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Intraspecific trait diversity in the marine dinoflagellate *Alexandrium ostenfeldii* and resulting effects on the community composition

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Abstract

The present thesis gives first indications of intraspecific facilitation in the toxin producing marine dinoflagellate *Alexandrium ostenfeldii*. The results show that modelling with trait averages in ecological modelling cannot cover the complexity of intra and interspecific interactions between several algal species and strains within a planktonic population.

The thesis research additionally confirms several extraordinary features of a population of *A. ostenfeldii* of the Ouwerkerkse Kreek in the Netherlands. The production of three types of phycotoxins – gymnodimines, spirolides and PSP-toxins - is shown for several strains of the Dutch population. The only other population of *Alexandrium* species where all three toxins could be detected was a population of *A. peruvianum* in Wickfort cove, USA. Furthermore it was shown in this thesis that the strains of the Dutch population own a very high allelopathic potency. In multispecies cultures they lyzed competitor species within hours of exposure. The exposure to the copepod *Acartia tonsa* also had no negative effect on the toxic dinoflagellate.

Despite the fact that strains of the Dutch population showed a small genetic variability very dense monospecific blooms were reported for the sampling location. The Dutch population of *A. ostenfeldii* is the population with the highest reported cell concentrations in nature to date. This research provides explanations how the traits of the population could have aided its development from a small seed population in a short time (<10 years) to a recurrent bloom species. Strains from the Dutch population were growing to higher cell concentrations in the mixed strain cultures and were able to lyze all co-cultured competitors.

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List of Abbreviations

12-Me GYM A	12-methyl gymnodimine
13-dm-27-hyd-SPX C	13-desmethyl-27-hydroxy spirolide C
ANOVA	analysis of variance
AON	*Alexandrium ostenfeldii* Netherlands (prefix for strains)
asqPCR	allelspecific quantitative PCR
bp	basepairs
C	cell concentration
CIT	cyclic imine toxins
d	day(s)
dcGTX	decarbamoyl gonyautoxin
dcNeo	decarbamoyl neosaxitoxin
dcSTX	decarbamoyl saxitoxin
div	cell divisions
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
EC_{50}	effect concentration where 50 % of the
FDA	fluorescein diacetate
FIN	Finnish *Alexandrium ostenfeldii* (prefix for strains)
gDNA	genomic DNA
GTX	gonyautoxin
GYM	gymnodimine A
h	hours
HPLC-FD	high-performance liquid chromatography with fluorescence detection
K	carrying capacity
LC-MS/MS	liquid chromatography–tandem mass spectrometry
M	magnification
m/z	mass-to-charge ratio
min	minutes
mL	milliliter
mM	millimolar
$MnCl_2$	manganese(II) chloride
N	equivalent per liter
Na_2EDTA	di-sodium ethylenediaminetetraacetate
Na_2MoO_4	sodium molybdate
NaH_2PO_4	monosodium phosphate
$NaNO_3$	sodium nitrate
NEO	neosaxitoxin
ng	nanogramm
OLS	ordinary least squares
P	Population size
PCR	polymerase chain reaction
pg	picogramm
psi	pound-force per square inch
PSP	paralytic shellfish poisoning
r	growth rate
RFU	relative fluorescence units

RNA	ribonucleic acid
RT	room temperature
s	seconds
SPX 1	13-desmethyl spirolide C
STX	saxitoxin
ukn	unknown (used for unknown compounds found during toxin analysis)
V	voltage
g	gravitational force or relative centrifugal force

1 Introduction

The marine primary production is dominated by microbial phytoplankton. Even though this group primarily comprises organisms that are hard to see with the naked eye, they have a major role in ecosystem functioning. Primary producers build the base of the marine food web and are therefore important drivers of the biological element cycling (Smayda 1997, Charlson et al. 1987). Furthermore, they can have massive effects on upper and lower trophic levels. Major members of the phytoplankton are eukaryotic microalgae, which encompass about 5000 described species (Sournia et al. 1991; Gerssen et al. 2010).

Some of these algal species appear locally or seasonally in large cell numbers and sometimes discolor the water by the formation of algal blooms. About 300 of all estimated phytoplankton species were reported to form blooms (Gerssen et al. 2010, Smayda 1997). In most cases this algal blooms are harmless and vanish within a short time, but sometimes they can have massive effects on the local ecosystem (Platt et al. 2003, Anderson et al. 2012).

1.1 Harmful algal blooms

Harmful algal blooms (HABs) although they are sometimes referred to as red tides can appear in various colors like red, green, brown or golden. The blooming species determines with its predominant pigments in, which color the water appears (Schofield et al. 1999, Sellner et al. 2003). HABs are natural phenomena, which can result of various reasons and might have been already reported in biblical times ("…and all the water in the Nile River turned to blood." Bible, Exodus 7:20). Sometimes they occur due to beneficial weather conditions for the blooming species or due to coastal upwelling. Especially in coastal regions they can be fueled by anthropogenic nutrient supply due to run off of agricultural fertilizers. (Smayda 2000).

HABs can have noxiously effects because of the massive amount of biomass in the upper water layers, which can lead to anoxic "dead" zones in deeper layers, if they start sink and to decay (Diaz and Rosenberg 2008, Dodds 2006). Sometimes the negative effects are caused by phycotoxins. Phycotoxins are secondary metabolites, which are produced by some algal species. They can be toxic for marine herbivores and other members of the marine food web. They can also have detrimental effects in upper trophic levels due to accumulation in the consumers, for example in fish, whales

and birds (Landsberg 2002, Turner et al. 2000, Durbin et al. 2002, Tester et al. 2000). HABs can attract attention with massive fish kills or because terrestrial mammals are effected by the consumption of toxic material (Castle et al. 2013). Humans can be negatively affected in many ways: first of all health effects like intoxication and respiratory irritation. The symptoms are various, depending on the toxin, which was taken up. Mostly the toxins are divided in groups by their effects in mammals and their origin. Popular symptoms after the consumption of shellfish are paralytic shellfish poisoning due to saxitoxins, amnesic shellfish poisoning due to domoic acid, neurotoxic shellfish poisoning e.g. due to brevetoxin, diarrheic shellfish poisoning e.g. due to okadaic acid and ciguatera fish poisoning e.g. due to ciguatoxin (Anderson et al. 2012, Kirkpatrick et al. 2004, Fleming et al. 2011). However fisheries and shellfish farmers experience reduced profits due fish kills and poisonous shell fish (Richlen et al. 2010, Kaartvedt et al. 1991). Livestock poisonings and kills, for example of cows have been reported too. Furthermore, the hospitality industry can have severe profit losses if the beaches are closed and swimming is prohibited. According to the NOAA (National Oceanic and Atmospheric Administration) harmful algal blooms have an estimated impact of at least 82 million dollars to the US economy every year. Harmful algal blooms are occurring worldwide and they are not bound to seasonal fluctuations or certain climate conditions (Anderson et al. 2012).

Reports about HABs have been increasing within the last decades, but also the severity and duration of such bloom events seems to increase. There are many possible reasons, which could cause the spreading of HAB species (Kim et al. 2012). For example the transport with ballast water of ships is an often discussed topic (Hallegreaf 1998, Kim et al. 2012). The climate change could also be a reason, as with warming oceans stratification increases, which favors several HAB species. Additionally the habitat of warm water organisms might shift to northern climes (Hallegreaf 2010, Kudela et al. 2015). The eutrophication of coastal waters with fertilizers by agricultural runoff, sewage disposal and due to aquaculture is most frequently linked to increased HAB events (Kudela et al. 2015). For example the expanded duration and geographical extent of harmful algal blooms at the coast of China have been linked to the increased use of fertilizers over the past two decades (Wang et al. 2009). Therefore the research on HABs and the causing species is of major interest worldwide. Despite this fact it is still not possible to efficiently predict the extent and persistence of such an algal bloom.

1.2 Modelling algal blooms

In the last two years great expenses in local long-term monitoring of weather conditions, water quality and movements, satellite data, frequent sampling and scientific modelling have already improved HAB forecasting that works at least for some species and waterbodies like the *Microcystis* blooms in Lake Erie (Michalak et al. 2013, Steffen et al. 2014). In other regions this forecasting is much more difficult as already the weather is alternating faster or there is scarce information about the water quality available. In some countries the waste and sewage disposal into the water of lakes and estuaries is still common (Wielgus et al. 2004; Huang et al. 2006). Furthermore, there are HAB species with only little information about life cycle and nutrition (Roelke et al. 2001). Other species are just not named consistently as there is no common guidelines on how to identify a species and assign it with phylogenetic information. Solely morphological information often leads to uncertain and ambitious taxonomies especially for eukaryotic algae, which show a high intraspecific diversity (John et al. 2003, Burkholder et al. 2006, Lundholm et al. 2006, Lilly et al. 2007). Scientists have reported about changing morphological characters of microalgae if grown in cultures over several generations (Leaw et al. 2005).

To improve the prediction of HABs it is necessary to share data and have standardized protocols on how to sample and identify a harmful algal species. Furthermore, it is essential to understand how environmental factors and species interactions influence the dynamics of algal populations (Smayda and Reynolds 2001). Big efforts have been already made to identify anthropogenic and physical factors, as the GEOHAB scientific summary shows (Kudela et al. 2015), whereas factors like intra- and interspecific facilitation are mainly disregarded by ecological models (Duggins 1981, John et al. 2014). Ecologists mostly focus on competition, predation, physical disturbance and physiological stress. This trend in ecology has been supported for several years, because influential ecologists like Darwin, Lotka, Volterra and MacArthur mainly ignored positive interactions between organisms (Bruno et al. 2003). Only in the last three decades ecological facilitation regained increasing attention. Research on Mycorrhiza and deep sea chemosynthesis has emphasized the significance of positive interactions in community structures (Johnson et al. 1997, Dubilier et al. 2008). Another prominent example for aquatic systems is the symbiosis of corals. In this symbiosis reef building polyps and dinoflagellates living in their tissue relate on each other (Trench, 1997).

However dinoflagellates are also the group of algae, which is most often associated with harmful algal blooms (Glibert et al. 2005). There are already some studies showing that inter- and intraspecific facilitation matters for harmful algal species like the cyanobacterium *Mycrocystis* or the haptophyte *Primnesium parvum* (van Gremberghe et al. 2009 ,Driscoll et al. 2013). Interspecific facilitation describes the interaction of two species, which benefits at least one of them without harming the other. Intraspecific facilitation is similar, with two different individuals or clones of one species interacting with each other. For toxic *Alexandrium* intraspecific facilitation was shown once by John et al. (2014). Therefore it is essential to include inter- and intraspecific facilitation into HAB modelling.

1.3 The dinoflagellate genus *Alexandrium*

About 75 % of all harmful algal species are dinoflagellates, which can produce a wide range of different toxins and other allelochemicals (Glibert et al. 2005, Murray et al. 2015). An especially well known and widespread toxic genus is *Alexandrium* (Halim), with populations detected in nearly every ocean basin (Murray et al. 2015). The type species of this genus, *Alexandrium minutum*, was described after a heavy bloom with water discolorations to red in the Harbor of Alexandria (Egypt, Halim 1960). Nowadays there are approximately 35 named species and several of them are highly studied. Species of this genus are reported to produce dense monospecific blooms with cell numbers higher than 10^3 cells * mL^{-1} (Anderson et al. 2012). At least six of them are known to produce paralytic shellfish poisoning (PSP) toxins and some others were described as slightly toxic (Murray et al. 2015). Especially the production of PSP-toxins seems to occur in an unexplained patchiness in several different groups of the genus. High varieties in both toxin content and profiles have been even found within the same populations e.g. for *A. fundyense* (John et al. 2014). Strains of this species were isolated from a natural population in the North Sea and characterized for growth rates and toxin production afterwards (former classified as *A. tamarense*, Alpermann et al. 2010). A high genetic diversity was also revealed in populations of this species and populations of *A. catenella* (Masseret et al. 2009). Higher cell toxin quota have been linked to certain stages of growth phases, including mid-exponential growth and stationary phase. (Murray et al. 2015). Several studies showed that chemicals exuded by copepods can induce the PSP-toxin production in *A. minutum* and *A. fundyense*. Cells of these species showed a several fold increase in cell toxin quota, if exposed

tominor concentrations of the copepod supernatant (e.g. Selander et al. 2006, Wohlrab et al. 2015). Most recently this bioactive compounds were identified and named copepodamides (Selander et al. 2015)

In addition to the PSP toxins gymnodimines and spirolides have been characterized for *A. ostenfeldii* and *A. peruvianum* (Cembella et al. 2000, Katikou et al. 2010). Lately *A. peruvianum* is considered as heterotypic synonym of *A. ostenfeldii* (Kremp et al. 2014). The ecological function of these spirolides and gymnodimines remains still unclear. Strains of *A. ostenfeldii* tend to produce either PSP-toxins or spirolides but rarely both groups (Suikkanen et al. 2013)

Several species of the *Alexandrium* genus have been described as allelopathically active (Tillman and John 2002, Murray et al. 2015). Allelochemicals are biochemicals with a certain function on other organisms in the environment of the producer. They can have detrimental or beneficial effects on the target organisms e.g. as attractor for pollinators or repellents against pests (Legrand et al. 2003). The lysis or immobilization of co-occurring plankton species was described for many *Alexandrium* species (Tillmann et al. 2007). These might be mechanisms of these species to compensate their rather low competitive abilities for nutrients (John et al. 2014). The lytic effect of harmful algal species is usually tested with a *Rhodomonas*-bioassay, as it was first explained in detail by Tillmann et al. in 2008. In the same article it was shown that *A. tamarense*, *A. minutum* and *A. ostenfeldii* were the most lytic among six tested species. The unusually high allelochemical potential of *A. ostenfeldii* was also shown in several other publications (detailed below).

1.4 *Alexandrium ostenfeldii* and its allelochemical potency

Alexandrium ostenfeldii mainly appears in temperate, coastal and brackish seawater, but the species was already isolated from several different locations all over the world (Figure 1). Most frequent reports of this species are from Western Europe, but it has also been detected in tropical waters like the bay of Kuching in Malaysia (as *Alexandrium peruvianum*, Lim et al. 2005) or the Callao Bay in Peru (as *Gonyaulax peruvianum* Balech and Mendiola, 1977). It usually co-occurs in low cell numbers with other phytoplankton (Cembella et al. 2000, John et al. 2003, Tillmann et al. 2014). Although they have relatively low growth rates and competitive abilities for nutrients, they can dominate the phytoplankton community by the formation of huge blooms (Kremp et al. 2009; Borkman et al. 2012; Hakanen et al. 2012). Hitherto the formation

of nearly monospecific harmful algal blooms was only observed in the Baltic Sea at several locations (since 2003) and in the Ouwerkerkse Kreek in the Netherlands

Figure 1 Locations where cells of *Alexandrium ostenfeldii* / *Alexandrium peruvianum* were isolated or described

blue dots – vegetative cells or cycts found in water samples, red dots - areas with bloom formation, with at least 1000 cells * mL^{-1} (as *A. ostenfeldii*: Aasen et al. 2005, Amzil et al. 2007, Burson et al. 2014, Brown et al. 2010, Cembella et al. 2001, Ciminiello et al. 2006, Gribble et al. 2005, Gu et al. 2013, Kaga et al. 2006, Kim et al. 2012, Konovalova 1991, Kremp et al. 2014, Nagai et al. 2010, MacKenzie et al. 1996, MacKinnon et al. 2006, Salgado et al. 2015, Suikkanen et al. 2013, Tillmann et al. 2014, Touzet et al. 2008; as *A. peruvianum*: Almandoz et al. 2014, Borkman et al. 2012, Bravo et al. 2006, Franco et al. 2006, Katikou et al. 2010, Kremp et al. 2014, Lim et al. 2005, Tomas et al. 2012)

(Kremp et al. 2009, Hakanen et al. 2012, Burson et al. 2012, Brandenburg et al. 2017). If this species appears in higher cell numbers, their bioluminescence in the night can be seen with the naked eye (Tortorec et.al, 2013).

The named phycotoxins are only found in minor concentrations in cell-free filtrate (John et al. 2001). They are stored intracellularly and can only act as toxins if the cells are lyzed or ingested by grazers (Ma et al. 2009). However, several studies have shown an extracellular effect of *A. ostenfeldii* on other species. In 1992 a tintinnid ciliate (*Favella ehrenbergi*) was observed to swim backwards to avoid the cells of *Alexandrium ostenfeldii* (Hansen et al. 1992). Another study in 2011 has shown a negative effect of cell suspensions and cell-free filtrate on two calanoid copepods (*Acartia bifilosa* and *Eurytemora affinis*). One of the copepod species was killed while the other one showed incapacitation (Sopanen et al. 2011). These cases could be an indicator that the chemicals are excreted to deterrence potential grazers, but they could also be produced by the algae to reduce competition by other algal species.

Tillmann et al. reported in 2007 the lysis of several marine protists if they were exposed to *A. ostenfeldii*. Similar results were observed in by Kremp et al (2014) and van de Waal et al. (2015). The cell-free filtrate of *A. ostenfeldii* induced shedding, encystment or lysis of different co-occurring dinoflagellate species (Kremp et al. 2014). All these findings could contribute to the fact that *A. ostenfeldii* is mixotrophic. Mixotrophy is the ability of an organism to utilize different sources of energy or carbon. Mixotrophic algae can use sun energy to build up their own organic compounds or they take these compounds up from their environment. This species can take up dissolved organic matter and was also reported to ingest bacteria or other smaller protists, like ciliates and dinoflagellates (Stoecker et al. 2006, Jacobson and Anderson, 1996). Due to the recent development of dense monospecific blooms, their various toxins and high allelochemical potency they are very interesting research subjects. Furthermore, it was not shown yet if they possess intraspecific facilitation or if their toxicity or allelochemical potency changes due to competition or grazing.

1.5 Recurrent *Alexandrium ostenfeldii* blooms at the coast of the Netherlands

In 2012 a harmful algal bloom was detected in the Dutch Ouwerkerkse Kreek in the Oosterschelde for the first time and since then annually (Burson et al. 2014; Brandenburg at el. 2017). The creek is a small pool of brackish seawater, which flows along a campground. The creek is used as drainage channel for the village Ouwerkerk

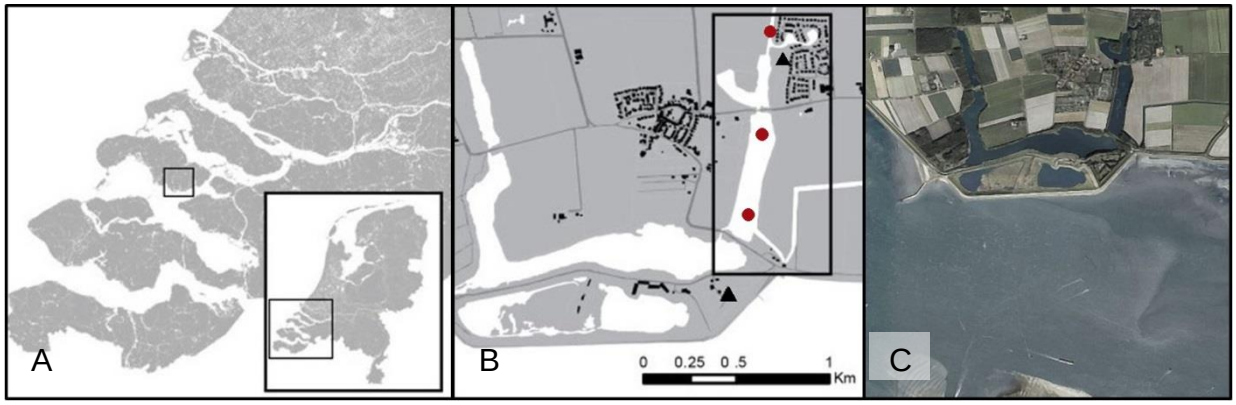

Figure 2: Location of the *Alexandrium ostenfeldii* bloom in the Ouwerkerkse Kreek, Netherlands.
A: Location of the village Ouwerkerk in the Netherlands; B: box: part of the creek with the bloom, red dots - Sampling locations in the creek, black tringles: camping grounds; C: satellite picture of the location, with the Oosterschelde river at the bottom (modified, Brandenburg et al. 2017)

and the local agriculture. A pumping station regulates the water level of the creek to prevent flooding by discharging into the Oosterschelde estuary (Burson et al. 2014). In

Figure 2 the area of the bloom formation is shown. The Oosterschelde estuary it is an area, which is used for the cultivation of oysters and the increased toxicity of the algal population leads to profit loss of the local fisheries and endangers residents and tourists (Burson et al. 2014; Van de Waal et al. 2015).

The severity of the bloom in August 2012 was only detected after a dog in the village Ouwerkerk died by poisoning. *Alexandrium ostenfeldii* was identified to be the blooming species after the analysis of water samples. The water was containing more than 1000 cells * mL^{-1} and both PSP-toxins and spirolides were detected. In the first year the bloom was eradicated by the addition of hydrogen peroxide (50 mg * L^{-1}) to prevent further damage to the environment (Burson et al. 2014).

In the following years up to 4500 *A. ostenfeldii* cells * mL^{-1} (in 2014) were detected in the creek. Temperatures were usually above 15 °C during the blooms and salinities ranging from 3.7 (summer 2015) to 20 were measured (Burson et al. 2014, Van de Waal et al. 2015, Brandenburg et al. 2017). Increased water temperatures and associated stratification of the water column as well as nutrient input may have caused the enormous cell growth, but this hypothesis is not proven yet (Brandenburg at el. 2017).

1.6 Aim of this thesis

The aim of this thesis is to test and demonstrate with the aid of basic ecological models if intraspecific facilitation matters in bloom formation and resilience. Therefore an artificial population, consisting of three algal species was set up and monitored under defined conditions in the laboratory. Emphasis was clearly laid upon the influence of trait diversity on the outcome of the incubation. The project should help to answer the following questions: What impact has the genotypic diversity on the success of *A. ostenfeldii* under competition and grazing pressure? Which traits of *A. ostenfeldii* dominate the population with and without competition and grazing pressure?

Competition was realized by co-culturing of two natural competitors of *A. ostenfeldii* – *Levanderina fissa* and *Chrysotila dentata* with the toxic dinoflagellate. *Levanderina fissa* was first isolated near the island of Lövö (Finland) and described as unarmoured dinoflagellate (Levander 1894). *Levanderina fissa* was reported to take up prey, like *Alexandrium pseudogonyaulax* through its sulcus (Moestrup et al. 2014, Blossom et al.

2012). Sometimes its prey was even bigger than the cells of this species, leading to distortions of the outer shape (Levander 1894). Like *A. ostenfeldii L. fissa* is a mixotrophic alga, but it was not shown to be toxic (Moestrup et al. 2014). However the nutritional mode of this species was never verified in the lab.

The haptophyte *Chrysotila dentata* was first isolated as *Pleurochrysis carterea var. dentata* from a saline lake in New Mexico (Johansen and Doucette 1988). Due to the diverse morphological life stages of the haptophyte, it was renamed several times (Andersen et al. 2015). The complex taxonomic history of the haptophytes complicates all efforts to describe them (Saez et al. 2003). According to Johansen (1988) *C. dentata* has non-motile, benthic life stages but in culture it most often appears as motile swarmer without scales or coccolith-bearing flagellated cells. It has been reported to have a purely autotroph nutrition (Johansen et al. 1988). The isolated strain used for this thesis work was identified via 18S cDNA sequencing in previous experiments. *Chrysotila dentata* clustered within a phylogenetic analyses best with sequences obtained by Andersen et al. (2014) from material sampled at the Isle of Wight (Ireland, GenBank accession no.: KJ020919.1).

Grazing pressure by *Acartia tonsa* was applied to the community in a second experiment. The calanoid copepod co-occurs with *A. ostenfeldii* in the Baltic Sea and the North Sea (Holste and Peck 2006; Fransz et al. 1991).

To answer the second question, the genetic diversity of the strain was determined first via microsatellite analysis. Dinoflagellates like *A. ostenfeldii* are haploid as vegetative cells, so one allele per strain can be expected in the monoclonal cultures. After the interaction experiment, the remaining strains and their ratio to each other was determined via allele-specific quantitative PCR (John et al. 2014, Löbbecke 2015). Additionally the experiments give the opportunity to analyze the toxin content and lytic effects of the different *A. ostenfeldii* strains, before and after competition and grazing. It shows, if there is any upregulation with one or the other interaction experiment. The toxin content of the different strains was determined by high-performance liquid chromatography (HPLC) measurements. As described by Krock et al. (2008) the hydrophilic PSP-toxins were measured via HLPC with fluorescence detection (HPLC-FD).The cyclic imine toxins, gymnodimines and spirolides, are in contrast lipophilic and will be determined via HPLC coupled with a triple-quadrupole mass spectrometry (LC-MS/MS) as decribed in Krock et al. 2008 and Kremp et al. 2014.

In a broader context the thesis work helps to answer the question: which traits are beneficial at the initiation of a bloom and how does the trait composition of the bloom changes over time?

2 Material and Methods

2.1 Chemicals

Chemicals and enzymes used in this study were purchased from AppliChem GmbH (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, Massachusetts, USA), Applied Biosystems, Life Technologies Corporation (Carlsbad, CA, USA), Qiagen (Hilden, Germany), Macherey Nagel GmbH & Co. KG (Düren, Germany), Merck KGaA (Darmstadt, Germany), Promega (Mannheim, Germany) and Sigma-Aldrich (Taufkirchen, Germany).

Deionized and purified Milli-Q water (Merck Millipore GmbH, Darmstadt, Germany) was used in all buffers and solutions, if not indicated otherwise. Seawater with a salinity of approximately 36 was sampled from the North Sea close to Helgoland, filtered (5 µm and 0.2 µm pore size) and stored at RT in the dark.

2.2 Medium and stock solutions

2.2.1 Seawater - K medium for the cultivation of protists and copepods

A seawater - K medium was used for the cultivation of all *Alexandrium ostenfeldii* strains, for the Cryptophyte *Rhodomonas baltica* as well as for the competitor strains of *Chrysotila dentata* SF1 and *Levanderina fissa* comp.05. The original K medium was described by Keller (Keller and Guillard 1985, Keller et al. 1987). The medium used in this thesis was modified from the original protocol. The components (listed in Table 1) were mixed with 1400 mL seawater and filled up with water to a final volume of 5 L to obtain a salinity of 10. The medium was sterile filtered through a 0.1 µm VacuCap® 90 Filter Unit (Pall Life Science, Dreieich, Germany).

Table 1: Composition of seawater - K medium

Ingredient	used amount for 1L
NH_4Cl	1.30 mg
$NaNO_3$	37.50 mg
NaH_2PO_4	2.50 mg
H_2SeO_3	0.60 mg
Trizma-Base	60.60 mg
Trace-metal solution	0.50 mL
Vita min solution	0.25 mL

2.2.2 Stock solutions

Table 2: Composition of trace-metal solution

Ingredient	Concentrations in mg * L^{-1}
$Na_2EDTA \times 2\ H_2O$	41 600
$FeCl_3 \times 6\ H_20$	3 150
$ZnSO_4 \times 7\ H_2O$	22
$MnCl_2 \times 4\ H_2O$	180
$Na_2MoO_4 \times 2\ H_2O$	6.6
$CoCl_2 \times 6\ H_2O$	10
$CuSO_4 \times 5\ H_2O$	4.9

Table 3: Composition of vita min solution

Ingredient	Concentrations in mg * L^{-1}
Vitamin B12	1
Thiamin HCl	50
Biotin	1

Table 4: Composition of FDA stock solution

Ingredient	Concentrations in mM
Fluorescein diacetate in DMSO	10

2.3 Algal culture techniques

2.3.1 Isolation of algal strains

The majority of algal strains used in this thesis were kindly provided by Sylke Wohlrab. They were isolated from an *Alexandrium ostenfeldii* bloom in the Ouwerkerkse Kreek (51°62' N, 3°99' E) in 2015. The sampling location is a small pool of brackish water in the Southwest of the Netherlands, which is connected to the Oosterschelde estuary. The cells were isolated as described in detail by Van de Waal et al. in 2015. In short - single cells were picked with a micro-pipette and cleaned five times with seawater - K medium. They were grown in 100 µL seawater - K medium in microplate wells. Successfully isolated clones were transferred to 40 mL culture flasks (Sarstedt, Nürnbrecht, Germany) and acclimated over several generations to the culture conditions described under 2.3.2. With one transfer the cultures were treated by a multi-antibiotic cocktail (Table 6) in seawater - K medium for 15 days in order to get axenic cultures (John et al., 2014).

Table 5: Composition of multi-antibiotic cocktail

Ingredient	Concentration in mg* mL^{-1}
Ampicillin	50
Gentamycin	3.3
Streptomycin	25
Chloramphenicol	1
Ciprofloxacin	10

Table 6 : provided algal strains

Strain	Source/ reference
Alexandrium ostenfeldii AON_01	S. Wohlrab, 2015, unpublished
Alexandrium ostenfeldii AON_02	
Alexandrium ostenfeldii AON_03	
Alexandrium ostenfeldii AON_04	
Alexandrium ostenfeldii AON_05	
Alexandrium ostenfeldii AON_06	
Alexandrium ostenfeldii AON_07	
Alexandrium ostenfeldii AON_08	
Alexandrium ostenfeldii AON_09	
Alexandrium ostenfeldii AON_10	
Alexandrium ostenfeldii AON_11	
Alexandrium ostenfeldii AON_12	
Alexandrium ostenfeldii AON_13	
Alexandrium ostenfeldii AON_14	
Alexandrium ostenfeldii AON_15	
Alexandrium ostenfeldii AON_26	
Alexandrium ostenfeldii AON_27	
Alexandrium ostenfeldii AON_28	
Alexandrium ostenfeldii AON_29	
Alexandrium ostenfeldii AON_30	
Alexandrium ostenfeldii AON_31	
Alexandrium ostenfeldii AON_32	
Alexandrium ostenfeldii AON_33	
Alexandrium ostenfeldii FIN_01	(Kremp at al. 2010)
Alexandrium ostenfeldii FIN_02	
Alexandrium ostenfeldii FIN_03	
Alexandrium ostenfeldii FIN_04	
Alexandrium ostenfeldii FIN_05	
Levanderina fissa comp.05	S. Wohlrab, 2015, unpublished
Chrysotila dentata SF1	
Rhodomonas baltica	AWI, ecological chemistry section, unpublished

Table 7: purchased copepod strain

Strain	Source/ reference
Acartia tonsa	(Holste and Peck, 2006)

2.3.2 Cultivation of algal strains

Cultures were incubated at 17 °C under a light intensity of about 100 µmol photons * m^{-2} * s^{-1}, provided by cool white fluorescent lamps at a light-dark cycle of 16 to 8 hours. The cells were harvested or a new culture flask was inoculated, when the previous culture reached nearly the end of the exponential growth phase – usually after about 25 days. For the inoculation 3 mL of the previous culture were transferred to 35 mL fresh medium in a new 40 mL cell culture flask (Sarstedt). As preparation for the mixed culture experiments (chapter 2.7) 500 mL cultures with 300 to 1300 cells * mL^{-1} were set up for the *A. ostenfeldii* strains and their two competitors in glass bottles (Schott AG, Mainz, Germany). These cultures were grown for 15 days until they were used for the experimental part.

Rhodomonas baltica cells were incubated under slight aeration for mixing. Cultures of 100 – 500 mL were set up in glass culture flasks (Schott AG, Mainz, Germany), as they were additionally used as nourishment for the copepods (paragraph 2.3.2).

2.3.3 Cultivation of *Acartia tonsa*

The copepod *Acartia tonsa* was incubated in sterile filtered diluted seawater (salinity of 10) under gentle aeration and a reduced light intensity to prevent algal growth. They were fed every day with a fresh inoculum of 50 mL of a dense *R. baltica* culture (approximately 10^6 cells * mL^{-1}). The medium was changed every 10 days to avoid accumulation of toxic waste products. Therefore the adult copepods were collected with a 200 µm pore size filter and transferred to the new medium (sterile filtered diluted seawater, salinity = 10). The eggs and the juveniles were as well transferred to a new glass bottle (Schott AG) of diluted seawater. A 50 µm pore size filter was used to recover them from the old culture. Only the upper layers of the culture were filtered and the sediment was discarded, to avoid the transfer of dead animals and feces.

For the grazing experiments (chapter 2.7.2) adult *Acartia tonsa* specimen were set with a pipette to sterile filtered diluted seawater (salinity = 10). They were starved for 24 hours to avoid the transfer of *R. baltica* cells. Four individuals were transferred to a well of a twelve-well plate (Sarstedt) in 5 mL of seawater - K medium. The complete content of a well was transferred to a flask of the three-species-culture (described in 2.7.2).

2.3.4 Harvesting of biomass and cell free supernatant

The biomass of cultures was harvested to extract genomic DNA and intracellular toxins. Therefore a dense culture was resuspended carefully and transferred to a 50 mL polypropylene centrifuge tube (Sarstedt). The sample was centrifuged for 15 min at 3220 x *g* at RT (Eppendorf centrifuge 5810R, Hamburg, Germany). The cell pellets of each culture were resuspended in 1 mL of seawater - K medium and split to two 2 mL screw cap micro tubes (Sarstedt). The screw cap micro tubes were centrifuged for 10minutes at 16100 x *g* (Eppendorf centrifuge 5415R, Hamburg, Germany). The supernatant was removed with a pipette. The biomass pellet and 10 mL of the supernatant were stored at -20 °C. The remaining supernatant was discarded.

2.3.5 Determination of cell concentrations

Cell concentrations were determined by the use of sedimentation chambers (Tillmann et al., 2008). Therefore the culture was mixed gently and 0.1 mL to 1 mL of the culture was transferred to a sedimentation chamber. The sample was fixed with 3 µL Lugol's solution (final concentration about 0.2 %). The cells sunk to the bottom of the chamber within 10 to 20minutes. They were counted with an inversed microscope (Zeiss Axiovert 40C, Carl Zeiss, Oberkochen, Germany) at magnifications of 200X, 100X or 50X. Subareas with at least 400 cells were counted per chamber. The cell concentration (*C*) in cells * mL^{-1} was calculated with the following equation:

$$C = \left(\frac{n}{100\ G}\right) \cdot 36{,}193\ M$$

In this equation *n* equals the number of cells counted, *G* equals the number of grids counted and *M* is the used magnification. Growth curves were obtained from three biological replicates of each strain with a start cell concentration of 400 to 800 cells * mL^{-1}. The cell concentration of each replicate was determined every three to four days for the *Alexandrium ostenfeldii* strains and every day for the competitor species *C. dentata* SF1 and *L. fissa* comp.05. Growth rates were calculated as explained in chapter 2.6.1.

Counting samples of 3 mL were taken every Monday and Thursday of the mixed cultures (in the competition experiment under 2.7.1). They were fixed with 10 µL Lugol's solution and stored in amber 4 mL – vials (Wheaton, Millville, New Jersey,

USA) at RT. Before the transfer of 1 mL to sedimentation chambers, the samples were mixed thoroughly.

2.3.6 Microscopical imaging of the algal strains

The algal strains were imaged via microscopy to compare their outer shape and roughly their size (not statistically evaluated). Therefore photos of the Lugol-fixed samples were taken with the Axiovert 200M (Carl Zeiss) at a magnification of 400X and the Progres GRYPHAX® ARKTUR microscope camera (JENOPTIK Advanced Systems GmbH, Jena, Germany).

2.4 Molecular biological methods

2.4.1 DNA extraction

The genomic DNA was extracted with the NucleoSpin® Soil kit by Macherey-Nagel according to the manufacturer's guideline with slight changes. The biomass pellets were resuspended in warm (65 °C) 700 µL SL1 buffer, transferred to a provided bead tubes and mixed with 150 µL Enhancer SX. The cells were lyzed by shaking the tubes with the MagNa Lyzer (Roche, Mannhein) at 5500 m * s^{-1} – first for 45 seconds and again for 30 seconds. The tubes were centrifuged for 2minutes at 11000 x *g* to reduce the formed foam (5415R, Eppendorf AG). After the addition of 150 µL Buffer SL-3 the tubes were vortexed for 5 minutes. An incubation step for 5minutes on ice followed to precipitate the proteins. Cell debris and beads were centrifuged down for 1minute at 11000 x *g* (5415R, Eppendorf AG). To remove remaining cell debris 700 µL of clear supernatant were filtered through a spin column for 1minute at 11000 x *g* (5415R, Eppendorf AG). To bind and purify the DNA, the supernatant was loaded to a silica membrane in the NucleoSpin® Soil Column. Therefore the filtrate was mixed with 250 µL SB loading buffer and stepwise filtered through the column at 11000 x *g* for 1 minute (5415R, Eppendorf AG). Three washing steps followed: first with 500 µL SB loading buffer, secondly with 550 µL of washing buffer SW1 and then two times with 700 µL washing buffer SW2. Each of these steps was done at 11000 x *g* for 30 seconds (5415R, Eppendorf AG). Remaining washing buffer was removed by another centrifugation step at 11000 x *g* for 2minutes (5415R, Eppendorf AG). The purified DNA was eluted with 35 µL elution buffer SE. Therefore the column was placed in a new 1.5 mL reaction vial (Eppendorf AG, Hamburg, Germany), incubated 1 min with the buffer and an open lid and then centrifuged for oneminute at 11000 x *g*

(5415R, Eppendorf AG). DNA extracts were stored at -20 °C or diluted to 5 ng * µL $^{-1}$ with PCR water and stored at 4 °C.

2.4.2 Measurement of DNA quantity and quality

The concentration of double-stranded DNA was measured photometrically with a Nano Drop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) at 260 nm. The purity of the genomic DNA was calculated from the ratio of the absorbance at 260 to 280 nm for protein and RNA contaminations. This ratio should be between 1.8 and 2.0. Contaminations with organic compounds or chaotropic salts appear in the ratio of 260 to 230 nm. For pure DNA this ratio is above 1.8 (manufacturer's guideline: Nano Drop Spectrophotometers, Thermo Scientific, 2010).

2.4.3 Amplification of the microsatellite loci by PCR

In previous studies with *Alexandrium ostenfeldii* seven microsatellite loci for strains from a bloom in Finland (Aosten-) were identified (Nagai et al., 2014). Another six microsatellite loci (OKNL_-) were found in Dutch strains, which were isolated from a bloom in the Oosterschelde at Ouwerkerk (Netherlands) in 2014 (personal communication: U. John). All *A. ostenfeldii* strains of this thesis were tested for the presence and size of the 13 microsatellite loci by amplifying the microsatellite loci via PCR. The used primers for each microsatellite locus are stated in Table 9. The PCR ingredients, which were used from the Type-it Microsatellite PCR Kit (Qiagen) and the thermocycler program are specified in the Tables 10 and 11 respectively.

Table 8: specific primers of the microsatellite loci in *A. ostenfeldii*

Microsatellite locus name	Primer name	Nucleotide sequence (5' → 3')	Annealing temperature in °C
Aosten10	Aosten10_For	*Fluo-GCGAGTGTGTTCTGGTAG	56.0
	Aosten10_Rev	GTCGGTCTGCAGTCTATAGCAAC	62.4
Aosten101	Aosten101_For	*Fluo-ATCACTCCACTTCAGATGGGTC	60.3
	Aosten101_Rev	GCTTCTGACTTGCATTGAATTGGTCAAA	62.2
Aosten126	Aosten126_For	*Fluo-AGCGGCTGTAACTGTATATGGC	60.3
	Aosten126_Rev	GCTTCTCAGCATGTCTCGAACTATCTGC	66.6
Aosten144	Aosten144_For	*Fluo-TAGCCTCCGCGTGTGTAACG	61.4
	Aosten144_Rev	CCAACACCGTCGTCTTAAACG	59.8
Aosten171	Aosten171_For	*Fluo-GTGATGCTGGTTATGGTGCTAA	58.4
	Aosten171_Rev	GCTTCTGGTACCACTCCCGTAGTAGACG	69.5
Aosten296	Aosten296_For	°Fluo-ACACCACTCGTGTACGATATGC	60.3
	Aosten296_Rev	GCTTCTGTCGCTCTTCTCTTGCCATT	64.8
Aosten359	Aosten359_For	°Fluo-ACACCACTCGTGTACGATATGC	58.0
	Aosten359_Rev	GCTTCTGAAGGCCAGAAACAAAGGATCT	65.1
OKNL_1	OKNL_1For	*Fluo-GCACCGCGCATACAAATTCC	59.4
	OKNL_1Rev	GAAGGCGGTGTTCTCGACAT	59.4
OKNL_2	OKNL_2For	*Fluo-CCCGATTTCCAGGGAGCATT	59.4
	OKNL_2Rev	AGAAAGGCTCGGGTGAAACC	59.4
OKNL_3	OKNL_3For	*Fluo-TAGCCTCGAGCCCAGTGCCG	65.5
	OKNL_3Rev	ATCGTGGACTGCGCGTCACT	61.4
OKNL_4	OKNL_4For	*Fluo-CGTGCAGCCTATGATCGCTA	59.4
	OKNL_4Rev	AATGCGCTGCCGAAGATAGT	57.3
OKNL_5	OKNL_5For	*Fluo-CAAGCTGGCCCGAAAACATC	59.4
	OKNL_5Rev	GATCATGGCGCTCTCGGTAG	61.4
OKNL_6	OKNL_6For	*Fluo-GACCGGCTTCACGCTCTATC	59.4
	OKNL_6Rev	TTAGCGCGGTCGGTCATTAG	61.4

For = forward primer, Rev = reverse primer, *Fluo = FAM label at the 5' end, °Fluo = HEX label at the 5' end

Table 9: PCR reaction mix for the microsatellite fragment analysis

ingredient	used amount for 1 reaction
DNA template	2 µL
Forward primer (10 ng * µL)	0.25 µL
Reverse primer (10 ng * µL)	0.25 µL
Q-solution	1.25 µL
TypeIt Master Mix (2x)	6.25 µL
PCR water	2.5 µL

Table 10: Cycler program for the PCR

Step	Temperature (°C)	Time inmin	
Initial denaturation	95	5.0	
Denaturation	95	0.5	x 35 cycles
Annealing	55	1.5	
Elongation	72	0.5	
Finnish	60	30.0	
Storage	8	∞	

2.4.4 Touch-down PCR to avoid stutter peaks

A stutter peak was always observed for the microsatellite locus Aosten144, which could lead to ambiguous results. To avoid this stutter peaks a touch-down PCR was performed. The PCR reaction mix (Table 9) was prepared as before. The thermocycler program is specified in Table 12.

Table 11: Cycler program for the Touch-down PCR

Step	Temperature (°C)	Time inmin	
Initial denaturation	94	5.00	
Denaturation	94	0.40	x 20 cycles
Annealing	61	0.33	
Elongation	70	0.50	
Denaturation	94	0.33	x 25 cycles
Annealing	54	0.20	
Elongation	70	0.50	
Finnish	70	15.00	
Storage	8	∞	

2.4.5 Detection of microsatellite length by capillary gel electrophoresis

After the PCRs the length of the microsatellites could be analyzed by capillary electrophoresis. Therefore the PCR products were diluted 1:5 for the Aosten microsatellite loci and 1:100 for the OKNL microsatellite loci with PCR-water. The diluted PCR products (1 µL) were mixed with 15 µL of the fragment analysis mix (Table 13) in a thin-walled 96 well-plate (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). The capillary Electrophoresis was done with the ABI 3130*xl* Genetic Analyzer (16 capillaries, 50 cm; Applied Biosystems). The forward primers were labelled with a fluorescent pigment, to obtain the length of each of the identified microsatellite loci and their presence in the strains. During the capillary electrophoresis the fluorescent signal was detected. The length was compared with the GeneScan™ 500 (-250) ROX™ length standard. The results for the single strains,

mixed cultures and standard series were analyzed with the GeneMapper® Software (Applied Biosystems). The signal intensity, which is given as peak area correlates positively with the amount of template for the PCR (Meyer et al. 2006, John et al. 2014). The standards for a comparison were prepared as explained in 2.4.6.

Table 12: Composition of the fragment analysis mix

Ingredient	used amount in µL
Hi-Di™ Formamide	15
GeneScan™ 500 (-250) ROX™	0.3

2.4.6 Allele-specific quantitative PCR

An allele-specific quantitative PCR was performed, to calculate the cell numbers of each *A. ostenfeldii* strain within the mixed cultures (Meyer et al. 2006, John et al. 2014). After the analysis of all microsatellite loci in all of the single strains, six strains were chosen for the mixed culture experiment (chapter 2.7) because of their unique microsatellite pattern. They could be distinguished with aminimum of three microsatellite loci. With the microsatellite Aosten10 it was possible to distinguish between the Finnish and Dutch strains. Alleles with a length of 362 bp were observed for the selected Dutch strains whereas the Finnish strains had a 390 bp allele.

For the discrimination of the Dutch strains from each other a combination of two microsatellite loci was analyzed. The microsatellite locus Aosten126 appeared as 326 bp allele in the strain AON_15, but was not detected in the two other strains. The microsatellite locus Aosten101 was observed in a length of 402 bp in the strains AON_13 and AON_15. In contrast the strain AON_27 has the same microsatellite as 397 bp allele. For the three selected Finnish strains a similar situation appeared. The microsatellite locus Aosten101 has a length of 402 bp in the strains FIN_01 and FIN_04, but just 382 bp in the strain FIN_02 (see results chapter 3.1).

The gDNA templates for the respective PCRs were either obtained from the standard series (chapter 2.4.7) or from the extraction of DNA from the mixed culture experiments (chapter 2.7). The microsatellite loci were amplified and analyzed as explained under 2.4.3 and 2.4.5. The primer pairs of the respective microsatellite loci (see above) were used. For the combination of primers for microsatellite loci Aosten126 and Aosten101 the amount of PCR H_2O water was reduced by 0.5 µL.

2.4.7 Standard series of DNA templates for the allele-specific quantitative PCR

As the amplification of the different templates and therefore the signal intensities can differ due to PCR bias, this calibration standards should help to calculate the actual ratios of the gDNA templates to each other. The peak area of the signals for the different alleles in the electropherogram should be increasing with increasing template for the according strains. To calculate the ratios of Finnish strains to the Dutch strains the standards were mixed in five ratios from 100 % AON: 0% FIN to 0 % AON: 100 % FIN with each other. The standards were prepared as detailed in Table 14.

Table 13: Standard mix for the discrimination of Finnish and Dutch strains

gDNA of Dutch strains in µL			gDNA of Finnish strains in µL		
AON_13	**AON_15**	**AON_27**	**FIN_01**	**FIN_02**	**FIN_04**
10	10	10	0	0	0
7.5	7.5	7.5	2.5	2.5	2.5
5	5	5	5	5	5
2.5	2.5	2.5	7.5	7.5	7.5
0	0	0	10	10	10

Table 14: Standard mixes for the discrimination of three different Dutch and three different Finnish strains

gDNA of Dutch strains in µL			gDNA of Finnish strains in µL		
AON_13	**AON_15**	**AON_27**	**FIN_01**	**FIN_02**	**FIN_04**
10	0	0	10	0	0
0	10	0	0	10	0
0	0	10	0	0	10
7.5	2.5	0	7.5	2.5	0
0	7.5	2.5	0	7.5	2.5
2.5	0	7.5	2.5	0	7.5
7.5	0	2.5	7.5	0	2.5
0	2.5	7.5	0	2.5	7.2
2.5	7.5	0	2.5	7.5	0
5	2.5	2.5	5	2.5	2.5
2.5	5	2.5	2.5	5	2.5
2.5	2.5	5	2.5	2.5	5
5	5	0	5	5	0
0	5	5	0	5	5
5	0	5	5	0	5
3.3	3.3	3.3	3.3	3.3	3.3

The standard series for the combined microsatellite loci analysis were prepared as stated in Table 15 in sixteen ratios. The analysis of each standard was performed in technical triplicates. Working solutions with 5 ng * μL^{-1} gDNA were used for the preparation of all standards. Subsequently all standards were used as template for an allele-specific quantitative PCR (see previous chapter).

2.5 Methods to quantify allelochemicals and phycotoxins

Several publications describe the allelopathic potential of *Alexandrium ostenfeldii*. Some strains produce the hydrophilic PSP-toxins others do not. Additionally this species has been found to produce the lipophilic gymnodimines and spirolides. Some strains of *A. ostenfeldii* even produce lytic compounds, which can directly kill competitors (Legrand et al. 2003, Cembella, 2003).

2.5.1 Extraction and analysis of PSP-toxins

PSP toxins (PSTs) were isolated from the biomass of each culture. The cell pellets were resuspended in 500 µL of acetic acid (0.03 M) and 0.9 g of FastPrep® lysis matrix D in 2 mL screw cap micro tubes (Sarstedt). The cells were desintegrated with the FastPrep FP120 (Bio101, Thermo Savant, Illkirch, France) at 6.5 m * s^{-1} for 45 seconds. Afterward the cell debris were centrifuged down for 10 min at 16100 x *g* (5415R, Eppendorf AG). The clear supernatant containing the toxins was filtered through a spin filter (pore-size 0.45 mm, Millipore Ultrafree, Merck Millipore) at 11000 x *g* for oneminute (5415R, Eppendorf AG). The clear supernatant was transferred to HPLC vials (Agilent Technologies, Ratingen, Germany), sealed with crimped caps and stored at -20 °C until the analysis by HPLC-FD.

The used HPLC system consists of the following components: degasser (G1379A), a quaternary pump (G1311A), an autosampler (G1229A), an autosampler thermostat (G1330B), a column thermostat (G1316A) and a fluorescence detector (G1321A; all Agilent Technologies, Ratingen, Germany). It was coupled with a PCX 2500 post-column derivatization system (Pickering Laboratories, Mountain View, USA). The chromatographic conditions used for each sample are stated in Table 16. The components of eluents A-P and B-P are stated in Table 17 and 18 respectively.

Table 15: Gradient elution condition for the quantification of the PSTs

Time in min	Eluent A-P	Eluent B-P
0	100 %	0 %
15	100 %	0 %
16	0 %	100 %
35	0 %	100 %
36	100 %	0 %
45	100 %	0 %

Table 16: Composition of eluent A-P

Ingredient	Concentration in mM
1-octanesulphonic acid	6
1-heptanesulphonic acid	6
Ammonium phosphate	40
Phosphoric acid to pH 7.0	
Oxolane	92,57

Table 17: Composition of eluent B-P

Ingredient	Concentration in mM
1-octanesulphonic acid	13
Phosphoric acid	50
Ammonium hydroxid to pH 6.9	
Acetonitrile	2850
Oxolane	185,13

The autosampler was set to 4 °C and a flow rate of 1 mL * min $^{-1}$. The analytes of an injection volume of 20 µL were separated on a 250 mm x 4.6 mm inner diameter, 5 µm, Lunca C18 reversed phase column (Phenomenex, Aschaffenburg, Germany) equipped with a Phenomenex SecuriGuard pre-column. After the elution the separated analytes were continuously oxidized with 10 mM of periodic acid in 550 mM ammonium hydroxide at a flow rate of 0.4 mL * min^{-1} in a reaction coil set at 50 °C. Subsequent, the eluate was acidified with nitric acid (0.75 N) at a flow rate of 0.4 mL * min. The toxins were detected by a dual monochromator fluorescence detector with an emission wavelength of 395 nm at an excitation of 333 nm. The PST standards (for saxitoxin (STX), neosaxitoxin (NEO), decarbamoyl analogues (dcSTX, dcNEO), gonyautoxins 1-4 (GTX1/4, GTX2/3), decarbamoyl gonyautoxins 2 & 3 (dcGTX2/3) and the n-sulfocarbamoyl PST B1 and C1/C2) came from the Certified Reference Materials Program, National Research Council in Halifax, Canada.

2.5.2 Extraction and analysis of lipophilic toxins

Gymnodimines and spirolides are lipophilic toxins. The extraction works similar as the extraction of PSP toxins (2.5.1), but pure Methanol was used as extraction buffer to resuspend the cell pellets. The following steps were performed as stated for PSP toxins in chapter 2.5.1. The HPLC vials were stored at -20 °C till the toxins were analyzed by HPLC-MS.

The cyclic imine toxins and spirolides were measured on an ABI-Scienx 4000 Q Trap triple-quatrupole mass spectrometer (Applied Biosystems) with a Turbo V ion source coupled to the HPLC system. The 1100 LC high performance liquid chromatograph (Agilent, Ratingen, Germany) was equipped with a solvent reservoir, in-line degasser (Pickering Laboratories, G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B) and a temperature-controlled column oven (G1316A). The used analytical C8 reverse phase column (50 mm × 2 mm) was packed with 3 µm Hypersil BDS 120 Å (Phenomenex, Aschaffenburg, Germany). The elution conditions used for each sample are stated in Table 19. Initial equilibration (10 minutes) was followed by a linear gradient from Eluent A-S to B-S. The final elution was performed at isocratic conditions for 10minutes before the return to initial conditions within 1minute. The procedure was performed at a flow rate of 0.4 mL * min^{-1}. The components of eluents A-S and B-S are stated in Table 20 and 21 respectively.

Table 18: Gradient elution condition for the quantification of the lipophilic toxins

Time inmin	Eluent A-S	Eluent B-S
9	95%	5 %
10	0 %	100 %
10	0 %	100 %
1	95 %	5 %

Table 19: Composition of eluent A-S

Ingredient	Concentration in mM
Formic acid	50
Ammonium formate	2

Table 20: Composition of eluent B-S

Ingredient	Concentration
Formic acid	50 mM
Ammonium formate	2 mM
Methanol	95 %

The following mass spectrometric parameters were used to detect the cyclic imine toxins (CIX) and the spirolides (SPX) (Table 22). Different multiple reaction monitoring (MRM) transitions were measured in positive ion-mode with dwell times of 50 ms (for CIX) and 40 ms (for SPX) per transition.

Table 21: MS parameters for the detection of lipophilic toxins

Parameter	Value for CIX	Value for SPX
Curtain gas	10 psi	20 psi
CAD gas	medium	medium
Ion-spray voltage	5500 V	5500 V
Temperature	0 °C	650 °C
Nebulizer gas	10 psi	40 psi
Auxiliary gas	0 psi	70 psi
Interface heater	On	On
Declustering potencial	50 V	121 V
Entrance potencial	10 V	10 V
Exit potencial	15 V	22 V
Collision energy	55 V	57 V

To quantify the toxin concentrations standards were measured with the same methods. For the spirolides standards with 1 pg * µL, 5 pg * µL, 10 pg * µL, 100 pg * µL and 1000 pg * µL 13-desmethyl spirolide C (SPX-1) were available. As standard for the cyclic imine toxins gymnodimine A in the concentrations 10 pg * µL, 50 pg * µL, 100 pg * µL, 500 pg * µL and 1000 pg * µL was used. The standards were purchased as certified reference material from the National Research Council, Halifax, Canada. Due to the lack of standards for derivatives of these two toxins, all concentrations of their derivatives were expressed as peak area equivalents respectively. Data acquisition and processing was performed with the Analyst Software (version 1.5, Applied Biosystems).

2.5.3 Detection and quantification of lytic compounds with a *Rhodomonas* bioassay

To quantify the lytic activity of the *A. ostenfeldii* strains, a *Rhodomonas* bioassay was used. The bioassay was of the present thesis was adapted from Ma et al. (2009) and Tillmann et al. (2009). It is a well-established method to quantify the lytic compounds of a variety of Alexandrium species. The cryptophyte *Rhodomonas baltica* is an especially sensitive microalga, which was used as target organism in the assay. If it is exposed to the substances excreted by some *A. ostenfeldii* strains the cells start to lyze. The amount of lyzed cells was detected with fluorescein diacetate (FDA). FDA is prone to cleavage by esterases and gets transformed into the fluorescent molecule fluorescein. The absorption maximum of this molecule is at 495 nm with an emission wavelength of 520 nm.

Esterases are versatile enzymes, which are found in almost every organism. They get released to the medium if the cells are lyzed. So the more cells are lyzed, the more esterases are released into the medium and the stronger the fluorescent signal of the fluorescein will be.

Prior to the bioassay the supernatants containing the lytic substances need to be diluted. A dilution series over seven dilution (1:1) steps was set up in small glass vials. To quantify the lyzed cells calibration standards were set up. Therefore a 50 mL culture of *Rhodomonas baltica* was first diluted to 200 000 cells * mL-1 with seawater - K-medium. Then the culture was split to two polypropylene centrifuge tubes (Sarstedt). In one tube all cells were lyzed with the Bandelin Sonopuls HD2070 ultrasonic homogenizer (BANDELIN electronic GmbH & Co. KG, Berlin, Germany). The tube was placed on ice and the culture was sonicated at a 50 % cycle at 55 to 60 % power for 5min. This sonication step was repeated twice with a pause of 1minute between each step for cooling. The cooling should avoid any protein denaturation.

For calibration, the lyzed and intact cells were mixed in 2 mL reaction vials (Eppendorf AG) to obtain standards with different percentages from 0 % to 100 % lyzed cells. For this, five steps while testing the supernatants and ten steps while testing the linearity of the fluorescence signal were prepared. The bioassay itself was performed in a black 96-well plate (Greiner BioOne, Kremsmünster, Austria) to prevent fluorescence interference from neighboring cavities. In the wells 75 µL of lytic substance or its dilutions were mixed with 25 µL of diluted *R. baltica* culture. To the standards 75 µL

seawater K - medium were added instead of the lytic substance. As blank 25 µL seawater K - medium was mixed with 75 µL of the lytic substances. To start the reaction of the esterases 50 µL of freshly prepared FDA solution was added to each well. The FDA solution was prepared by diluting the FDA stock solution in seawater K-medium to a concentration of 9 µM. The plate was covered and incubated in the dark for at least 20 hours at room temperature (RT). The fluorescence was measured with the Berthold plate reader. The used program of the device is given in the Table 23.

Table 22: measurement program for the Berthold plate reader

Step	time
Shake	5 s
Pause	1 s
Measure at 454 nm	till end

With the obtained calibration curve it was possible to calculate the percentage of lyzed cells per treatment. Then the EC_{50} values were calculated with a dose-response curve, by plotting the percentage of intact R. baltica against the logarithmic-scaled *A. ostenfeldii* concentration. The EC_{50} value is the concentration of *A. ostenfeldii* cells where half of the target cells are lyzed (Tillmann et al. 2007).

2.6 Bioinformatical methods and modelling

2.6.1 Determination of growth rates and carrying capacities of single strains

The growth rate of the six chosen strains was determined by recording the cell concentrations over a time frame of 25 days. Therefore three culture replicates were set up with a start cell concentration of 500 cells * mL^{-1}. The cell concentrations were determined after every third or fourth day. Maximum growth rates and carrying capacity were estimated by fitting an exponential function through all replicate cell counts in the respective time periods according to

$$\frac{dP}{dt} = rP \cdot \left(1 - \frac{P}{K}\right).$$

Where *r* refers to the maximal growth rate, *P* refers to the population size - in this case the cell density, *t* to the time and *K* to the carrying capacity. The parameters *r* and *K* were estimated by the 'ordinary least squares regression (OLS)' method. The calculation was performed in the R software with the R package 'growthrates' (Petzoldt, 2016).

2.6.2 Determination of interaction coefficient α of the competitors

In a stable coexistence at least two different organisms live in the same habitat using the same resources but without extinguishing each other. Sometimes this coexistence is mirrored in oscillating cell numbers. The Lotka-Volterra interaction model was used to estimate the growth of *Levanderina fissa* comp.05 and *Chrysotila dentata* SF1 in co-culture. The Lotka-Volterra interaction model is explained with the following equations:

$\frac{dP_1}{dt} = r_1 P_1 \cdot \left(1 - \frac{(P_1 + \alpha_{12} P_2)}{K_1}\right)$ and $\frac{dP_2}{dt} = r_2 P_2 \cdot \left(1 - \frac{(P_2 + \alpha_{21} P_1)}{K_2}\right)$.

Here α represents the interaction coefficient of both species to each other. It is the effect of species 2 on the population of species 1 as α12 and α21 respectively the effect species 1 has on the population of species 2. This model is an extension of the logistic growth model and includes parameters to describe the effect of the co-occurring species on their respective population growth. To estimate the interaction parameters *L. fissa* comp.05 and *C. dentata* SF1 were mixed and incubated in two-species cultures with each other. The start cell numbers were set up in different ratios as given in Table 24. In each culture a different situation was expected to evolve – either species 1 dominates over species 2 (culture A), species 2 dominates over species 1 (culture B) or the species live in a stable coexistence (culture C). As ample nutrient an light are provided by the culture conditions, the start numbers of cells were estimated with the Lotka-Volterra interaction model with the assumption that the interaction parameters (α) are set by the ratios of carrying capacities (K1/K2; K2/K1). So that species with higher carrying capacities have less impact on the co-occurring species than lower carrying capacity species (Taylor et al. 2005). The assumed development of the abundance of each species in co-cultures was then estimated by numerically solving the two differential equations with different start parameters (start concentrations of the cells at the beginning of the experiment). Maximum growth rate and carrying capacities were obtained from single growth experiments (described in 2.6.1). Solving of the differential equations was done with the R package ‘deSolve’ (Soetaert et al., 2010). Then cultures were set up in triplicates and monitored over nine days. The subsequent cell numbers were determined as described before (chapter 2.3.5) every second or third day.

Table 23: Start cell concentrations for the three different two-species cultures

culture	species 1 (*L. fissa* comp.05)	species 2 (*C. dentata* SF1)
A	3280	70
B	770	490
C	1620	300

From the recorded data, actual interaction coefficients were estimated by parameter fitting using the ordinary differential equations from the Lotka-Volterra interaction model. Solutions that significantly minimized predicted vs experimental residual were considered as new parameters for the further experiments. The parameter fitting was done using the Levenberg-Marquardt routine with the R package 'minpack.lm' (Elzhov et al. 2015).

2.6.3 Development of a three-species model

With the estimated interaction parameters it was possible to develop a simple three-species model by the introduction of a third equation and a third interaction coefficient in the Lotka-Volterra interaction model for *A. ostenfeldii*:

$$\frac{dP_3}{dt} = r_3 P_3 \cdot \left(1 - \frac{(P_3 + \alpha_{32} P_2 + \alpha_{31} P_1)}{K_3}\right).$$

The interaction term ($\alpha_{x3} P_3$) for species 3 was also included in the first equations for species 1 and 2 with

$$\frac{dP_1}{dt} = r_1 P_1 \cdot \left(1 - \frac{(P_1 + \alpha_{12} P_2 + \alpha_{13} P_3)}{K_1}\right) \quad \text{and} \quad \frac{dP_2}{dt} = r_2 P_2 \cdot \left(1 - \frac{(P_2 + \alpha_{21} P_1 + \alpha_{13} P_3)}{K_2}\right).$$

This model was used to plan the mixed culture experiment, under the assumption that all missing interaction parameters (α_{13}, α_{23}, α_{32} and α_{31}) can be determined with the respective carrying capacities (e.g. with $\alpha_{13} = K_3 * K_1^{-1}$). The resulting model was used to estimate at, which start cell concentrations a stable coexistence between the three species over a time period of 20 days could be expected. The average of the growth rates of the six *A. ostenfeldii* strains was set as maximal growth rate (r_3) for this species. The same was applied for the carrying capacity K_3. Solving of the differential equations was done with the R package 'deSolve' (Soetaert et al., 2010). The cultures were set up as explained in chapter 2.7.1.

2.7 Mixed culture experiments

In the second part of this thesis mixed cultures with three algal species were set up and monitored. As it is common to use the average traits of a population to model the outcome of a certain scenario the results should reveal the reliability of this practice. Therefore the average effect of the six *A. ostenfeldii* strains on the community was determined and compared with the effect of the strain mix on the community. The following two experiments were set up for this purpose.

2.7.1 Competition experiment

The competition experiment should help to explain the effect of the different *Alexandrium ostenfeldii* strains on two of their competitors. It should clarify if the average effect of single strains on the community differs significantly from the effect several strains combined have on the community. Thus the three species were mixed in nine different culture set-ups (A-I), in five replicates each as stated below. The tenth set-up (J) with five replicates was used as control without any of the *A. ostenfeldii* strains. The aimed start cell numbers were estimated from a three-species model as explained in 2.6.3.

Table 24: start cell concentrations for the competition experiment

	aimed concentrations in cells * mL^{-1} of							
Culture ID	***Chrysotila dentata***	***Levanderina fissa***	***Alexandrium ostenfeldii***					
	SF1	**comp.05**	**AON_13**	**AON_15**	**AON_27**	**FIN_01**	**FIN_02**	**FIN_04**
A	650	250	510	0	0	0	0	0
B	650	250	0	510	0	0	0	0
C	650	250	0	0	510	0	0	0
D	650	250	0	0	0	510	0	0
E	650	250	0	0	0	0	510	0
F	650	250	0	0	0	0	0	510
G	650	250	170	170	170	0	0	0
H	650	250	0	0	0	170	170	170
I	650	250	85	85	85	85	85	85
J	650	250	0	0	0	0	0	0

The subsequent cell concentrations of each species in the mixes were determined every Monday and Thursday within the incubation time of 20 days (as explained in

chapter 2.3.5). With the obtained cell counts maximal growth rates were estimated with the non-parametric “smoothing splines”-function of the R package ‘growthrates’ (Petzoldt, 2016). Furthermore, the real overall population growth of the three species and interaction parameters of the *A. ostenfeldii* strains were estimated for comparison. Interaction coefficients were estimated as described in 2.6.2. The overall population growth was calculated as population increase (ΔP) over the experimental time frame with the equation: $\Delta P = \frac{P_{end-}P_{start}}{P_{start}}$.

Additionally to the cell counting samples toxin and DNA samples were taken of the cultures at the beginning and the end of the experiment. No toxin samples were taken of the cultures H, I and J at the end of the experiment. Instead the cultures were used to set up the following copepod grazing experiment (sea chapter 2.7.1). The DNA was used to determine the ratios of the *A. ostenfeldii* stains to each other as it is explained in paragraph 2.6.1. The toxins were analyzed to see if the cell toxin quota were influenced due to the competition or if it stayed constant.

2.7.2 Copepod grazing experiment

At the end of the competition experiment the culture set-ups H, I and J were used further for the grazing experiment. This experiment should help to understand, how the community changes due to grazing. Furthermore, it should verify if the *A. ostenfeldii* strains would show an induced defense behavior and/ or facilitate each other under grazing pressure.

The approximately 52 mL that were left of each replicate were combined in a 1 L glass bottle (Schott AG) per treatment. They were filled up to 760 mL each with fresh seawater - K medium. DNA, counting and toxin samples were taken of each mix as start point for the grazing experiment. Afterwards the remaining volume was split equally to 8 new culture flasks (Erlenmeyer form). To four of them the prepared copepods of the species *Acartia tonsa* were set (explained under 2.3.3). The volumes of the controls were equalized with the same amount of diluted, sterile filtered seawater (salinity =10). The 24 cultures were incubated for 5 days at the culture conditions for algae (mentioned in paragraph 2.3.1). Samples for cell counts were fixed with Lugol’s solution at the second and the fifth day. The cell concentrations were calculated as before (chapter 2.3.5)

At the last day the copepods were filtered out of the cultures and the volume was split to two 50 mL polypropylene centrifuge tubes (Sarstedt). The biomass of the algae was harvested as explained in chapter 2.3.4. For the analysis of the lytic compounds (chapter 2.5.3) 10 mL of supernatant was transferred to a new polypropylene centrifuge tube (Sarstedt), the rest was discarded. Cell pellets and supernatant were stored at -20 °C.

The cell pellets were used for toxin (see chapter 2.5) and DNA extractions (see chapter 2.4.1) as explained in the according paragraphs. The results will show whether or not the cell toxin quota changed due to the competition and grazing pressure and, which of the *A. ostenfeldii* strains was grazed mostly.

2.8 Statistical analysis of experimental data

Differences in the overall population growth of the algal strains in the competition experiment (three-species cultures) were tested with a two-way ANOVA. Fixed factors were culture form (mixed strains versus single strains), origin (Dutch versus Finnish *A. ostenfeldii* strains) and strain. Differences in growth rates of the algal strains in single species culture versus three species cultures were either evaluated with a two sample t-test or with a two-way ANOVA and the fixed factors strain and origin.

The differences in the population growth under grazing pressure were compared for all three species with a two-way ANOVA with the control. Culture condition (grazed versus control) and incubation time were applied as fixed factors. All ANOVAS were followed by post-hoc comparison of the means with Tukey's HSD in the R Software.

3 Results

3.1 Analysis of the microsatellite loci of each strain

The microsatellite loci were analyzed for all 38 *A. ostenfeldii* strains and showed similar results for all strains from the same origin. The genetic diversity of the Finnish strains was in comparison with the Dutch strains higher. For the Aosten microsatellite loci at least two different alleles were detected each. The OKNL_ microsatellite loci were almost present in all strains, but mostly only one allele was found. The found alleles are listed in Table 25, an absent microsatellite locus is indicated with a zero.

Table 25: Found alleles in the *A. ostenfeldii* strains as length of the microsatellite loci in basepairs (bp)

Strain	Aosten						OKNL_					
	10	101	126	171	296	359	1.1	2.4	3.1	4.1	5.1	6.1
AON_1	0	0	0	0	264	0	208	166	277	138	254	236
AON_2	0	0	0	0	0	0	208	0	0	0	0	0
AON_3	0	0	326	0	264	376	208	166	277	138	254	236
AON_4	0	402	0	0	264	0	208	168	277	138	254	0
AON_5	363	0	0	0	264	376	208	172	277	138	254	236
AON_6	363	0	326	0	264	376	208	166	277	138	254	0
AON_7	0	0	326	0	0	376	208	166	277	138	254	0
AON_8	0	0	0	0	264	376	0	166	276	0	0	236
AON_9	363	0	0	0	264	376	208	166	276	138	254	236
AON_10	0	0	0	0	264	0	208	166	277	138	254	236
AON_11	363	0	0	0	264	376	208	166	277	138	254	236
AON_12	363	0	0	0	264	376	208	172	277	138	254	233
AON_13	363	402	0	0	264	376	208	166	277	138	254	236
AON_14	363	402	0	0	264	376	208	166	277	138	254	236
AON_15	363	402	326	0	264	376	208	166	276	138	254	236
AON_16	363	402	0	0	264	376	208	166	277	138	254	236
AON_19	0	402	0	0	264	376	208	166	276	138	254	236
AON_20	0	402	0	0	264	0	208	166	276	138	254	236
AON_24	363	402	326	0	264	376	208	166	276	138	254	236
AON_25	363	402	0	0	264	376	208	166	277	138	254	236
AON_26	0	402	0	0	0	376	208	166	276	138	254	236
AON_27	363	397	0	0	264	376	208	166	277	138	254	236
AON_28	363	402	0	0	264	376	208	166	277	138	254	236
AON_29	363	402	326	0	264	376	208	166	276	138	254	236
AON_30	363	402	0	0	264	376	208	166	276	138	254	236
AON_31	363	402	326	0	264	376	208	166	276	138	254	236
AON_32	363	0	326	0	264	0	0	0	276	138	254	236
AON_33	363	402	0	0	264	0	0	0	277	0	254	236
FIN_01	390	402	355	358	266	375	208	166	277	138	254	236
FIN_02	390	382	353	358	259	382	208	166	277	138	254	236
FIN_03	0	413	355	362	259	382	0	172	277	0	0	236
FIN_04	390	402	353	358	259	375	208	166	277	138	254	236
FIN_05	390	392	345	358	259	382	208	166	277	138	254	0

Grey labelled strains showed the highest genetic diversity and were selected for the further experiments

As the microsatellite loci Aosten171 was not found in any of the Dutch strains, it was omitted from further discussions. The same applied to all of the OKNL microsatellite loci, as almost no differences between the strains were detected. The microsatellite locus Aosten144 was not detected in the Finnish strains and showed up as stutter peaks in the Dutch strains. The manufacturer's guideline of the Type-it Microsatellite PCR Kit suggests a touch-down PCR in this case. In this method the annealing temperature is higher in the first set of cycles to prevent unspecific binding. After several cycles the temperature is decreased as the probability of unspecific binding is decreased with increasing amount of specific template. This PCR was performed as explained in chapter 2.4.4, but it could not solve the problem – the stutter peak was still observed. Therefore this microsatellite locus was omitted too (results not shown).

The number of found alleles ranged from one to five, but the highest diversity for the Dutch strains was three different alleles (for the locus OKNL_2.4) for the 38 analyzed strains. The Finnish strains showed the highest genetic diversity in the microsatellite locus AON_101 with four different alleles in just five clones.

According to the results of the remaining microsatellite loci the following ten strains were chosen to record the growth rates and cell toxin quota of: the Dutch strains AON_13, AON_14, AON_15, AON_16 and the strain AON_27 as well as all five Finnish strains. These ten strains had the most differences in the alleles of the microsatellite loci and could be discriminated with the least number of analyzed microsatellite loci.

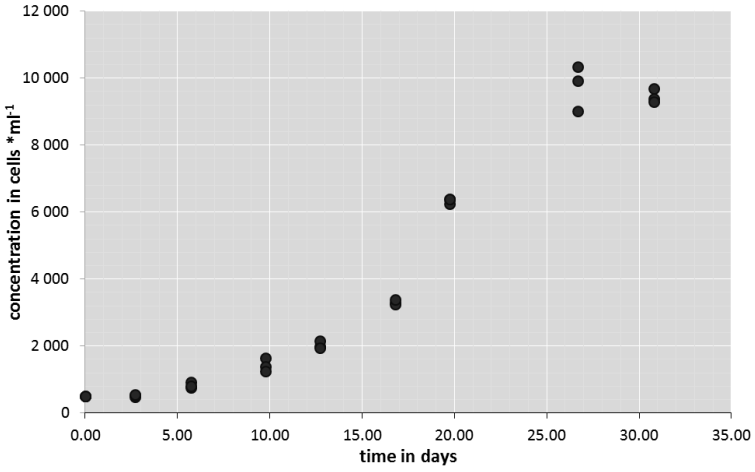

Figure 3: cell counts of the Dutch *A. ostenfeldii* strain AON_15
Each point represents the cell count of one of three replicates.

3.2 Physiological and morphological characterization of ten *A. ostenfeldii* strains

The growth curves obtained from the cell counts of the monoclonal cultures were similar between all *A. ostenfeldii* strains. The cultures usually had a lag phase of three to five days and reached the end of the exponential growth after about 25 days. Only the Finnish

strain FIN_05 reached the stationary growth phase already at day 21 of the incubation. The growth curve of the Dutch strain AON_15 is illustrated in Figure 3 as example.

The calculated growth rates and carrying capacities of each strain are listed in Table 26. The carrying capacity indicates the highest cell number reached. The Finnish strains with 0.227 divisions * d^{-1} on average grew slightly faster than the Dutch strains, at approximately 0.225 divisions * d^{-1}. In average the ten monitored strains divided 0.226 * d^{-1}. The Finnish strain FIN_05 grew the fastest at 0.285 cell divisions * d^{-1}, but it also had the lowest carrying capacity (13664 cells * mL^{-1}) compared with the other Finnish strains. The slowest strain AON_14 grew at 0.196 cell divisions * d^{-1}. In average the Finnish strains reached cell numbers up to 25700 cells * mL^{-1}, whereas the Dutch strains with an average carrying capacity of 11800 cells * mL^{-1} grew only half as dense. The lowest carrying capacity was calculated for the Dutch strain AON_16 with 8568 cells * mL^{-1}.

Table 26: Growth rates and carrying capacities of the ten *A. ostenfeldii* strains

Strain	Growth rate in div * day $^{-1}$	Carrying capacity in cells * mL $^{-1}$
AON_13	0.22	11600
AON_14	0.20	12500
AON_15	0.24	10500
AON_16	0.25	8570
AON_27	0.22	15900
FIN_01	0.21	23800
FIN_02	0.23	29300
FIN_03	0.20	32400
FIN_04	0.21	29100
FIN_05	0.26	13700
L. fissa comp.05	0.33	29700
C. dentata SF1	0.48	722100

Values are given as mean of three independent replicates, carrying capacities were rounded to hundreds

The two competitors *Levanderina fissa* comp.05 and *Chysotila dentata* SF1 grew considerable faster than the *A. ostenfeldii* strains. The haptophyte *C. dentata* grew at 0.48 divisions * d^{-1} more than double as fast as the *A. ostenfeldii* strains. This competitor was also able to reach cell numbers more than 20-times higher than the *A. ostenfeldii* strains, but it was also much smaller than the dinoflagellate – as can be seen in Figure 4. The dinoflagellate *L. fissa* comp.05, at 0.33 cell divisions * d^{-1}, grew slightly faster than *A. ostenfeldii* and could also reach slightly higher cell concentrations (29710 cells * mL^{-1}). *L. fissa* comp.05 could be easily distinguished

from *A. ostenfeldii* because of its distinct cell morphology and different outer shape (Figure 4, panel K). *Levanderina fissa*, a non-toxic, non-thecate dinoflagellate was not as round as *A. ostenfeldii* and had an especially large sulcal groove, which incised the hypotheca deeply. Cells of all morphologically analyzed strains are shown in Figure 4. Stained with Lugol's solution the cells of *L. fissa* comp.05 appeared usually a bit darker than the cells of *A. ostenfeldii*, whereas the haptophyte *Chrysotila dentata* SF1 was not stained as strongly.

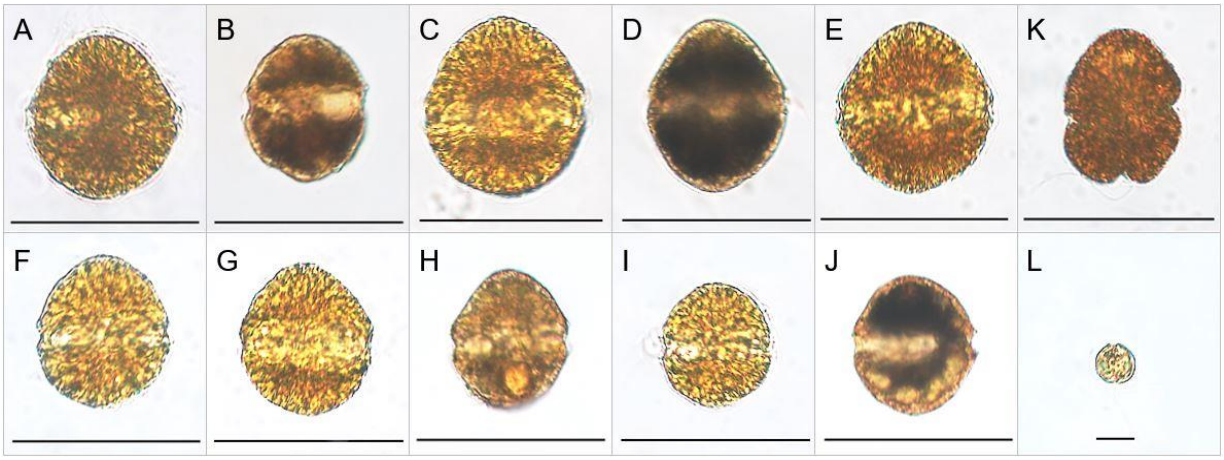

Figure 4: Algal cells of the morphologically analyzed strains in this thesis
A - J: *A. ostenfeldii* cells; cells of strain AON_13 to FIN_05 respectively; A - E strains from the Netherlands, F - J strains from Finland; K: *Levanderina fissa* comp.05; L: *Chrysotila dentata* SF1; the cells were stained with Lugol's solution, pictures were taken with the 200M Axiovert inverted microscope (Carl Zeiss) and the PROGRES GRYPHAX® AKTUR microscope camera (JENOPTIK Advanced Systems GmbH) at magnification 400X; scale bars A - K: 50 µm, L: 10 µm

3.3 Phycotoxins of the ten selected *Alexandrium ostenfeldii* strains

The analysis of the phycotoxins revealed more differences between the *A. ostenfeldii* strains from the different origins. The Dutch strains had an overall higher PSP-toxin content than the Finnish strains. The PSP toxin content of the different strains varied strongly, as it is shown in Figure 5. The Dutch strains had an overall higher PSP toxin content than the Finnish strains. Only strain AON_16 had a lower PSP-toxin content, which was comparable to the Finnish strains with about 3 pg * cell^{-1}. The PSP-toxin content of the other Dutch strains was above 17 pg * cell^{-1}. Strain FIN_05 with about 15 pg PSP-toxins * cell^{-1} had a higher toxin content than the other strains from the same origin. The lowest toxin content was observed for FIN_03 with about 1 pg * cell^{-1}, whereas the Dutch strain AON_13 showed a 60-fold higher toxin content.

Similar results were achieved for the cyclic imine toxins. The lipophilic toxin content was higher for the Dutch compared Finnish strains (Figure 6). Only a small amounts of

gymnodimines (> 0.1 pg * cell^{-1}) were detected for the Finnish strains. In contrast, the Dutch strains had a toxin content of about 2 pg * cell^{-1} for the cyclic imine toxins. The ratio of gymnodimines to spirolides was similar in all Dutch strains (40-60%), only strain AON_14 differed from the others. This strain had a considerable higher amount of spirolides than gymnodimines. The highest cyclic imine toxin content was found in strain AON_13, the strain that also contained the highest amount of PSP-toxins. All *A. ostenfeldii* strains showed a lower content of cyclic imine toxins than PSP-toxins. For the competitor *Levanderina fissa* comp.05 negligible amounts (< 0.0003 pg * cell^{-1}) of gymnodimines were detected. Beside that no other toxins were detected in the two competitor strains.

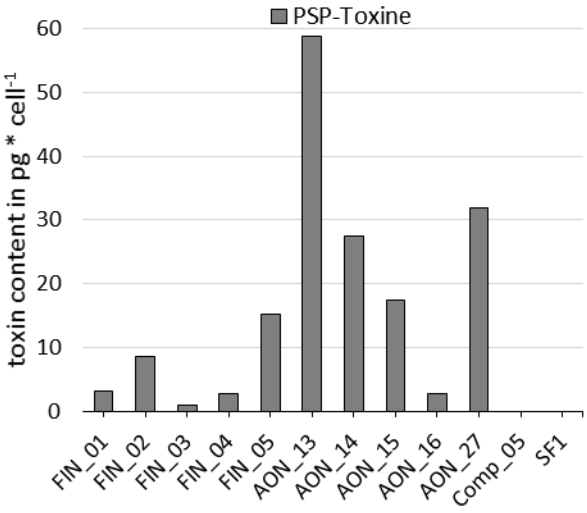

Figure 5: PSP-toxin quota of the analyzed algal strains

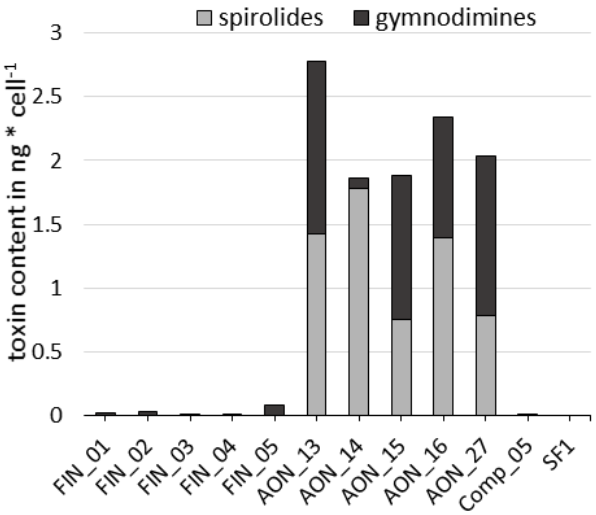

Figure 6: Cyclic imine toxin content of the analyzed algal strains

The different toxin profiles for the PSP toxins and the cyclic imine toxins of the ten strains and the competitor strains are shown in Figure 7 and Figure 8 respectively. Especially pronounced is the difference of toxin profiles between the strains of the different origins (Figure 7). For the PSP-toxins all Dutch strains showed a considerable high proportion of the derivative C1/C2, but it was not detected in the Finnish strains. Saxitoxin (STX), gonyautoxin (GTX) 3 and gonyautoxin 2/3 were detected in all ten strains. Gonyautoxin 2 was detected in all of the Dutch strains and in the Finnish strains FIN_01, FIN_02 and FIN_05. The derivative B1 was only detected in the Dutch strains AON_14 and AON_27.

The cyclic imine toxin profile were even more distinct between the strains of the two origins than the profiles for the PSP-toxins (Figure 8). Different derivatives of gymnodimine were detected for the Finnish than for the Dutch strains. In the Dutch

strains gymnodimine A (GYM) and 12-methyl gymnodimine A (12-Me GYM A) and small amounts of an unknown derivative (m/z 524) were found. The lipophilic toxin composition in the Finnish strains was completely different with small amounts of three unknown derivatives of gymnodimines (m/z 524, 510 and 508). The last one was not detected in the strain FIN_03, which had in general less toxins than the other Finnish strains.

The complexity of the following experiments was reduced by the selection of six *A. ostenfeldii* strains from the analyzed ones. The selection criterions were growth and toxin production within one group of origin and the quality of the microsatellite markers. The strains FIN_01, FIN_02 and FIN_04 were selected from the Finnish strains. The strains FIN_03 was not considered because it showed the lowest toxicity. The strain FIN_05 had the highest toxicity of the Finnish strains, but did not reach as high cell numbers (carrying capacity). From the Dutch strains AON_13, AON_15 and AON_27 were selected for the following experiments, as they showed the highest similarities in the toxin profiles. AON_16 was the least toxic strain from the Netherlands in comparison of the PSP toxins. The strain AON_14 had a different cyclic imine toxin profile than the other Dutch strains – it produced in contrast much more spirolides (> 90 %) and less gymnodimines.

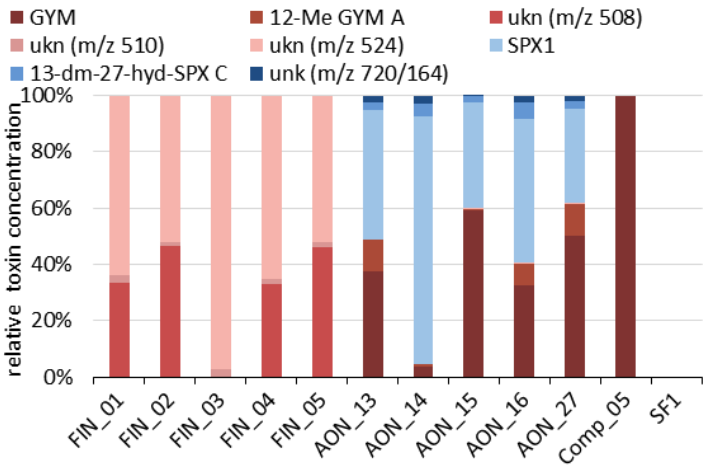

Figure 7: Cyclic imine toxin profiles of the analyzed algal strains

Figure 8: PSP-toxin profile of the analyzed algal strains

3.4 The allelopathic potency of the *A. ostenfeldii* strains

The allelopathic potency of the six selected *A. ostenfeldii* strains was quantified by a FDA-bioassay with *Rhodomonas baltica*. The automated bioassay showed under the applied conditions a direct proportionality between the amount of lyzed cells and fluorescent signal (shown as linear regression in Figure 9). Nevertheless the bioassay could not be applied to quantify the lytic activity of the *A. ostenfeldii* strains. The results of the FDA bioassay were not in line with the observations from the three species cultures (see chapter 3.6.2 and 3.6.3).

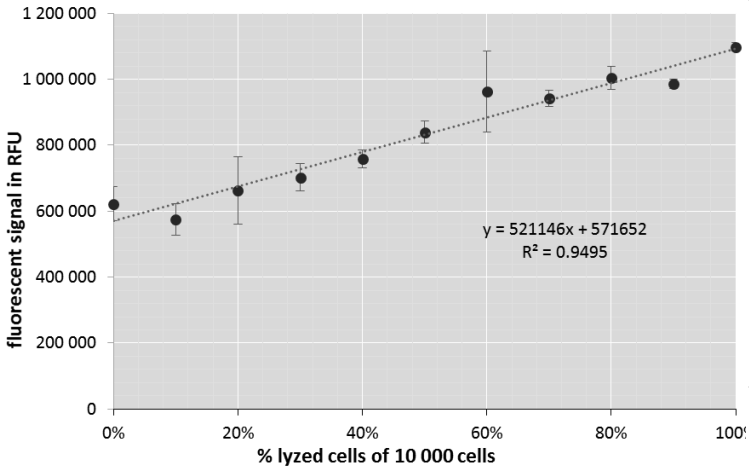

Figure 9: Calibration line for the FDA-bioassay
Each point represents the mean of six fluorescence reads (in relative fluorescence units =RFU) after the incubation of *R. baltica* standards with FDA for 23 h; error bars indicate the SD (*n*=6)

Due to several reasons the results were considers as incorrect – the fluorescent signal of the measured samples was lower as the signal for 0 % of lyzed *R. baltica* cells and the fluorescent signal of the highest dilution of the supernatant was higher as the fluorescence of the highest concentrated sample. In Figure 10 the fluorescence obtained from the different dilution of supernatant is shown for strain AON_13. Calculating the amount of lyzed cells from the fluorescent signal would result in negative values. Due to these differences no EC_{50} value could be calculated. Reasons for this failure were not found yet and need to be discussed (see chapter 4.1.3).

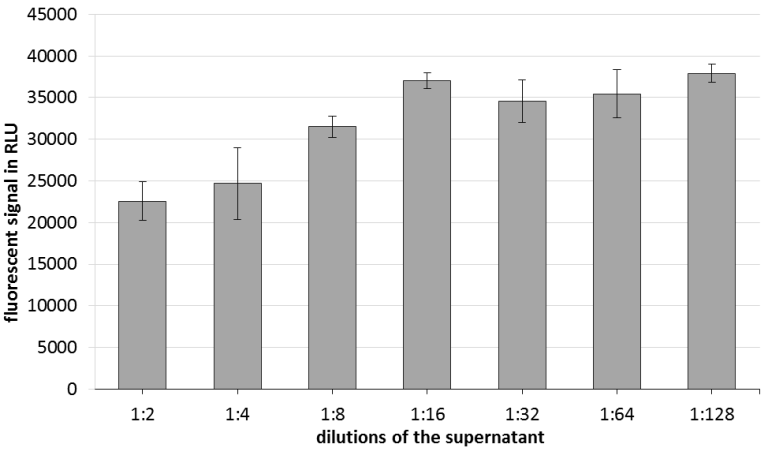

Figure 10: Fluorescence measured for the supernatant dilutions after the incubation with *R. baltica*

3.5 Results of the two-species cultures of the competitor strains

Two-species cultures with different ratios of the two competitors were set up to reveal the interaction. The obtained results of these two-species cultures suggested the predation of the potentially mixotroph dinoflagellate *L. fissa* on the haptophyte *C. dentata.* Despite the high growth rates of the haptophyte *C. dentata* was not able to reach higher cell numbers than *L. fissa* in any of the cultures. Figure 11 shows the previously predicted and the actually observed growth curves of the two competitor species. Prediction aimed for three different situations: either one of the species outperforming the other (A and B) or a stable coexistence (C). A stable coexistence was however most closely resembled in culture B with start cell number of 770 cells * mL^{-1} of *L. fissa* and 490 cells * mL^{-1} of C*. dentata*. After recording the cell concentrations for fifteen days it was possible, to estimate the real interaction coefficients of the two species to each other. The apparent grazing of the mixotroph *L. fissa* on the haptophyte *C. dentata* resulted in a very high interaction coefficient α_{12} and a much smaller one for the other way around (α_{21}). The estimated values are stated in Table 27 below. With these values the three-species model was implemented as it is explained under 2.6.3.

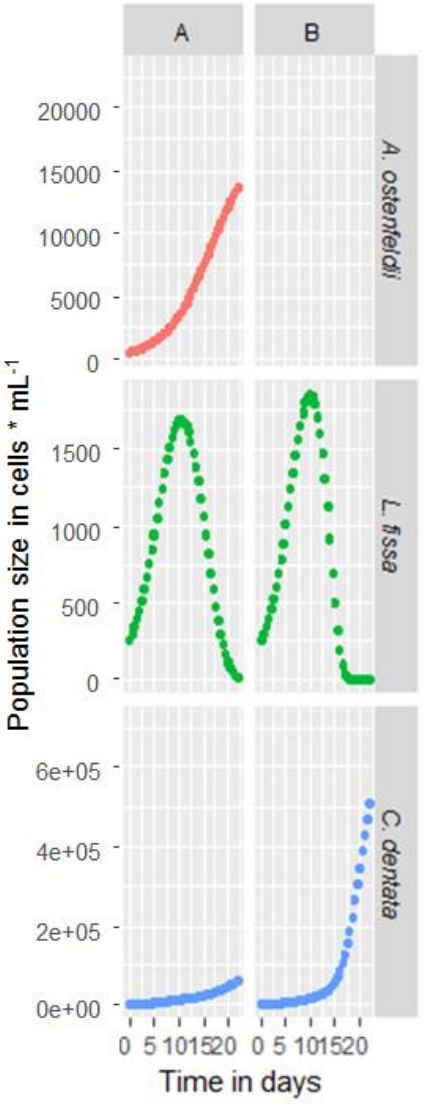

Figure 11: predicted growth curves for competition experiment with three algal species

A: three species cultures - growth predicted with average carrying capacity of *A. ostenfeldii, B* – control cultures without *A. ostenfeldii, interaction coefficients were derived from the two species cultures of the competitors und the carrying capacities*

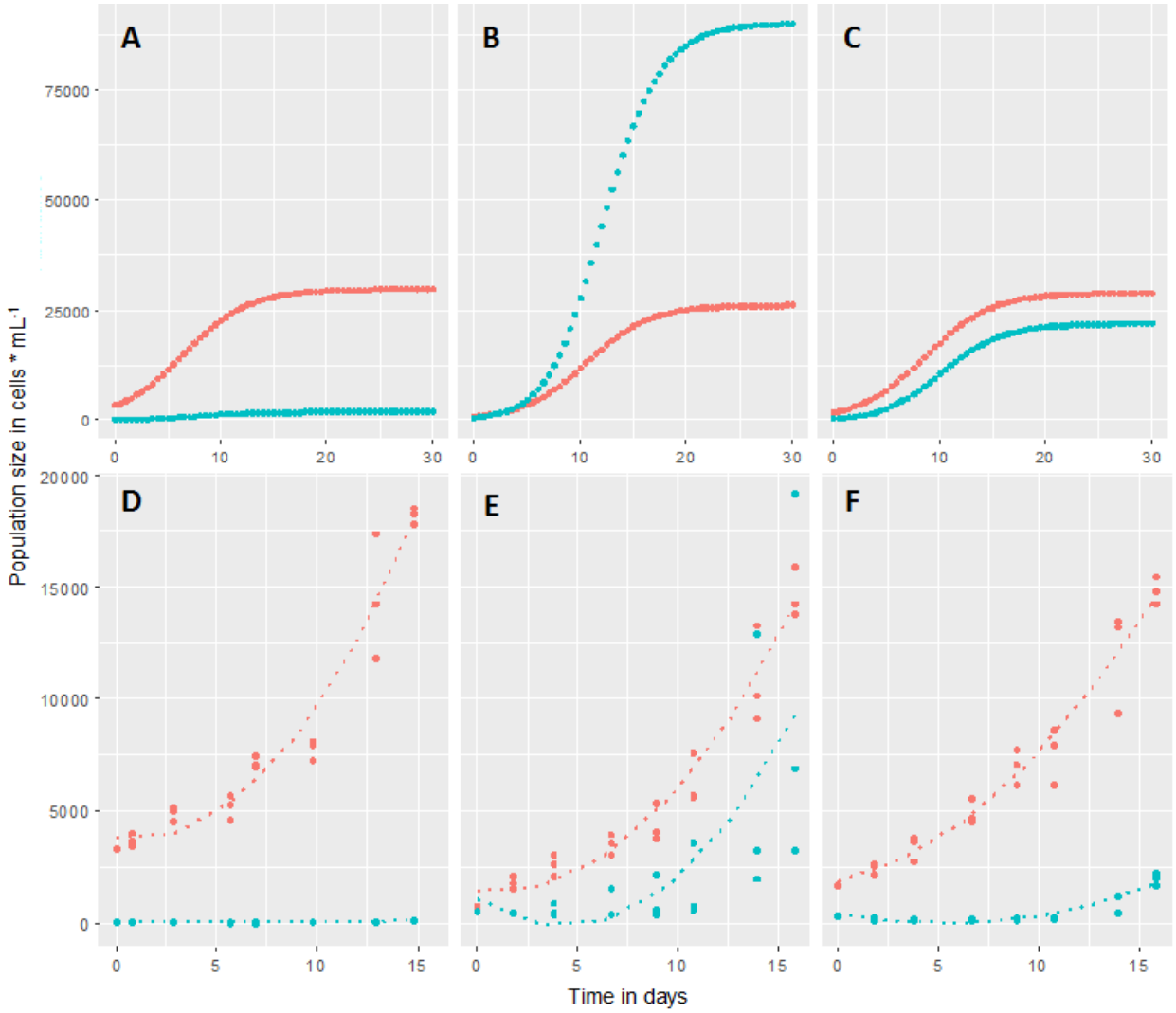

Figure 12: Growth curves of the two competitor strains in the two-species cultures
Red dots indicate the cell numbers of *L. fissa*, and turquoise dots the cell numbers of *C. dentata*; A - C: predicted growth curves with the interaction coefficients calculated from the carrying capacities obtained from the single species cultures, D - F: obtained growth curves from the two species cultures A and D: start population size – 3280 *L. fissa* and 70 *C. dentata*, B and E: start population size - 770 *L. fissa* and 490 *C. dentata*, B and E: start population size - 1620 *L. fissa* and 300 *C. dentata*

Table 27: calculated interaction coefficients from the two-species cultures of the competitors

Interaction coefficient	value
α_{12}	3.71
α_{21}	619.92

3.6 Modelling of three species cultures and the observed results from the mixed culture experiments

To implement a three species model despite the missing interaction parameters, some assumptions were made: The third species *Alexandrium ostenfeldii* only competes with the other two species for space without any additional features. Assuming this, it

is possible to calculate the missing interaction coefficients through the according carrying capacities with $\alpha_{13} = K_1 * K_3^{-1}$ for example. The carrying capacities of the two competitors were estimated from the growth curves of the strains in a previous experiment and are stated in chapter 3.2. As carrying capacity for the *Alexandrium ostenfeldii* strains the average value of the carrying capacities of the six selected strains was set in (K_3 = 18403). The following interaction coefficients were calculated for the three-species model.

Table 28: Calculated interaction coefficients from th carrying capacities of the three species

Interaction coefficient	value
α_{13}	1.61
α_{31}	0.62
α_{23}	39.24
α_{32}	0.03

With these values it was possible to estimate at, which initial cell numbers a stable coexistence between the three species can be expected. The cell numbers are given in Table 24 in chapter 2.7.1.The predicted growth curves of the three species in a mixed culture with the initial cell numbers as calculated are shown in Figure 12. Panel A shows the growth curves for the three species in the mixed culture with all six *A. ostenfeldii* strains. Panel B of Figure 12 shows the expected growth curves of the two competitor strains without the addition of *A. ostenfeldii*.

Despite the high growth rates of *C. dentata* SF1 the growth seems strongly restricted by the presence of *A. ostenfeldii* in the three species cultures. Whereas the competitor *C. dentata* SF1 is only slightly constrained by the presence of *A. ostenfeldii* and reaches nearly the same cell concentrations as in the control. However according to this model the three species culture were set up and monitored.

3.6.1 Calibration for the allele-specific quantitative PCR with the standard mixtures of the gDNA templates

To determine the proportion of the different strains in the mixed cultures to each other an allele-specific quantitative PCR (asqPCR) was performed. The analysis of the calibration standards with the defined ratios of the strain gDNA however did not result in a good linear regression (R^2 = 0.63 or 0.34). The obtained peak areas for the microsatellite Aosten10 are given in Table 29 with the according amount of DNA template. The resulting calibration curves are shown in Figure 14. As the correlation

coefficient for both curves was below 0.8 the curves could not be taken to calculate the ratios of the different strains to each other. Furthermore, the ratios of the peak areas to each other did not stay consistent within the replicates, so it was not possible to calculate the ratios from the average peak areas either. Similar results were obtained if the microsatellite locus Aosten296 was analyzed instead. For this microsatellite loci three peaks at the size of 259bp, 264 bp and 266bp were expected in the mix of the different template DNAs. As two alleles with 259bp and 266 bp were observed for the Finnish strains, the peak area of these both alleles was added together for the proportion of the Finnish strains. The third peak area at 264 bp should correlate with the amount of DNA of the Dutch strains in the mix. In fact the correlation of peak area with DNA template from the different origin was even worse (R^2 = 0.59, graph not shown).

Due to time constrains no further efforts were made to get calibration curves for the other microsatellite loci. Reasons for the failure and possible improvements need to be discussed (see chapter 5.2)

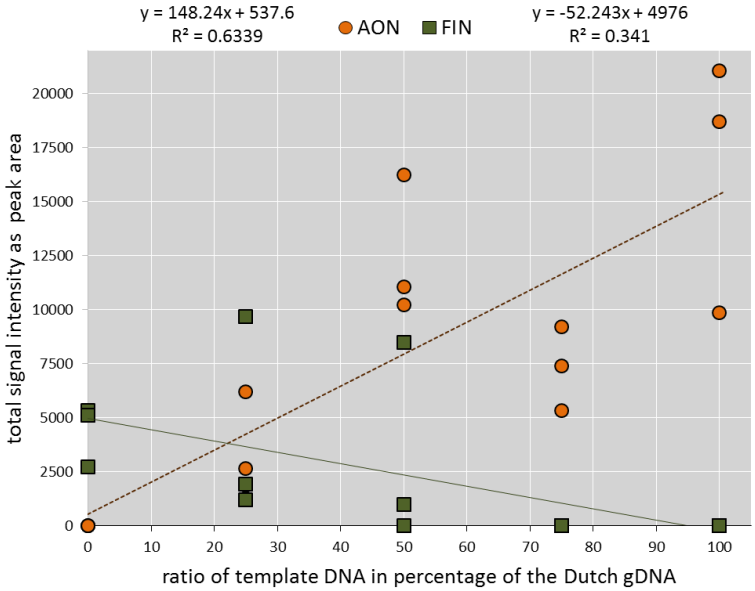

Figure 13: Observed peak areas of the two alleles for the microsatellite Aosten10 in the gDNA standards and the resulting calibration lines

Orange dots show the peak areas obtained for the Dutch allele with 363 bp and the dotted line is the according calibration line (R^2 = 0.6339, equation above the plot), green squares show the peak areas obtained for the Finnish allele with 390 bp the according calibration line is drawn (R^2 = 0.341)

Table 29: Results obtained from the allele specific quantitative PCR of the gDNA standards

Replicate:		**1**		**2**		**3**	
Ratio of gDNA from strains of both origins in the standards		**Peak area of the fluorescent signal for the alleles**					
		363 bp	**390 bp**	**363 bp**	**390 bp**	**363 bp**	**390 bp**
0 % AON	100 % FIN	0	5343	0	2733	0	5105
25% AON	75 % FIN	6206	1213	1279	1937	2633	9670
50 % AON	50 % FIN	10223	963	16240	8494	11047	0
75 % AON	25 % FIN	7403	0	5324	0	9209	0
100 % AON	0% FIN	21082	0	18728	0	9872	0

3.6.2 The effect of single strains of *A. ostenfeldii* on the three-species community

The nine three-species cultures and the control culture of the competitors were monitored for twenty days to determine the real interaction parameters of *A. ostenfeldii* on the two competitor species. For each set-up the cell concentrations of three replicates were determined every two to three days. The population growth of the three species in the competition experiment is shown in Figure 14 (page 50). From that Figure the different effects between the Dutch and the Finnish *A. ostenfeldii* strains on the two competitors can be seen. The single Dutch strains (panel A B and C) already had a strongly negative effect on both competitors, whereas the effect of the Finnish strains was not as pronounced. The Dutch *A. ostenfeldii* strains lead in fact to lysis of the two competitors, so no competitor cells were observed anymore after two days.

In the cultures D, E and F the growth of *L. fissa* was slightly constrained by *A. ostenfeldii* and *C. dentata* SF1 grew even better as in the control. So the effect of the Finnish *Alexandrium ostenfeldii* strains on the haptophyte was actually positive, which was represented by the real interaction coefficient as negative value. The Dutch strains grew in comparison to the Finnish strains in the mixed cultures to higher average cell concentrations whereas it was exactly the other way around in the single cultures (chapter 3.2). So the growth of the Finnish *Alexandrium ostenfeldii* strains was more strongly restricted by the presence of the competitor strains than the growth of the Dutch strains.

Considering the growth rates of the single strain cultures, the Finnish strains grew in comparison to the Dutch strains in the competition experiment significantly slower (p = 0.002). The single strains Fin_02 and FIN_04 grew the slowest, but for the mixed strain culture of the three Finnish strains the higher growth rate was observed. The growth rate of strain AON_27 was least affected by competition. This strain solely showed no significant difference to the single species cultures.

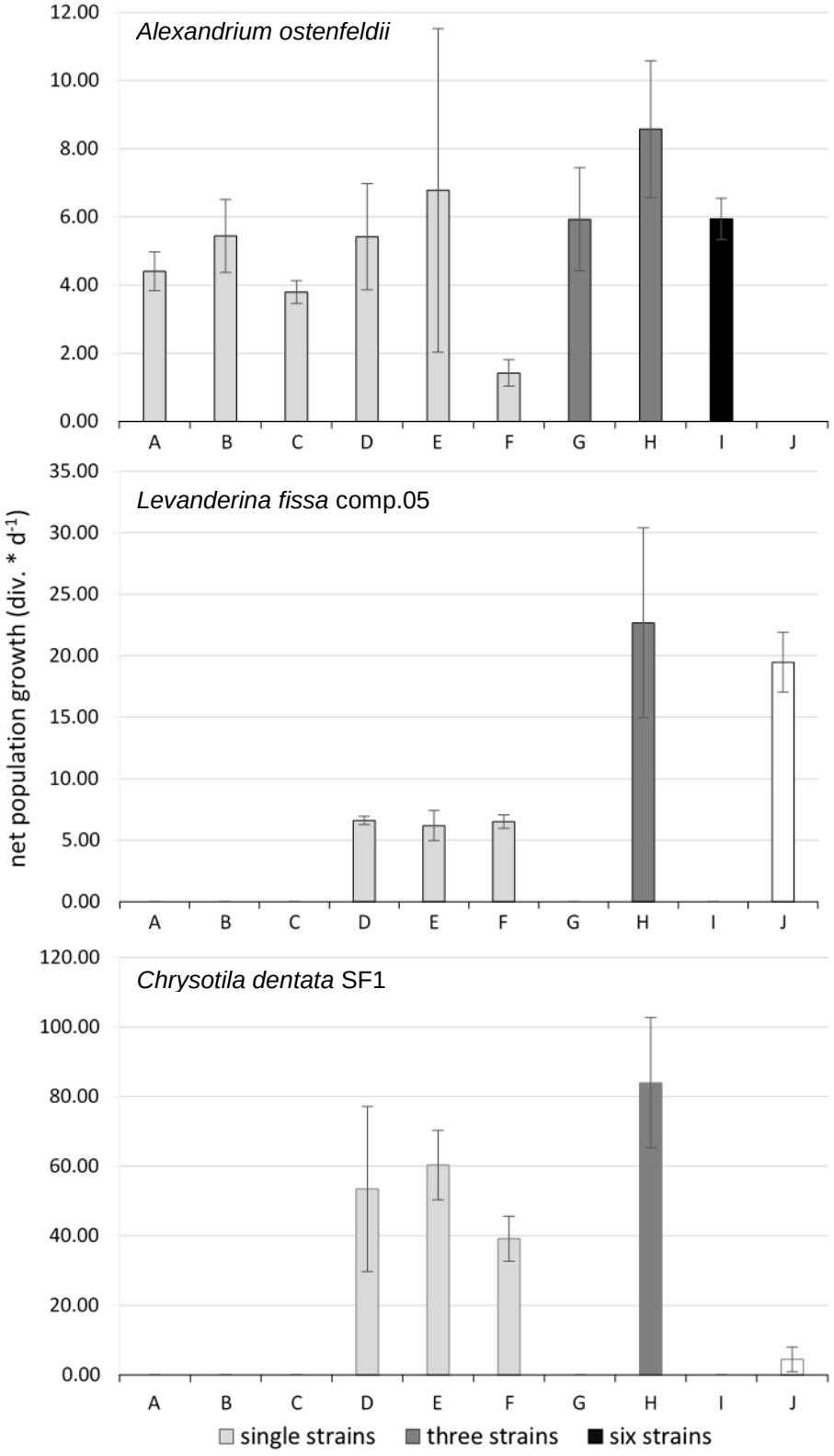

Figure 14: Population growth of the three algal species in the competition experiment
On the x-axis: the culture IDs of the set ups according to the table 24 in chapter 2.7.1; culture A – C: with one of the Dutch *A. ostenfeldii* strains; culture D – F: with one of the Finnish *A. ostenfeldii* strains; culture G: all three Dutch strains, culture H: all three Finnish strains, culture I: all six *A. ostenfeldii* strains, culture J: control of the two competitors (*L. fissa* and *C. dentata*) without the addition of *A. ostenfeldii*; error bars indicate standard deviation (*n* = 3)

3.6.3 Effect of the mixed *A. ostenfeldii* strains on the community

The effect of the mixed *A. ostenfeldii* strains from the both origins on the competitors was similar to the single strains of the respective origins: the Dutch strains had a strong negative effect on the competitors. In contrast the Finnish strains had only a negative effect on the dinoflagellate *L. fissa*. The positive effect of *A. ostenfeldii* on the competitor *C. dentata* was even more pronounced as the haptophyte reached even higher cell numbers within the twenty days.

The mixed Dutch strains of *A.*

ostenfeldii (culture G) reached again higher cell numbers than the mixed Finnish strains (culture H). In the mixed cultures with all six *A. ostenfeldii* strains (culture I) the toxic dinoflagellate grew to similar cell concentrations as in the cultures with all three Dutch strains. The effect of the six *A. ostenfeldii* strains on the competitors was the same as in the culture containing just Dutch strains – the competitors were lyzed within the first days of the incubation. The different response and abundance of the competitor strains to the *A. ostenfeldii* strains from the different origin can be also seen in Figure 15. Panel A shows the community of the culture set-up G, which contained initially both competitors and the three Dutch *A. ostenfeldii* strains. After an incubation time of three days none of the competitors were left in the culture. Panel B shows the community of the culture set-up H at the last time point (day 20). There are still both competitors visible in the sample. As the asqPCR was not successful, it was not possible to evaluate the ratios of the Dutch to the Finnish *A. ostenfeldii* strains in the mixed strain culture. Differences in population growth of each strain in the mixed strain community could not be compared either.

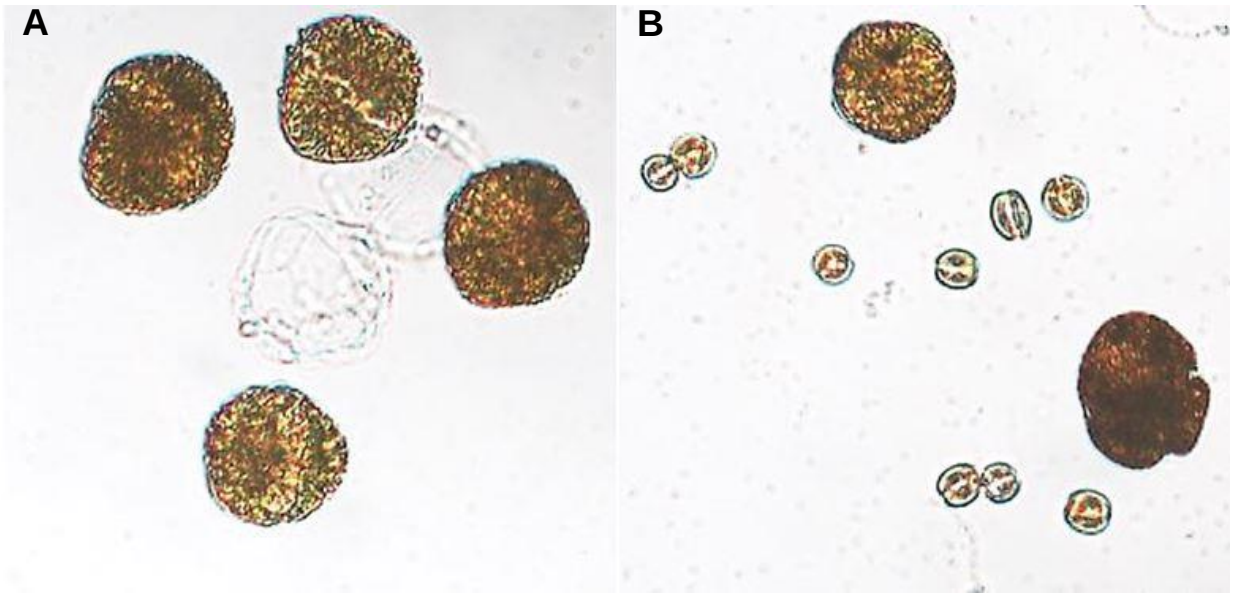

Figure 15: The three-species culture either with the Dutch or with the Finnish strain mixture
Panel A: shows the algal community after incubation with the Dutch *A. ostenfeldii* strains, there were no competitors visible; panel B displays the algal community after the incubation with the Finnish A. ostenfeldii strains, both competitors were still viable; cell were stained with Lugol's solution, magnification: 400X at Axiovert 200M (Carl Zeiss) and PROGRES GRYPHAX ARKTUR microscope camera (JENOPTIK Advanced Systems GmbH)

The obtained growth curves of all species were different to the previously predicted growth curves. In contrast to the predictions, *L. fissa* grew only in cultures, were no Dutch *A. ostenfeldii* strains were added. The same was observed for the other competitor, but in contrast to the predictions, it was positively influenced by the Finnish

A. ostenfeldii strains and achieved higher cell numbers if one of these strains was present.

3.7 Results of the copepod grazing experiment

The competition experiment with the three algal strains was followed by a grazing experiment with the copepod *Acartia tonsa*. The cell counts of the algal species showed a totally different response of each species to the grazing. Figure 15 shows the cell counts obtained for the three algal species at the first, the third and the fifth day. The cell numbers obtained for *C. dentata* were divided by ten to show them together with the other competitors in one chart per condition and culture. Culture H still contained all three algal strains, whereas in culture I the two competitor strains were already ruled out by *A. ostenfeldii*. As the asqPCR could not be applied, it is not possible to solve, which strains of *A. ostenfeldii* were still in the culture. Culture J only contained the two competitor strains of *C. dentata* and *L. fissa*. The population growth of each species were compared for the culture conditions (control versus grazed) and the time points statistically.

In culture H *A. ostenfeldii* was less affected by the copepods than *L. fissa*. The cell numbers of both dinoflagellate species were reduced compared to the control, but only for *L. fissa* the difference was significant. The haptophyte *C. dentata* was only slightly impaired by grazing pressure: the statistical analysis revealed no significant difference between the controls and the grazed cultures (for culture set-up I and J, n = 3, p =0.47 and p=0.76).

In culture I the cell numbers of *A. ostenfeldii* were still increasing despite the grazing. In fact the species grew even better with the addition of the copepods compared to the control. The population growth showed significant differences between the grazed culture and the control (p = 0.016), but the differences were only observed at the last day of the incubation.

In the culture J without *A. ostenfeldii* no differences were observed for the abundance of the haptophyte *C. dentata* (p=0.7). Only *L. fissa* was affected by the grazing pressure and showed significant differences in population growth between the culture conditions (p=0.003). The dinoflagellate *L. fissa* was most affected by the grazing through the copepod *Acartia tonsa* whereas the response of *A. ostenfeldii* was rather positive. Possible reasons will be discussed. After the grazing experiment it was not

validated whether or not the copepods were still healthy or showed any behavioral abnormities.

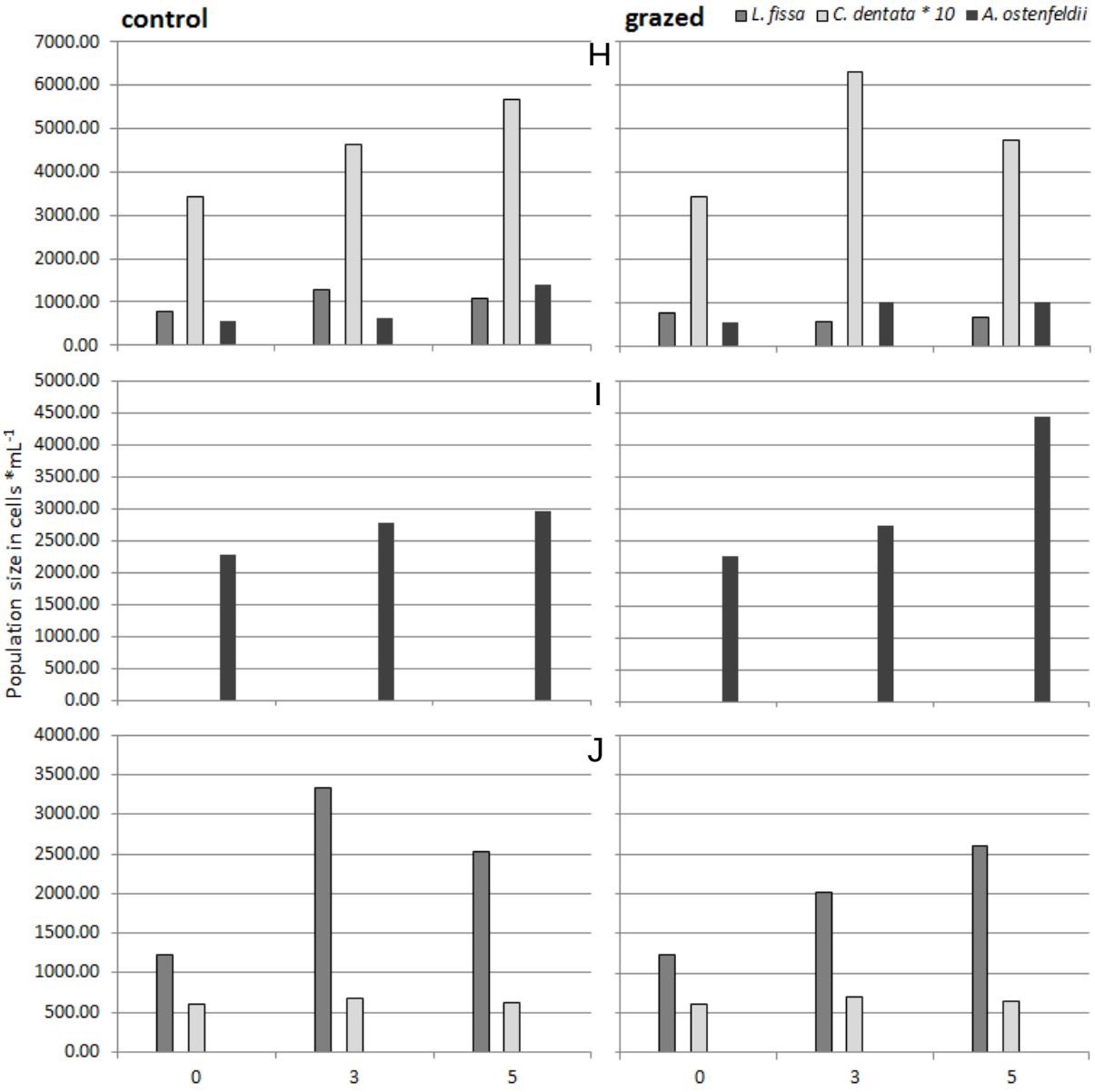

Figure 16: Population size of the algal species with and without the addition of copepods
Panels are labeled according to the culture ID the cultured had in the previous experiment: H contained all three species, but only the Finnish *A. ostenfeldii* strans; I contained all three species, with all strains of *A. ostenfeldii*, J contained the two competitor species *L. fissa* and *C. dentata;* cell numbers of *C. dentata* are divided by 10. the X-axis shows the incubation time in days

3.8 Comparison of the toxin data before and after the experiments

The toxins PSP-toxin concentration measured before and after the competition and the grazing experiment resembled almost the previous observations (see 3.3). In contrast for cyclic imine toxins (CIT) much higher values were measured during the competition than for the single strains. The results can be found as charts in Figure 17. The highest CIT value was measured for culture B at the beginning of the competition

experiment (38.86 pg * cell^{-1}) and is therefore doubtful. The second highest value was with about 12 pg * cell^{-1} was not even half as concentrated, but compared to the initial concentration of the according *A. ostenfeldii* strain still six times as high. The increase of gymnodimines was especially pronounced for the Dutch strains and the Dutch strain mix (culture G). The concentration of spirolides was not as strongly increased, which can be seen in the toxin profiles (Figure 17, panel D). At the end of the competition experiment the values were again lower (up to 6.26 pg * cell^{-1}). In the grazing experiment similar values as at the beginning were measured. The control mostly showed a bit higher CIT concentrations. For the Finnish strains only slight changes were recognized - over the complete time the CIT concentrations stayed below 0.5 pg * cell^{-1}.

The concentrations measured for the PSP toxins were ranging from 0 to 99.18 pg * cell^{-1}. The highest value was measured for the strain mix at the beginning of the competition experiment. The concentrations measured for the three Dutch strains at the beginning of the competition experiment were about 34 pg * cell^{-1}. This value was about the average concentrations measured before for the single strain cultures. The Finnish strains contained still less PSP- toxins. For culture D and F the cell toxin quota was even below the detection limit. At the end of the competition experiment the cell toxin quota were for most cultures higher as at the beginning (16.5 to 61.2 pg * cell^{-1}). Even the Finnish strains had PSP-toxin concentrations up to 41.3 pg * cell^{-1}. During the grazing experiment the changes in the cell toxin quota were not as distinct. The cell PSP toxin quota for culture H with the mixed Finnish strains was decreasing within the five days. For both culture conditions (grazed and control) similar concentrations were measured at the end of the experiment. In culture I (all six *A. ostenfeldii* strains) the cell PSP toxin quota was increasing, but only for the control. In the grazed culture I the value stayed constant. The PSP toxin profiles showed almost no difference between the different conditions and time points. Only the differences between the different origins of *A. ostenfeldii* strains were visible, but they were similar to the profiles obtained for the single strain cultures (chapter 3.3). For culture J, which only contained the two competitor strains no toxins were detected over the whole experimental period.

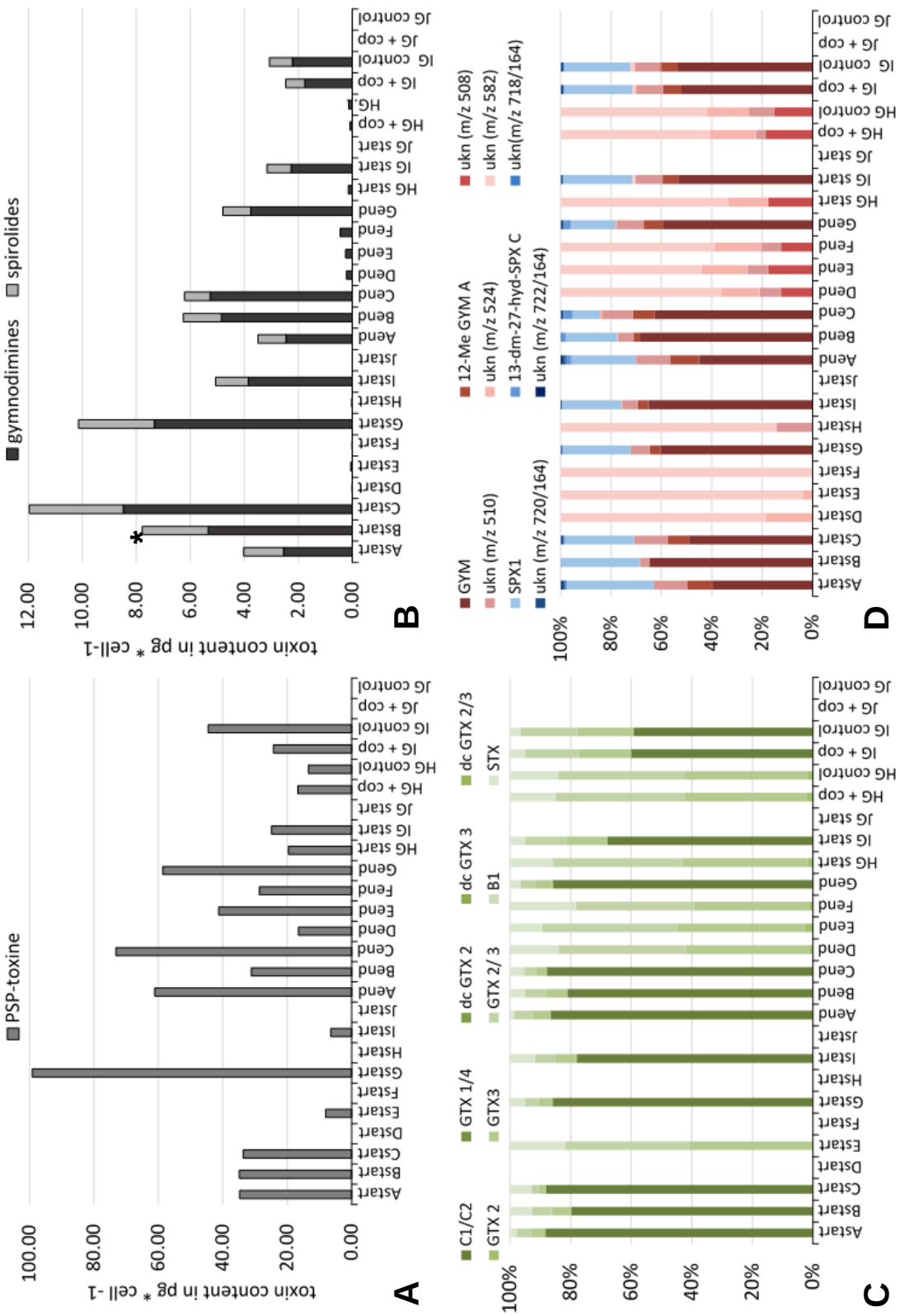

Figure 17: Toxin contents and profiles of the culture extracts before and after the experiments

A: cell PSP toxin quota, B: cell CIT quota, C: PSP toxin profile, D: gymnodimine and spirolide toxin profile; The abrs are labelled with the Culture IDs (according to Table 24 in chapter 2.7.2 A – J and the timepoint when the sample was taken, either at the strat or the end of the experiment; HG to JG indicate the samples of the grazing experiment with control for the non-grazed and + cop for the grazed cultures (with copepods), toxins with the prefix ukn are not described yet (unknown)

Note: values of CITs for Bstart were divide by five to show them in the same chart (asterisk)

4 Discussion

4.1 Differences between the *A. ostenfeldii* strains

4.1.1 Different genotypes – higher similarities in Dutch than in Finnish strains

The genotypes of the analyzed *A. ostenfeldii* strains were all quite similar. The different strains could be barely distinguished via microsatellite loci the microsatellite fragments (alleles) either had the same length or were not present (null allele). Several publications report about the enormous intraspecific genetic diversity in blooms of the genus *Alexandrium* (Nagai et al. 2004, 2006, Alpermann et al. 2006, 2010, Masseret et al. 2009). This diversity was not observed in the strains isolated from the bloom in the Ouwerkerkse Kreek. The Finnish strains in contrast showed a slightly higher genetic diversity.

In contrast to the results obtained for other *A. ostenfeldii* populations and other species of this genus the Dutch *A. ostenfeldii* strains of this thesis seemed genetically rather homogenous. For example six microsatellite loci in a selection of 48 strains of an *A. tamarense* population yielded five to nine different alleles (Alpermann et al. 2006). The seven microsatellite loci analyzed in this thesis yielded two to 12 different alleles in a selection of 40 strains of *A. ostenfeldii* from Föglö in Finland (Nagai et al. 2014). In the present thesis the maximal number of different alleles for these microsatellite loci was much lower.

Homogenous populations like they are shown in this thesis were only reported in small subpopulations in the Baltic Sea, which differentiated due to prevailing current systems and local habitat conditions (Tahvanainen et al. 2012). Because of the low intraspecific diversity, the population in the creek must have originated from a very small seed population. Obviously, the population in the creek perhaps did not have the initial stock diversity and time to differentiate into several lineages. Where exactly the cells came from remains an open question, especially as the creek is not directly connected to the North Sea. The water of the creek is released to the Oosterschelde estuary and not pumped in (Burson et al. 2015), so this option is debarred.

4.1.2 Differences between the populations in toxicity and toxin profiles

The results achieved in this thesis by toxin analysis showed that strains from the same origin showed similar toxin profiles and only small differences in the cell toxin quota. The Finnish *A. ostenfeldii* strains were overall less toxic than the Dutch strains. The Dutch strains produced PSP toxins and additionally several derivatives of the cyclic imine toxins. According to the literature the production of both spirolides and PSP toxins is uncommon. Usually strains of *A. ostenfeldii* tend to produce either one or the other group of phycotoxins (Suikkanen et al. 2013, Salgado et al. 2015). For example, the Finnish strains did not produce spirolides whereas strains from Denmark only produced spirolides and no PSP toxins (Kremp et al. 2009, Medhioub et al. 2011). The only case were all three toxins (PSP toxins, gymnodimines and spirolides) were measured in one population of *Alexandrium* was in Wickfort cove isolates of *Alexandrium peruvianum* (Borkman et al. 2012).

The average cell PSP toxin quota was similar to previously measured values from strains of the same location in the Netherlands (Van de Waal et al. 2015). For the Finnish strains the PSP toxin contents were lower in comparison to the strains from the Netherlands and the amounts of gymnodimines were barely measurable (>0.1 pg * $cell^{-1}$). Conform to the literature about strains from Finland no spirolides were detected (Kremp et al. 2012, Salgado et al. 2015). For Baltic strains the PSP toxin contents were overall low compared to the Dutch strains as reported by several studies (Salgado et al. 2015, Kremp et al 2012).

The amounts of cyclic imine toxins were in the range of previously reported values with 0,4 to 89,5 pg SPX * $cell^{-1}$ (Tillmann et al. 2007). Strains from Denmark had 0.4 to 4.9 pg * $cell^{-1}$ of spirolides (Medhioub et al. 2011). Up to 13 pg * $cell^{-1}$ gymnodimines were reported for Dutch strains (Van de Waal et al. 2015). In the cited publication (Van de Waal et al. 2015) the strains also showed a high intraspecific variability in cell toxin quota for all three toxin groups. The results of the present thesis can only confirm this variability for the cell toxin quota of the PSP toxins. The present thesis also suggests that the PSP toxin profiles in *A. ostenfeldii* are rather stable phenotypic characters within populations of the same origin. The toxin profiles did not change from monocultures to the mixed cultures and there were also no differences observed to the grazing experiment. In current publications it was shown that the toxin profiles within *A. ostenfeldii* can vary dependent on the culture

conditions (Van de Waal et al. 2013). The toxin profiles were also found to be no sufficient parameter to reveal the origin of a strain (Alpermann 2009).

The analysis of the cyclic imine toxins revealed slight differences in cell toxin quota as well as in the toxin profiles within the populations under the different culture conditions. Due to climate change it was already analyzed in several studies whether or not there is a correlation between physical factors and toxin production. The saxitoxin production was reported to elevate with rising CO_2 levels or temperature (Kremp et al. 2012). Martens et al. (2016) showed a negative correlation between the cyclic imine toxin content and the salinity. Another study gives account of changing toxin profiles in *A. ostenfeldii* if incubated under different culture conditions (salinity, light, medium in Otero et al. 2010). However for *A. ostenfeldii* there were no studies conducted yet that show a correlation between competition and toxin production. In the present thesis results were achieved, which indicate a positive correlation between toxin production and competition. Due to time constrains it was not possible to validate this indications statistically. Furthermore, there are results showing a negative correlation between cell spirolide quota and cell concentration (Medhioub et al. 2011). If the detected increase of cyclic imine toxins at the beginning of the competition experiment resulted from the dilution of the cultures and lower cell concentrations needs to be proven.

4.1.3 Lytic activity of the Dutch strains

One of the major observations was that none of the competitors survived if incubated with a Dutch *A. ostenfeldii* strain. In contrast to the literature no such effect was observed for the five Finnish strains (Hakanen et al. 2014). The strains obtained for this thesis work were isolated from the same origin as the strains in the study of Hakanen et al. (2014). In this study a clearly negative effect of cell free-filtrate on the dinoflagellate *L. fissa* was observed. The cells were immobilized and started to lyze within an hour. The literature also shows a stronger lytic activity of the Dutch strains compared to the Finnish strains. The EC_{50} values were 180 to 500 cell * mL^{-1} and 250 to 1600 cells * mL^{-1} respectively (Hakanen et al. 2014, Van de Waal et al. 2015).

Unfortunately it was not possible during this thesis work to quantify the lytic potential of the *A. ostenfeldii* strains with the Fluorescein diacetate (FDA) bioassay. The FDA-bioassay applied in this thesis work was described in detail by Machado and Soares

(2012). FDA is a non-fluorescent, hydrophobic molecule, which can diffuse easily through cell membranes. Esterases cleaving the acetate residues from the molecule transform it into the fluorescent, hydrophilic fluorescein (Machado and Soares, 2012, Franklin et al. 2001). Their results suggest linear decreasing fluorescence intensity with an increasing amount of esterase-inhibiting copper. Instead of the chlorophyte *Pseudokirchneriella subcapitata* the cryptophyte *Rhodomonas baltica* served as target organism of the bioassay in this thesis. In contrast to the results obtained by Machado and Soares, previous results of *R. baltica* cells showed a proportional increase of fluorescence with the amount of lyzed cells (C. Bruhn, personal communication). An explanation for these contrary results could be that the algal cells in the test of Machado and Soares stayed intact, whereas the *R. baltica* cells were lyzed. If the initial molecule is already transformed into fluorescein, before it diffuses into the cells it cannot pass the membrane that easily anymore. As result the fluorescent signal will be stronger the more esterases are released to the medium by the lysis of cells.

There are several possibilities why the FDA bioassay in the end did not work out. One explanation could be that there were already esterases in the supernatant of *A. ostenfeldii*. In this case the fluorescent signal of the analyzed samples would have been even stronger. Additional fluorescence would have been subtracted with the blanks from the samples. The fluorescence from the blanks was however even stronger as for the standards with 100 % lyzed *Rhodomonas baltica* cells, which resulted in negative values of lyzed cells.

Another alternative explanation could be that the pH of the supernatant was different as from the standards. The fluorescence of the fluorescein is highly pH dependent, so slight pH changes can have a strong effect and reduce the fluorescent signal of fluorescein (Wanandy 2005; Martin and Lindqvist 1975). The pH effect on the fluorescein was however not regarded in this thesis and there are no data about pH changes in the cultures available. However the group of Van de Waal in the Netherlands reported about increasing pH values with preceding incubation times (personal communication, Karen M. Brandenburg). Similar data, the increase of pH with increasing cell numbers, were also reported in other studies (Hansen 2002). The determination of the pH change in the cultures and the pH of the supernatant would clarify if the pH was the reason for the failure.

A third hypothesis is that the lytic compounds excreted by *A. ostenfeldii* inhibit the esterase activity, similar to the effect of copper in the tests of Machado and Soares (2012). The inhibition of the esterases would explain why the fluorescent signal in the sample with less concentrated supernatant of *A. ostenfeldii* is higher. This hypothesis could be tested with the measurement of esterase-supernatant mixtures and standards containing heat inactivated and active esterases.

4.2 Failure of the asqPCR calibration

In order to apply asqPCR for reliable quantitative strain differentiation in mixed culture assemblages equal and high efficient standard curve for the target strains have to be generated (John et al. 2014). Unfortunately, this was not possible for the assay used in this study. The results of the calibration standard did not result in a linear regression for the peak areas, although the approach were performed as described before (Löbbecke 2015). One explanation could be that the gDNA contained potential contaminations, so that the concentration measured was not the actual gDNA concentrations. The ratio of 260 to 280 nm of the spectrophotometric measurement showed value of approximately 2.0. This ratios gives the concentration of nucleic acids to proteins, so it can include RNA (Sambrock and Russel 1989). In the study of John et al. (2015) the DNeasy plantmini Kit (Quiagen) was used for the DNA extraction and in the thesis work of Löbbecke (2015) the NucleoSpin Plant II kit (Macherey-Nagel) was applied. Both of the named kits contain a RNAse A treatment step, which was not included in the kit applied in the present thesis work. If the gDNA stocks were containing RNA and DNA the actual amount of DNA was lower than the measured values, this would however lead to lower peaks in all the replicates of standards as they were derived from the same gDNA stock and the ratio of the peak areas would have stayed the same. However if the RNA content of the later analyzed samples would have varied the standards were useless.

An alternative explanation could be that there were organic solvents or salts contaminating the gDNA stocks. This compounds can inhibit or influence the efficiency and or stringency of the DNA polymerase during the PCR or the annealing of the primers and lead to varying results as they influence the different primers differently (Malke, 1990). The kit applied in the thesis work might not have worked as

sufficiently to remove such compounds in the purification steps. Improvement can be probably achieved by an additional purification step of the gDNA extracts.

4.3 Modelling with trait averages of a population

The adapted simple Lotka-Volterra interaction model applied in this thesis work aided the laboratory experiments but was not adequate to show the interaction of three species. The growth and interaction of two species could be predicted sufficiently as figure 19 (supplements) show, but it did not cover the complexity of the interactions in the three-species cultures. The model was implemented by the assumption that *A. ostenfeldii* has a neutral effect on the competitor species but the results showed that it was clearly not the case. The developed model also predicted much higher cell concentrations for all species than actually observed, most likely because trait averages were set in. The applied model was especially insufficient to resolve the lytic activity of the Dutch *A. ostenfeldii* strains as no parameter was incorporated describing the allelopathy. An improvement could be achieved by the implementation of allelopathic terms, as it was suggested by Sole et al. (2005).

Furthermore, the different traits of the *A. ostenfeldii* strains had totally different effects on the two competitor strains. Separations of the different strains / traits in the (improved) model (figure 19) already lead to predictions which were closer to the obtained results. Problems occurred anyhow in the calculation of the final interaction parameters and lead to the assumption that the interaction did not stayed constant over time.

4.3.1 Inter- and Intraspecific facilitation

The significant increase of growth rates and population growth in the mixed strain cultures compared to the single strain cultures indicates an intraspecific facilitation between the strains (Bruno et al. 2003, John et al. 2014). Due to the failure of the asqPCR assay it was not possible to show the impact of the single strains in the strain mixtures on the communities. However the toxin profiles suggest a higher contribution of the Dutch strains on the six strain community (culture I) as this profiles were more similar to the profiles of the single Dutch strains (Figure 17). The Dutch strains with their lytic compounds and/or toxins possibly reduced any competition and grazing pressure and therefore enhanced the performance of the

entire *A. ostenfeldii* population. John et al. (2014) reported that the mixture of a toxic with a non-toxic *A. tamarense* strain lead to grazing protection of the non-toxic strain and in an higher overall population growth.

Additionally it was observed that the cell concentrations of *C. dentata* were increasing if Finnish *A. ostenfeldii* strains were present. This result suggests that the lytic activity of *A. ostenfeldii* has a stronger impact on the non-thecate dinoflagellate *L. fissa* than on the haptophyte. In the previous two-species cultures it was found that the cell concentrations and growth of *C. dentata* were heavily reduced by the presence of the dinoflagellate *L. fissa*. This is an indication for the mixotrophic nutrition of *L. fissa*. In the three species culture the growth of *L. fissa* was impaired by *A. ostenfeldii* and therefore leads to a promotion of the haptophyte *C. dentata*. This result is a demonstration of the interspecific facilitation. The results cannot show, if the Finnish *A. ostenfeldii* strains also feed on *C. dentata*, but the Dutch strains had a negative effect on all co-cultured species, which could explain their fast population extension in the Ouwerkerkse Kreek even if the seed population was just transferred to the creek recently.

4.4 Different effects of grazing by copepods on the populations

The different response of the three algal strains on the grazing pressure shows that *A. ostenfeldii* was the least susceptible species. The highest growth rate was observed for *A. ostenfeldii* in the culture with the copepod. The smaller haptophyte *C. dentata* did not show any response to the grazing. The growth of *L. fissa* was reduced strongest due to the grazing pressure. These results suggest that the copepod preferred the bigger dinoflagellates and did not graze much on the small haptophyte. As the non-toxic *L. fissa* was most affected, the copepod probably was deterred by *A. ostenfeldii*. The culture purely consisting of *A. ostenfeldii* validates this assumption as no decrease in cell numbers was observed for this culture. The increased population growth observed for this culture in comparison to the control might have resulted from bacterial input as food for the mixotroph alga. The *Acartia tonsa* specimens were not treated with antibiotics beforehand so bacteria were probably transferred with them to the algal cultures. Although no bacteria were observed microscopically in these cultures with the Dutch strains there were plenty in the other cultures after the addition of the copepods.

If the copepods were still viable after the exposure to *A. ostenfeldii* was not determined, but reduced cell numbers of *L. fissa* prove their grazing at least for the cultures without any Dutch strains. Sopanen et al. (2011) demonstrated a clearly negative effect of Finnish strains on two co-occurring copepod species. The presence of Finnish *A. ostenfeldii* in the three species culture might have even supported the competitors. The results achieved did not prove this hypothesis, as the initial cell numbers of the competitors in the cultures was different.

4.5 Mixed cultures vs. bloom conditions

Blooms of *A. ostenfeldii* are usually observed in the summer month, when the temperatures are rising. During this time the water becomes often stratified, especially in smaller water bodies due to the rising surface water temperatures. As dinoflagellates are favored by such conditions and the amount of grazer in the water column decreases with decreasing prey of the spring bloom, they start to rise (Brandenburg et al. 2017). Compared to co-occurring microalgae *A. ostenfeldii* has several advantages – it is mixotrophic and can take up smaller cells or particulate organic matter (Jacobson and Anderson 1996). Additionally *A. ostenfeldii* is able to produce certain allelochemicals and phycotoxins, which can help to reduce grazing as well as competition pressure (Tillmann et al. 2007, Sopanen et al. 2011, Hakanen et al. 2014). The present thesis work showed that the highly toxic Dutch population of *A. ostenfeldii* was least affected by the competition or grazing pressure. The Dutch population, represented by three selected strains, was able to reach higher cell numbers than the Finnish population. This observation fits the current literature, as the booms observed in the Netherland had up to 4.5 * 10^3 cells * mL^{-1}, whereas from blooms in the Baltic Sea just cell concentrations of 10^3 cells * mL^{-1} were reported for *A. ostenfeldii* (Van de Waal et al. 2015, Kremp et al. 2009).

The culture conditions in the thesis work may resemble only some conditions within a natural bloom. The physical factors provided by the culture conditions were chosen to fit natural bloom conditions best. In the Ouwerkerkse Kreek the temperature was usually above 15 °C during the bloom periods (Brandenburg et al. 2017). Therefore 17 °C were provided for cultures in this thesis. The algal isolates were adapted to a salinity of 10. *Alexandrium ostenfeldii* has a high salinity tolerance and grew at salinities ranging from 6 to 34, but the highest growth rates were observed at

salinities of from 10 to 22 (Martens et al, 2016). Higher PSP and cyclic imine toxin contents were found at a salinity of 10 (Martens et al. 2016). For Finnish strains best growth conditions were between salinities of 6 and 10 (Kremp et al. 2009). The cultures were not shaken or incubated on a plankton wheel, because the highest cell densities in the Ouwerkerkse Kreek were observed at reduced wind speeds and precipitations, which resulted in a stable water column (Brandenburg et al. 2017). Furthermore, species of the genus *Alexandrium* are prone to turbulence and form temporary cysts under minimal disturbance (Bolli et al. 2007, Cembella et al. 2000).

The biological environment of the *A. ostenfeldii* blooms was represented by two algal competitors and a grazer. The competitors were isolated from the same habitat as *A. ostenfeldii* and the copepod *Acartia tonsa* co-occurs with it in the Baltic Sea and in the North Sea (Holste and Peck 2006; Fransz et al. 1991). Under natural conditions *Acartia tonsa* has a variety of prey, with preferred particle sizes of 7 – 250 µm (Berggreen et al. 1988). The three algal species lay within this range and represented possible food. The bacterial abundance during the blooms was not taken into account. In comparison to the bloom condition this can really make a difference as both dinoflagellates *A. ostenfeldii* and *L. fissa* were reported to be mixotrophic. Bacteria for the cultures are however hard to select and their fast adaption to different conditions difficult to predict. Furthermore they can be potential parasites or pathogens of the algae and harm them. The chosen condition mirrored the natural bloom conditions as good as possible hence the results of this thesis can be assigned to natural populations of *A. ostenfeldii* with slight chances and help to understand the inter and intraspecific interactions in such a bloom better.

5 Conclusion and Outlook

The work of this thesis could show that *A. ostenfeldii* possess population traits that vary massively between strains from different origins. A high genetic variability like it was reported for other algal strains was however not detected within the populations. Especially the Dutch strains showed a relatively low genetic variability and very similar toxin profiles, which implies that the population developed from a small seed population. The geographical origin of the population in the Ouwerkerkse Kreek was not resolved, as it would include the genetic analysis of several populations of *A. ostenfeldii*.

Despite the high similarities within the Dutch population the strains seemed to facilitate each other under competition and grazing pressure and enhanced the overall population performance. These results indicate the importance of intraspecific facilitation in the initiation of a bloom. The asqPCR that was previously proven to be a sufficient method for the differentiations of strains in a strain mixture (John et al. 2016), was not working in these thesis. The failure of the calibration due to contaminated gDNA stocks will be easily solved by another purification step e.g. with an RNase A treatment and an additional size exclusion chromatography.

Results from the asqPCR could validate whether the growth of the Finnish strains in the six strain mixture was supported by the Dutch strains or not. Furthermore it will clarify if the ratio of the Dutch strains was like the toxin profiles suggest in fact higher in the six strain population.

The results of the two-species cultures showed that the cell concentrations of the small haptophyte *C. dentata* were heavily reduced by the dinoflagellate *L. fissa*. In culture with the Finnish *A. ostenfeldii* strains the growth of *C. dentata* was probably supported due to the lytic activity of *A. ostenfeldii* on *L. fissa*. The interaction of *A. ostenfeldii* and *C. dentata* is an example for intraspecific facilitation of the dinoflagellate. This positive effect should be validated with a mixed culture of *A. ostenfeldi*i and the haptophyte *C. dentata*. This experiment could reveal, if there is indeed a positive effect of the Finnish *A. ostenfeldii* strains on the haptophyte or if the dinoflagellate actually feeds on the smaller algal.

The results from the grazing experiment with *Acartia tons*a showed a negative effect on *L. fissa* but not on *A. ostenfeldii*. As the population of the mixed strain culture

performed even better with the copepods it is proposed that the bacteria transferred with the copepods served as food for the mixotrophic dinoflagellate. Testing this hypothesis could be done by the incubation of *A. ostenfeldii* with and without unfiltered supernatant of the copepods. The cell count will show if there is an increased population growth due to the bacteria of the copepod culture.

The possible deterrence or incapacitation of the grazer *Acartia tonsa* by *A. ostenfeldii* was not analyzed in this thesis. The effect of the different algal species and strains on the copepods should be compared with the aid of exposure experiments as they were performed by Sopanen et al. (2011).

The results from the supposed experiments and the result of this thesis together will be a helpful source of information for an improved modelling of *A. ostenfeldii* populations. The current result already prove, that a mixture of different strains does not act as their respective average and that different traits in the population can have totally different effects on the community. If the analyzed populations possess a higher variety of different traits the intraspecific facilitation might be even more pronounced than it was shown in this thesis work.

The result could be as well applied for other species of the genus *Alexandrium* and therefore help to predict HABs of this genus better. In general it should be taken into account for ecological modeling that the observation can differ strongly from the predictions, if intra and interspecific facilitation is not taken into account.

6 References

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7 Supplements

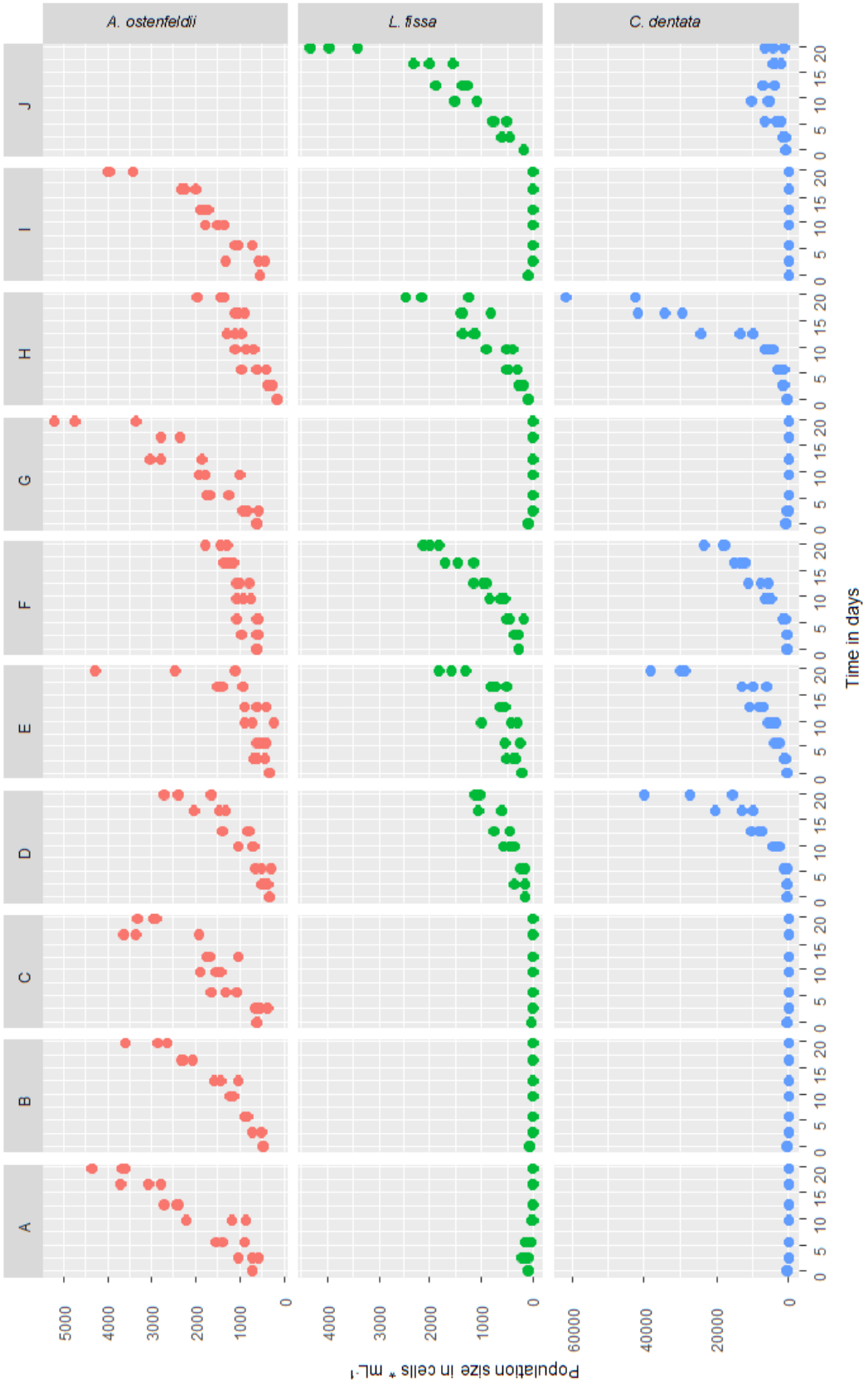

Figure 18: Observed cell concentrations of the three species cultures in the competition experiment

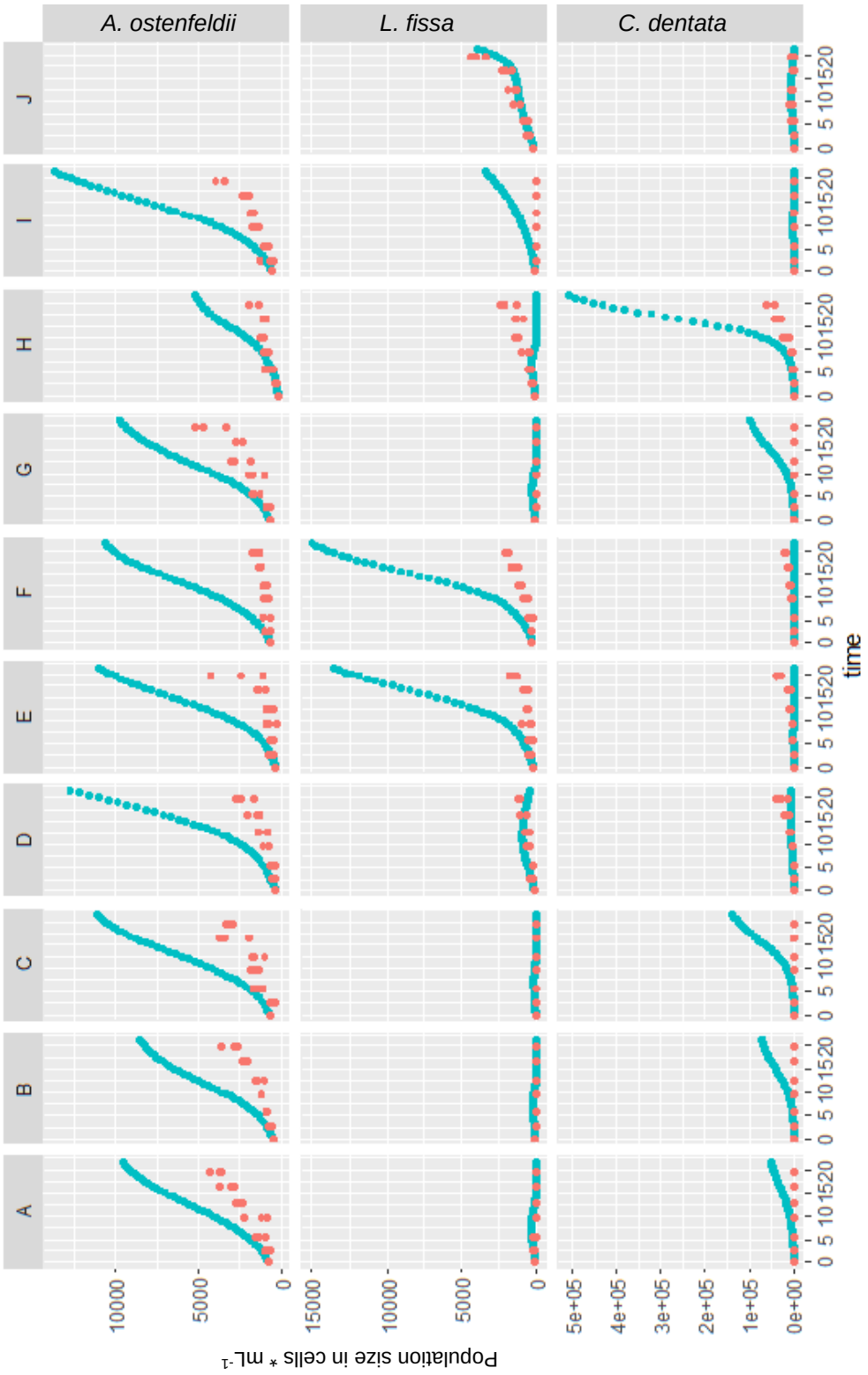

Figure 19: Cell concentrations of the three species cultures in the competition experiment – observed vs. predicted

red dots show the cell concentrations that were actually observed during the competition experiments, turquoise dots show the cell concentrations with the adapted three species model

8 Avowal of independently writing

I hereby confirm that I have written the present thesis independently and that I did not use more than the indicated materials and sources. Furthermore, it is confirmed that this thesis nor any parts of it, were ever part of an exam before.

Date, Place	Signature
14th of March 2017, Bremen	