waters. The formation of localised patches and thin-layers (which can also be observed for *Alexandrium* spp. in culture flasks grown under non-turbulent conditions), within which *Alexandrium* can increase cell numbers to abundances high enough to deter potential grazers and competitors by accumulation of allelochemicals, may therefore nevertheless constitute a key factor for bloom initiation (Tillmann *et al*., 2008).

Chapter III

4. CHAPTER III: Growth, competition and grazing control of the potentially harmful dinoflagellate *Lingulodinium polyedrum* in a natural plankton community

4.1. Abstract

Population dynamics of potentially harmful dinoflagellates are regulated both 'bottom-up' by resources (e.g. concentrations of dissolved nutrients) and 'top-down' by zooplankton grazing. In the present study I investigated 'bottom-up' and 'top-down' effects on the population dynamics of the red tide dinoflagellate *Lingulodinium polyedrum* in coastal waters of Southern California, USA. Laboratory experiments were conducted using a natural plankton community off the coast of Los Angeles. I manipulated concentrations of dissolved nutrients by adding phosphate, nitrogen or both nutrients to natural seawater containing a natural *L. polyedrum* population and investigated its growth under different nutrient conditions. In a second approach, the effect of the presence of potential competitors and consumers was investigated on the growth and competitive behaviour of an *L. polyedrum* population that was spiked into 2 different size fractions of a natural plankton community; i.e., < 20 µm (excluding consumers) and < 110 µm (including consumers). In an additional treatment, I added the heterotrophic dinoflagellate consumer *Noctiluca scintillans* to both size fractions. *L. polyedrum* responded positively to P and N additions, but was not able to successfully compete with diatom competitors, which dominated the initial plankton community. Natural zooplankton grazers (<110 µm) decreased the nanophytoplankton (< 20 µm) and thus indirectly promoted the growth of *L. polyedrum* by competitive release. In turn, the heterotrophic dinoflagellate *N. scintillans* exerted a strong grazing pressure on the *L. polyedrum* population, indicating that *L. polyedrum* bloom formation can be hampered by zooplankton grazing. A subsequent laboratory experiment investigating grazing of *N. scintillans* on a gradient of increasing cell concentrations of *L. polyedrum*, supported these results and suggested that *N. scintillans* grazing is not only important in inhibiting bloom formation under low *L. polyedrum* concentrations, but may also play a role for bloom demise at high *L. polyedrum* cell densities.

4.2. Introduction

Harmful dinoflagellate blooms are a common phenomenon in coastal upwelling regions such as the Californian eastern boundary current system (Kudela *et al*., 2010; Trainer *et al*., 2010). Common bloom forming dinoflagellate species along the coast of Southern California include *Lingulodinium polyedrum*, *Prorocentrum micans*, *Gymnodinium* spp., and *Ceratium* spp. (e.g. Anderson *et al*., 2008; Horner *et al*., 1997; Schnetzer *et al*., 2007). These blooms are most common during mid- to late summer in stratified conditions after the coastal upwelling season (Horner *et al*., 1997).

In the past decades there has been a significant research focus on key factors leading to harmful dinoflagellate blooms throughout the world. Despite a substantial effort to understand bloom dynamics of different species by monitoring combined with operational modelling, it is still challenging to predict these high biomass blooms. Bloom dynamics are influenced by a complex interplay between the physical, chemical and biological factors involved (Anderson, 1995; Davidson *et al*., 2014; GEOHAB, 2011) making the parameterization in numerical models extremely difficult (Berdalet *et al*., 2015). Abiotic factors such as solar radiation and essential nutrient availability are the two main factors controlling phytoplankton production in the marine environment (Lalli and Parsons, 1997); together with temperature, salinity and surface currents, they form the “bottom-up” factors that control the growth rate of phytoplankton populations (Lalli and Parsons, 1997; Smayda and Reynolds, 2003). Many studies on bloom formation of harmful dinoflagellates to date have focused on such factors, e.g. dissolved inorganic nutrients and irradiance, that might stimulate the growth and favour the dominance of dinoflagellates in both laboratory experiments and in the field (e.g. Dagenais Bellefeuille *et al*., 2014; Hu *et al*., 2011; Kudela and Cochlan, 2000; Kudela *et al*., 2008). In contrast, “top-down” control of phytoplankton growth includes losses due to predation (e.g. Banse, 1994; Lehman, 1991), but can also arise from other biotic factors, like parasite and viral attack (Alves-de-Souza *et al*., 2015; Coats and Park, 2002; Park *et al*., 2004). In addition to top-down and bottom-up controls, a third type, ‘sideways’ controls, can be important (Fuhrman and Hagstrom, 2008). Sideways control refers to resource competition and to the direct negative effects on other competing phytoplankton i. e. allelopathy (Kaiser, 2011).

The primary grazers on HAB species are microbial pathogens (including algicidal bacteria, infectious viruses, and parasites, Coats and Park, 2002), microzooplankton (< 200 mm), mesozooplankton (> 200 mm), benthic invertebrates, and fish. Microzooplankton comprises heterotrophic dinoflagellates and ciliates, including tintinnids and aloricate ciliates (Stoecker and Evans, 1985; Turner, 2006), while copepods and cladocerans are the major mesozooplankton grazers (Turner, 2006). Particularly, protozoan grazers (i.e. heterotrophic dinoflagellates and ciliates) can provide an effective "top down" control on HAB species compared to metazooplankton because of their faster growth rates (Admiraal and Venekamp, 1986; Strom and Morello, 1998). In the Southern California Bight (SCB) protistan microzooplankton is dominated by ciliates (approximately 23 – 32% of the total microzooplankton biomass, Beers and Steward, 1971) and can consume up to 20% of the total phytoplankton biomass (Heinbokel, 1978) and thus the protozooplankton can be assumed to be the main consumers of the autotrophic pico- and nanoplankton in the SCB (Azam *et al*., 1983).

The reduction of grazing pressure, especially from microzooplankton, is recognized as one of the key factors facilitating phytoplankton bloom formation (Irigoien *et al*., 2005; Stoecker *et al*., 2008). For instance, grazing pressure can be reduced by physical or chemical perturbations that can break down strong predator - prey links (Irigoien *et al*., 2005) or by spatial and temporal variability in microzooplankton grazing pressure that can provide ''windows of opportunity'' for growth of dinoflagellates (Stoecker *et al*., 2008). Furthermore, feeding preferences of particular grazers and thus grazer community composition can play an important role for the promotion of dinoflagellate blooms. Selective feeding of planktonic consumers (e.g. phagotrophic protists) can provide a competitive advantage for bloom forming species (Caron *et al*., 2004). That is, the consumption of other phytoplankton (e.g. nano- or pico-phytoplankton) removes potential competitors, while rejection of the bloom forming dinoflagellates allows for an accumulation of the latter in the plankton community.

Mixotrophy (the combination of phototrophy and phagotrophy) is another factor that may favour bloom-forming dinoflagellates. Many of them are known to be mixotrophic and thus may also remove competitors by feeding on other phytoplankton, facilitating their dominance in a plankton community (e.g. Adolf *et al*., 2006; Burkholder *et al*., 2008; Stoecker, 1999). Various studies have thus hypothesized that blooms of some

dinoflagellates are closely related to their mixotrophic capability (Burkholder *et al*., 2008; Glibert *et al*., 2009; Jeong *et al*., 2005b).

L. polyedrum (Stein) Dodge is a typical high biomass bloom-forming dinoflagellate species in coastal upwelling regions (Allen, 1946; Holmes *et al*., 1967; Kahru and Mitchell, 1998; Kudela and Cochlan, 2000a). Blooms of up to 1 Mio cells L^{-1} (Kudela and Cochlan, 2000, SCCOOS, 2013, http://www.sccoos.org/) have been associated with fish and shellfish mortality events due to oxygen depletion when high biomass blooms accumulate in enclosed harbours or bays (Horner *et al*., 1997). In the SCB blooms of *L. polyedrum* are common from April to November and are associated with calm, warm weather conditions (Allen 1946, Holmes *et al*. 1967, Eppley and Harrison 1975), the formation of a shallow (< 10 m) nutrient-deplete mixed surface layers, a steep thermocline and nutrient-rich deeper water masses (Eppley and Harrison, 1975). However, blooms can also occur under conditions, presumably fuelled by high nutrient freshwater runoff (Hayward *et al*., 1995; Kudela and Cochlan, 2000; Kudela *et al*., 2008). *L. polyedrum* produces yessotoxin, a hepato- and and cardiotoxin (Armstrong and Kudela, 2006; Paz *et al*., 2004); however, no human health issues or marine mammal deaths associated with yessotoxins have been reported in this area yet (Caron *et al*., 2010). *Noctiluca scintillans*, a common heterotrophic dinoflagellate, has been described as an effective grazer of large *L. polyedrum* blooms in the SCB (Goldstein, 2011; Howard, 1996; Torrey, 1902), and thus extensive *N. scintillans* blooms often appear in the same area a few weeks after *L. polyedrum* blooms (Howard, 1996).

In recent years *L. polyedrum* has been studied extensively in laboratory and field experiments and has become a model organism for dinoflagellate physiology and ecology (Lewis and Hallet, 1997). These studies focused on different aspects of *L. polyedrum* bloom dynamics with regard to its nutrient uptake kinetics (e. g. Kudela and Cochlan 2008, Eppley *et al*., 1969), vertical migration to prevail in stratified water (Eppley and Harrison, 1975; Moorthi *et al*., 2006), the effect of turbulence on its growth and division rates (Juhl *et al*., 2000; Sullivan *et al*., 2003), its mixotrophic tendencies (Jeong *et al*., 2005) as well as grazing on *L. polyedrum* (e.g. Jeong *et al*., 1999, 2001, 2002).

Despite major advances in understanding its ecology based on laboratory experiments using monocultures or communities with just a few species, the complexity of biological and environmental interactions in the field still hampers

predictions about where and when bloom events will occur. Thus it requires an even deeper knowledge of the dinoflagellates' various ecological traits and adaptive strategies that influence its population dynamics in the field and may lead to bloom formation (Smayda and Reynolds, 2003; Smayda, 1997b). For instance, patterns observed in lab experiments with monocultures or a few species can differ greatly from patterns in complex natural plankton communities, comprising a greater variety of competitors, consumers and potential prey organisms. Therefore, experiments with an increased complexity, i.e. using natural plankton communities, are crucial to evaluate whether mechanisms that determine population dynamics in controlled lab experiments also play a role in a more natural environment with a highly diverse plankton community and thus a wider array of interacting biotic and abiotic environmental factors.

This study aims to fill this void and to understand interactive effects of nutrient supply (bottom-up control) and grazing (top-down control) on the population dynamics of *L. polyedrum* in a natural plankton community from coastal waters of the SCB. I conducted three laboratory experiments in which I manipulated dissolved nutrient concentrations and grazer abundances (natural grazers and *N. scintillans* from a culture) in a natural plankton community off the coast of Los Angeles containing *L. polyedrum*. In Exp. 1, I added different nutrients (phosphate, ammonium, nitrate, trace metals and vitamins) to the natural plankton community to investigate the effects of altered resource levels on competition and population dynamics of *L. polyedrum*. In a second approach (Exp. 2 and Exp. 3), I investigated the effect of potential consumers on growth and competitive behaviour of *L. polyedrum* by using different size fractions of a natural plankton community (< 20 µm, excluding consumers, and < 110 µm, including consumers) and by the addition of the heterotrophic dinoflagellate *Noctiluca scintillans*. In a subsequent laboratory experiment (Exp. 4) using laboratory cultures I investigated the grazing effect of *N. scintillans* on a gradient of different cell concentrations of *L. polyedrum* in order to find a particular threshold concentration above which *N. scintillans* is not able anymore to control the population growth of *L. polyedrum*.

4.3. Materials and Methods

Dinoflagellate cultures and collection of natural seawater

Cultures of *L. polyedrum* and *N. scintillans* were isolated from the Southern California coast by the Caron Laboratory (Caron Laboratory, USC, Los Angeles) and were maintained in f/2 medium without silicate (Guillard and Ryther, 1962). The food source for *N. scintillans* was *L. polyedrum*. Stock cultures were grown at 18°C, a light intensity of 75 µmol photon m^{-2} s^{-1} and a 12:12 light:dark cycle in a walk-in temperature-controlled incubator. Natural seawater from the coast was used for media preparation, filtered through a 0.2 µm filter and autoclaved for sterilization. Cultures were non-axenic, but the experiments were set up and sampled under sterile conditions to minimize bacterial and other contaminations. For Experiments 1-3, water samples (20 L) containing natural plankton were collected from surface water at Cabrillo Harbor, south of Los Angeles, California, in October 2013. For transport to the laboratory, the water was stored in 20 L carboys under dark and cool conditions. Upon return to the laboratory, the seawater was immediately filtered through a 110 µm mesh to remove larger grazers. The remaining plankton assemblage was then examined by light microscopy (Zeiss Axiovert) and the dominant taxa were identified to gain a general overview of the plankton composition as well as to estimate the natural *L. polyedrum* and grazer abundances. Afterwards, the seawater was filtered into different size fractions for different experimental manipulations (see below).

Experimental design

Experiment 1: Competitive ability of *L. polyedrum* in a natural plankton community under different nutrient regimes

After filtration (110 µm), the natural plankton was manipulated by adding different nutrient sources, resulting in six different experimental treatments: Control (no addition), Control + (addition of trace metals (TM) and vitamins (vit)), +P (addition of PO_4^{3-}, TM and vit), +N (addition of NO_3^- or NH_4^+, TM and vit), and ALL (addition of all nutrients, see Table 4-1). The trace metal and vitamin solutions were prepared according to the f/2 medium (Guillard and Ryther, 1962) and contained thiamine (vit B_1), cyanocobalamin (vit B_{12}) and biotin. All treatments were set up in triplicates in 1.2 litre polycarbonate containers with an experimental volume of 500 ml (seawater

containing natural plankton < 110 µm), and incubated under constant environmental conditions in an incubator (see above). Samples were taken every second day for microscopic cell counts (preserved with 1% formol) and the experiment was terminated after 6 days. Phytoplankton organisms were assigned to broad taxonomic groups based on morphological examination (diatoms, dinoflagellates and other flagellates). Only *L. polyedrum* was identified at species level. The initial natural cell density of *L. polyedrum* was approximately 1×10^3 cells L^{-1} at the beginning of the experiment.

Table 4-1: Different nutrient treatments and concentrations applied in the nutrient addition experiment (Exp. 1)

Treatment	Limitation	Trace metals & vitamins	PO_4^{3-} 3 µmol l^{-1}	NO_3^- 50µmol l^{-1}	NH_4^+ 50µmol l^{-1}
C (control)	potentially N and P	---	---	---	---
C+ (control + vit and TM)	potentially N and P	X	---	---	---
ALL	---	X	X	X	X
$+PO_4^{3-}$	Nitrogen/ Ammonium	X	X	---	---
$+NO_3^-$	Phosphorus	X	---	X	---
$+NH_4^+$	Phosphorus	X	---	---	X

Experiments 2 and 3: Grazing control and competitive ability of *L. polyedrum* in a natural plankton community.

Experiment 2 was set up as a pre-experiment to Experiment 3 to test whether smaller phytoplankton (<20 µm) may serve as a food source for *L. polyedrum*, or rather acts as competitor for dissolved nutrients. Before the setup of the experiment, a 50 ml water sample was settled and counted for *L. polyedrum* cell abundance. Water was then prepared by reverse filtration of 20 L seawater through a 20 µm mesh size filter. The control was set up by further filtering the previously filtered seawater through a 0.2 µm nucleopore membrane filter. No nutrients were added. Because natural *L. polyedrum* abundances were less than 1,000 cells L^{-1}, the experimental water was spiked with *L. polyedrum* from a lab culture (isolated from the coast of Southern California and maintained as described above) to provide a final concentration of approximately 100 cells ml^{-1} (simulating low bloom abundance as defined here

between 1 and 1,000 cells ml^{-1}). Some smaller zooplankton, notably ciliates, were still included in the <20 µm treatments and may have grazed some of the phytoplankton, but their abundance was low (< 100 L^{-1}), and attempts to remove such protozoa would have caused unwanted changes in the ambient plankton community (e.g. removal of larger dinoflagellates). All treatments were set up in triplicate, resulting in 6 experimental units, using 1.2 litre poly-carbonate bottles with an experimental volume of 500 ml. The bottles were sampled every second day for determining the abundances of *L. polyedrum* and other phyto- and zooplankton. The experiment was terminated after 12 days.

Experiment 3 was set up in accordance with experiment 2, but with an additional size fraction (<110 µm) and two additional grazer treatments (<20 µm+ and <110 µm+, Table 4-2). The size fraction < 20 µm was supposed to contain mainly phytoplankton competitors/prey while the < 110 µm size fraction aimed to also include protozoan and metazoan grazers. In the first trial of the experiment, the additional grazer treatments were set up by adding a concentrated natural grazer assemblage, which was generated by reverse filtering the natural plankton assemblage through a 110 µm mesh. However, it turned out that the concentrated natural grazer assemblage also included high cell densities of large diatoms, which also accumulated in this concentration process. Thus, these two additional grazer treatments were dismissed and only the 3 replicates for the < 20 and < 110 µm treatments were kept and run together with the second set-up of this experiment (see below). These additional treatments were then later used for the regression analysis of *L. polyedrum* growth rate in response to grazer abundance (Figure 4-5, see below). In the second set-up of this experiment, the grazer treatments <20 µm+ and <110 µm+ (Table 4-2) were incubated without additional natural grazers, but by adding the heterotrophic dinoflagellate grazer *N. scintillans* from a culture into the natural plankton community.

Seawater was filtered into three size fractions (< 0.2, 20 and 110 µm) and spiked with approximately 100 *L. polyedrum* cells ml^{-1} from a culture, as natural *L. polyedrum* abundances were again < 100 cells L^{-1}. While the control (< 0.2 µm) was set up in triplicate, the 20 µm and the 110 µm fractions were set up with 6 flasks, respectively, and half of them were spiked with *N. scintillans* from a culture (10 cells ml^{-1}), resulting in three replicates per experimental treatment (Table 4-2). Samples of 20 ml were taken after1, 2, 4, 5 and 7 days from each flask for the whole experimental duration (7 days) and preserved with 1% Formol for enumeration of phytoplankton and

zooplankton. Subsamples (10 ml) were settled in sedimentation chambers for 12 h before light microscopic analysis. The dominant taxa (species or taxonomic groups) were identified and enumerated using an inverted Zeiss Axiovert microscope at a magnification of 10 to 40x, focusing on diatoms and dinoflagellates >15 µm. Additionally, 2 ml subsamples were taken and preserved with 1% Formol on experimental day 1, 4 and 7 for flow-cytrometric (Accuri flow cytometer, BD, San Jose, CA) analysis of the pico-phytoplankton. These samples were stored at -80°C until analysis.

Table 4-2: Experimental setup of experiment 3

Treatment	Filter size	Addition
Control	0.2 µm	*L. polyedrum*
<20 µm	20 µm	*L. polyedrum*
<20 µm (+)	20 µm	*L. polyedrum* + *N. scintillans*
<110 µm	110 µm	*L. polyedrum*
<110 µm (+)	110 µm	*L. polyedrum* + *N. scintillans*

Experiment 4: Functional and numerical response of *N. scintillans* grazing on *L. polyedrum*

A subsequent functional and numerical response experiment (Exp. 4) was conducted to estimate the grazing impact of *N. scintillans* on a gradient of five different *L. polyedrum* cell concentrations ranging from 800 to 12,800 cells ml^{-1} (800, 1600, 3200, 6400 and 12800 cells ml^{-1}). Prior to the experiment, *N. scintillans* cells were starved for 48 h in 200 ml Falcon culture flasks containing f/2 medium and a low concentration (ca. 80 - 100 cells ml^{-1}) of remaining *L. polyedrum* cells. Approximately 100 *N. scintillans* cells were then transferred into Erlenmeyer flasks containing 100 ml medium and the experimental *L. polyedrum* concentrations (resulting in approximately one *N. scintillans* cell ml^{-1}). The experiment was set up in triplicate and incubated at 18°C, 60 µmol photon $m^{-2}s^{-1}$ on a 12:12 light:dark cycle. Samples (5 ml volume) were taken every second day and preserved with Lugol´s iodine solution at 1% final concentration; the experiment was terminated after 13 days. Cell numbers were determined using an inverted microscope (Leica DM IL).

Analytical procedures

All data were analysed using the software R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria, 2014).

Growth rates (μ) of *L. polyedrum* and *N. scintillans* for each treatment were calculated using the formula:

(1) $\mu\ (d^{-1}) = \frac{(\ln(C_2) - \ln(C_1))}{(t_2 - t_1)}$

where C_1 and C_2 are cell numbers at the beginning (t_1) and the end (t_2) of the exponential growth phase (t in days). To convert *L. polyedrum* cell numbers into biovolume, species-specific cell volumes were calculated by assuming a geometrical shape of a sphere (Hillebrand *et al*., 1999). The spherical diameter of *L. polyedrum* (32.7 ± 0.4 μm) was determined microscopically by measuring n= 30 individual dinoflagellate cells. Final biovolume per ml^{-1} was calculated by multiplying single cell volume (18.3 x 10^3 μm^3) with corresponding cell counts.
One way analyses of variances (ANOVA) were performed to test the effect of nano-phytoplankton presence (in < 20 μm filtered SW) on *L. polyedrum* maximum cell density (MCD) and growth rate compared to the 0.2 μm filtered control (experiment 2) as well as to test the effect of the two different plankton size fractions (< 20 μm and < 110 μm) on *N. scintillans* cell density (experiment 3). For experiment 3, interactive effects of size fraction (< 20 μm and < 110 μm) and grazer presence (*N. scintillans*) on final cell numbers of *L. polyedrum*, pico-phytoplankton and cyanobacteria were tested using a two-factor ANOVA. The level of significance was defined at $p < 0.05$.

Whenever ANOVA's showed significant effects, the significant differences among treatment levels were determined using a TukeyHSD post hoc test. All data were examined for normal distribution. Homogeneity of variances was tested using the Bartlett's test. Data that failed to meet these criteria were log transformed as indicated in text or table legends.

To test the effect of natural zooplankton abundances in different size fractions without *N. scintillans* on *L. polyedrum* (experiment 3), the growth rate (μ d^{-1}) of *L. polyedrum* in each experimental unit (6 replicates < 20 μm and 6 replicates of the <110 μm treatment) was plotted against the corresponding zooplankton abundance and the correlation was determined using a Spearman rank order correlation.

Additionally, for experiment 1 and 3, log response ratios (LRR) were calculated to quantify the proportionate change that resulted from the experimental manipulation (Hedges *et al*., 2015). Here, LRR is the natural-log proportional change in the means ($\bar{X}$) of a treatment (T) and control group (C):

(2) LRR= $ln\,(\bar{X}_T/\bar{X}_C)$

Accordingly, the experimental effect was measured by dividing the biovolume of *L. polyedrum* (μm^3 ml^{-1}) in the different filtration and grazer treatments by the *L. polyedrum* biovolume in the 0.2 µm filtered seawater control. A student's t-test was performed to test for statistically significant differences from zero as well as between the log response ratios of different treatments for both experiments.

For the numerical and functional response experiment (experiment 4) the ingestion (IR) and clearance rate (CR) for each *L. polyedrum* cell concentration was calculated over the exponential growth phase of *N. scintillans* (day 5 – 13) using a modification of the method used by Frost (1972). The grazing rate (g) describes the differences between the *L. polyedrum* growth rate (µ) in monoculture and in mixed culture (µ*) with the grazer *N. scintillans* (eq. 3).

(3) $g = \mu - \mu^*$

Considering the exponential growth of the grazer and the prey during the time interval $t_2 - t_1$, the mean grazer (N) and prey (L) cell numbers $\langle C \rangle$ for t_2 - t_1 were calculated using equation 4. This value for the mean grazer cell number was then used to calculate the clearance rate (CR, eq. 5) and the ingestion rate (IR, eq. 6).

(4) $\langle C_{L,N} \rangle = \frac{C_1^* \left[e^{(\mu - g)(t_2 - t_1)} - 1\right]}{(\mu - g)(t_2 - t_1)}$

The clearance rate (CR) is given by (eq.5) where V is the volume (ml), g the grazing coefficient and $\langle C_N \rangle$ the mean grazer cell number in the time interval t_2-t_1.

(5) $CR = \frac{V \times g}{\langle C_N \rangle}$

(6) $IR = \langle C_L \rangle \times CR$

Afterwards, the average *N. scintillans* growth rates (eq. 1), the ingestion (eq. 6) and grazing rates (eq. 3) were plotted against the 5 different initial cell concentrations of *L. polyedrum* and the maximum values were calculated by fitting the data points to a sigmoidal curve using the non-linear model fit in R. After statistical examinations, corresponding graphs were created with the software Sigma plot (version 11.0, from Systat Software, Inc., San Jose California, USA).

4.4. Results

Competitive ability of *L. polyedrum* in a natural plankton community under different nutrient regimes

After 48 h of incubation, *L. polyedrum* cell numbers increased compared to the control (C, untreated seawater) when vitamins, trace metals and NO_3^-, were added (LRR significantly different from zero, t-test, $p< 0.05$, Figure 4-1A). However, the *L. polyedrum* cell numbers significantly decreased when all nutrients were added together (LRR 'All' significantly different from zero, t-test, $p< 0.05$, Figure 4-1A). Diatoms dominated the phytoplankton community at the beginning of the experiment, while *L. polyedrum* cell numbers were low (< 1000 cells L^{-1}), as the cell numbers of other dinoflagellates (e.g. *Prorocentrum* spp., *Ceratium* spp., *Akashiwo* spp., *Alexandrium* spp., data not shown). There were non-significant trends of increased diatom biovolume when an N source was added (positive LRR, All, NO_3^- or NH_4^+), while under N limitation, when only PO_4^{3-} was added, diatoms responded negatively compared to the control (negative LRR, t-test, $p > 0.05$, Figure 4-1B). After 4 days of incubation *L. polyedrum* densities decreased (to < 200 cells L^{-1}) in all treatments and no cells could be observed in the 'All' and $+NH_4^+$ treatment anymore.

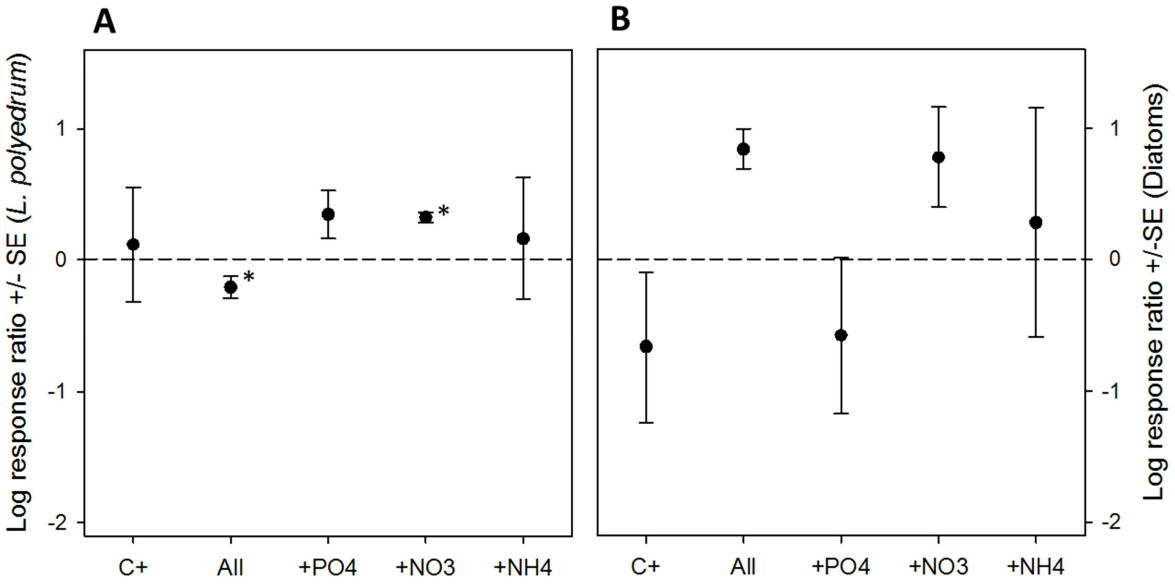

Figure 4-1: Log response ratio ± SE (n = 3) of *L. polyedrum* (A) and diatom (B) biovolume in the 5 different treatments C+ (control plus TM & vit.), All (all nutrients), $+PO_4$, $+NO_3$ and $+NH_4$ 48 h after nutrient addition (experiment 1). Asterisks indicate statistically differences from zero based on the one-sample t-test ($p< 0.05$).

Grazing control and competitive ability of *L. polyedrum* in a natural plankton community

In experiment 2, *L. polyedrum* was negatively affected when grown together with the < 20 µm fraction of the natural plankton community. The maximum cell density (MCD) of *L. polyedrum* at the end of exponential growth (after 7 days) was significantly lower when grown with nanophytoplankton (< 20 µm) compared to the 0.2 µm filtered seawater control (ANOVA, $F_{1,4}=48.36$, $p<0.005$). *L. polyedrum* cell numbers increased in both, the control and the nano-phytoplankton treatment, with similar growth rates (0.28 ± 0.005 in the nano-plankton treatment and 0.37 ± 0.04 d^{-1} in the control, not statistically different, $p > 0.05$); however, the stationary growth phase was reached after 5 days in the treatment with nano-phytoplankton and after 7 days in the control (Figure 4-2).

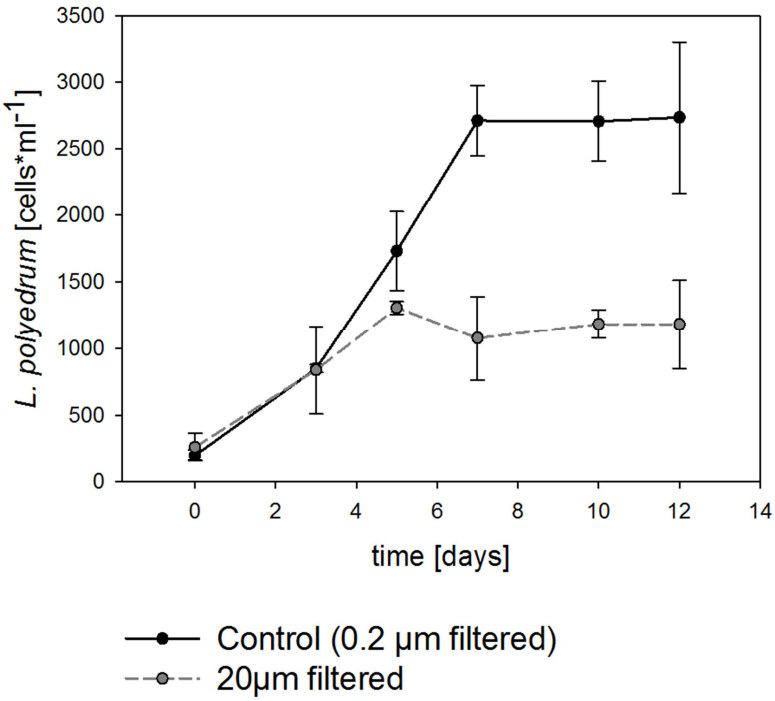

Figure 4-2: Growth curves of *L. polyedrum* (cells ml^{-1}) in the 0.2 µm filtered control and in the 20 µm filtered treatment containing the natural nano-phytoplankton community (experiment 2). Data are presented as means ± 1 SD (n=3).

In experiment 3, the addition of the heterotrophic dinoflagellate *N. scintillans* significantly reduced the final cell density of *L. polyedrum*, while the different plankton size fractions (< 20 µm, <100 µm) had no effect on *L. polyedrum* MCD (no significant interaction, Table 4-3). In the first two days of the experiment, *L. polyedrum* was able to grow in all treatments with similar growth rates (no significant differences among different treatments, ANOVA, $F_{3,8}$=3.55, p > 0.05). The stationary growth phase was reached in the two treatments without *N. scintillans* after 4 days (Figure 3 C, D), afterwards the *L. polyedrum* abundances declined in the < 20 µm fraction and remained stable in the < 110 µm plankton fraction. In both plankton size fractions with *N. scintillans* cell numbers started to decline after two days (Figure 4-3 E, F). *N. scintillans* was able to maintain positive population growth with no significant differences in the MCD between both filtrations treatments (<20 and <110 µm) at the end of exponential growth (Figure 4-3 A, B, ANOVA, $F_{1,4}$=0.687, p > 0.05), indicating no negative effect due to competing zooplankton). At the beginning of the experiment the microzooplankton in the <110 µm filtered fraction consisted mainly of heterotrophic dinoflagellates (e.g. *Protoperidinium* sp., *Dinophysis* sp.), ciliates

(tintinnids) and copepod nauplia, while in the < 20 µm fraction only small tintinnids were observed (size 20 – 50 µm, these individuals might have passed the filter due to their elongated shape, data not shown).

Final pico-phytoplankton cell numbers (after 7 days) were significantly affected by *N. scintillans* addition and there were indications of an effect of the different plankton size fractions (p = 0.065, Table 4-3). The significant interaction term of both factors indicated that the two factors were not independent from each other (Table 4-3). Pico-phytoplankton was significantly higher in the < 110 µm fraction compared to the < 110 µm fraction with *N. scintillans* (TukeyHSD, p = 0.012, Figure 4-3 H, J) as well as compared to the <20 µm fraction (TukeyHSD, p = 0.014).

Cyanobacteria abundances were not significantly affected by the *N. scintillans* addition or by the different plankton size fractions (data not shown, Table 4-3).

Table 4-3: Results of a two-factorial ANOVA testing the effects of size fraction (<20 and <100 µm) and grazer addition (*N. scintillans*) on the final cell density of *L. polyedrum*, picophytoplankton and cyanobacteria. The table gives degree of freedom (df) for each factor, its F-ratio and significance level (p).

Response	Factor	df	F	p
Cell numbers	size fraction	1	2.6	0.145
(*L. polyedrum*)	*N. scintillans*	1	53.35	*< 0.0001*
	interactions	1	3.004	0.121
Cell numbers				
(picophytoplankton)	size fraction	1	5.038	0.065
	N. scintillans	1	9.192	*< 0.05*
	interactions	1	18.651	*< 0.05*
Cell numbers				
(cyanobacteria)	size fraction	1	0.198	0.668
	N. scintillans	1	0.006	0.94
	interactions	1	12.139	*< 0.05*

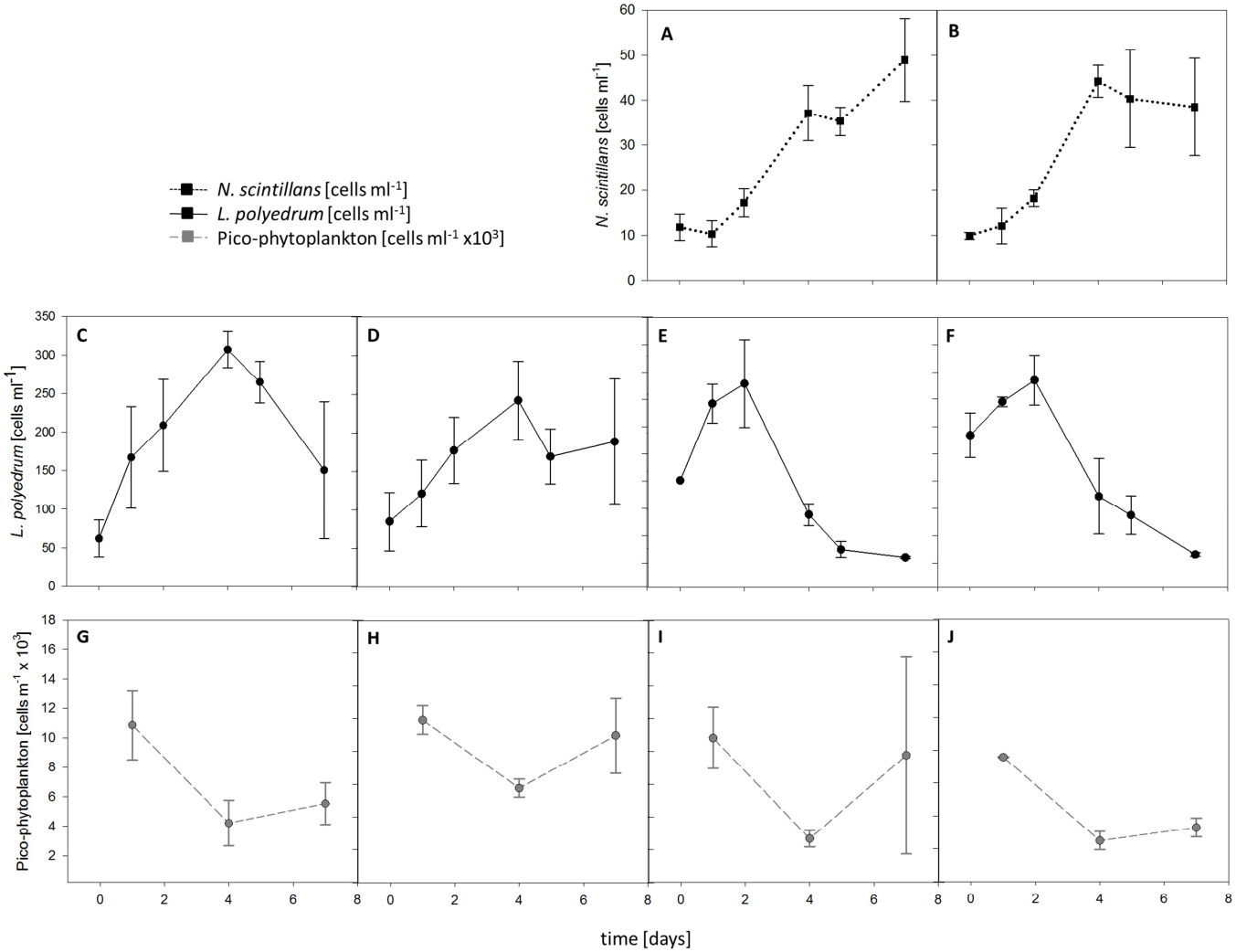

Figure 4-3: Growth curves of *L. polyedrum*, *N. scintillans* and picophytoplankton (cells ml^{-1}) in different size fractions of the plankton community (experiment 3). Data points present means ± SD (n=3).

To better illustrate the effect of the different treatments on *L. polyedrum,* the average effect size of *L. polyedrum* biovolume (log response ratio (LRR), Figure 4-4) was calculated. While the addition of *N. scintillans* had a significantly negative effect on *L. polyedrum* biovolume in both plankton size fractions (LRR significantly different from zero, one-sample t-test, $p < 0.005$), the LRR of the two filtration treatments without grazer addition (< 20 µm and < 110 µm) were not different from zero (one-sample t-test, $p > 0.05$, Figure 4-4). *L. polyedrum* biovolume was slightly negatively affected in the < 20 µm treatment, indicating that *L. polyedrum* growth was negatively affected by competing phytoplankton. In the < 110 µm treatment *L. polyedrum* biovolume was as high as in the control (indicated by a log response ratio of about 0).

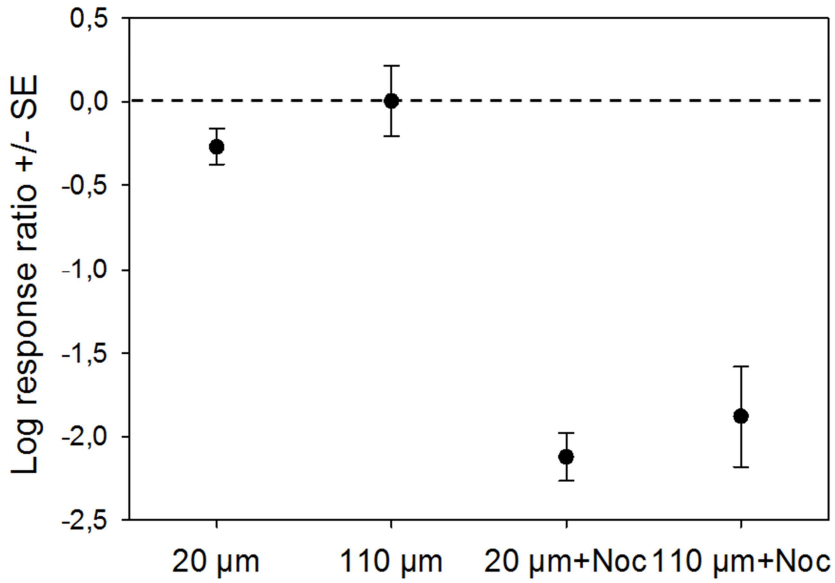

Figure 4-4: Log response ratio ± SE (n = 3) of final *L. polyedrum* biovolume in the 2 different plankton size fractions (< 20 µm and < 110 µm) with and without *N. scintillans* addition compared to the 0.2 µm filtered control (experiment 3). A negative log response ratio means there was a negative effect in the treatment compared to the control.

Taking both plankton size fractions containing natural zooplankton without *N. scintillans* (<20 µm and <110 µm, 6 replicates each from both experimental set-ups of Exp. 3, see Material and Methods) into account, *L. polyedrum* growth rate [d^{-1}] was significantly positively correlated with zooplankton cell abundances (Figure 4-5, Spearman rank order correlation, $R^2 = 0.4347$, $p < 0.05$), indicating an indirect positive effect of microzooplankton presence on *L. polyedrum* growth.

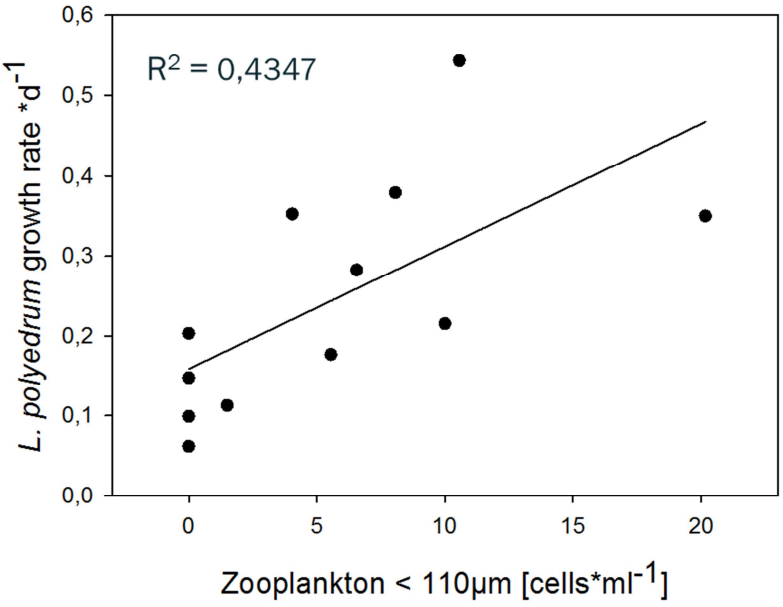

Figure 4-5: *L. polyedrum* growth rate d^{-1} depending on zooplankton < 110 µm presence in the two treatments containing natural zooplankton (<20 µm and <110 µm) of experiment 3. The correlation of (R^2 = 0.4347) is statistically significant ($p<0.05$), n = 6.

Functional and numerical response of *N. scintillans* grazing on *L. polyedrum*

The ingestion rate (uptake of prey cells $grazer^{-1}$ day^{-1}) of *N. scintillans* feeding on *L. polyedrum* was density dependent and significantly increased with increasing *L. polyedrum* cell concentration (Figure 4-6A, non-linear fit, t= 6.196, p< 0.001) up to a maximum rate (I_{max}) of 8.06 ± 1.3 cells ind^{-1} day^{-1} (0.18 µg C ind^{-1} day^{-1}). At mean prey concentrations higher than 6,000 cells ml^{-1}, however, its ingestion rate declined. *N. scintillans* exhibited the highest clearance rate at low concentrations of *L. polyedrum*, which steeply declined with increasing *L. polyedrum* concentrations (between 8 x 10^{-3} and 0.6 x 10^{-3} ml ind^{-1} day^{-1}, data not shown). The growth rate [d^{-1}] of *N. scintillans* increased with *L. polyedrum* concentration and reached its maximum with 0.57 ± 0.0381 d^{-1} (non-linear fit, t = 14.86, p = 0.0045, Figure 4-6C) at a mean prey concentration of 2,490 cells ml^{-l}. The grazing rate (grazing impact on the growth of the prey population) of *N. scintillans* on *L. polyedrum* was significantly negatively correlated with *L. polyedrum* cell concentrations (spearman rank order correlation, R= - 0.95, p< 0.0001, Figure 4-6B). However, only after 5 days *N. scintillans* cell numbers were high enough to reduce the population growth of *L. polyedrum,* in the

lower cell density treatments (treatment N1, N2 and N3). In the two highest cell density treatments (N4 and N5) containing the grazer *N. scintillans*, *L. polyedrum* was able to maintain positive population growth during the first five and seven days of the experiment, respectively.

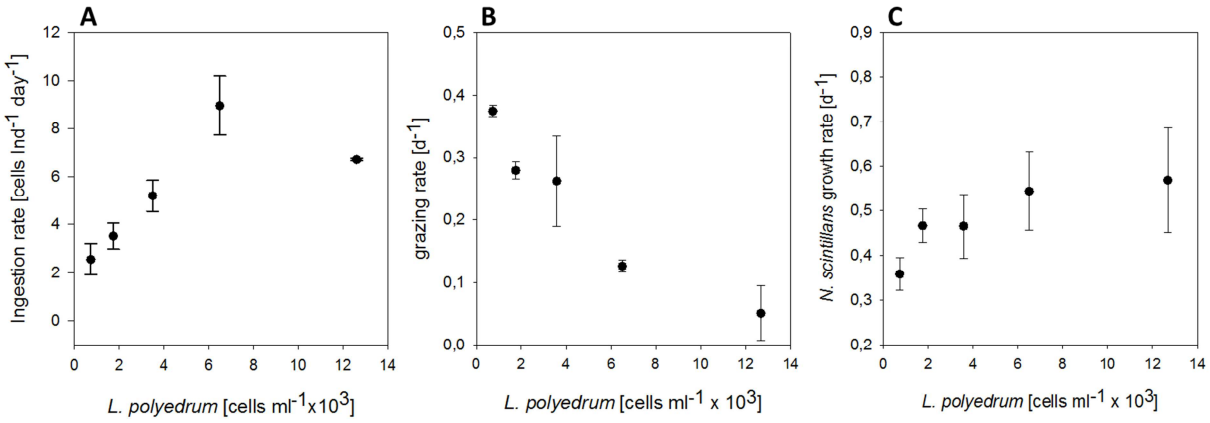

Figure 4-6: Ingestion rate, grazing rate and *N. scintillans* growth rate (means ± SE, n=3) in 5 different *L. polyedrum* concentrations (experiment 4). The rates were calculated during the exponential growth phase of *N. scintillans* (day 5-13).

4.5. Discussion

Overall, my experiments revealed that *L. polyedrum* cell numbers increased with phosphate and nitrogen additions in a natural plankton community; however, the dinoflagellate was not able to successfully compete with co-occurring diatom competitors, which dominated the initial plankton community, in any of the nutrient treatments. The impact of microzooplankton grazers on *L. polyedrum* depended on consumer selectivity and thus grazer composition; while *L. polyedrum* was strongly controlled by *N. scintillans*, natural microzooplankton mainly consisting of tintinnids and copepod nauplii had an indirect positive effect on *L. polyedrum*, presumably by feeding on other phytoplankton, which lead to competitive release of *L. polyedrum*.

Competitive ability of *L. polyedrum* in a natural plankton community under different nutrient regimes

The addition of macronutrients (NH_3^- or PO_4^{3-}) slightly increased the cell numbers of *L. polyedrum* compared to the untreated seawater control 'C' (positive log resp. ratio). However, *L. polyedrum* abundances decreased when all nutrients were added together ('All'), which was presumably due to an increase in diatom abundances in this treatment, which in turn might have had a negative effect on *L. polyedrum* due to increased competition for dissolved nutrients. These log response ratios were determined after an incubation time of 48 h and thus only reflect potential short-term effects of nutrient additions on *L. polyedrum* in a natural plankton community. After 48 h, *L. polyedrum* abundances (as well as other dinoflagellates) decreased in all nutrient treatments. These results are in agreement with previous studies on bottom-up effects on dinoflagellates in natural plankton communities. Piehler *et al*. (2004) observed in a study conducted in Pamlico Sound (North Carolina, USA) that dinoflagellates (not specified) did not positively respond to various nutrient additions (which were in similar concentration ranges as used in this study), but rather decreased in the treatment where all nutrients were added together (similar to my ALL treatment), in which they were outcompeted by diatoms. Kremp *et al*. (2008) showed in mesocosm experiments using natural phytoplankton communities that the addition of N and P generally stimulated the growth of diatoms; the dominance of dinoflagellates (*Woloszynskia halophile*), however, strongly depended on the size of the inoculum's population and the relative abundance of co-occurring diatoms. Thus, independent of nutrient additions, dinoflagellates were only able to out-compete diatoms when they were initially dominant. In the present experiment, the phytoplankton community was initially dominated by diatoms, while dinoflagellate cell numbers were low (< 1000 cells L^{-1}), which might explain why *L. polyedrum* was not able to compete successfully with the remaining phytoplankton assemblage.

It is generally assumed that dinoflagellates exhibit low affinity for nitrate and ammonium relative to diatoms (Smayda, 2000, 1997b). However, analyses of N (NO_3^-, NH_4^+ and urea) kinetics during a dense bloom of *L. polyedrum* from California revealed that the bloom could meet its entire nutritional N requirement from ambient urea and ammonium concentrations and that *L. polyedrum* was capable of competing with typical coastal phytoplankton such as diatoms under high N conditions (Kudela

and Cochlan, 2000). Additionally, previous field studies on the distribution and bloom dynamics of *L. polyedrum* in the Southern California Bight related high *L. polyedrum* cell abundances to both high levels of nutrient input to near surface waters via upwelling (Eppley and Harrison, 1975) and freshwater runoff (Hayward *et al*., 1995; Kudela and Cochlan, 2000; Kudela *et al*., 2008) as well as to more stratified and lower nutrient conditions (Holmes *et al*., 1967; Shipe *et al*., 2008). Thus, it has been suggested that bloom forming dinoflagellates in upwelling systems seem to be adapted for both low or pulsed nitrate supplies, and moderate to high ammonium (Kudela *et al*., 2010). Dagenais Bellefeuille *et al*. (2014) showed that the adaptation to N-deplete conditions allows *L. polyedrum* cells that are re-exposed to N to survive at high cell densities for a longer time period. In the field, conditions of N stress are more likely to be encountered by this species than continuous exposure to high N since nitrate has previously been shown to be the limiting nutrient for phytoplankton production in the Southern California Bight (Shipe *et al*., 2008). These adaptations could help blooming populations survive and maintain their high density for long periods of N stress in the environment. This demonstrates that relating *L. polyedrum* cell abundances in coastal waters to particular nutrient conditions is not straightforward, as its bloom dynamics are complex and also seem to be influenced by other environmental factors such as temperature and hydrodynamics (Shipe *et al*., 2008), as well as by its unique eco-physiological properties (i.e. nutrient retrieval vertical migrations, mixotrophy, lower nutrient affinity and the ability to use different forms of nutriens e.g. Smayda 1997). Thus, extrapolating short-term laboratory results to field conditions from upwelling systems is difficult because there are numerous factors other than nutrients involved in species succession and dominance.

In addition, the type of nutrients that are available can be an important factor in stimulating the growth of *L. polyedrum*. The present results indicated that in addition to macronutrients also vitamins and TM (C+) can increase *L. polyedrum* cell numbers. While in the present experiment the cell numbers of *L. polyedrum* were only slightly increased compared to the control when vitamins and TM were added, the growth rates of other dinoflagellates e.g., *Gymnodinium* sp. (Takahashi & Fukazawa, 1981), and *Prorocentrum micans* (Iwasaki, 1989) during blooms off the Japanese coast have been found to be significantly stimulated by additions of

vitamins B_1 or B_{12} in combination with N, P, and/or Fe. This supports the hypothesis that also vitamins can have important ecological relevance for harmful algal blooms (e.g. Iwasaki, 1989; Takahashi & Fukazawa, 1981).

Effects of potential consumers and competitors on *L. polyedrum* growth and competitive ability

The experiments investigating the population dynamics of *L. polyedrum* in different size fractions of a natural plankton community indicated that *L. polyedrum* was negatively affected by the presence of phytoplankton competitors < 20 µm. In contrast to the < 20 µm fraction, *L. polyedrum* cell numbers did not show a strong decrease at the end of the experiment in the < 110 µm fraction and ended up in similar cell numbers compared to the control. Microzooplankton abundance was positively correlated with the growth rate of *L. polyedrum.* This indicated that natural zooplankton grazers (< 110 µm), which mostly consisted of tintinnids, heterotrophic dinoflagellates, and copepod nauplii, decreased the competing nanophytoplankton (< 20 µm) and thus indirectly promoted the growth of *L. polyedrum* by competitive release.

Microzooplankton (< 200 µm) grazing is generally accepted as being the main predatory pressure on marine planktonic primary production, as microzooplankton may consume up to 60–70% of this production (Calbet and Landry, 2004). Tintinnids in particular, which were the most abundant component of the natural microzooplankton community (<110 µm) in the present study, have been shown to be important consumers of nanophytoplankton in the Southern California Bight (e.g. *Tintinnopsis* sp., *Eutintinnus pectinis*; Heinbokel and Beers, 1979; Heinbokel, 1978). Some of the present microzooplankton grazers, also ciliates, were shown to actively feed on *L. polyedrum* in monoculture experiments (Jeong, 1999; Jeong *et al*., 2002). However, prey preferences of ciliates are extremely variable among different species and mostly depend on prey size (e.g. Heinbokel and Beers, 1979; Tillmann, 2004). The heterotrophic dinoflagellate *Protoperidinium* sp., which was also present (in low numbers) in the plankton community in this study, preferably feeds on diatoms over dinoflagellates, including *L. polyedrum* (Buskey, 1997). As *L. polyedrum* abundances did not decrease in the <110 µm fraction compared to the control, and as microzooplankton abundances were even positively correlated with *L. polyedrum*

growth rates, it is likely that the specific microzooplankton community present here selectively fed on other phytoplankton in the natural plankton community, thus reducing competition for dissolved nutrients and promoting the growth of *L. polyedrum*.

These “windows of opportunity” (Stoecker and Gustafson, 2002; Stoecker *et al*., 2008) or “loopholes” (Irigoien *et al*., 2005) for bloom forming species can arise, for instance, when decreased copepod abundances release microzooplankton from grazing control and increase top-down control of small phytoplankton (Granéli and Turner, 2002). This could in turn release large dinoflagellates from competition at the same time when control of smaller phytoplankton increases (Stoecker *et al*., 2008). Likewise, Caron *et al*. (2004) showed for the bloom forming pelagophyte *Aureococcus anophagefferens,* that selective feeding of planktonic consumers (e.g. phagotrophic protists) on other competing species provided a competitive advantage for the bloom forming species.

Additionally, factors other than trophic cascading can reduce grazing on bloom forming species. Once a bloom is established, toxicity of the bloom-forming organisms, allelopathic compounds, high pH, or poor food quality for micrograzers can reduce grazing pressure (Irigoien *et al*., 2005; Mitra and Flynn, 2006; Stoecker *et al*., 2008; Sunda *et al*., 2006). *L. polyedrum* is able to produce yessotoxin (Armstrong and Kudela, 2006; Paz *et al*., 2004); however, no adverse effects (toxic or allelopathic) on direct grazers or indications for low food quality of *L. polyedrum* have been demonstrated, yet (e.g. Jeong and Latz, 1994; Teegarden, 1999).

In contrast to the natural grazer community, the heterotrophic dinoflagellate *N. scintillans* had a strong grazing impact on the *L. polyedrum* population, indicating that grazing control of the dinoflagellate strongly depends on zooplankton community composition. These results are consistent with previous field observations. The decline of a massive *L. polyedrum* bloom in 1995 was associated to *Noctiluca* sp. grazing, which resulted in a subsequent *Noctiluca* bloom (Hayward *et al*., 1995). *L. polyedrum* was also shown to be suitable food for the tintinnid *Favella ehrenbergii*, which selectively preys on dinoflagellates (Stoecker *et al*., 1981), as well as for the heterotrophic dinoflagellate *Protoperidinium* (Jeong *et al*., 1994) and for the mixotrophic dinoflagellate *Fragilidium* (Jeong *et al*., 1999b). This suggests that some phagotrophic protists are indeed capable of using *L. polyedrum* as food and of controlling its abundances, which may potentially inhibit bloom formation. While most

of these previous grazing experiments (e. g. Stoecker 1981, Joeng *et al*., 1994, 1999) were mainly conducted under monospecific bloom conditions or using monocultures of *L. polyedrum*, the present study clearly demonstrated that depending on the composition of the zooplankton community heterotrophic dinoflagellates can have a significant grazing impact on *L. polyedrum*, even in a complex natural plankton community, when alternative prey is available. In turn, other grazers such as ciliates preferred other phytoplankton over *L. polyedrum*, even though such grazers exhibited high grazing rates on *L. polyedrum* in monoculture laboratory experiments (Jeong *et al*., 1999; 2002; Stoecker 1981).

Functional and numerical response of *N. scintillans* feeding on *L. polyedrum*

N. scintillans exhibited a positive growth rate when feeding on *L. polyedrum* and reached its maximum at a mean prey concentration of 2,490 cells ml^{-1}. Its ingestion rate (i.e. the number of cells taken up by each grazer per time unit) increased with increasing *L. polyedrum* cell concentration and were similar to those reported for *N. scintillans* feeding on other algae such as *Alexandrium minutum* (Frangópulos *et al*., 2011), *Tetraselmis tetrathelle* and *Gymnodinium nagasakiense* (Lee and Hirayama K., 1992), *Chatonella antiqua* and *Heterosigma akashiwo* (Nakamura, 1998). Furthermore, the maximum ingestion rate (I_{max}) obtained in this study was comparable to the I_{max} reported for the heterotrophic dinoflagellate *Polykrikos kofoidii* feeding on *L. polyedrum* (~ 24 ng C ind^{-1} d^{-1}, Jeong *et al*., 2001).

N. scintillans has previously been described as an effective grazer of large *L. polyedrum* blooms in the Southern California Bight (Torrey, 1902; Howard, 1996, Goldstein, 2011) and extensive *N. scintillans* blooms were observed to appear in the same area a few weeks after an *L. polyedrum* bloom (Howard, 1996). These studies and the present results support the hypothesis that *N. scintillans* is capable of substantially limiting the growth of *L. polyedrum* not only in laboratory feeding trials, but also in the field, and thus can play an important role in the inhibition or regulation of high biomass dinoflagellate blooms. However, there were also indications of reduced grazing pressure on *L. polyedrum* at higher cell densities. *N. scintillans* grazing rate (i.e. grazing impact on the prey population) was negatively correlated

with *L. polyedrum* cell concentration, indicating that the growth rate of *L. polyedrum* was less affected by *N. scintillans* grazing at higher cell concentrations. Further, *N. scintillans* started its exponential growth phase only after 5 days after incubation, and reached maximum growth at a prey concentration of ~ 2,500 prey cells ml^{-1}, above which its growth did not further increase. Despite *N. scintillans* reaching higher growth rates than those of the prey population (0.57 d^{-1} compared to 0.06 - 0.1 d^{-1} for *L. polyedrum*), grazing might not be sufficient to control the growth of *L. polyedrum* once a certain prey cell concentration is reached. Jeong & Latz (1994) reported similar maximum growth rates for the heterotrophic dinoflagellate *Protoperidinium cf. divergens* on *L. polyedrum* (0.363-.484 d^{-1} at 1100 – 1500 prey cells ml^{-1}); however, in contrast to their study, the growth of *N. scintillans* did not decrease after maximum growth rate was reached. The time delay in *N. scintillans* growth (5 days before exponential growth started), as well as the decreasing grazing rate with increasing prey cell concentration could cause an uncoupling between grazer and prey dynamics, which has been described as an important factor for phytoplankton bloom formation (see above, e.g. Stoecker *et al*., 2008; Buskey 2008, Irigoien *et al*., 2005, Mitra and Flynn, 2006). A lag between the growth of phytoplankton and grazer populations, or some other factors depressing the abundances of potential grazers, can cause phytoplankton populations to be temporarily released from grazer control and enables them to reach bloom densities (Buskey, 2008). Possible factors that can cause a suppression of growth and ingestion rate of protozoan grazers at high prey densities can be the adverse effects on protistan growth due to low oxygen concentrations, when the increase in phytoplankton biomass leads to an increase in respiration rates and total oxygen demand, and to higher pH (Buskey, 2008). In addition, dynamic, patchy distribution of dinoflagellate and grazer populations in coastal areas may cause an initiation of blooms even when average potential microzooplankton grazing coefficients are relatively high (Stoecker *et al*., 2008). The present study demonstrated that *N. scintillans* is able to control the growth of *L. polyedrum* up to a certain cell concentration and thus can play an important role during pre-bloom phases and for bloom termination. However, the presented data also suggest that above a certain prey threshold concentration *N. scintillans* might no longer be capable of controlling the growth *L. polyedrum*. It can therefore be assumed that initiation of *L. polyedrum* blooms depend on the at least temporary

suppression of grazer populations, or the temporal or spatial uncoupling of grazer and prey populations, to allow *L. polyedrum* to grow until bloom levels are achieved.

General Discussion

5. GENERAL DISCUSSION

The objective of this thesis was to investigate abiotic and biotic factors that influence the bloom dynamics of the two potentially harmful dinoflagellate species *Lingulodinium polyedrum* and *Alexandrium catenella*. In particular, I focused on their competitive abilities and species-specific adaptive strategies such as mixotrophy and allelopathy, which have been suggested in previous studies to be important factors in determining the bloom dynamics of potentially harmful dinoflagellates (e.g. Burkholder *et al*., 2008; Stoecker, 1999; Stoecker *et al*., 2006; Tillmann and Hansen, 2009; Tillmann *et al*., 2009).

Overall, my experimental results revealed that the population dynamics of these dinoflagellates are influenced by a complex interplay of environmental abiotic factors such as inorganic dissolved nutrients, species-specific strategies to avoid competition and grazing, and plankton community structure. However, the specific adaptive strategies and mechanisms facilitating their growth differed fundamentally for the two dinoflagellates and included both direct and indirect interactions among different trophic levels of the plankton community. In order to gain a predictive understanding under which environmental conditions these two different HAB species are likely to become dominant in a plankton community and form blooms it is crucial to consider these direct and indirect interactions in a food web context.

In my thesis, I identified the following factors and adaptive strategies to influence the population dynamics of the two dinoflagellates investigated at different trophic levels:

1) nutrient storage capacities
2) mixotrophy (removal of potential competitors outweigh metabolic costs for switching nutritional modes)
3) allelopathy, negatively affecting competitors and grazers
4) zooplankton grazing (important loss factor or potentially facilitating factor, depending on grazer identity and grazing preferences)

Nutrient storage capacities

In the first part of this thesis (**chapter I**) I demonstrated that *L. polyedrum* was able to maintain high cell densities in a gradient of increasing N and P concentrations over the entire time of the experiment irrespective of nutrient concentrations and ratios due to luxury uptake of nitrogen and phosphorus after acclimation to lower nutrient conditions. Cellular nutrient storage, which was not related to growth rate, allowed *L. polyedrum* to draw on nutrients mobilized from internal storage pools. This ability enables *L. polyedrum* to thrive even at low nutrient conditions and thus to survive in high cell densities until the nutrient conditions become more beneficial for growth, e.g. via upwelling events, which can be an important factor for bloom initiation and persistence of *L. polyedrum*. Compared to *L. polyedrum*, *A. catenella* cell density was negatively affected by low nutrient conditions. Additionally, *A. catenella* did not exhibit the luxury nitrate uptake that was observed for *L. polyedrum*, however, there were indications of phosphorus storage capacities. These results support previous studies that showed a wide variability for HAB dinoflagellates in responding to different nutrient concentrations and ratios (e.g. Glibert *et al*., 2012; Jauzein *et al*., 2010; Kudela *et al*., 2010). Therefore, in order to predict potential consequences of anthropogenically altered nutrient conditions (e.g. through fertilizers, sewage, or coastal aquaculture) as well as of input of dissolved inorganic nutrients via upwelling in coastal waters, we require specific information on the responses of particular HAB dinoflagellate species or strains common to the coastal waters of interest to altered nutrient concentrations and ratios. My results also indicate that absolute nutrient concentrations or nutrient imbalances do not seem to be directly related to the growth of the dinoflagellates, as trophic interactions seem to be much more complex. Therefore, predicting bloom formation only based on dissolved nutrient concentrations is particularly difficult, or even impossible. Instead, competitive strategies such as nutrient storage capacities, but also mixotrophy and allelopathy need to be considered in the context of nutrient dynamics in order to fully understand dinoflagellate population dynamics and their impact on planktonic food webs. These implications are in line with theoretical studies emphasizing the importance of internal nutrient concentrations (Flynn, 2010) and adaptive strategies (e.g Smayda, 2000, 2002; Smayda and Reynolds, 2001, Glibert, 2016) for dinoflagellate bloom formation.

Mixotrophy and allelopathy

Furthermore, results presented in **chapter I** provide an important step forward regarding our understanding of the mixotrophic and allelopathic capabilities of the two dinoflagellate species investigated. Besides being beneficial for reducing co-occurring phytoplankton, these strategies also affect the cellular nutrient concentrations of the dinoflagellates. Both of them were able to ingest a variety of different prey organisms, but had a particularly strong negative impact on the pico-phytoplankton *Ostreococcus sp.* irrespective of nutrient limitation. However, there was only little benefit for the dinoflagellates from growing with prey in terms of biomass production. Instead, their cellular nutrient contents indicated initial metabolic 'costs' for switching nutritional modes from photosynthesis to phagotrophic feeding, before having a benefit from ingested prey. These initial metabolic costs were too high for feeding to be sufficient to enhance the growth rate under these experimental conditions. Nevertheless, switching trophic modes may be more efficient and thus more beneficial for the dinoflagellates compared to simultaneous phototrophy and phagotrophy (Stoecker, 1998). Overall, the results presented in **chapter I** indicate that nutrient storage capacities and mixotrophy may favour dinoflagellates in environments that provide temporarily or spatially heterogeneous nutrient conditions such as coastal upwelling systems.

Preliminary tests to differentiate between the effects of phagotrophic feeding and allelopathy of both dinoflagellates (**chapter I**) revealed that *A. catenella* has a strong lytic effect on target cells, while *L. polyedrum* does not, demonstrating that different strategies are used by different dinoflagellates to gain a competitive advantage. Phagotrophic feeding (*L. polyedrum*) and the continuous release of allelochemicals causing cell lysis (*A. catenella*), or a combination of both, can provide an indirect benefit for the mixotrophic dinoflagellates by reducing competition for dissolved inorganic nutrients. Furthermore, the uptake of organic nutrients, both by the ingestion of whole prey cells or by taking up dissolved organic matter resulting from cell lysis, supports the dinoflagellates internal nutrient contents and might help them to thrive at high cell numbers even under low nutrient conditions.

Given the high variability of species- and strain-specific allelopathic potencies of *Alexandrium* sp. (Tillmann and John, 2002; Tillmann *et al*., 2009, Hakanen *et al*., 2014), results presented in **chapter II** provide important new information on the

toxicity and allelopathic activity of the North American strain of *A. catenella.* My results clearly showed that *A. catenella* whole cell culture and cell free filtrate immobilised potential prey cells and caused a density dependent steep decline in competitor and grazer cell numbers. Since the allelopathic effects of *A. catenella* on co-occurring algae and grazers are density dependent, allelopathy is more likely to contribute to bloom persistence when cell densities are high rather than to bloom initiation when cell densities are low (Jonsson *et al*., 2009). However, even at low average cell concentrations, spatial variation in cell concentration may be high. Under natural conditions, patches of increased *A. catenella* cell abundances can occur when cells are being transported onshore during relaxation-favourable winds (Langlois and Smith, 2001; Price *et al*., 1991) and thus accumulate in shallow nearshore waters. The formation of localised patches and thin-layers, within which *Alexandrium* can increase cell numbers to abundances high enough to deter competitors and grazers by accumulation of allelochemicals, may therefore nevertheless constitute a key factor for bloom initiation (Tillmann *et al*., 2008).

My results further demonstrated that the allelopathic potency of *A. catenella* depends on the initial structure of the plankton community. On the one hand, allelochemicals produced by *A. catenella* can distinctly shape natural phytoplankton communities by affecting different members of the community in different intensities. On the other hand, high biomass of other members of the plankton community can weaken allelopathic effects. For example, mixed culture experiments with *A. catenella*, one phytoplankton competitor (*Tetraselmis* sp.) and the metazoan grazer *B. plicatilis* showed that the negative effect of excreted allelochemicals on both grazers and competitors was quickly masked by the high biomass of *Tetraselmis* and accordingly *Tetraselmis* became the superior competitor for dissolved nutrients. Thus, in combination with decreasing *A. catenella* concentrations the high biomass which was formed by *Tetraselmis* and *B. plicatilis* probably led to a quick removal of allelopathic compounds from the medium (Ma *et al*., 2009) and thus may have weakened the negative allelopathic effect.

Zooplankton grazing

Metazoan and protozoan grazing can be an important loss factor for HAB dinoflagellates irrespective of their toxin content (investigated in studies presented in **chapters II** and **III**). The metazoan grazer *B. plicatilis* (rotifer) fed actively on two

similarly toxic *Alexandrium* species (*A. catenella* and *A. tamarense*, **chapter II**) and the protozoan grazer *N. scintillans* (heterotrophic dinoflagellate) exerted a strong grazing pressure on an *L. polyedrum* population, even when alternative food was available (**chapter III**). *A. catenella* caused a density dependent negative effect on *B. plicatilis,* which was mainly caused by extracellular allelochemicals. However, long-term toxic effects also seemed to be possible, both releasing the dinoflagellate from grazing pressure. Another indirect positive feedback mechanism of grazing for HAB dinoflagellates was identified in the study presented in **chapter III**. In a natural plankton community zooplankton grazers (<110µm) selectively fed on nanophytoplankton (< 20 µm) and thus indirectly promoted the growth of *L. polyedrum* by competitive release. Even through the natural plankton community was artificially manipulated and spiked with *L. polyedrum* and *N. scintillans* cultures, the results of this experiment clearly demonstrated that depending on the composition of the plankton community some grazers such as *N. scintillans* can have a significant grazing impact on *L. polyedrum*, while others such as ciliates prefer other phytoplankton over *L. polyedrum*, thus potentially facilitating its competitive success. Thus, in accordance with previous studies (Stoecker *et al*., 2008; Buskey 2008, Irigoien *et al*., 2005), that describe the temporal or spatial uncoupling of grazer and prey dynamics, as an important factor for phytoplankton bloom formation, it can be assumed that the initiation of *L. polyedrum* blooms depend on the at least temporary suppression of grazer populations, to allow *L. polyedrum* to grow until bloom levels are achieved.

5.1 Dinoflagellate dynamics in a food web context

In summary, the diagram in Figure 5-1 shows that there are many interacting processes on different levels of the food web that influence and are influenced by potential HAB dinoflagellates. For example, the availability of dissolved inorganic nutrients (different ratios and concentrations) affects dinoflagellates and other phytoplankton in different ways (bottom-up factors). This leads to competition for the limiting nutrients. Based on lower growth rates and nutrient affinities, dinoflagellates might not be the dominant phytoplankton competitors under certain conditions. However, the dinoflagellates possess a range of strategies such as mixotrophy and allelopathy that may offset these ecological disadvantages (sideways factors).

Phagotrophic feeding on competitor (prey) populations reduces competition for limiting nutrients (and light), thus indirectly benefitting the dinoflagellates (Figure 5-1, dashed yellow arrow). The production and release of allelopathic substances that act lytically on sensitive co-occurring phytoplankton species also reduces competition (only shown for *A. catenella*, Figure 5-1, dashed green arrow) and in combination with mixotrophic tendencies lysed cells might supplement nutritional demands of the dinoflagellates by the uptake of DOM. Thus mixotrophy does not only affect competing phytoplankton, but is also relevant for the dinoflagellates' nutrition, providing additional organic nutrient sources (bottom-up control).

Further, heterotrophic protistan and metazoan grazers are able to feed on the dinoflagellates and can pose an important loss factor, even at high dinoflagellate cell concentrations. Similar to the reduction of competitors, dinoflagellates have also evolved strategies that negatively influence higher levels of the food web. These strategies include allelopathic effects on both protozoan and metazoan grazers as well as possible toxic effects on metazoan grazers, both leading to a reduction of grazing pressure (Figure 5-1, dashed green arrow). Thus, when dinoflagellates exhibit such species-specific traits that lead to avoidance of grazing, other co-occurring phytoplankton may face an increased grazing pressure, providing the HAB dinoflagellates with a competitive advantage (Figure 5-1, positive feedback due to reduced competition, dashed brown arrow). This indicates that grazing control of HAB dinoflagellates strongly depends on zooplankton community composition; factors such as feeding preferences and sensitivity to allelopathic substances / toxins determine if the growth of the HAB species can be controlled or not.

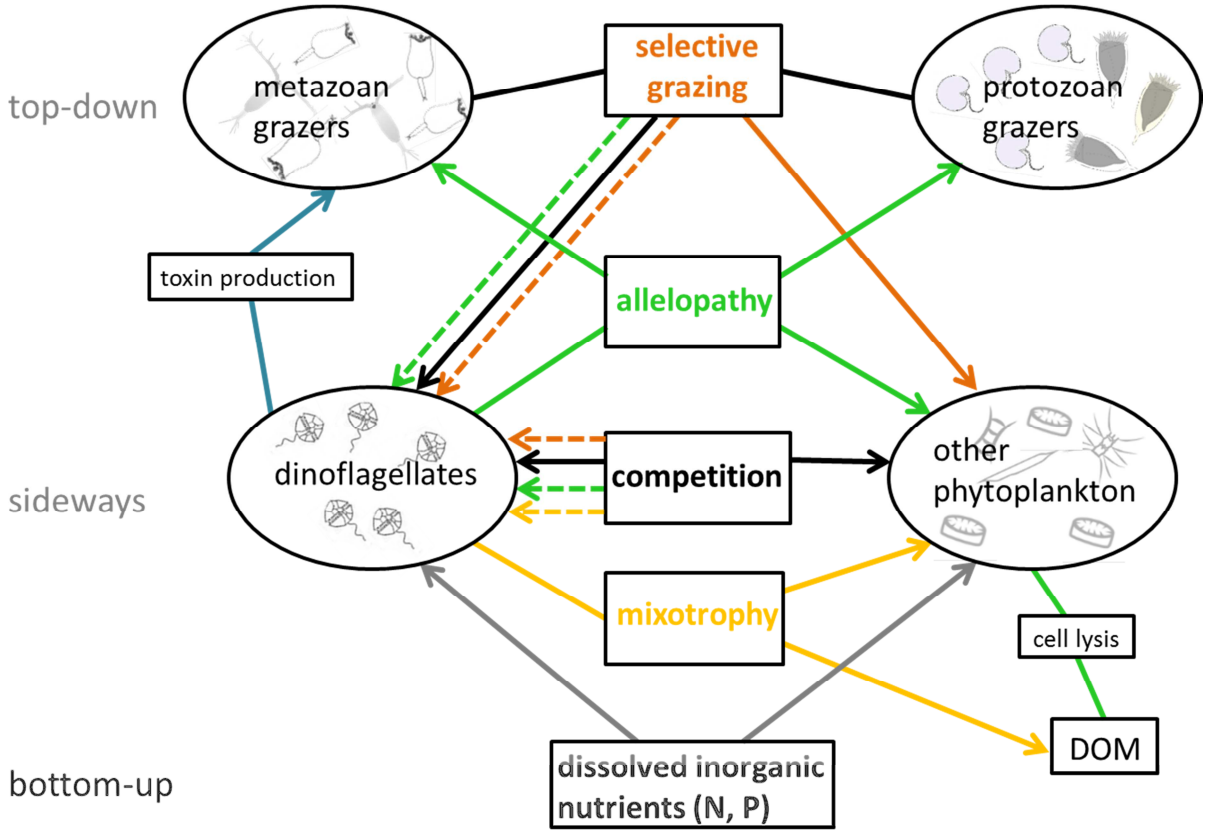

Figure 5-1: Conceptual summary of the network of interactions between HAB dinoflagellates, other phytoplankton and their grazers that were investigated in the present thesis. Black arrows indicate trophic interactions that inhibit dinoflagellate growth, while coloured arrows show the trophic interactions that may lead to a positive feedback facilitating dinoflagellate growth (brown: selective grazing; green: allelopathy; blue: toxin production; yellow: mixotrophy; grey: dissolved inorganic nutrients) Solid arrows indicate direct effects on food web components, and dashed arrows indirect positive feedbacks on the dinoflagellates. Bottom-up control refers to effects of dissolved inorganic and organic (DOM) nutrients; sideways control depicts the interactive effects of allelopathy, phagotrophic feeding/mixotrophy and competition between dinoflagellates and other phytoplankton species; top-down control describes the effects of zooplankton. Symbols of the organisms are own illustrations or redrawn from the University of Maryland Center for Environmental Science Integration and Application Network symbol library.

5.2 Limitations of the study and future perspective

This thesis provides valuable insights into different factors (abiotic and biotic) that influence the bloom dynamics of the HAB dinoflagellates tested here; however, there are limitations which need to be addressed.

Results presented in **chapter I** indicate that the determination of ingestion rates in feeding assays, observed prey uptake using staining methods (i.e. CMFDA staining) under a light microscope and the comparison of phototrophic and mixotrophic growth rates can provide valuable information on the mixotrophic ability of the dinoflagellates. However, this information may not help to answer the question of whether phagotrophic feeding provides competitive or physiological benefits for the dinoflagellates under different nutrient conditions. Previous studies have shown such benefits for various dinoflagellate species, for example *L. polyedrum* showed higher mixotrophic growth compared to purely phototrophic growth (Jeong *et al*., 2005), but these were only short-term effects and could not be supported by the present study which was conducted over longer time periods (12 - 21 d). This highlights the importance of measuring cellular nutrient concentrations and ratios, photosynthetic rates and chlorophyll content in future studies to gain a better understanding of the costs required for switching nutritional modes and thus identifying conditions that benefit biomass production on a longer time scale. A number of other factors should also be considered in future studies. Firstly, the available prey concentration should be taken into account, this is important as the present study indicated that the prey concentrations offered were not sufficient to promote the growth rate and biomass production of the dinoflagellates under lower nutrient concentrations. The prey disappeared too quickly in the mixed culture with the dinoflagellates in the present experiment and thus it would be preferable to provide a continuous prey concentration, e.g. in a continuous 2-stage chemostat-system, when measuring the benefit of phagotrophic feeding in terms of biomass production.

For *A. catenella* in particular, it was difficult to measure the nutritional benefits resulting from lysed prey cells. It has been suggested in previous studies that the lysis of protistan cells due to allelochemicals may increase the dissolved organic material (DOM) pool in the seawater similarly to lysis due to viruses or bacteria, as the cell content is released as highly available DOM for bacteria (Bratbak *et al*., 1998; Brussaard *et al*., 2007; Fuhrman, 1992; Middelboe *et al*., 2003). In order to understand the role of allelopathy as ‘pseudo-mixotrophy’ by enlarging the nutrient pool for allelopathic donor species (Roy, 2009) it is important to investigate the different possible mechanisms involved, i.e. direct ingestion of whole prey cells, uptake of immobilized prey cells or dissolved organic matter to determine the benefits for *A. catenella*.

Experiments that were conducted to investigate the role of grazing (**chapter II** and **III**) revealed the importance of considering the whole plankton community. In particular, results from the mixed culture experiments in **chapter II** demonstrated that the intensity of allelopathic effects is not only dependent on the donor species concentration, but also varies depending on the target density. However, these mixed culture experiments were conducted using only one concentration level for the donor and the target species, which does not present direct evidence for the importance of allelopathic activity for *A. catenella* bloom formation. Furthermore, experiments need to be conducted with a variety of different donor and target cell concentrations to determine which threshold conditions might favour *A. catenella* dominance in a plankton community.

The more complex experiments using a natural plankton community in **chapter III** clearly demonstrated that depending on the composition of the zooplankton community, heterotrophic dinoflagellates can have a significant grazing impact on *L. polyedrum*, even in a complex natural plankton community when alternative prey is available. In turn, other grazers such as ciliates preferred other phytoplankton over *L. polyedrum.* This indicates that data derived from two-species (one consumer, one prey) feeding trials cannot easily be transferred to a more complex natural plankton community with a variety of other prey and grazer organisms available. Therefore, in order to gain a predictive understanding of environmental conditions under which zooplankton grazing may inhibit bloom formation or even contribute to bloom demise, more complex experimental approaches are required taking into account specific zooplankton feeding preferences in a natural plankton community and thus demonstrating the variety of different direct and indirect potential grazing effects. This is in accordance with previous studies (Pitcher *et al*., 1991) that show that HAB prediction at the species-level is challenging. For instance, the increase in dinoflagellate blooms in California is dependent on environmental forcing, but the particular species that becomes dominant is not predictable (Kudela *et al*., 2010). Thus, the community composition as a whole should be monitored, rather than specific organisms or HAB species and that higher predictive capability would evolve from modelling the plankton assemblage (Estrada *et al*., 2003). In order to realise this, co-occurring non-HAB species common to the coastal waters of interest should be included into longer term HAB studies. Thus, artificial communities should be simulated allowing for direct interactions among species to provide a better

integrative assessment of relative success of HAB dinoflagellates. These advanced studies may also provide important information in the context of expected climate change. Climate driven changes (e.g. in stratification, ocean acidification, alteration of nutrient availability due to altered vertical mixing and runoff) may affect HAB species, non-HAB species and their grazers – but in different ways, thus altering community structure and food web dynamics (Wells *et al*., 2015). A number of studies have focused on the topic of HAB dinoflagellates and climate change (e.g. Flores-Moya *et al*., 2012; Fu *et al*., 2010; Kremp *et al*., 2012; Tatters *et al*., 2013; Van De Waal *et al*., 2014; Van de Waal *et al*., 2013). However in most studies, the focus is usually on a few environmental factors, single biological properties (photosynthesis, growth, nutrient uptake), or selected "pet" species. Complex factor and species interactions are rarely covered by these types of experiments.

5.3 How to gain more predictability for unpredictable dinoflagellate bloom dynamics?

The specific information gained on the complex interactions between the HAB dinoflagellates, other competing phytoplankton species and grazers under various nutritional conditions, including mixotrophic and allelopathic abilities of the two dinoflagellates, are crucial to enhance our predictive power of the bloom dynamics of these HAB species, especially in the face of anthropogenically induced environmental changes that may alter their bloom dynamics. In recent years a major research effort has been dedicated to the development of models that can help to predict harmful algal blooms. These attempts range from exploratory theoretical and conceptual models focusing on different aspects of environmental factors such as nutrient concentrations and ratios (e.g. Flynn, 2010), and physiological aspects of the HAB species such as mixotrophy (e.g. Stoecker 1998; Mitra and Flynn, 2010) to applications in natural systems with HAB phenomena (McGillicuddy *et al*., 2005; He *et al*., 2008). Although sufficient data is still lacking to fully test these models, they could be helpful for the design of future experimental work which could be structured to test specific models. In addition, physiological models can also shed light on factors that are directly relevant for the development of effective HAB management strategies.

Further improvements in HAB modelling have been achieved by coupling detailed biological models with hydrographic models. For example McGillicuddy *et al*. (2005) and He *et al*. (2008) have modelled *Alexandrium* sp. bloom dynamics in the Gulf of Maine region by combining a hydrographic model that can realistically simulate water movement over a large region with a second model simulating the germination of *Alexandrium* cysts from seedbeds. The hydrographic model includes factors which can influence ocean currents such as winds, tides, stratification, river runoff, and large-scale forcing from the open ocean. The biological model takes into account the factors that can influence the subsequent growth of the HAB population such as temperature, salinity, light, and nutrients. The values for the timing and rates of cyst germination and cell growth were derived from laboratory experiments on cultures of *A. fundyense*. A good record of reproducing observations has been achieved with these models and they have been used extensively for looking at past events to understand underlying mechanisms (He *et al*., 2008; Y. Li *et al*., 2009), as well as for weekly nowcasts and forecasts (looking forward 3–4 days) and even seasonal or annual forecasts (McGillicuddy *et al*., 2011).

A different recent approach by Jeong *et al*. (2015) categorizes HAB bloom dynamics into four conceptual models, based on HAB species nutrient acquisition strategy (i.e., inorganic nutrient uptake, mixotrophy), behaviour (e.g., vertical migration), and biological interactions with their communities. These four models increase in complexity from exclusively autotrophic organisms (1) to including swimming behaviour (2), mixotrophic organisms (3) and included biological interactions such as predator-prey interactions and allelopathic effects (4). With respect to the costs and time required to acquire the relevant model parameters for HAB species, correctly choosing one of these four conceptual models for the HAB causative species can help to increase the accuracy of HAB prediction. This approach also includes the complex food web interactions shown for the two dinoflagellates in this thesis. The present data could be used in model (3) and (4), which include the adaptive strategies mixotrophy and allelopathy and thus can be suitable for predicting dinoflagellate population dynamics.

Furthermore, a part of the data collected in this thesis will be incorporated into a mathematical model describing the population dynamics of *A. catenella* and a competing non-toxic phytoplankton species in the presence of a zooplankton grazer (*B. plicatilis*). This model will include the negative effects of allelopathic compounds

on phytoplankton and the zooplankton grazer, as well as ingestion rates and growth rates of particular species as determined in chapter II of this thesis. Additionally, it will include the weakening of the allelopathic effect as a result of high biomass of other members of the plankton community and thus will simulate the donor and target density dependency of the allelopathic effects. This model will be a valuable tool for theoretically investigating the effects of *A. catenella* on competitors and grazers depending on allelopathic potency, donor and target concentrations and food web configuration, and will help to further understand the complex effects of allelochemically active HAB dinoflagellates on different levels of the food web.

6. SUMMARY

Harmful algal blooms (HABs), particularly those dominated by dinoflagellates, are widespread in marine ecosystems and can pose dramatic effects on human health and industry, affecting coastal fish- and shellfish farms as well as tourism by prohibiting swimming in affected areas. Understanding the factors that regulate HAB bloom dynamics is necessary in order to predict and possibly prevent these blooms and has therefore become an important major research focus in the past decades. This requires an in-depth knowledge of the environmental factors, species-specific traits and adaptive strategies that determine when and under which conditions certain HAB species are able to dominate the phytoplankton community and form blooms. However, for most HAB species, these factors and specific traits driving trophic interactions are still poorly understood.

In this thesis I focus on trophic interactions of two different potentially harmful dinoflagellates, *Lingulodinium polyedrum* and *Alexandrium catenella*, both originating from the Southern California Bight, USA. I conducted a series of laboratory experiments to investigate their competitive ability under different nutrient conditions and potential grazing control by zooplankton consumers, taking into account species-specific adaptive strategies such as mixotrophy and allelopathy.

Chapter I describes microcosm experiments which were conducted to investigate the impact of different nutrient conditions and prey availability on the mixotrophic and allelopathic capabilities of these two dinoflagellates. The results indicated that besides being beneficial for reducing co-occurring phytoplankton, these strategies also affect the cellular nutrient concentrations of the dinoflagellates. Both dinoflagellate species were able to ingest a variety of different prey organisms, but had a particularly strong negative impact on the pico-phytoplankton *Ostreococcus* sp. irrespective of nutrient limitation. However, there was only little benefit for the dinoflagellates from growing with prey in terms of biomass production. Their cellular nutrient contents indicated initial metabolic ‘costs’ for switching nutritional modes from photosynthesis to phagotrophic feeding, before having a benefit from ingested prey. In additional tests to differentiate between the feeding impact on prey and potential allelopathic effects, *A. catenella* showed strong lytic activity, while *L. polyedrum* did not. This indicates that *L. polyedrum* is able to use phagotrophic

feeding to reduce competitors, while for *A. catenella* allelochemicals are likely play a more important role for escaping competition and becoming dominant in the phytoplankton community.

Allelopathic effects of *Alexandrium catenella* on both phytoplankton competitors and on the metazoan consumer *Brachionus plicatilis* were further investigated in **chapter II**. The results clearly showed that *A. catenella* whole cell culture and cell free filtrate immobilised potential prey cells and caused a density dependent steep decline in competitor and grazer cell numbers. Since the allelopathic effects of *A. catenella* on co-occurring algae and grazers are density dependent, allelopathy is more likely to contribute to bloom persistence when cell densities are high rather than contributing to bloom initiation when cell densities are low. This was supported by mixed culture experiments including *B. plicatilis* and the non-toxic chlorophyte *Tetraselmis*, in which the negative effect of *A. catenella* was 'diluted' by high *Tetraselmis* cell abundances, i.e. *Tetraselmis* was able to counteract the negative allelopathic effect on *B. plicatilis* in mixed culture.

In **chapter III**, 'bottom-up' and 'top-down' effects on the population dynamics of *L. polyedrum* in coastal waters of Southern California, USA, were investigated. I manipulated concentrations of dissolved nutrients by adding either phosphate, nitrogen or both nutrients to a natural plankton community containing an *L. polyedrum* population, and investigated its growth under these different nutrient conditions. The results indicated that *L. polyedrum* was not able to successfully compete with diatom competitors, which dominated the initial plankton community under high nutrient conditions. In a second approach, I investigated the effect of potential competitors and consumers on the growth and competitive success of an *L. polyedrum* population that was spiked into 2 different size fractions of a natural plankton community; i.e., < 20 µm (excluding consumers) and < 110 µm (including consumers). In an additional treatment, I added the heterotrophic dinoflagellate consumer *Noctiluca scintillans* to both size fractions. Depending on the composition of the plankton community some grazers such as *N. scintillans* can have a significant grazing impact on *L. polyedrum*, while others such as ciliates prefer other phytoplankton over *L. polyedrum*, thus potentially facilitating its competitive success.

In conclusion, the results of this thesis substantially enhance our understanding of the complex interactions between potentially harmful dinoflagellates, other competing

phytoplankton species and grazers under various nutritional conditions. The specific information gained on these interactions, including mixotrophic and allelopathic abilities of the two dinoflagellates, are crucial to enhance our predictive power of the bloom dynamics of these HAB species, especially when considering anthropogenically induced environmental changes that may alter their bloom dynamics.

7. ZUSAMMENFASSUNG

Schädliche Algenblüten (Harmful Algal Blooms, HABs), insbesondere solche, die durch Dinoflagellaten verursacht werden, sind ein weit verbreitetes Phänomen in marinen Ökosystemen. Diese Algenblüten können sowohl dramatische Auswirkungen auf die menschliche Gesundheit haben, als auch ökonomische Verluste verursachen, wenn in Küstenregionen Fisch- und Muschelzuchten oder die Tourismusbrache betroffen sind. Um Fortschritte in der Vorhersage und Prävention dieser Blüten zu erzielen, sind fundierte Kenntnisse der Umweltfaktoren, artspezifischen Eigenschaften und Anpassungsstrategien, die bestimmen, wann und unter welchen Bedingungen bestimmte HAB Arten die Phytoplanktongemeinschaft dominieren, von besonderer Bedeutung. Allerdings sind diese Faktoren für die meisten blütebildenden Dinoflagellaten nach wie vor kaum bekannt.

In der vorliegenden Dissertation untersuche ich die trophischen Interaktionen der beiden potenziell schädlichen Dinoflagellaten, *Lingulodinium polyedrum* und *Alexandrium catenella*. Beide verursachen regelmäßig schädliche Algenblüten in der Südkalifornischen Bucht, USA. In Laborexperimenten untersuchte ich die Konkurrenzfähigkeit beider Dinoflagellaten in Abhängigkeit von verschiedenen Nährstoffbedingungen, potentieller Fraßkontrolle durch Zooplankton und den Anpassungsstrategien Mixotrophie und Allelopathie.

Die Mixotrophie (Kombination von Phototrophie und Heterotrophie in einem Organismus) und die Allelopathie (Produktion von Sekundärmetaboliten, die eine meist hemmende Wirkung auf andere Organismen haben) der beiden Dinoflagellaten unter verschiedenen Nährstoffbedingungen stehen im Fokus von **Kapitel I.** Die Ergebnisse zeigen, dass diese beiden Strategien nicht nur vorteilhalft für die Reduzierung von co-existierenden Phytoplanktonkonkurrenten sind, sondern auch einen Einfluss auf die zellulären Nährstoffkonzentrationen der Dinoflagellaten haben. Obwohl beide Dinoflagellaten eine Vielzahl von verschiedenen Beuteorganismen ingestierten, und auch unabhängig von Nährstofflimitation einen negativen Effekt auf die Prasinophyceae *Ostreococcus* sp. hatten, war der Nutzen für die Dinoflagellaten in Bezug auf die Biomasseproduktion eher gering. Stattdessen ließen die zellulären Nährstoffkonzentrationen anfängliche metabolische "Kosten" vermuten, die durch die Umstellung von Photosynthese auf Phagotrophie entstehen. Zusätzlich wurde die

lytische Aktivität der beiden Dinoflagellaten getestet. Während *A. catenella* einen starken lytischen Effekt auf Beuteorganismen hatte, konnte dies für *L. polyedrum* nicht gezeigt werden. Dies deutet darauf hin, dass verschiedene Dinoflagellaten Arten auch unterschiedliche Strategien anwenden um einen Konkurrenzvorteil zu erlangen.

Eine detailliertere Untersuchung der allelopathische Effekte von *Alexandrium catenella* auf Konkurrenten und den Konsumenten *Brachionus plicatilis* (Metazoa) führte ich in **Kapitel II** durch. Die Ergebnisse zeigen deutlich, dass sowohl eine *A. catenella* Zellkultur als auch zellfreies Filtrat potentielle Beuteorganismen immobilisiert und eine Zelldichte-abhängige Reduzierung der Konkurrenten und Konsumenten verursacht. Diese Dichte-abhängige allelopathische Wirkungen von *A. catenella* auf Konkurrenten und auf Konsumenten lässt vermuten, dass die Produktion von allelopathischen Substanzen eher die Blütedauer von *A. catenella* verlängern kann, wenn die Zellkonzentration bereits hoch ist, als dass sie zur Entstehung von Blüten beiträgt.

In **Kapitel III** zeigte ich, dass der kompetitive Erfolg von *L. polyedrum* in einer natürlichen Planktongemeinschaft aus der Südkalifornischen Bucht, stark von der initialen Zusammensetzung der Gemeinschaft abhängt. Ich manipulierte die Konzentration gelöster Nährstoffe durch Zugabe von Phosphat, Stickstoff oder beiden Nährstoffe und konnte zeigen, dass *L. polyedrum* unter hohen Nährstoffbedingungen nicht in der Lage war, erfolgreich mit Diatomeen, die die anfängliche Planktongemeinschaft dominierten, zu konkurrieren. In einem zweiten Versuchsansatz untersuchte ich inwieweit natürliche Konkurrenten und Konsumenten die Konkurrenzfähigkeit von *L. polyedrum* beeinflussen. Dazu ließ ich eine *L. polyedrum* Laborkultur in zwei verschiedenen Größenfraktionen einer natürlichen Planktongemeinschaft (<20 µm, ohne Konsumenten und <110 µm einschließlich Konsumenten) wachsen. In einem weiteren Ansatz fügte ich den heterotrophen Dinoflagellaten *Noctiluca scintillans* zu beiden Größenfraktionen zu. In Abhängigkeit der Zusammensetzung der Planktongemeinschaft konnten Konsumenten wie zum Beispiel *N. scintillans* einen starken Fraßdruck auf *L. polyedrum* ausüben, während andere natürliche Zooplankton Konsumenten (z.B. Tintinniden) kleineres Nano-Phytoplankton bevorzugten und so möglicherweise *L. polyedrum* einen Wachstumsvorteil verschafften.

Die Ergebnisse dieser Arbeit erweitern wesentlich unser Verständnis der komplexen Interaktionen zwischen potenziell schädlichen Dinoflagellaten, co-existierenden Phytoplanktonarten und Zooplanktonkonsumenten unter verschiedenen Nährstoffbedingungen. Besonders im Hinblick auf zukünftige anthropogen verursachte Umweltveränderungen sind die gewonnenen Informationen über die mixotrophen und allelopathischen Eigenschaften der beiden untersuchten Dinoflagellaten unter veränderten Nährstoffbedingungen wichtig, um die Vorhersagbarkeit der Blütedynamiken dieser HAB Dinoflagellaten zu verbessern.

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9. SUPPLEMENTAL MATERIAL

Table 9-1: Summary of dinoflagellate final nutritional states (at the end of exponential growth) from all experimental treatments in experiment 2 B in chapter I ([a] mono- and [b] mixed culture)

	Lingulodinium polyedrum				*Alexandrium catenella*			
initial N:P (µmol L^{-1}) of media	320:20	160:10	80:5	32:2	320:20	160:10	80:5	32:2
final N:P ratio of media (molar)	7.2±1.5 [a] 8.2±1.9 [b]	5.7±1.8 [a] 5.8±1.5 [b]	3.2±0.1 [a] 2.4±0.9 [b]	3.5±0.9 [a] 4.2±0.6 [b]	33.8±0.1 [a] 29.0±2.9 [b]	7.6 ±3.6 [a] 6.5 ±2.0 [b]	4.2 ±2.0 [a] 2.1±1.3 [b]	6.5±5.8 [a] 1.5±1.3 [b]
total C content (pg $cell^{-1}$)	590 384	618 418	454 421	457 439	303.8 182.4	302.4 196.1	310.6 174.7	304.3 207.5
total N content (pg $cell^{-1}$)	95 64	74.5 50	49 32	35.3 25	36.1 16.4	35 19.5	29.1 14.5	23.1 20.4
total P content (pg $cell^{-1}$)	7 3.1	2.2 1.9	2.6 1.4	0.9 1.1	2.3 1.2	1.8 1.4	1.6 0.9	1.6 0.9
final cell C:N ratio (molar)	5.6±0.04[a]; 6 ±0.03[b]	8.3±0.1[a]; 8.4 ±0.7[b]	9.3±0.3[a]; 13± 1.2[b]	13.2±1.4[a]; 17.3±0.07[b]	8.4± 0.2[a]; 9.4± 0.5[b]	8.6± 0.1[a]; 10.1±0.1[b]	10.6±0.1[a]; 12.1±0.5[b]	13.1±0.1[a]; 14.4 ±0.02[b]
Final cell C:P ratio (molar)	108.6±4.2[a]; 162 ±65.3[b]	207.1±11.5[a]; 211 ±2.1[b]	350.7±54[a]; 298± 21.3[b]	349.5±71[a]; 497.7±63.4[b]	136.5±33.6[a]; 121± 20.6[b]	135.3± 4.6[a]; 137±12.4[b]	193±22[a]; 194.4±24[b]	206.3±59[a]; 220±6.3[b]

Table 9-2: Specific growth rate (d^{-1}), maximum cell density (MCD) and change of maximum cell density (%) when growing with prey of both dinoflagellates (in [a)] mono- and [b)] mixed treatment) under four nutrient conditions (experiment 2B, chapter I).

	L. polyedrum			*A. catenella*		
Nutrient treatment	Growth rate (d^{-1})	MCD (cells ml^{-1})	Change in MCD (%)	Growth rate (d^{-1})	MCD (cells ml^{-1})	Change in MCD (%)
N4	0.13±0.01[a]; 0.17 ±0.02[b]	$2.88x10^3$ $3.20\ x10^3$	9.9	0.17± 0.01[a]; 0.16± 0.01[b]	$1.34\ x10^4$ $1.12\ x10^4$	-19.48
N3	0.18±0.03[a]; 0.16 ±0.03[b]	$3.59\ x10^3$ $3.28\ x10^3$	-9.6	0.16± 0.01[a]; 0.17±0.01[b]	$7.39x10^3$ $1.09\ x10^4$	32.03
N2	0.11±0.01[a]; 0.11± 0.02[b]	$3.34\ x10^3$ $3.11\ x10^3$	-7.3	0.12±0.01[a]; 0.15±0.004[b]	$5.77\ x10^3$ $5.96\ x10^3$	3.06
N1	0.08±0.02[a]; 0.08±0.03[b]	$2.42\ x10^3$ $2.49\ x10^3$	2.8	0.08±0.003[a]; 0.08 ±0.01[b]	$2.98\ x10^3$ $2.79\ x10^3$	-6.64

Table 9-3: Summary of prey (*Ostreococcus* sp.) final nutritional status, as measured at the end of exponential growth of experiment 2B ([a] dinoflagellate filtrate [b] dinoflagellate cells)

	Ostreococcus sp. *L. polyedrum* filtrate [a] / cells [b]				*Ostreococcus* sp. *A. catenella* filtrate [a] / cells [b]			
initial N:P (µmol L^{-1}) of media	N4	N3	N2	N1	N4	N3	N2	N1
final N:P ratio of media	26.6 ± 2.03 [a] 43.8 ± 2.9 [b]	109.7 ± 6.6 [a] 48.3 ± 1.4 [b]	n.d. [a] n.d. [b]	20.7 ± 16.6 [a] 16.5 ± 7.2 [b]	26.9± 1.8 [a] 27.6 ±1.6 [b]	64.7 ±3.9 [a] 56.5± 5.2 [b]	26.8± 15.3 [a] 111.4 ±41.8 [b]	91.6±29.0 [a] 16.1± 6.2 [b]
growth rate µ (d^{-1})	0.51 ± 0.03	0.55 ± 0.02	0.14 ± 0.25	-0.33 ± 0.06	0.38 ± 0.02	0.28 ± 0.02	0.10 ± 0.05	-0.13 ± 0.08
final cell C:N ratio (molar)	8.9 ±3.3 [a] 6.4 ±1.1 [b]	9.5 ±1.2 [a] 7.9 ±0.4 [b]	9.5± n.d. [a] 7.1 ± 0.5 [b]	8.2 ±n.d. [a] 6.3 ±0.1 [b]	7.4 ± 0.4 [a] 8.2 ± 1.0 [b]	7.1 ± 0.2 [a] 8.1 ± 0.6 [b]	7.6 ± 0.4 [a] 8.3 ± 2.1 [b]	7.9 ± 2.1 [a] 8.2 ± 0.7 [b]
Final cell C:P ratio (molar)	75.9 ±35.1 [a] 59.2± 8.8 [b]	88.3± 11.0 [a] 93.5± 7.1 [b]	81.4 ±13.1 [a] 85.9± 3.0 [b]	83.1 ±12.1 [a] 94.4± 11.7 [b]	93.3 ± 2.4 [a] 61.9 ± 5.5 [b]	122.6 ± 22.8 [a] 88.5± 3.4 [b]	127.9 ± 7.5 [a] 91.9 ± 16.9 [b]	105.1 ± 49.9 [a] 113.2 ± 47.1 [b]
Final cell N:P ratio (molar)	10.2 ±0.02 [a] 9.3± 0.7 [b]	9.3 ±1.2 [a] 11.8± 0.4 [b]	9.5 ± n.d. [a] 12.4± 0.4 [b]	11.4 ±n.d. [a] 14.9± 2.0 [b]	12.7 ± 0.5 [a] 7.6 ± 0.4 [b]	17.7 ± 4.9 [a] 9.3 ± 2.3 [b]	16.7± 0.3 [a] 11.1 ± 0.7 [b]	12.6 ± 5.7 [a] 13.5 ± 4.5 [b]

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11. ERKLÄRUNG

Erklärung gemäß § 12 (2) der Promotionsordnung der Fakultät V Mathematik und Naturwissenschaften der Universität Oldenburg vom 04. September 2014.

Hiermit versichere ich, dass ich die von mir vorgelegte Dissertation mit dem Titel „“ selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Abbildungen und Tabellen – die anderen Werken im Wortlaut oder dem Sinn nach entnommen habe, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht worden ist sowie, dass ich solche Veröffentlichungen vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Leitlinien guter wissenschaftlicher Praxis der Carl von Ossietzky Universität Oldenburg wurden befolgt. Es wurden keine kommerziellen Vermittlungs- oder Beratungsdienste im Zusammenhang mit dem Promotionsvorhaben in Anspruch genommen. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Dr. Stefanie Moorthi und Prof. Dr. Helmut Hillebrand betreut worden.

Oldenburg, 27.04.2016 _____

(Michaela Busch)