

**Characterization of grazer-induced
responses in the marine dinoflagellate
*Alexandrium tamarense***

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Hiermit erkläre ich, Sylke Wohlrab, dass ich die Arbeit mit dem Titel:

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Summary

Harmful algal blooms (HABs) have increased worldwide over the last several decades. The characterization of processes that promote the ecological success of toxic algae species that form such blooms has therefore gained immense importance. The HAB-dinoflagellate *Alexandrium tamarense* causes outbreaks of Paralytic Shellfish Poisoning (PSP) due to the synthesis of the highly neurotoxic alkaloid saxitoxin and several of its analogues (Paralytic Shellfish Toxins, PSTs). While the ecological function of PST production is still unknown and their allelochemical nature is mainly supposed due to the negative effects they have on some copepod species, there are other unknown secondary metabolites produced by *A. tamarense* which are definitely known to possess allelochemical properties. These compounds are distinct from PSTs and have a lytic effect on co-occurring competitors and protistan grazers. The investigations in this thesis focus on the analysis of mechanisms that promote the success in grazer interactions related to secondary metabolite synthesis in *A. tamarense*. The implementation of functional genomic tools enabled a detailed characterization of processes that are induced due to the presence of grazers in *A. tamarense*. Such induced processes that either decrease grazing pressure and/or provide an advantage over co-occurring species in the presence of grazers can strongly promote a species' success.

Within this thesis work an increase in PST content due to the presence of copepods and their cues was demonstrated to occur in *A. tamarense*. Behavioral investigations on the fitness of the copepods and the analysis of gene expression patterns in *A. tamarense* showed that toxin production only increases when the copepod represents a threat in terms of grazing pressure. Co-evolution therefore seems to have driven the ability of *A. tamarense* to recognize threatening grazers based on their cues and in turn drove selection for less susceptible grazers. The results further show that the increased toxin synthesis in the presence of cues from copepods is accompanied by a reduction in cell-chain length in *A. tamarense* and that both responses are reversible. Hence the inducing cue is well suited to track ambient grazer concentrations. In addition, the response of *A. tamarense* towards this cue appeared to be more complex than previously thought, i.e. they comprise trait changes at the morphological and physiological level. It was further demonstrated that this induced defense response against copepods is realized in

different ways in two *A. tamarensis* strains. Hundreds of genes were differentially expressed in response to trace amount of copepod cues, with a large discrepancy between the two *A. tamarensis* strains tested. Furthermore, the two genotypes seem to express different target-defended phenotypes that are, however, both able to enhance defense against copepods, but with varying costs and efforts as inferred from the transcriptomic analysis. As a consequence, the induced defense response implies different trade-offs for each *A. tamarensis* strain investigated. A further discrepancy was observed for the physiological realization of the increased PST synthesis, which points towards a convoluted regulation of toxin synthesis in *A. tamarensis*.

Transcriptomic analysis of the response of *A. tamarensis* towards a protistan grazer (*Polykrikos kofoidii*, Dinophyceae) showed that a large number of genes are associated with this species interaction. Lytic compound production seems therefore to be only one of several traits that promote the success of *A. tamarensis* in the presence of such grazers. Endocytic processes, outer-membrane properties and cell cycle progression might be influenced additionally due to the presence of a protistan grazer. The investigated differences in the gene expression of the lytic and non-lytic strain without grazers points towards a maintenance of high allelic variation in natural *A. tamarensis* populations perhaps as a consequence of large scale gene duplications.

The transcriptional analysis of the grazer-induced responses provided the basis for the successive establishment of a gene library derived from a high-throughput sequencing platform. The annotation of the final data indicated a comparable high abundance of genes involved in signal transduction processes and secondary metabolite biosynthesis, affirming that species interactions might have a major influence on the selection of genomic features.

In conclusion, the fact that co-occurring grazers have the potential to influence several traits in *A. tamarensis* indicates that a sophisticated range of responses that enable *A. tamarensis* strains to succeed under diverse grazing pressure has been selected for through evolution. These multifaceted grazer-induced responses may have further consequences on the community structure that go beyond simple ingestion and thus loss of cell cohorts from the population. Investigation of co-evolutionary driven adaptations in addition to grazer behavior as well as mesocosm studies might contribute substantially to the ongoing search for

conceptual models to describe the formation and development of harmful algal blooms.

Zusammenfassung

Schädliche Algenblüten (Harmful algal blooms, HABs) haben in den letzten Jahrzehnten weltweit zugenommen. Die Charakterisierung von Prozessen, die den ökologischen Erfolg schädlicher Algenarten fördern, wurde daher zu einem Thema von zunehmender Bedeutung. Der HAB-Dinoflagellate *Alexandrium tamarense* verursacht weltweit Ausbrüche des sogenannten „Paralytic Shellfish Poisoning“ (PSP) durch die Synthese des stark neurotoxischen Alkaloids Saxitoxin und dessen Molekülanaloge (Paralytic Shellfish Toxins, PSTs). Während die ökologische Relevanz der PST-Synthese immer noch rätselhaft ist und deren allelochemische Eigenschaften sich hauptsächlich durch negative Auswirkungen auf einige Copepoden-Arten äußern, sind weitere, von *A. tamarense* produzierte unbekannte Sekundärmetabolite, eindeutig allelochemisch aktiv. Diese Substanzen lassen sich deutlich von den PSTs abgrenzen und wirken lytisch auf co-existierende Konkurrenten und protistische Prädatoren. Die Untersuchungen in dieser Arbeit konzentrieren sich auf die Analyse von Mechanismen die im Zusammenhang mit der Synthese von Sekundärmetaboliten stehen und somit möglicherweise den Erfolg in Prädator-Interaktionen unterstützen. Die Implementierung funktioneller genomischer Methoden erlaubte eine detaillierte Beschreibung von Prozessen, die durch die Anwesenheit eines Prädatoren in *A. tamarense* induziert werden. Solche induzierten Prozesse die zu einer Reduktion des Fraßdrucks oder eventuell zu einem Vorteil gegenüber co-existierenden Arten in der Anwesenheit von Prädatoren führen, können wesentlich zum Erfolg einer Art beitragen.

In dieser Arbeit konnte demonstriert werden, dass die Anwesenheit von Copepoden und deren Botenstoffe eine Erhöhung des PST-Gehaltes in *A. tamarense* erzeugen. Verhaltensbeobachtungen bezüglich der Fitness der Copepoden sowie eine Analyse der Genexpressionsmuster in *A. tamarense* zeigten, dass die Toxinproduktion nur dann zunimmt, wenn die Copepoden eine Gefahr hinsichtlich des Fraßdrucks darstellen. Co-evolution führte möglicherweise zu der Fähigkeit *A. tamarenses* gefährliche Prädatoren an ihren Botenstoffen zu erkennen und im Gegenzug zu einer Selektion weniger sensibler Prädatoren. Des Weiteren zeigen die Ergebnisse in dieser Arbeit, dass die Zunahme der Toxinsynthese in Gegenwart der Botenstoffe der Prädatoren von einer Reduktion in der Zellkettenlänge in *A. tamarense* begleitet wird und, dass beide Reaktionen reversibel sind. Daher ist der

induzierende Botenstoff sehr gut dazu geeignet, die umgebende Dichte an Prädatoren anzuzeigen. Die Reaktion von *A. tamarensis* auf diesen Botenstoff scheint zudem komplexer zu sein als bisher angenommen und umfasst Veränderungen der Morphologie und Physiologie. Des Weiteren konnte ich zeigen, dass diese induzierte Verteidigungsreaktion gegen Copepoden in zwei *A. tamarensis* Zelllinien unterschiedlich verwirklicht wird. Hunderte an Genen waren unterschiedlich reguliert als Reaktion auf Spuren von Copepoden-Botenstoffen und es zeigte sich eine große Diskrepanz diesbezüglich bei den beiden getesteten *A. tamarensis* Zelllinien. Zudem scheint es, als ob diese beiden Genotypen unterschiedlich zielgerichtet-verteidigte Phänotypen darstellen, welche jedoch beide ihre Verteidigung gegenüber Copepoden verstärken können. Die Transkriptions-Analysen zeigten, dass dies für den jeweiligen Genotyp mit unterschiedlichen Kosten und Aufwand verbunden ist. Demzufolge kann die induzierte Verteidigungsreaktion unterschiedliche Kosten-Nutzen-Kompromisse für die jeweilige *A. tamarensis* Zelllinie beinhalten. Ein weiterer Unterschied hat sich in der physiologischen Umsetzung der erhöhten PST-Synthese gezeigt, was auf eine verschachtelte Regulation der Toxinsynthese in *A. tamarensis* hindeutet.

Die Transkriptionsanalysen, die der Reaktion von *A. tamarensis* auf einen Protisten-Prädatoren (*Polykrikos kofoidii*, Dinophyceae) zugrunde liegen, zeigten, dass eine hohe Anzahl von Genen mit dieser Interaktion verbunden ist. Die Produktion lytischer Substanzen ist demnach möglicherweise nur eine von vielen Eigenschaften, die den Erfolg *A. tamarensis* in der Gegenwart solcher Prädatoren fördert. Endozytische Prozesse, Eigenschaften der äußeren Zellmembran sowie das Fortschreiten des Zellzyklus, werden wahrscheinlich durch die Gegenwart des Protisten-Prädatoren beeinflusst. Die beobachteten Unterschiede in der Genexpression zwischen der lytischen und nicht-lytischen Zelllinie von *A. tamarensis* deuten auf eine Aufrechterhaltung einer hohen Variation an Allelen in natürlichen *A. tamarensis* Populationen hin, welche möglicherweise das Resultat umfangreicher Genduplikationen sind.

Die Transkriptionsanalysen der Prädatoren-induzierten Reaktionen stellten die Grundlage für eine sukzessive Erstellung einer Gen-Bank aus einer Hochdurchsatz-Sequenzierungsplattform dar. Die Annotation der finalen Daten zeigte eine vergleichsweise hohe Abundanz an Genen die in der Verarbeitung von Signalen und der Biosynthese von Sekundärmetaboliten beteiligt sind und bekräftigt somit, dass Interaktion zwischen Arten einen großen Einfluss auf die Selektion an Funktionen im Genom hat.

Schlussfolgernd kann gesagt werden, dass co-existierende Prädatoren das Potential haben mehrere Eigenschaften in *A. tamarensis* zu verändern und dies impliziert, dass die Evolution eine Reihe von hochentwickelten Reaktionen selektiert hat, welche den *A. tamarensis* Zelllinien Erfolg unter verschiedenen Fraßdrücken gewähren. Diese vielfältigen Prädatator-induzierten Reaktionen können zusätzliche Konsequenzen für die Struktur der Gemeinschaft haben, die weiterreichender sind als nur die durch den Konsum verursachten Verluste von Zell-Kohorten aus der Population. Untersuchungen von co-evolutiv beeinflussten Adaptionen und zusätzliche Verhaltensbeobachtungen in Prädatoren sowie Mesokosmos-Studien könnten grundlegend zu der anhaltenden Suche nach Konzepten beitragen, die die Formation und Entwicklung von schädlichen Algenblüten beschreiben.

Abbreviations

ANOVA	Analysis of variance
ASP	Amnesic Shellfish Poisoning
ATP	Adenosine triphosphate
B1	n-sulfocarbamoyl PST
BLAST	Basic Local Alignment Search Tool
C1/C2	n-sulfocarbamoyl PST
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CDPK(s)	Calcium-dependent protein kinase(s)
CFP	Ciguatera Fish Poisoning
cNMP	Cyclic nucleotide monophosphate
cRNA	Complementary RNA
CTPs	Cytidine triphosphate
Cy-3	Cyanine-3
Cy-5	Cyanine-5
DAG	Diacylglycerol
DAGK	Diacylglycerol kinase
dcGTX2/3	Decarbamoyl gonyautoxins 2 & 3
dcNEO	Decarbamoyl neosaxitoxin
dcSTX	Decarbamoyl saxitoxin
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
DSP	Diarrhetic Shellfish Poisoning
dw	Dry weight
EBI	European Bioinformatics Institute
ESTs	Expressed sequence tags
FC	Fold change
FLD	Fluorescence detector
G x E	genotype-by-environment
GESA	Gene Set Enrichment Analysis
GF/F	Glass fiber filter
GO	Gene Ontology
GTX1/4	Gonyautoxins 1 & 4
GTX2/3	Gonyautoxins 2 & 3
GUMACC	Gothenburg University Marine Culture Collection
HAB/HABs	Harmful Algal Bloom(s)
HCl	Hydrochloric acid

HLPs	Histone-like proteins
HMM	Hidden Markov Model
HPLC	High performance liquid chromatography
KAAS	KEGG Automatic Annotation Server
KEGG	The Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Orthology
KOG	Eukaryotic orthologous groups
LC-FD	High-performance liquid chromatography with fluorescence detection
LSU	Large ribosomal subunit
MAPK(s)	Mitogen-activated protein kinase(s)
MAPKK(s)	Mitogen-activated protein kinase kinase(s)
Mb	Megabases
MIAME	Minimum Information About a Microarray Experiment
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NEO	Neosaxitoxin
NGS	Next-generation sequencing
NSP	Neurotoxic Shellfish Poisoning
OD	Optical density
PA	Phosphatidic acid
PCR	Polymerase chain reaction
PEEK capillary	Polyether ether ketone capillary
PFD	Proton flux density
PKA	Protein kinase A
PKS	Polyketide synthase
PSP	Paralytic Shellfish Poisoning
PST(s)	Paralytic Shellfish Toxin(s)
qPCR	Quantitative PCR
rDNA	Ribosomal DNA
RM	Repeated measure
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RP	reverse phase
RP-genes	Ribosomal protein-genes
RT	Reverse transcription
SD	Standard deviation
SL	Spliced Leader
ss-cDNA	Single stranded complementary DNA
STX	Saxitoxin
THF	Tetryhydrofuran

1. Introduction

1.1 Species Interactions

Interactions between species play a central role in the evolution and diversification of life (Thompson 1999). Many contemporary consequences of species interactions are so pervasive that their contribution to the Earth's biodiversity is easily overlooked: e.g., mitochondria and the emergence of the eukaryotic cells, chloroplast that gave rise to all plants, dinoflagellates and the development of coral reefs, lichens and terrestrial plant succession, gut bacteria and animal digestion (Thompson 1999). Contrary to a species' adaptation to its physical environment, adaptation to another species can drive reciprocal and rapid evolutionary responses that result in natural selection for co-evolved species. The theory that species interactions may indeed contribute primarily to evolution rather than adaptations to the environment was first put forward in the 1970s by Van Valens' "Red Queen Hypothesis" (Van Valen 1973). With the quote from Alice in Wonderland (Lewis Carroll) "*It takes all the running you can do, to keep in the same place*" he illustrated that any species must continually evolve to survive, because other species are constantly evolving (Van Valen 1973, Liow et al. 2011). However, it was only in 2010 that scientists experimentally verified Van Valens hypothesis by demonstrating that most natural selection will arise from co-evolutionary species interactions (Paterson et al. 2010).

The world's oceans are the point of origin of the contemporary biodiversity and the habitat of various photosynthetic microorganisms, collectively termed "Phytoplankton". Around 45% of the annual oxygen production and carbon fixation arises from these microscopic organisms though they only account for less than 1% of the Earth's photosynthetic biomass (Field et al. 1998, Falkowski et al. 2004). This ratio of a small amount of biomass that converts large amounts of carbon is the result of an average weekly turn-over rate whereas the average turn-over rate of land plants is 10 years (Falkowski et al. 1998, Finazzi et al. 2010). High mortality rates due to predation, viral and parasitic attack, autocatalysed cell death or sinking in the ocean interior cause these high turn-over rate in the phytoplankton. Consequently, adaptations that promote a species survival in such a "mortality environment" are strongly driven by species-interactions (Smetacek et al. 2004). Co-

evolution, though still poorly investigated in phytoplankton, hence will have a strong influence on a species ability to thrive in its environment (Hamm & Smetacek 2007).

1.2 Top-down and bottom-up control

The growth rate of a phytoplankton population hence is expressed in the species' ability to gather resources and avoid becoming a resource itself. Solar radiation that drives photosynthesis and essential nutrient availability are the two main factors controlling phytoplankton production in the sea (Lalli & Parsons 1997). Together with temperature, salinity and surface currents, they make up the *abiotic* world of a phytoplanktonic organism and as such the “bottom-up” factors that influence intrinsic controlled species specific growth rates in the euphotic zone (Lalli & Parsons 1997, Reynolds 2006). The bottom-up factors hence determine a species gross growth rate. Opposed to this are “top-down” factors, controlling phytoplankton growth that arises from the *biotic* environment. Such “top-down” factors include competition for nutrients and lights and particularly the losses due to predation, parasite and viral attack. Those loss factors hence reduce a species' gross growth rate and, together with advective inputs, define the population net growth rate (Fig. 1.1).

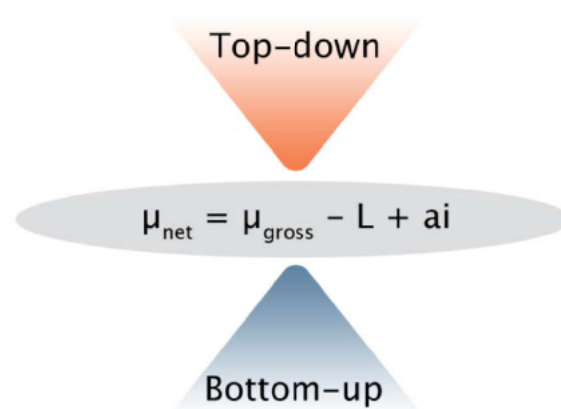


Fig.1.1: Influence of bottom-up and top-down factors to the net population growth rate for any phytoplankton species. μ_{net} = net growth rate; μ_{gross} = gross growth rate; L = losses (including top-down processes, respiration, sinking and advection); a_i = advective inputs (Thompson et al. 2008).

Both, bottom-up and top-down control lead to the evolution of a vast array of different adaptations to minimize constraints and losses arising from those processes. Adaptations are however often limited by trade-offs, act reciprocally and feedback on other species, resulting in the selection of co-evolved species and hence leading to different evolutionary trajectories. The contemporary species composition in the phytoplankton mirrors such attempts to adapt by a variety of morphologically and physiologically differing species (Smetacek et al. 2004, Hamm & Smetacek 2007).

1.3 Harmful algal blooms

An algal “bloom” in general is the result of an increase towards a high concentration, or a higher concentration compared to the background distribution of cells of any species within the phytoplankton community. As such, the effects of bottom-up and top-down processes are reduced for specific species, creating favorable conditions for an optimal growth that exceeds losses (Thompson et al. 2008). Increased net growth rates finally result in the occasional dominance of a particular species (monospecific blooms), a strong increase in the species abundance compared to the average abundance without dominance, or an increase of a group of species (Masó & Garcés 2006). Such blooms of particular species can be annually re-occurring e.g. the diatom spring-blooms, and provide the basis of the food web to feed the various marine life forms. Other blooms are exceptional events reflecting an unpredictable temporal and spatial distribution e.g. various dinoflagellate blooms (Smayda & Reynolds 2003).

A phytoplankton bloom is considered to be a “Harmful Algal Bloom” (HAB) if it impairs human health, socioeconomic interests or components of aquatic ecosystems (Anderson et al. 2012b). The term “HAB” designates therefore more a societal concept than a scientific definition and covers a broad spectrum of biological phenomena usually caused by phytoplanktonic microalgae. Yet, benthic microalgae and non-photosynthetic species that do not typically “bloom” but are able to cause harm are also counted as HAB taxa (Landsberg 2002). In addition, the term has also been applied to a harmful mass occurrence of seaweed (macroalgae) (Anderson et al. 2012b). Harmful algal blooms affect virtually every coastal region of the world and are commonly referred to as “Red Tides” due to the potential of some species to discolor the water. Among the ~5.000 species of marine phytoplankton there are at least 300 species which can occur in such high densities to discolor the

water, while only ~80 species have the potential to produce toxic or noxious substances and most of these are dinoflagellates (Hallegraeff 2003). In general, three different types of HAB species can be distinguished for marine habitats based on their detrimental effects (Hallegraeff 2003):

- I. Species that cause water discolorations, slime or foam and therefore deter tourism and recreational activities. Under certain circumstances such blooms can grow so dense that they cause indiscriminate kills of fish and invertebrates through oxygen depletion.
- II. Species that produce potent toxins that can accumulate through the food web and cause intoxication of humans. The toxins produced by these species are classified with reference to the human toxin syndrome they cause (Cembella 2003, Falconer 1993):
 - Paralytic Shellfish Poisoning (PSP); caused by marine dinoflagellates.
 - Diarrhetic Shellfish Poisoning (DSP); caused by marine dinoflagellates.
 - Amnesic Shellfish Poisoning (ASP); caused by marine diatoms.
 - Ciguatera Fish Poisoning (CFP); caused by marine dinoflagellates.
 - Neurotoxic Shellfish Poisoning (NSP); caused by marine dinoflagellates.
 - Cyanobacterial toxin poisoning; caused by marine/brackish cyanobacteria.
 - Estuarine associated syndrome; caused by aerosols from marine dinoflagellates.
- III. Species that are harmful to fish and invertebrates by damaging or clogging their gills and thus disrupt the oxygen exchange mechanisms at the respiratory membranes. They are, however, not assumed to be toxic to humans.

The occurrence of harmful algal blooms are completely natural phenomena which have occurred throughout recorded history. However, there is now a general scientific consensus that HABs have globally increased in frequency, magnitude and geographic extent over the past few decades (Anderson 1989, Hallegraeff 1993, 2003, Anderson et al. 2012b). This increase is attributed to both an apparent increase due to growing scientific awareness of toxic species, as well as increased utilization of coastal waters for aquaculture; and a “true” increase due to the stimulation of phytoplankton blooms by cultural eutrophication, unusual

climatological conditions, the transport of species via ships' ballastwater or due to the movement of shellfish stocks (Hallegraeff 1993, 2003).

1.4 Dinoflagellates

Marine dinoflagellates are the primary culprits in harmful algal bloom formation due to the production of toxins with a global impact on human health and their negative impact on ecosystem- functioning and service (Kellmann et al. 2010), i.e. 5 out of the 7 classical seafood poisoning syndromes described above are caused by dinoflagellates. Dinoflagellates are a unique group of eukaryotes with various features that deviate from the "conventional view" of cellular mechanisms and are therefore worth mentioning in terms of their biology and not only due to their ability to produce a wide array of toxins:

Dinoflagellates are an ancient group of organisms that emerged in the early Neoproterozoic (Escalante & Ayala 1996, Baldauf 2000), ~900 million years ago, spanning two supercontinental cycles. With the development of shallow continental shelves in the Mesozoic (~150 Ma ago) the dinoflagellates experienced a rapid radiation and most of the lineages have persisted through to the present day (Fensome et al. 1996, Taylor 2004). Dinoflagellates have evolved diverse lifestyles ranging from free-living benthic or planktonic species within marine and fresh waters to symbiotic and parasitic species that live within the tissue, cytoplasm or nucleoplasm of other organisms (Taylor 1980, Hackett & Anderson 2004). The great majority of the present dinoflagellates are marine planktonic species (Taylor 2004) and even though they are microscopic, we become aware of them due to their ability to discolor the water and/or bioluminescent within the water when mechanically stimulated (Fig. 1.2). Marine planktonic dinoflagellates comprise species capable of photosynthesis as well as heterotrophs to an approximate equal amount (Taylor 2004), whereas many photosynthetic species are not purely autotroph and can act as facultative mixotrophs (Jeong et al. 2005, 2010). The photosynthetic dinoflagellates are distributed worldwide and are the second most abundant group in the phytoplankton following diatoms (Lalli & Parsons 1997).



Fig. 1.2: Left: bioluminescence caused by dinoflagellates due to mechanical stimulation (©Ned Potter, ABC News, New York). Right: "Red Tide" algae streaks in Alaska waters (©Don Pitcher, Alaska Stock Images/National Geographic Stock).

Heterotrophic dinoflagellates, as their photosynthetic counterpart, contribute worldwide and significantly to the zooplankton, where they even sometimes dominate marine micrograzer communities (Tillmann & Hesse 1998, Levinsen & Nielsen 2002, Yang et al. 2004, Vargas & Martínez 2009). Diverse feeding modes are observed among those heterotrophic dinoflagellates: phagotrophic dinoflagellates engulf whole cells and nematocysts can be used to capture them, peduncles (thin, tube-like extensions) are deployed to penetrate prey and withdraw the contents, a feeding veil or a pallium can be extruded from the cell and envelopes the prey item that is digested within (Jacobson & Anderson 1992, Hackett & Anderson 2004, Lee et al. 2008).

Dinoflagellate genomes are among the largest of any organisms ranging from 3-250 pg DNA per cell (Spector 1984), corresponding to approximately 3,000 – 250,000 Mb for a haploid cell (in comparison, the haploid human genome is 3,180 Mb) (Hackett & Anderson 2004). In accord with their large genome size are the numbers of chromosomes that range from 4 to 200 in a haploid cell depending on the species (Bhaud & Guillebault 2000). These chromosomes are morphologically similar to each other (Loeblich 1976) and remain permanently condensed throughout the cell cycle (Dodge 1964, Hackett & Anderson 2004). The chromosome structure appears to be the result of DNA self-assembly into liquid crystals (Livolant & Bouligand 1978, Gautier et al. 1986, Bouligand & Norris 2001). Loops of DNA extend from the condensed liquid-crystalline chromosomes and are stabilized with dinoflagellate specific histone-like proteins (HLPs) (Sala-Rovira et al. 1991, Chan & Wong 2007). These loops are either condensed or open, depending on the amount of

associated HLPs and are thought to be the place where gene transcription takes place since the interior of the chromosomes is likely too dense for that (Sigee 1983). Dinoflagellates are also the only eukaryotes where a large proportion of the DNA base thymine (up to 70%) has been replaced by hydroxymethyluracil (Rae & Steele 1978). This base normally occurs in eukaryotes only as the result of oxidative DNA damage, yet gets quickly repaired, and its role in dinoflagellate nuclear biology remains unknown (Boorstein et al. 1989, Wisecaver & Hackett 2011).

The large genome of dinoflagellates is mainly constituted of repetitive and non-coding DNA with an estimated gene coding percentage of 1.8% for the smallest, and 0.05% for the largest documented genome (Hou & Lin 2009, Jaeckisch et al. 2011). Thus, the numbers of genes are estimated to lie between ~38,000 to ~88,000, depending on the genome size (Hou & Lin 2009), whereas by comparison the human genome is considered to have ~25,000 genes (Southan 2004). The large gene content of dinoflagellates does not, however, necessarily account for the number of unique genes producing distinct gene products, as some genes have been shown to occur in tandem arrays encoding identical proteins (Liu & Hastings 2006, Bachvaroff & Place 2008) and are likely a result of successive gene duplications (Zhang & Lin 2003). Gene copy numbers within such arrays range from 30 – 5000 (Hou & Lin 2009) and seem to correlate with the RNA expression level of those genes (Bachvaroff & Place 2008). Intergenic spacer regions between such arrays lack recognizable transcription factor binding sites, hence the expression of the whole array is assumed to be regulated from an upstream promoter (Wisecaver & Hackett 2011). The mRNA of dinoflagellates can therefore be polycistronic as in prokaryotes and kinetoplastids yet differs from the latter two in that it carries multiple copies of a single gene (Bachvaroff & Place 2008, Lukes et al. 2009). A recent study, however, questioned the existence of polycistronic mRNA at least in the dinoflagellate *Lingulodinium* sp. (Beauchemin et al. 2012).

Another feature in dinoflagellates that deviates gene expression from the canonical view is “*trans*-splicing”: every mRNA carries a 5’spliced leader (SL) sequence which is added by *trans*-splicing and might be involved in the resolution of polycistronic mRNAs as well as in mRNA stability and translatability (Maroney et al. 1995, Satou et al. 2006, Palenchar & Bellofatto 2006, Lukes et al. 2009). The spliced leader sequence is conserved across all dinoflagellates and its discovery enabled conducting dinoflagellate-specific metatranscriptomic studies within their natural habitats (Lin et al. 2010). The subsequent discovery of relict and truncated spliced-leader sequences at the 5’end of expressed genes as well as in the genomic

DNA of diverse dinoflagellates pointed towards an astonishing process: expressed and trans-spliced genes can be reverse-transcribed into cDNA and reintegrated into the genome where they can again exhibit the next cycle of expression, trans-splicing and re-integration (Slamovits & Keeling 2008, Jaeckisch et al. 2011).

Taken together, this unusual and large genome of dinoflagellates seems to be a highly dynamic one, permitting large gene duplications, reflected by gene arrays and mRNA recycling events. Gene duplication events are a major source of evolutionary innovation (e.g. Haldane 1933, Muller 1935, Byrne & Blanc 2006, Aravind et al. 2006, Lynch 2007, Conant & Wolfe 2008, Kaessmann 2010) and the dynamic genome of dinoflagellates might therefore be a premise not only for their ability to produce a wide array of secondary metabolites (Kellmann et al. 2010), but more generally speaking, for their evolutionary success.

1.5 The *Alexandrium tamarense* species complex

The genus *Alexandrium* is one of the most prominent HAB genera in terms of potency of its toxins, diversity, and distribution (Fig. 1.3; Anderson et al. 2012a). Toxic or otherwise harmful effects are attributed to at least half of all defined species within this genus (Anderson et al. 2012a). Furthermore, the genus *Alexandrium* shows a toxigenic diversity not found in any other HAB genus with three different families of known toxins being produced – saxitoxins, spirolides, and goniodomins (Anderson et al. 2012a). Saxitoxin is the most significant of these toxins in terms of its impacts and is responsible for outbreaks of paralytic shellfish poisoning (PSP), the most widespread of the HAB-related poisoning syndromes (Fig. 1.3; Anderson et al. 2012a).

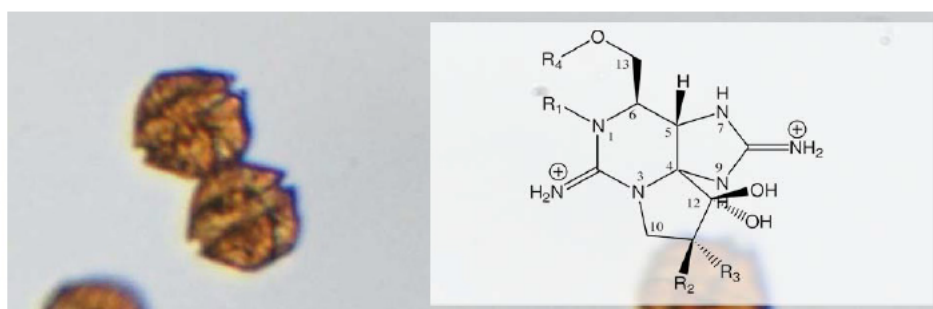


Fig. 1.3: A two cell chain of *Alexandrium tamarense* (left) and the molecular structure of saxitoxin (right). R_x refers to positions where different residuals can be added and form saxitoxin analogues.

Saxitoxin is the basic molecule and all other paralytic shellfish toxins (PSTs) are structural analogs of saxitoxin. Saxitoxin blocks the sodium channels and disrupts the transmission of action impulses in excitable cells causing PSP in humans. PSP is a neurological affliction starting with a prickly feeling in the lips, tongue and extremities followed by numbness. With the further progression of intoxication, it becomes difficult to co-ordinate muscles and depending on the severity, PSP can lead to death due to respiratory paralysis (Schantz 1960, Prakash et al. 1971). The distribution of events where PSTs were detected in fish or shellfish has tremendously increased in the last decades (Fig. 1.4), leading to globally coordinated monitoring programs (Anderson et al. 2012b).



Fig. 1.4: Increase of events from 1970 to 2009 where paralytic shellfish toxins were detected in shellfish or fish (Anderson et al. 2012b).

While saxitoxin can also be produced by other dinoflagellates (*Gymnodinium catenatum* and *Pyrodinium bahamense*) as well as some limnic cyanobacteria (*Anabena* spp., *Aphanizomenon* spp., *Cylindrospermopsis* spp. & *Lyngbya* spp.) their impact on paralytic shellfish poisoning outbreaks lags far behind those caused by *Alexandrium* spp. (Anderson et al. 2012a).

Within the genus *Alexandrium*, the *Alexandrium tamarense* species complex appears to be the most widely dispersed and occurs in many locations worldwide, however, it is largely absent from the equatorial tropics (Lilly et al. 2007, Anderson et al. 2012a). The *Alexandrium tamarense* species complex is a phylogenetically unresolved cluster of three morphotypic species: *A. tamarense*, *A. fundyense* and *A. catenella* (John et al. 2003). Based on the phylogenetic relationships between their large ribosomal subunit rDNA, isolates of the *A. tamarense* species complex cluster

more on the basis of geographic origin than on their morphotaxonomy (Scholin et al. 1995, John et al. 2003). Yet, more recent studies indicate a global distribution for most groups and hence question the concept of geographic ribotype assignment (Fig. 1.5; Lilly et al. 2007, Brosnahan et al. 2010, Anderson et al. 2012a). The ability to produce PSTs, however, seems to be consistent within different clades and is most likely only a characteristic of members in Group I and IV (Fig. 1.5; John et al. 2003, Lilly et al. 2007, Brosnahan et al. 2010).

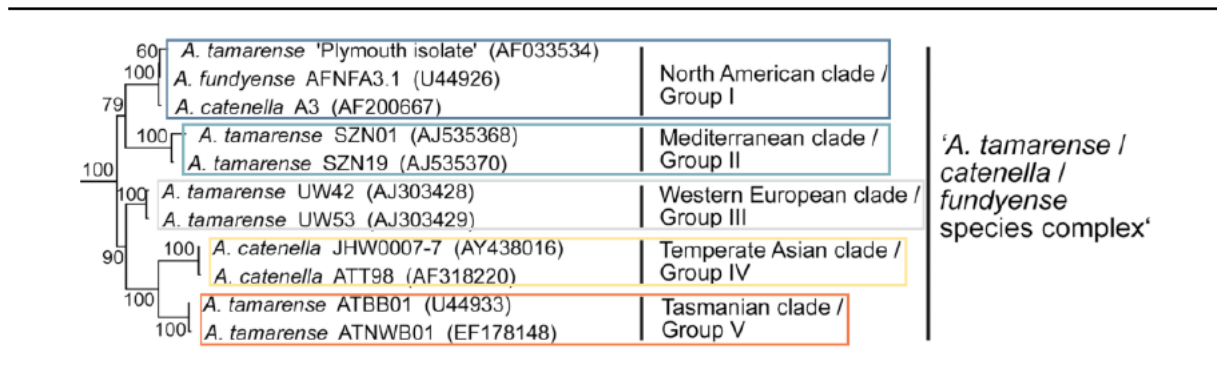


Fig. 1.5: Phylogenetic tree inferred by maximum likelihood analysis of the partial LSU rDNA of species designated to the *A. tamarense* species complex (Anderson et al. 2012a).

Organisms within the *Alexandrium tamarense* species complex maintain a meroplanktonic life cycle with haploid vegetative cells and diploid motile and non-motile zygotes (Fig. 1.6; Wyatt & Jenkinson 1997, Anderson 1998). At the beginning of the annual growth cycle, diploid planomeiocytes hatch from resting hypnozygotes. Meiotic division, followed by mitotic division, subsequently leads to the formation of haploid, vegetative cells that reproduce asexually and form part of the marine phytoplankton (Wyatt & Jenkinson 1997, Anderson 1998). Vegetative cells can also transform into non-motile, pellicle cysts under stressful conditions. Such pellicle cysts can rapidly switch back to the motile stage when conditions improve. At the end of the planktonic population growth phase, and often triggered by “adverse” conditions, mitotic division ceases and gametogenesis is induced (Brosnahan et al. 2010). The resulting non-dividing gametes are competent to conjugate and can fuse to form planozygotes (Brosnahan et al. 2010). Planozygotes can persist for days or weeks before returning to haploid mitotic cell growth or metamorphosing into hypnozygotes (Fig. 1.6; Pfiester & Anderson 1987, Figueroa & Bravo 2005, Figueroa et al. 2006).

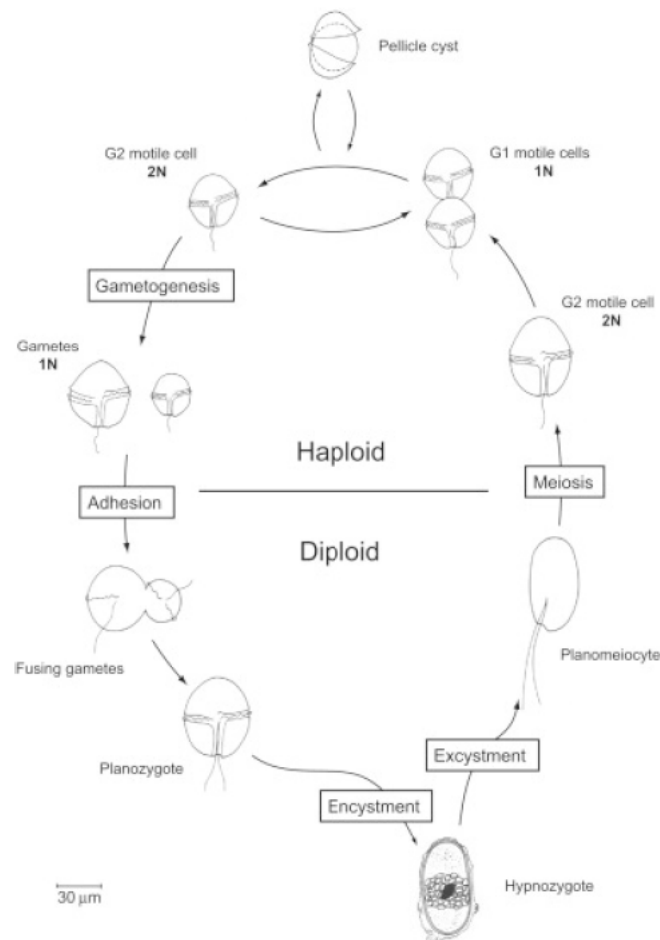


Fig. 1.6: Schematic life cycle representation of members of the *A. tamarense* species complex (Brosnahan et al. 2010)

Hypnozygotes have to pass through an obligatory dormancy period before they are able to hatch (Anderson 1998). Those hypnozygotes are a significant life stage in the ecology of *Alexandrium* spp. as they form “seedbeds” that provide an inoculum for harmful blooms (Anderson 1998). The hypnozygotes are able to hatch even after 100 years of dormancy (Miyazono et al. 2012) and are subject to longshore or offshore advective transport and the mixing of populations (Franks & Anderson 1992, Anderson 1998). In modern times, hypnozygotes facilitate the worldwide dispersal of toxic *Alexandrium* spp. strains through ship ballastwater.

1.5.1 Toxic blooms of *Alexandrium tamarense*

Members of the *Alexandrium tamarense* species complex are often considered to be “background” taxa and are most often outnumbered by co-occurring phytoplankton species (Anderson 1998). However, in some areas such as the north American East Coast, concentrations ranging from 40,000 to > 700,000 cell L⁻¹ have been frequently reported (Cembella et al. 2002, Anderson et al. 2005b, Martin et al. 2005). Nevertheless, *A. tamarense/fundyense/catenella* can cause harmful effects even at low cell concentrations (Hallegraeff 2003) and their ability to temporarily dominate the phytoplankton is frequently observed. The general mechanism of how harmful blooms of *Alexandrium* spp. are formed is complex and far from understood (Anderson et al. 2012a). Once vegetative cells enter the water column, their net growth rates are heavily affected by bottom-up and top-down processes (Anderson et al. 2012a). Although many of these interactions remain uncharacterized, some common characteristics can be linked to several blooms of *Alexandrium* species. Besides species specific physiological adaptations and their vertical nutrient retrieval migrations, physical forcing by water fronts, i.e. due to river plumes or heavy rainfall that generate a highly stratified water column are a contributing factor (Therriault et al. 1985). Large scale coastal current systems that distribute local populations through various water bodies that might favor population growth is also recognized as an important factor for *Alexandrium* spp. bloom formation (Franks & Anderson 1992, Anderson et al. 2005a, 2005c). Finally, stable and calm weather is a premise for large proliferation of *Alexandrium* spp. cells because the division of the vegetative cells is sensitive to turbidity. These physical factors are however often temporally more stable than any *Alexandrium* bloom and therefore cannot explain the bloom dynamics. In addition, blooms in certain areas (Cape Cod salt ponds, USA) seem to be controlled mainly by salinity and temperature and optimal growth conditions determined from laboratory cultures differ from natural bloom conditions (Anderson et al. 1983). Local adaptations to various environmental factors thus seem to trigger *Alexandrium* spp. bloom formation. Their large global dispersal into different habitats hence makes it difficult to generalize about abiotic factors that control those HABs (Alpermann 2009, Anderson et al. 2012a).

Given the complexity of, and difficulties in, disentangling bottom-up effects that facilitate the formation of an *Alexandrium* spp. HAB it should not be surprising that the other side, namely the top-down processes, exhibit the same if not even a

higher level of complexity. Indeed, species-specific interactions in competing for nutrients, as well as predator-prey and pathogen-host interactions are powerful sources in driving annual species succession as well as species' evolution (Fuhrman 1999, Smetacek et al. 2004, Jancek et al. 2008, Chambouvet et al. 2008). The observed correlation between HAB and shellfish-borne toxicity or fish kills have fostered the notion that such blooms develop because the blooms species produce toxins (Tillmann & John 2002). Great attention has been paid to the idea that PST production in *Alexandrium* spp. might be an adaptation for grazer defense (Tillmann & John 2002). In fact, grazing is the sole most important loss factor for phytoplankton organisms and, on average, more than 80% of the marine phytoplankton production is consumed by herbivores (Cyr & Pace 1993, Calbet 2001, Calbet & Landry 2004). Mesozooplankton, and in particular copepods consume around 10 - 40%, and the microzooplankton consumes around 60 - 70% of the primary production (Calbet 2001, Calbet & Landry 2004). Grazers can therefore have a potential impact on preventing or terminating blooms which, in turn, may be favored when grazing control is out-of balance concerning phytoplankton growth (Turner & Tester 1997).

1.5.2 Grazing by microzooplankton

PSTs were considered for a long time to be responsible for observed negative effects of *Alexandrium* spp. on microzooplankton. However, Tillmann & John (2002) demonstrated that the toxic effects of *Alexandrium* spp. on a protistan grazer (*Oxyrrhis marina*) are independent of PST and arise from extracellular substances that can cause cell lysis. It is now evident that *Alexandrium* spp. produce additional, yet uncharacterized compounds with lytic capacities which are responsible for the negative effects on competitors and protistan grazers (Fistarol et al. 2004, Ma et al. 2009, 2011, Tillmann & Hansen 2009, Yang et al. 2010, Hattenrath-Lehmann & Gobler 2011). Due to the negative effects that these compounds bear on co-occurring species, they are referred to as "allelochemicals". Allelochemicals are secondary metabolites that mediate "allelopathic" effects, an expression derived from the Greek "*allelon*", "*of each other*" and "*pathos*", "*to suffer*". The concept of allelopathy was originally defined by the Viennese botanist Hans Molisch (1937) to describe any inhibitory or stimulatory effect of one plant on another distinct plant, mediated by the release of chemicals. While this concept has been widely investigated in terrestrial plant communities (Rice 1974, 1984), allelopathic interactions in phytoplankton ecology have only been recently considered to explain

patterns of dominance or changes in community structure that do not emerge from competitive or trophic interactions (Cembella 2003, Gross 2003, Legrand et al. 2003).

The benefits to *Alexandrium* spp. in producing allelochemicals that act lytically on co-occurring species seem obvious: reduced competition for nutrients and light as well as relief from grazing pressure. Furthermore, due to exerted mixotrophic tendencies (Jeong et al. 2005, 2010), lysed species might, either directly or indirectly via stimulated bacterial growth, supplement nutritional demands of the producer (Smayda 1997). These benefits are also considered to be major strategies to offset ecological disadvantages derived from low nutrient uptake- and growth rates, characteristics that are intrinsic to most photosynthetic flagellates such as *Alexandrium* spp. (Smayda 1997). The assumption that allelochemicals play an important role in the formation of HABs is therefore an appealing hypothesis. However, released allelochemicals might become quickly diluted in the water when cells move and could benefit not only the producer (Lewis 1986, Jonsson et al. 2009, Hattenrath-Lehmann & Gobler 2011). Since dilution effects diminish with increased density of the producer cells, allelopathy in *Alexandrium* spp. is more likely to contribute to bloom maintenance via positive feedback mechanisms, rather than to bloom initiation when cell densities are low (Jonsson et al. 2009). However, it should be considered that density and not abundance determines the positive feedback of the allelochemical interaction. An aggregated distribution pattern can therefore enhance benefits at low cell concentrations (Durham & Stocker 2012).

The effects of the allelochemicals produced by *A. tamarensis* are dependent on the concentration of *Alexandrium* cells, the concentration of the target cells (or more generally, the density of all absorbing particles) and the target species, as some species are far more resistant to the allelochemicals than others (Tillmann et al. 2007, 2008, Tillmann & Hansen 2009, Hattenrath-Lehmann & Gobler 2011). Hattenrath-Lehmann & Gobler (2011) showed in field and laboratory experiments that *A. fundyense* can cause a decline in the densities of autotrophic nanoflagellates and diatoms, yet might also benefit the growth of all other dinoflagellates, and therefore could be an important biotic factor contributing to the dominance of dinoflagellates after the spring diatom bloom in temperate ecosystems. Whether or not *Alexandrium* spp. become superior competitors in a system due to the excretion of allelochemical substances depends therefore strongly on the community composition, which in turn, is the result of multiple, complex and co-occurring processes. In addition, we lack substantial knowledge concerning detailed

observations about the feedbacks gained from those allelochemicals produced within the *Alexandrium* species complex.

1.5.3 Grazing by copepods

Investigations on the effects of PSP on mesozooplankton grazers have primarily focused on copepods and have resulted in a scope of controversial results regarding both the ingestion or rejection of PSP-toxic *Alexandrium* spp. cells and the effect of the toxins on the copepods (Turriff et al. 1995, Teegarden & Cembella 1996, Turner et al. 1998, Cembella 2003, Colin & Dam 2003; and see Table 1.1).

Table 1.1: Summarized results of studies that compared effects of sole versus mixed diets of PST-containing *Alexandrium* spp. on copepods. Feeding rates: (-) reduced rates compared to mixed or control species; (+) higher rates compared to sole diet or equal to control species. Prey selection: (+) species was selected for or against (-), but still ingested at low rates; + in both columns indicates no preference. Effects: (-) was worse compared to mixed or control diet; (+) effect was better than sole diet or indifferent from control diet. NJ: New Jersey; ME: Maine (Adopted from Colin & Dam 2003).

Algal species <i>Test species / Control species</i>	Copepod species	Feeding rates		Prey selection		Effects		Type	Source
		Sole diet	Mixed diet	Test alga	Control alga	Sole diet	Mixed diet		
<i>Alexandrium excavatum / Thlassiosira weissflogii</i>	<i>Calanus finmarchicus</i>	-	+	-	+				Turriff et al. (1995)
<i>Alexandrium fundyense / Alexandrium tamarense</i>	<i>Acartia tonsa</i>	-	+	-	+	-	+		Teegarden (1999)
<i>Alexandrium fundyense / Alexandrium tamarense</i>	<i>Centropages hamatus</i>	-	+	-	+	+	+		Teegarden (1999)
<i>Alexandrium sp. / Tetraselmis sp.</i>	<i>Acartia tonsa</i>	-	-	-	-	-	-	Ingestion	Colin & Dam (2002)
<i>Alexandrium sp. / Tetraselmis sp.</i>	NJ <i>Acartia hudsonica</i>	-	-	-	-	-	-	Ingestion	Colin & Dam (2003)
<i>Alexandrium sp. / Tetraselmis sp.</i>	ME <i>Acartia hudsonica</i>	+	+	+	+	+	+		Colin & Dam (2003)
<i>Alexandrium minutum / Alexandrium minutum</i>	<i>Acartia clausii</i>	-	+	+	+	-	+	Mortality / Fecundity	Barreiro et al. (2006)

The interaction between copepods and PSP-toxin producing *Alexandrium* spp. is therefore highly variable depending on the *Alexandrium* strain investigated, and can vary greatly among zooplankton species. The assumption that natural co-occurrence of the copepod species and its toxic prey item could be an important determinant in the outcome of those interactions was previously raised by Runge (1992) and Ives (1985), however only explicitly shown by Colin and Dam (2003 & 2005) who compared the ingestion rates over time, as well as respiration rates as fitness parameter for historically exposed and non-exposed strains of *Acartia hudsonica*. They concluded that copepods that co-exist with PSP-toxic *Alexandrium*

spp. can become resistant to the toxins and such a resistance can include different mechanisms: behavioral avoidance or metabolic resistance that increases the rate at which toxins are broken down or a decreased sensitivity towards the toxins (Taylor 1986, Colin & Dam 2003). Behavioral avoidance of copepods against toxic prey was shown by i.e. Turriff et al. (1995) and Teegarden (1999); species specific differences in toxin accumulation by i.e. Turriff et al. (1995) and Teegarden et al. (2003); differences in toxin retention by i.e. Teegarden & Cembella (1996); variation in depuration rates by i.e. Teegarden (1999) and Guisande et al. (2002) and copepod-species specific toxin transformations by i.e. Shimizu (1978) and Teegarden et al. (2003). Behavioral avoidance strategies against toxic algae (i.e. Turner & Tester 1997, Teegarden 1999, Teegarden et al. 2008) can, however, not be linked definitively to PSTs because they are based on correlative evidence and may well be confounded by other traits, correlated to PST levels. More concrete examples of counter-adaptation are described by Avery and Dam (2007) and Chen (2010). Avery and Dam (2007) demonstrated that the resistance to PSP-toxic *Alexandrium* spp. by the copepod *Acartia hudsonica* carries a cost in the absence of these toxic algae. Their results suggest a heterozygote advantage in the resistant trait, and in turn, that this heterozygote advantage hampers the fixation of the trait within the population (Avery & Dam 2007). In addition, the frequency of heterozygotes appears to increase with the degree of historical exposure to toxic *Alexandrium* spp. (Chen 2010). A mutation at one of the isoforms of their voltage-gated sodium channels might be responsible for this resistance (Chen 2010, Dam & Haley 2011). This mutated sodium-channel seems to function as a kind of “saxitoxin scavenger” and is thought to be leaky if no saxitoxin is bound (Chen 2010). The counter-adaption in the case of *A. hudsonica* is therefore a double-edged sword and less elaborate than described adaptations within shellfish, where the sodium-channel mutation disables the binding of saxitoxin at the extracellular side at no apparent cost (Bricelj et al. 2005, Connell et al. 2006). These two examples illustrate that counter-adaptations to saxitoxin can evolve with different trade-offs and different levels of elaborateness, even when targeting the same functional gene. If more and more counter-adaptations are detected, further structural radiations or a decline or loss of saxitoxin production may occur since this metabolite would lose its selective advantage. Hence, we know that non-PST producers occur with PST producing species (Lilly et al. 2007) with a recent indication, that these non-producers still harbor the putative *sxt*-genes in their genome (Stüken et al. 2011, Hackett et al. 2012). This either indicates a purifying selection of PST production in *A. tamarense* or an ongoing attempt of complementing the saxitoxin biosynthesis pathway in

different populations/ribotypes. Generally speaking, PSTs cannot be said to have evolved with a particular purpose. They are present either because they confer a selective advantage, or they are biologically neutral with respect to evolutionary developmental processes. The latter constrains our attempt to clearly assign an ecological function to this group of secondary metabolites. In addition, further putative functions have been proposed for PSTs, including physiological (e.g., ion homeostasis or nitrogen storage (Cembella 1998, Pomati et al. 2004, Soto-Liebe et al. 2012) and autecological functions (pheromone activity (Wyatt & Jenkinson 1997) and impact on associated bacteria (Jasti et al. 2005).

The interaction between copepods and *Alexandrium* spp. becomes even more complex when considering that at least some strains of *Alexandrium* spp. are able to induce increased PST production in the proximity of copepods (Selander et al. 2006, Bergkvist et al. 2008): *Alexandrium minutum* increased its cellular PST content up to > 25-fold due to the presence of naturally occurring concentrations of copepods which, in turn, is correlated with an increased resistance to copepod grazing (Selander et al. 2006). This response towards copepods was induced by “waterborne-cues” or “infochemicals” present in the water, and no direct contact between *A. minutum* and the copepod *Acartia tonsa* was necessary to elicit the response (Selander et al. 2006). Further experiments showed that this induced response in *Alexandrium minutum* is not of a general nature against copepods and that mostly, also here, co-existence is necessary for the algal cell to recognize its predator (Bergkvist et al. 2008). In a recent study, Selander et al. (2011) added that *A. tamarense* cells split off their cells from chains and swim mostly solitarily and more slowly in the presence of copepods. This behavioral response, or “stealth behavior”, led to a reduced encounter rate with the rheotactic copepod grazer *Centropages typicus*, which tracks its prey using hydrodynamic signals (Selander et al. 2011). This stealth behavior can also reduce the encounter rates with copepods using chemical prey detection, simply because more cells leak more chemical signals (Selander et al. 2011; Fig. 1.7).

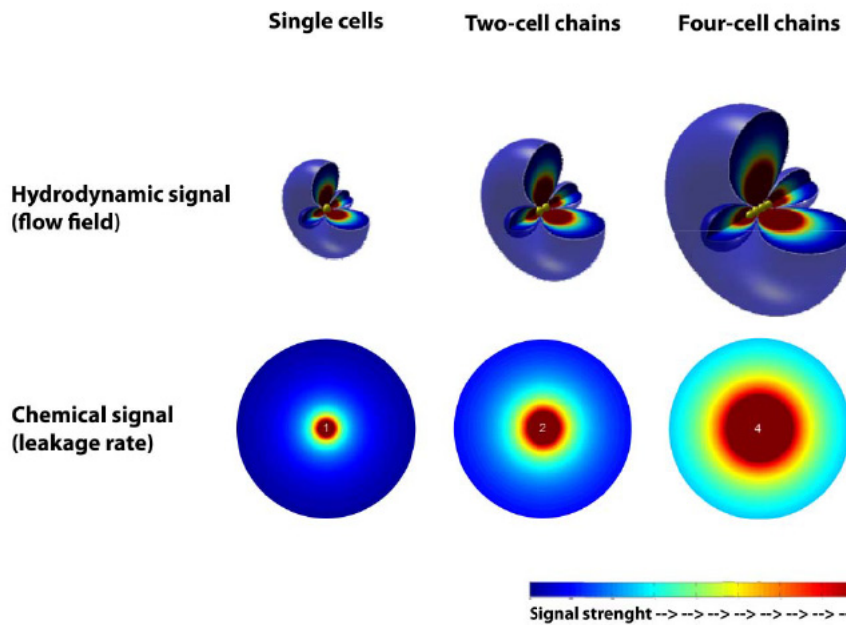


Fig. 1.7: Comparison of hydrodynamic- and chemical signals generated from single cells and two or 4 cell-chains (model provided by Erik Selander, University of Gothenburg).

1.4 Induced defense

In contrast to the previously described allelochemical mediated cell lysis of microzooplankton grazers that can be defined as a permanently expressed, constitutive defense mechanism, the interaction of copepods and *Alexandrium* spp. strongly implies that *Alexandrium* spp. is able to induce defense in the presence of the grazer. Inducible defense in phytoplankton was first described for the freshwater green algae *Scenedesmus subspicatus* (Hessen & Van Donk 1993). In the presence of the water flea *Daphnia magna*, *S. subspicatus* increased the proportion

of cells in colonies as well as the proportion of armored cells (Hessen & Van Donk 1993). The recognition of plastic-induced defenses in phytoplankton has increased until then, and the flexibility of algal species in terms of their morphology, growth form, biochemical composition and production of toxic and deterrent compounds has been interpreted as defense mechanisms against grazers (Van Donk et al. 2011). Induced defense is a form of phenotypic plasticity and theoretically four factors are considered as prerequisites for the evolution of induced defenses (Harvell & Tollrian 1999):

- I. “The selective pressure of the inducing agent has to be variable and unpredictable but sometimes strong. If the inducer is constantly present, permanent defenses should evolve;
- II. A reliable cue is necessary to indicate the proximity of the threat and activate the defense;
- III. The defense must be effective;
- IV. A major hypothesis about the advantage of an inducible defense is cost saving. If a defense is inducible, it could incur a cost that offsets the benefit of the defense. If there is no trade-off, it is widely postulated that the trait will be fixed in the genome” (Harvell & Tollrian 1999).

The main factors that trigger chemical cue associated induced defense in marine phytoplankton are thought to be mechanical damage, herbivore presence and direct grazing, however there is a tremendous lack of knowledge regarding the exact mechanisms governing defense induction (Van Donk et al. 2011). In addition, the detection of costs that arise from an inducible defense system has been a major challenge (i.e. DeWitt et al. 1998, Tollrian & Harvell 1999, van Kleunen & Fischer 2007, Van Donk et al. 2011) and such costs have to be considered to be expressed in several ways (Table 1.2). Indeed high costs of plasticity may have been purged out by evolution (DeWitt et al. 1998, van Kleunen & Fischer 2007) and low costs may remain undetected, particularly in a relatively high quality environment, such as that provided in the laboratory (Van Donk et al. 2011).

Table 1.2: Type of costs that can occur as a consequence of an induced defense response (Tollrian & Harvell 1999).

Type of Cost	Subtype	Origin
Plasticity costs	Genetic costs	Genetic correlation (e.g. negative pleiotropy)
	Infrastructure costs	Costs of providing mechanisms to respond (e.g. receptors)
Allocation costs	Construction costs	Resources and energy for building the defense (e.g. synthesis of secondary metabolites)
	Maintenance costs	Resources and energy for maintaining the defense
	Operation costs	Resources and energy for using defenses (e.g. energy for escape)
Self-damage costs		Costs caused by self-damage or for protection against self-damage (e.g. autotoxicity)
Opportunity costs		Long-term consequences of allocation or developmental constraints
Environmental costs		Interaction of the defense with the environment, often in variable environments (e.g. defense to one predator is disadvantageous in the presence of another predator)

1.5 Secondary metabolites

Both the grazer induced defense response and the allelopathic interactions are mediated by chemicals produced by the respective species. In the case of the induced defense response, this additionally includes an increase in PSTs. Such interactions are centralized in the discipline of chemical ecology that aims to untangle the relationships between the structure and function of metabolites, and how these affect organisms in the environment, controlling co-existence and co-evolution of species (Cembella 2003). Of primary focus in chemical ecology are

therefore “secondary metabolites”. The term secondary metabolite comprises all metabolites that are non-essential to growth and development as defined by Julius Sachs in 1873, whereas a primary metabolite, or primary metabolism is essential in this context (Sachs 1873, Hartmann 1996; see Table 1.3 for main characteristics).

Table 1.3: Characterization of primary and secondary metabolism (Hartmann 1996)

PRIMARY METABOLISM	SECONDARY METABOLISM
<i>Growth and development of the individual</i>	<i>Interaction of the individual with its environment</i>
<ul style="list-style-type: none"> • indispensable • uniform • universal • conservative 	<ul style="list-style-type: none"> • dispensable for growth and development • indispensable for survival of a population • unique • diverse • adaptive

Enzymes for the biosynthesis of secondary metabolites most probably arose from duplication events, starting with genes controlling primary metabolism (Pichersky & Gang 2000, Aubourg et al. 2002, Ober 2005, Weng et al. 2012). Mutations in such duplicated genes can lead to substrate permissiveness and a subsequent constriction of their substrate recognition site, due to selection that acts on their novel products, leaving such mutations non-silent (Ober 2005, Weng et al. 2012). Hence, new compounds can arise due to gene duplication events while maintaining the functions of the ancestral genes (Pichersky & Gang 2000, Ober 2005, Weng et al. 2012). In addition, horizontal gene transfer can add to a species' make-up of enzymes and secondary metabolites (i.e. Boucher & Doolittle 2000, Schmitt & Lumbsch 2009). Structural radiation of secondary metabolites is then the next step in adding diversity and new functions of compounds. Indeed, the great majority of secondary metabolites are produced by basic biosynthetic routes from which numerous derivatives are formed through enzymatic transformations (Hartmann 1996, Cembella 2003, Weng et al. 2012). The resulting key-metabolites are then further modified to secondary level key intermediates that can further diversify (Hartmann 1996). A remarkable plethora of secondary metabolites arose due to different combinations of modification and diversification, and therefore it is not surprising that these chemicals are able to fulfill specialized and species-specific functions. Consequently, secondary metabolites are used as chemical cues at every level in the marine system and can determine whether the species consume, fight with, run from, or mate with the creature next to them (Hay 2009).

Communication with each other and sensing the environmental are remarkably abundant in the sea and many species rely on chemical cues that lead to a sophisticated chemical language (Hay 2009, Pohnert 2010).

The biosynthesis of secondary metabolites in *Alexandrium tamarense* (PSTs and the unknown allelochemicals) that can act as forces for selection and community composition, however, further underlies genotypic and phenotypic variation within those traits. Alpermann et al. (2010) showed that within a single population, an enormous diversity exists between different strains for cellular PST content, PST profiles and the allelopathic potency. Species interact in their natural community with a multitude of different species throughout their life cycle, including predators, pathogens, mutualists, competitors and conspecifics. Hence, traits such as the production of secondary metabolites can have varying effects on co-occurring species and can affect multiple interactions simultaneously. Additionally selection might be density dependent (Lankau & Strauss 2008): at high densities species will mostly interact with conspecifics, whereas at low densities they will mostly interact with heterospecifics. Genetic variation in such ecological important traits is therefore thought to be maintained due to fluctuations in the community composition over time and space (Lankau & Strauss 2008). Such a genetic variation does however lead to various “genotype-by-environment” (G x E) interactions, making species interactions studies even more complex as only effects from reciprocal co-evolution would suffice (Agrawal 2001). Plastic responses, such as the induced defense response consequently add a dynamic component to the complex web of species interactions, which in turn can be overlaid with a web of infochemicals (Agrawal 2001, Dicke 2006).

1.6 Genes involved in species interactions

Species interactions as well as the infochemicals they emit or perceive which influence the outcome of those processes on the ecological community are therefore complex systems (Werner & Peacor 2003, Dicke 2006). On a mechanistic level, these complex systems are an integration of complexity at different levels: The community, composed of interacting species with variable phenotypes; the complexity of infochemicals emitted by each organism in the community; the composition of phenotypes with different traits; the interactions of proteins that determine traits and phenotypes; and finally the transcriptome and genome that shapes each phenotype (Dicke 2006, Bailey et al. 2009). Given that causality, an

organisms' genome and transcriptome are the very origin of processes that shape complex biological systems. Transcriptomic changes are therefore substantial, dynamic and highly variable depending on the environmental conditions. They furthermore contribute significantly to the phenotype expressed, which in turn provides feedback to the community composition (see i.e., Schlichting & Smith 2002, Dicke 2006, Landry et al. 2006, Aubin-Horth & Renn 2009, Bailey et al. 2009, Pavey et al. 2012). In that sense, it is the external environment and the cues received within, that alter the internal environment by changes in gene expression and protein interactions that can be recognized at different levels: biochemically, morphologically and/or behaviorally (Schlichting & Smith 2002).

Recent developments in next-generation sequencing (NGS) have opened the door to high-throughput and large-scale genomic and transcriptomic surveys that were previously limited to "model-organisms" (e.g. *Escherichia coli*, yeast *Saccharomyces cerevisiae*, plant *Arabidopsis thaliana*, fruit fly *Drosophila melanogaster*, nematode *Caenorhabditis elegans*, corn *Zea mays*, mouse *Mus musculus* and zebrafish *Danio rerio*) (Jackson et al. 2002, Pavey et al. 2012). These model species were selected based on particular genetic and developmental features and for ease of growth in the laboratory rather than on their ecological or evolutionary importance (Jackson et al. 2002). Consequently, many ecological important pathways and processes remain poorly investigated, simply because they are not represented in model organisms (Jackson et al. 2002, Pavey et al. 2012). Applying large scale transcriptomic analyses to ecological important organisms such as toxin producing dinoflagellates, in ecologically and evolutionarily relevant settings, such as grazing and species interactions, will therefore not only broaden our understanding of molecular mechanisms that enable such responses, it will also contribute to the identification of genes and networks that are somehow linked to ecological settings uninvestigated so far, that lay outside the model-species approach and that display a strong selective pressure.

Despite the fact that genomic and transcriptomic investigations have increased exponentially in recent years, the capacity to interpret this abundance of data lags far behind and a large fraction of genes remains without any annotation (Pavey et al. 2012). The interpretation of genomic and transcriptomic data from non-model organisms rests in the establishment of homologies between genomic features of traditional model organisms, where data are mainly derived from non-ecological purposes (Pavey et al. 2012). However, due to the continuous and detailed characterization of genes involved in a wide array of pathways and processes (for an

overview see i.e. “The Kyoto Encyclopedia of Genes and Genomes” (KEGG; <http://www.genome.jp/kegg/>), homology based annotation approaches indeed contributed significantly to our understanding of basic processes in a phylogenetically diverse range of species. In gratitude to such approaches, we are able to dissect the proportion of conserved processes in ecological genomic studies which indeed have profound effects on the organisms’ physiology and its ability to thrive in a certain environment. Many genes that respond to the environment are generally characterized by a high diversity, proliferation via duplication events and rapid rates of evolution (Berenbaum 2002). These characteristics lay the basis for responses emanating from variable external conditions (Berenbaum 2002). Gene duplications are thought to account for 90% of a eukaryote’s genome (Gough et al. 2001) and subsequent diversifying selection of duplicated genes can create sequences too divergent from their ancestor to assign any function based on homology. The expression of genes under diversifying selection subsequently exposes their effect to the processes of natural selection. Selected beneficial mutations have the potential to be fixed in the population. The frequency of such events is even higher in haploids as *Alexandrium* spp. are, since the effects cannot be masked by the alternative allele (Mable & Otto 1998). In addition, many genes specifically expand not only within species, but also at the kingdom level. Annotating genes in the context of the environment, in which they are expressed, can thus add fundamental knowledge to the understanding of the functions and processes with which a gene is associated (Aubin-Horth & Renn 2009, Pavey et al. 2012). While this is maybe a dissatisfying answer to the question concerning the nature of genes associated with responses to certain biotic settings in the environment, one should consider the expression of those genes and their variability as the key players that keep the species on stage in the evolutionary theater.

2. Aim and outline of the thesis

2.1. Justification and aim of the thesis

Interactions between the red tide dinoflagellate *Alexandrium tamarense* and grazers (copepods and protists) are hypothesized to be influenced by infochemicals and lytic substances present in the water. The response of *Alexandrium tamarense* to such interactions, until now, has been defined by the characterization of traits like PST content, the lysis of the grazer, net growth and ingestion rates and, more recently, in shifts in cell chain lengths. Accordingly, our knowledge about mechanisms that trigger or are correlated to these responses is restricted to these traits. Yet a better characterization of the molecular context of these responses could increase our understanding concerning the impact that the expression of such traits has on *A. tamarense*. Finally this would result in a better understanding about the capacity of *A. tamarense* to cope with grazing as one top-down process, an emerging aspect in harmful algal bloom formation and persistence. Understanding how grazers influence a species success (despite consumption effects) therefore demands a more complete view on traits that change in the presence of a grazer. Moreover, we lack basic knowledge about how cues are perceived and translate into a response that has been selected to be the most appropriate through evolution. Subsequently, we don't know how species' interactions have contributed to shape the functional genome. While there is likely no single gene (and even if there were, we don't know its nature) whose change in expression has the power to define environmentally triggered changes and to adjust the phenotype, high throughput gene expression techniques that enable quantitative assay of gene expression at a genomic scale are valuable tools in dissecting such responses. The overall aim of the thesis was, therefore, to obtain a more detailed characterization of processes that underlie, and result from species interactions with grazers in *Alexandrium tamarense*.

2.2 Outline and approach of the thesis

Recent scientific literature has provided little information and inconsistent experimental outcomes concerning copepod grazer-induced PST level alterations in *Alexandrium* spp. Hence, context and inducing cues remain to be determined. While the chemical analysis of the inducing cues is beyond the scope of this thesis, I aimed to analyze the molecular mechanisms that these cues elicit in *A. tamarense*. I assumed that the cues recognized by *A. tamarense* elicited a response at the transcriptomic level through receptor stimulation and the consequent transcriptional changes would be measurable. In addition, I was interested in identifying a potential co-evolutionary background in order to interpret the grazer induced defense response in a broader context. To achieve these goals, I investigated the impact of naturally co-occurring copepod species on PST production and, *vice versa*, the effect of PSTs on the different copepod species. Responses of *A. tamarense* towards copepod grazers could therefore be interpreted based on a common history of co-existence, eliminating previous uncertainties of this variable. The ability of different copepod species to handle their toxic prey can give us additional information on the impact that each species might have on an *A. tamarense* population, and hence the strength of evolutionary forces that select for counter-adaptations to each species. Therefore, in **Publication I** the effect of three different copepod species with different feeding strategies and their waterborne-cues was investigated on *A. tamarense* in an attempt to assess and better characterize grazer-induced increases in PST content in *A. tamarense*. Microarrays were used to identify genes that respond to the presence of copepods and their cues. In order to estimate the effect of *A. tamarense* on each copepod species, the fitness of the copepods was investigated after the experiments and their internal PST levels were measured.

A main prerequisite for the evolution of induced defenses is that cues that underlie such responses should be reliable and indicate the proximity of the threat. The cues that emanate from copepods hence should be suited to track ambient grazer densities. In order to analyze these cue characteristics, the kinetics of the copepod-induced defense response was investigated and is presented in **Publication II**. Media- and copepod-replacement experiments were performed to follow the induced defense response and cue attenuation over time.

The high genotypic variability in *A. tamarense* population leads to major differences in the expression of ecologically important traits that are known to influence top-down processes. Yet, the outcome of the grazer-induced defense response can vary depending on the strain used. In addition, the experimental set-up can have an influence on the transmission of the inducing cue. In **Publication III**, the induced defense response towards a copepod grazer was therefore evaluated with a broad experimental set-up for two *A. tamarense* strains that differ in their phenotype but originate from the same population. I was interested in whether two different genotypes that express a different phenotype are able to change their expression of traits towards the defended phenotype and, how they realize it. As the in Publication I presented results of transcriptomic changes have been proven to be a useful complement for the interpretation of the induced defense response, I aimed to further broaden the knowledge that can be gained from the implementation of functional genomic approaches. To ensure that the majority of genes that respond to the presence of copepods are present on the microarray, a pooled subsample of the RNA isolated from the experiments was sequenced on a high-throughput sequencing platform (454). With this transcriptomic analysis I aimed to characterize how the defense response gets realized in different genotypes/phenotypes and to identify genetic fingerprints of associated costs. In addition, the analysis of genes in biotic interactions is a useful approach to link unknown genes to an ecological context in which they are expressed and hence helps to explore the function of such genes. Since the presence of a copepod grazer represents an ideal system to study PST related gene expression changes I further analyzed a potential correlation between the gene that initiates the PST biosynthesis pathway (*sxtA*) with the respective phenotype. Such a correlation or the absence of it can provide valuable insights into the regulation of PST-biosynthesis in *Alexandrium* spp., the genus responsible for the most paralytic shellfish poisoning outbreaks worldwide.

Microzooplankton grazers can contribute as much as mesozooplankton grazers like copepods to population losses in the phytoplankton. *A. tamarense* species are able to reduce this top-down pressure by producing allelochemicals that can lyse such co-occurring grazers. However, the strength of this lytic effect is dependent on the *A. tamarense* strain. Different genotypes per environment interactions are therefore evident. Yet it is unknown if the presence of microzooplankton grazers have further impacts on *A. tamarense* and hence its ability to succeed under pressure of biotic interactions. The earlier analysis (Publication I & III) of the induced defense response towards copepods revealed that

the response elicits changes of many traits at the gene expression level. I was therefore interested if the interaction between microzooplankton grazers is as multifaceted as the response of *A. tamarense* towards copepod grazers. Such complex responses that go beyond consumption and mortality effects may indeed contribute to the complex succession patterns observed in the phytoplankton. Particularly, I was interested in the reaction of a defended versus a non-defended strain of *A. tamarense* towards a microzooplankton grazer that actually grazes on the population. In **Publication IV**, I investigated possible feedbacks gained from the presence or absence of lytic compound production in two *A. tamarense* strains exposed to cues from a sympatric protist grazing on *A. tamarense*. To ensure that observed results in the lytic strain are due to an active production of lytic compounds, both strains were washed and placed in fresh media prior to the experiment. Since the implementation of microarrays provided valuable information in the two above outlined studies with the copepod-grazers, transcriptional changes were also thought to give insights into trait changes in this study. Analogous to the previously performed experiment (Publication III), high-throughput sequencing (454) of RNAs derived from these treatments was carried out to ensure that induced changes in gene expressions are covered by probes on the microarray. Additionally, this functional genomic approach allowed to assess differences in the genes expressed by the lytic and non-lytic *A. tamarense* strain.

The studies outlined above consequently opened up the possibility to gain insights into the functional genome of *A. tamarense*. The analysis of protein coding genes and their diversification will allow for the identification of processes that are crucial for the ability of *A. tamarense* to thrive under various conditions. Central to this aim is the identification of PST associated genes which can contribute to our understanding of the molecular background of PST biosynthesis in *Alexandrium* spp. **Publication V** presents results from the assembly and annotation of a comprehensive transcription based gene library. A conventional clone-based EST-library was therefore expanded by two high-throughput sequencing (454) runs. This further allowed building a resource for transcriptomic investigations of *A. tamarense*. The established database was characterized using four different annotation databases and screened for putative PST biosynthesis genes.

3. Publications

This thesis is organized into 5 core chapters, each corresponding to separate publications. The candidate is the first author of 4 of the 5 publications.

3.1 List of publications and declaration of contribution

Publication I

Wohlrab S., Iversen M.H., John U. (2010)

A molecular and co-evolutionary context for grazer induced toxin production in *Alexandrium tamarense*.

PLoS ONE 5(11): e15039.

DOI:10.1371/journal.pone.0015039

The candidate designed the experimental setup in collaboration with the coauthors. The candidate isolated the copepod species, performed cell counts, performed RNA isolations and microarray hybridizations, analyzed the microarray results, annotated the regulated genes and prepared the manuscript.

Publication II

Selander E., Fagerberg T., **Wohlrab S.**, Pavia H. (2012)

Fight and flight in dinoflagellates? Kinetics of simultaneous grazer-induced responses in *Alexandrium tamarense*

Limnol.Oceanogr., 57(1), 2012, 58-64

DOI: 10.4319/lo.2012.57.1.0058

The candidate took part in performing the experiments, interpretation of the data and discussions on the manuscript.

Publication III

Wohlrab S., Selander E., Glöckner G., John U.

Grazer induced reaction norms - a transcriptomic comparison.

Submitted to BMC Biology.

The candidate designed the experimental setup in collaboration with the coauthors. The candidate isolated the copepod species, performed cell counts and cell-chain size estimations, performed RNA isolations and microarray hybridizations, analyzed the microarray results, annotated the regulated genes, performed the RT-qPCR analysis and prepared the manuscript.

Publication IV

Wohlrab S., Tillmann U., Cembella A., John U.

Mapping genotypic trait alterations in *A. tamarensis* in response to a protistan grazer.

To be submitted

The candidate designed the experimental setup in collaboration with the coauthors. The candidate performed cell counts, RNA isolations and microarray hybridizations, analyzed the microarray results, annotated the regulated genes and prepared the manuscript.

Publication V

Wohlrab S., John U.

Assembly and functional annotation of a comprehensive expression based gene library to study biotic interactions in the marine dinoflagellate *Alexandrium tamarensis*

To be submitted

The candidate performed the annotation and analysis of the gene library and prepared the manuscript.

3.2 Further publications

The candidate is a co-author on two further publications not included in this thesis

Jaeckisch N., Yang I., **Wohlrab S.**, Glöckner G., Kroymann J., Vogel H., Cembella A., John U., (2012)

Comparative genomic and transcriptomic characterization of the toxigenic marine dinoflagellate *Alexandrium ostenfeldii*.

PLoS ONE 6(12):e28012.

DOI:10.1371/journal.pone.0028012.

Van de Waal D.B., Eberlein T., John U., **Wohlrab S.**, Rost B.,

Impact of elevated pCO₂ on PSP toxin production by *Alexandrium tamarensis*

Submitted to Harmful Algae.

4. A molecular and co-evolutionary context for grazer induced toxin production in *Alexandrium tamarense*

4.1 Abstract

Waterborne cues from copepod grazers have been previously found to induce a species-specific increase in toxin content in *Alexandrium minutum*. However, it remains speculative in which context these species-specific responses evolved and if it occurs in other *Alexandrium* species as well. Here we investigated the response of *Alexandrium tamarense* in terms of toxin content and gene expression changes to three copepod species (*Calanus helgolandicus*, *Acartia clausii*, and *Oithona similis*) and their corresponding cues. We further analysed ingestion rates, toxin content and fitness of the copepods after grazing on *A. tamarense*. Our results show that *A. tamarense* reacts to all copepod species and their cues through transcriptomic changes but a significant increase in intracellular toxin content is only observed in the presence of the least impaired grazer (*Calanus helgolandicus*). Grazer-specific responses towards copepods and their cues are therefore not restricted to one *Alexandrium* species and potentially a result of a predator-prey co-evolution. Through the annotation of the differentially regulated genes we gained insights into the underlying molecular processes which could trigger the different outcomes of these species-specific responses in *Alexandrium tamarense*. We propose that the regulation of serine/threonine kinase signaling pathways has a major influence in directing the external stimuli i.e. copepod-cues, into different intracellular cascades and networks in *A. tamarense*. Our results show that *A. tamarense* can sense potential predating copepods and respond to the received information by increasing its toxin production. Furthermore, we demonstrate how a functional genomic approach can be used to investigate species interactions within the plankton community.

4.2 Introduction

Dinoflagellates of the genus *Alexandrium* possess a high ecological impact due to their ability to form Harmful Algal Blooms associated with Paralytic Shellfish Poisoning (PSP). PSP is a threat to marine aquaculture and shellfish consumers,

occurring worldwide with increasing frequency and distribution (Hallegraeff 1993). PSP is caused by an accumulation of highly potent neurotoxic alkaloids, the Paralytic Shellfish Toxins (PSTs), in the marine food web. Accumulations of PSTs can induce mass death of fish (Mortensen 1985, Cembella 2003), mortalities among marine mammals (Durbin et al. 2002, Doucette et al. 2006), and cause human intoxication via consumption of contaminated shellfish (Prakash et al. 1971).

Saxitoxin is one of ~two dozen naturally occurring PSTs and was structurally characterized in 1975 (Schantz et al. 1975). Despite the early discovery of the first PST, the ecological function that PST may play in marine dinoflagellates remains unclear (Cembella 2003). It has been suggested that they serve a function in nitrogen storage (Cembella 1998), possess pheromone activity (Wyatt & Jenkinson 1997), have an impact on associated bacteria (Jasti et al. 2005), and act as defense compounds (Turner & Tester 1997). Yet, there is no evidence that PSTs are involved in allelopathic interactions, including interactions with heterotrophic micrograzers in plankton food webs (Fistarol et al. 2005, Tillmann et al. 2007). One may therefore not exclude the fact that a single metabolite may have multiple ecological functions. If we consider metabolic energy costs associated with biosynthesis and modification of secondary metabolites, it seems intuitive that natural selection would favor metabolites with multiple functions (Wink 2003). Several investigations have suggested that the PST function as defense compounds against copepods (reviewed in Turner et al. 1998). However, studies focusing on the influence of copepod grazing on dinoflagellates have shown both (1) high ingestion rates of toxic *Alexandrium* with no adverse effects on the grazers as well as (2) enhanced mortality of the grazer (Bagøien et al. 1996, Teegarden & Cembella 1996). Thus, it seems that grazing experiments are highly dependent on the *Alexandrium* strain investigated, as well as on the grazer species (Turner & Tester 1997). Parallel investigations concerning the effects of toxin producing cyanobacteria on zooplankton grazers are described for freshwater ecosystems (Tillmanns et al. 2008). *Microcystis* spp. strains, for example, can differ significantly in their toxin content (Sivonen & Jones 1999) and the potential predator (*Daphnia* spp.) shows different levels of impairment upon grazing, potentially due to differences in detoxification abilities or adaptations to the toxins following post-exposure (Pflugmacher et al. 1998, Gustafsson & Hansson 2004). Also, naturally occurring *Alexandrium* populations are composed of different strains, producing different amounts of PST, and possessing different PST profiles (Alpermann et al. 2010). Such genotypic and phenotypic diversity could lead to varying results among grazing

studies. In addition, copepods show differences in toxin accumulation rates and in the ability to transform the toxins into less toxic derivatives (Teegarden et al. 2003). It has also been observed that copepods, which have been historically exposed to *Alexandrium* blooms, are less affected by PST compared to copepods originating from regions devoid of *Alexandrium* (Colin & Dam 2005). Further, some copepods are able to adapt to the toxins within a few days of exposure and can develop resistance towards the PST (Avery & Dam 2007), while other studies observed selective feeding on non-toxic *Alexandrium* over toxic cells by copepods (Teegarden 1999). However, the selective feeding only seems to apply when there is high cell concentration of *Alexandrium* (Teegarden et al. 2008). The range of observed adaptations by copepods exposed to PST suggests a predator-prey co-evolution, and may support the hypothesis that PST acts as defense compounds. Additional evidence of PST acting as grazer defense compounds is given by Selander et al. (2006), who demonstrated that PST content was increased in *Alexandrium minutum* after exposure to waterborne cues from copepods, which correlated with a decreased copepod grazing. Further, Bergkvist et al. (Bergkvist et al. 2008) found that *A. minutum* only increased its PST production significantly when exposed to waterborne cues from two out of three different copepods, which indicates that toxin production might be target specific.

This study aimed to investigate the effects on PST content in *Alexandrium tamarense* when exposed to copepod grazing or the waterborne cues from copepods. By collecting the copepods from the same geographic regions as the origin of the *A. tamarense* strain used, we assumed that the copepods had a history of co-existence with *A. tamarense*. Co-existence has been proposed to be a driver of chemical cue specific responses towards copepods in *A. minutum* (Bergkvist et al. 2008). Assuming that one of the ecological functions of PST is defense against predation, exposure of *A. tamarense* cells to actively grazing copepods or only their waterborne cues should induce an increase in PST production by *A. tamarense*. The impact of PST on the copepods, post exposure to *A. tamarense*, was estimated by relating their internal PST concentration, ingestion rates and behavioral response to the toxins. The response of *A. tamarense* after exposure to copepods and potential waterborne cues was assessed via screening of gene expression patterns through microarray analyses for all treatments and related to the PST measurements. This functional genomic approach allowed us to trace cue perception to changes in gene expression, since potential waterborne cues recognized by *A. tamarense* may alter gene expression through receptor stimulation.

4.3 Material and Methods

Phytoplankton

The dinoflagellate *Alexandrium tamarense* clonal strain was isolated in May 2004 from the North Sea coast of Scotland (Alpermann et al. 2010), grown in K-medium (Keller et al. 1987) prepared from filtered North Sea water (0.2 μm ; salinity ~ 33) in a light-dark cycle (14:10 h) and diluted with K-medium to a concentration of ~ 500 cells mL^{-1} prior to the experiment. The used clonal strain *A. tamarense* A5 contains intermediate toxin content in comparison with 9 other *A. tamarense* strains (see “clone 5” in Tillmann et al. 2009).

Zooplankton

Female copepods were collected with vertical WP2 net hauls (200 μm) from 50 m depth to the surface in the North Sea during 12-29 August 2007. *Oithona similis* and *Calanus helgolandicus* were collected off the coast of Scotland and *A. clausii* was collected off the northwest coast of Denmark. All copepods were starved for 24 hours in K-medium prepared from filtered seawater (0.2 μm ; salinity ~ 33) at 15 °C in darkness before each experiment. To avoid anoxia in the 20 L containers the seawater was gently bubbled with air (5-10 cm between each bubble). Females were chosen because they release metabolites that attract males and function as a chemical trail potential perceived by *A. tamarense* in this experiment (Bagøien & Kiørboe 2005, Kiørboe et al. 2005).

Grazing experiments

To investigate if the presence of copepods has an effect on the toxicity of *A. tamarense* we incubated the clonal strain in two types of K-medium prepared from filtered (0.2 μm) seawater collected *in situ*; **a**) K-medium with no further manipulation (control treatment) and **b**) K-medium in which copepods had been kept for 24 hours and potentially released metabolites during the incubation (copepod-cue treatment). In each experiment *A. tamarense* was incubated in 1.15 L bottles at a concentration of approximately 4×10^6 cells L^{-1} . This concentration was necessary to ensure enough material for downstream analysis (PST determination and gene expression analysis). Nine bottles were incubated for each treatment, three bottles containing copepods, three bottles containing the K-medium with potential copepod-cues (treatments) and three bottles without copepods or any trace of them

(control). For copepod containing bottles, we either added (per bottle) 10 *C. helgolandicus* females, 15 *A. clausii* females or 30 *O. similis* females. The K-medium was consequently pretreated with potential copepod cues from the corresponding number of copepod females. All copepod species for this experiment were dominant species in the waters investigated. The feeding range of the selected copepod species is within the right range to enable them grazing on *A. tamarensis* cells (3 - 100 μm for *C. helgolandicus* (Harris 1994, Iversen & Poulsen 2007), 18 - 60 μm for *A. clausii* (Berggreen et al. 1988) and 20 - 50 μm for *O. similis* (Svensen & Kjørboe 2000)). We determined the ingestion rates of three different species of adult copepod females (*C. helgolandicus*, *A. clausii* and *O. similis*) feeding on *A. tamarensis* in the treatments (see Fig. 2). Incubations were run for 48 h in the dark on a plankton wheel rotating at ~ 1 rpm at 15 °C. Ingestion rates were calculated using the equations of Frost (1972).

Cell harvesting

Culture bottles were taken off the plankton wheel after 48 h, carefully rotated overhead 7 times to ensure equal mixing of the culture. Of each culture, 10 mL were taken out with a sterile pipette tip and preserved in acidic Lugol's iodine solution for cell counts. Afterwards the culture was poured through a 100 μm mesh followed by a 10 μm mesh to filter out the copepods and to sample the cells. The copepods were transferred into a container with sterile-filtered sea water for further examination of their condition. The 10 μm mesh containing the cells was washed out into a 50 mL collection tube with sterile sea water and adjusted to a volume of 40 mL. From this concentrated cell culture 4 mL were subsampled for toxin analysis and the remaining 36 mL were immediately centrifuged at 4 °C for 5 min for RNA samples. Cell pellets were immediately mixed with 1 mL 60 °C hot TriReagent (Sigma-Aldrich, Steinheim, Germany), transferred to a 2 mL cyrovial containing acid washed glass beads. Cells were lysed with a Bio101 FastPrep instrument (Thermo Savant Illkirch, France) at maximum speed (6.5 m s^{-1}) for 45 s. Afterwards, cells were frozen in liquid nitrogen and stored at -80 °C until further use.

Copepod condition

All copepods were individually examined under a stereomicroscope at the end of the experiment. The escape response of each copepod was assessed by observing its ability to avoid being drawn up into a pipette. If the copepods showed escape response and normal swimming behavior they were categorized as "not

affected”. Copepods without any escape response and copepods without heart beat were categorized as “affected”. The proportion of affected versus non affected individuals was compared within the group for every species over all three replicates.

Counting procedure

Lugol’s iodine-fixed *Alexandrium* cells were counted with an inverted microscope after sedimentation of 3 x 1 mL. In both initial and final samples, all cells in the 1 mL were counted.

RNA isolation

For RNA isolation, the suspended cell lysate was thawed on ice. After thawing, 200 μ L of pure chloroform was added to each vial and vortexed for 15 s. The mixture was incubated for 10 min at room temperature and afterwards centrifuged for 15 min at 4 °C at 10000 x *g*. The upper aqueous phase was transferred to a new vial, filled up with an equal volume of 100% isopropanol, vortexed and incubated for 2 h at -20 °C to precipitate the RNA. The RNA-pellet was collected by 20 min centrifugation at 4 °C and 10000 x *g*. The pellet was washed with 70% EtOH, air dried and dissolved with 100 μ L RNase free water (Qiagen, Hilden, Germany). The RNA-sample was further cleaned with the RNeasy Kit (Qiagen) according to the manufacturer’s protocol for RNA clean-up including on-column DNA-digestion. RNA quality check was performed with a NanoDrop ND-100 spectrometer (PiqLab, Erlangen, Germany) for purity and the RNA Nano Chip Assay with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) was just to examine the integrity of the extracted RNA. Only high quality RNAs (OD 260/280 >2 and OD 260/230 > 1.8) as well as with intact ribosomal peaks (obtained from the Bioanalyzer readings) were used for further experiments.

Gene expression analysis with microarrays

A. tamarense total RNA samples derived from the copepod treatment and the waterborne cues treatment were hybridized against the respective control treatment. The microarray hybridization procedure was carried out with 500 ng total RNA from each sample and the Agilent two color low RNA Input Linear Amplification kit (Agilent Technologies, Santa Clara, USA). Prior to the labeling, the Agilent RNA Spike-In Mix (Agilent) was added to the RNA. The RNA was reverse transcribed into cDNA, amplified towards cRNA and labeled following the ‘Agilent

Low RNA Input Linear Amplification Kit' protocol (Agilent). Dye incorporation rates (cyanine-3 and cyanine-5) and cRNA concentrations were measured with the NanoDrop ND-100 spectrometer (PeqLab). Hybridization was performed onto 4 x 44k microarray slides at 65 °C for 17 h (Agilent). The microarrays contained 60 mer oligonucleotide probes designed by Agilent's E-array online platform from three *Alexandrium* spp. EST libraries. For each hybridization, 825 ng of cyanine-3 and cyanine-5 labeled cRNA were deployed. After hybridization, microarrays were disassembled and washed according to manufacturer's instructions (Agilent). Microarrays were scanned with an Agilent G2565AA scanner. Raw data were processed with the Agilent Feature Extraction Software version 9.1.3.1 (FE). Array quality was monitored with the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07. The array design, raw data and normalized data and the detailed experimental design are MIAME compliant and deposited in a MIAME compliant database (ArrayExpress at the EBI; <http://www.ebi.ac.uk/microarray-as/ae/>; ID: E-MEXP-2874). Testing for differentially expressed genes was performed with the GeneSpring GX software platform version 11 (Agilent) with the implemented T-test against zero statistics combining biological replicates. Genes were considered to be differentially expressed when test statistics reply *p*-Values were less than 0.05 and calculated fold changes between the control and the treatment exceeded 1.5.

PST analysis

The 4 mL concentrated cell solution was centrifuged for 15 min at maximum speed. The water was poured out and the cell pellet was transferred with 1 mL of sterile seawater to a 2 mL tube and centrifuged for another 10 min at maximum speed. The seawater was subsequently removed with a pipette and the pellet was transferred with 2 x 250 µL of acetic acid (0.03 N) into a FastPrep tube (Thermo Savant, Illkirch, France) containing 0.9 g of lysing matrix D. Cells were lysed by reciprocal shaking in a Bio101 FastPrep instrument (Thermo Savant) at maximum speed (6.5 m s⁻¹) for 45 s. and centrifuged afterwards for 15min at 13.000 x *g* and 4 °C. From the supernatant, 400 µL were passed through a spin filter (pore size 0.45 µm) by centrifugation for 30 sec. at 3000 x *g*. For the copepods the same procedure was applied with the following changes: after removing the remaining seawater from the copepods, some liquid nitrogen was filled in the tube and the copepods were crushed. After evaporation of the nitrogen the acetic acid was filled in the tube. The samples were further processed as described for the cell pellet samples. The filtrate was injected into the HPLC/FLD equipped with a fluorescence detector The PST

analysis was carried with an LC1100 series liquid chromatograph (Agilent Technologies) coupled with a PCX 2500 post-column derivatization system (Pickering Laboratories, Mountain View, USA). The HPLC system consisted of a 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). The chromatographic conditions are summarized in Table 4.1.

Table 4.1: Gradient elution conditions for the quantification of the PSTs. Eluent A: 6 mM 1-octanesulphonic acid and 6 mM 1-heptanesulphonic acid in 40 mM ammonium phosphate, adjusted to pH 7.0 with diluted phosphoric acid and 0.75% THF for the gonyautoxin group. Eluent B: 13 mM 1-octanesulphonic acid in 50 mM phosphoric acid adjusted to pH 6.9 with ammonium hydroxide and 15%(v/v) of acetonitrile and 1.5% of THF for the saxitoxin group.

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

The autosampler was cooled to 4 °C and set to a flow rate of 1 mL min⁻¹. The injection volume was 20 µL and the separation of the analytes was performed 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. The eluate from the column containing the separated analytes was 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 0.75 N nitric acid at a flow rate of 0.4 mL min⁻¹. The toxins were detected by a dual monochromator fluorescence detector (λ_{ex} 333 nm; λ_{em} 395 nm). 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.) were purchased from the Certified Reference Materials Program, National Research Council, Halifax, Canada.

4.4 Results

Cellular PST content of *A. tamarensis*

Exposure to *C. helgolandicus* individuals and water borne cues caused the largest increase in *A. tamarensis* PST content per cell compared to the control (Fig. 4.1). Incubation with *C. helgolandicus* individuals raised the cellular PST level of *A. tamarensis* by 100%, and incubation with waterborne cues from *C. helgolandicus* resulted in 60% higher PST content per cell, compared to that of the control incubations. The differences between the three treatments were significant (ANOVA: $n = 3$; $p < 0.05$) whereby both the test treatments differed significantly from the control treatment, and also from each other (Holm-Sidak method: $p < 0.05$). In contrast, no significant change in total PST content of *A. tamarensis* was observed when exposed to *O. similis* individuals or *O. similis* waterborne cues (ANOVA: $n = 3$; $p > 0.05$). Similarly after exposure to *A. clausii*, no significant changes in cellular PST concentrations of *A. tamarensis* could be verified (ANOVA; $n = 3$; $p > 0.05$).

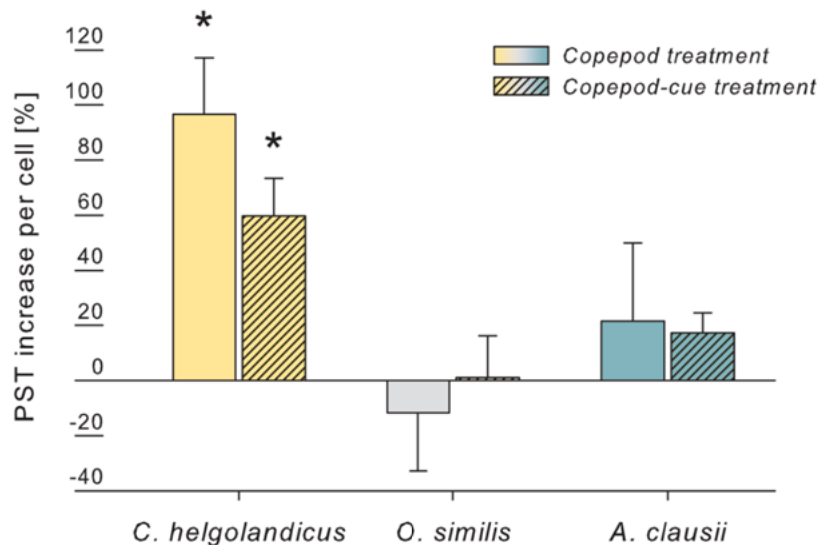


Fig. 4.1: PST content of *A. tamarensis* cells after 48h exposure to live copepod and/or waterborne cues. Differences in PST content are denoted in percentage compared to that of the control. Bars are grouped according to the respective copepod species tested. The first bar of each group represents the treatment with copepods, the second bar represents the treatment with the copepod waterborne cues and the third bar represents the control treatment ($n = 3$ each). Bars marked with asterisks show a significantly higher PST content compared to the control (ANOVA, $p < 0.05$).

Ingestion rates and PST content of the copepods

C. helgolandicus had two-fold higher average ingestion rates of *A. tamarensis* cells compared to the average ingestion rates of both *O. similis* and *A. clausii*, which had ingestion rates of ~50 and ~60 cells female⁻¹ h⁻¹, respectively (Fig. 4.2). Though no significant differences due to high standard deviations could be verified for the determined ingestion rates (ANOVA: $p > 0.05$).

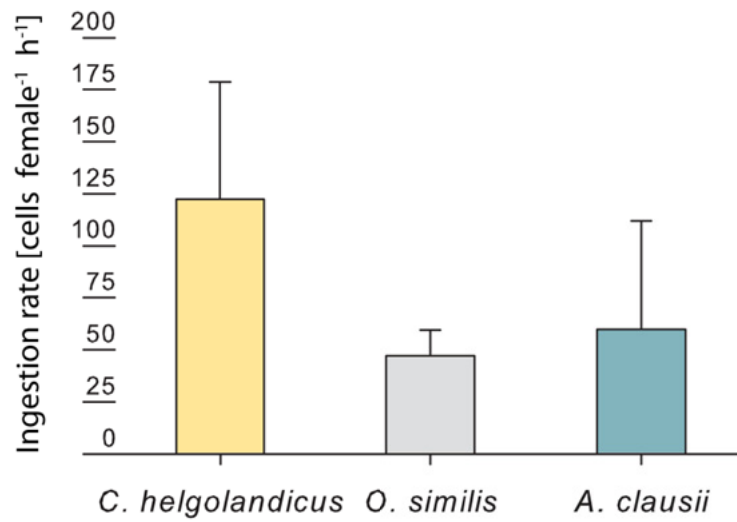


Fig. 4.2: Ingestion rates of the copepod species determined after 48h. Ingestion rates calculated after Frost (1972) and standard deviations, reported as ingested cells per copepod species per hour (*C. helgolandicus* n = 30, *O. similis* n = 90 and *A. clausii* n= 45).

The lowest weight-specific PST content was found in *C. helgolandicus*, which had an average weight-specific PST content 3.7 and 2.5 times below the average weight-specific PST content for *O. similis* and *A. clausii*, respectively (Fig. 4.3).

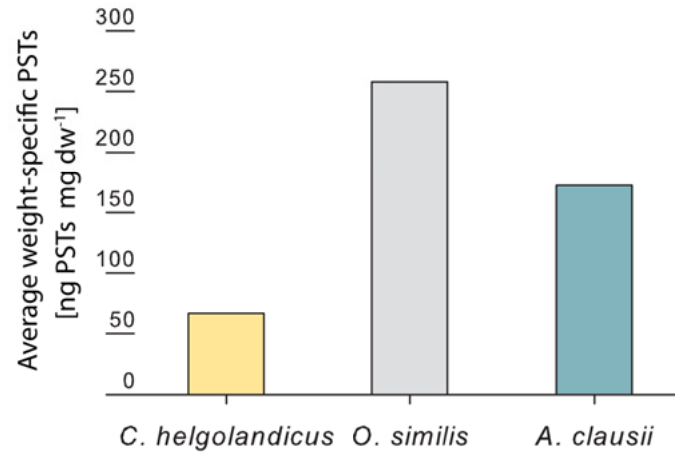


Fig. 4.3: Average PST content of the copepod species determined after 48h. PST measurements taken out with pooled copepods (excluding dead individuals) of every group (*C. helgolandicus* n = 24, *O. similis* n = 67 and *A. clausii* n = 25). Dry weight (dw) of copepods was determined through interpolation with the equations of Uye (1982).

Behavioral response of the copepods

A significantly higher proportion of unaffected copepods was only observed among the *C. helgolandicus* individuals (Students *t*-test, n = 3 $p < 0.05$). After 48 h incubation with *Alexandrium* cells, 57% of the *C. helgolandicus* individuals showed an escape response, interpreted as normal response behavior, while 29% showed no escape response and 14% of *C. helgolandicus* died during the incubation. In contrast, *O. similis* individuals revealed a significantly higher proportion of affected copepods (Students *t*-test, n = 3 $p < 0.05$): 86%. Here the group of affected copepods is composed of 65% individuals with no escape response and of 21% dead individuals. No noticeable change in escape behavior and therefore no effect were observed for 14% of the *Oithona* individuals. *Acartia* individuals exposed to *A. tamarense* showed an equal mean distribution of not-affected and affected copepods (50% each). Due to the standard deviation no significance could be determined (Students *t*-test, n = 3 p

> 0.05) (Fig. 4.4). Affected individuals were composed of 10% with no escape response and 40% dead copepods.

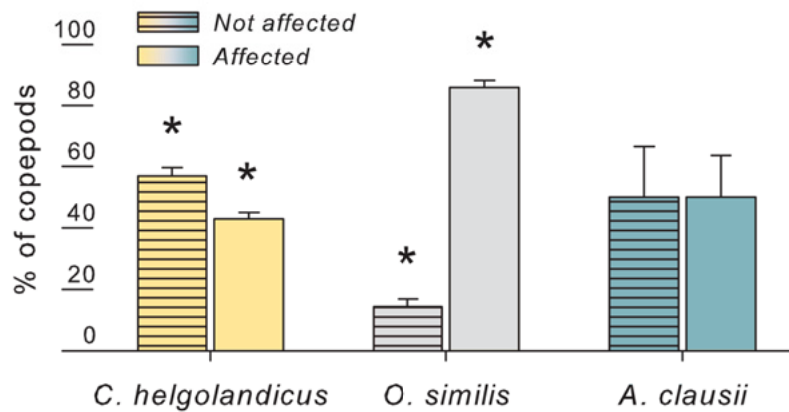


Fig. 4.4: Copepod condition after 48h exposure to *A. tamarensis* cells. Results are grouped according to species (*C. helgolandicus* n = 30, *O. similis* n = 90, *A. clausii* n = 45). The first bar displays the percentage of individuals with no noticeable change in their response behavior (not affected). The second bar represents the percentage of disoriented and dead individuals (affected). Asterisks mark significant differences in the amount of non affected and affected individuals (Students *t*-test, n = 3 $p < 0.05$).

Gene expression profiles of *A. tamarensis*

A significant change in the gene expression compared to the control treatment was observed for every treatment (Student's *t*-test against control treatment; $p < 0.05$; Fig. 4.5). Additionally, overlaps in responsive genes could be identified for every treatment with copepod individuals and respective waterborne cues. The highest numbers of up-regulated genes were observed in the treatments containing *C. helgolandicus* individuals and *C. helgolandicus* waterborne cues whereas the lowest numbers were observed in the treatments containing *O. similis* individuals and *O. similis* waterborne cues. Numbers of up-regulated genes observed

from the *A. clausii* treatments were intermediate. The numbers of down-regulated genes were highest for the treatments with *A. clausii* individuals and waterborne cues, followed by the treatment with *C. helgolandicus* individuals.

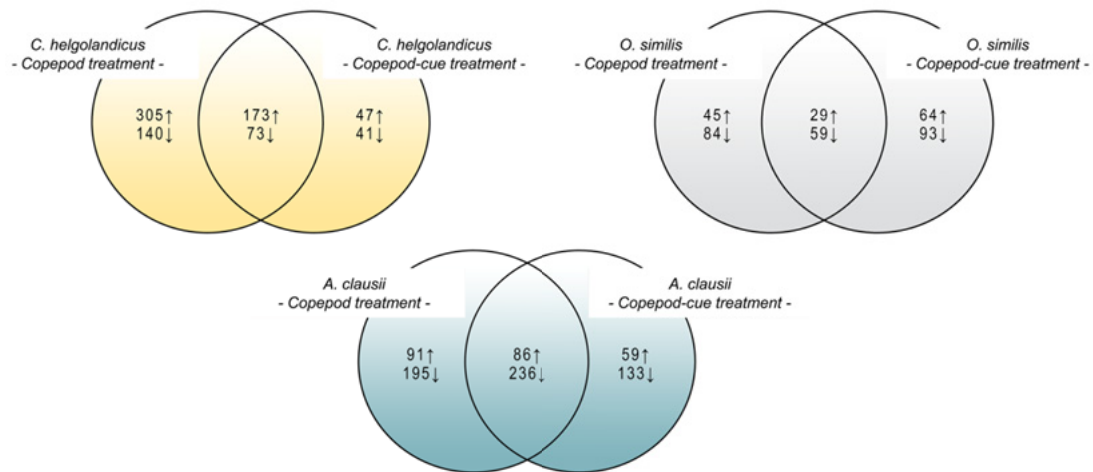


Fig. 4.5: Venn diagram of responsive ESTs after 48h exposure to different copepod species and cues. Numbers of responsive ESTs are separated into up- (↑) and down- (↓) regulated ESTs. The intersections displays the number of ESTs regulated in both treatments. Identification of regulated ESTs is based on microarray hybridizations and evaluated with a Student's *t*-test against a control treatment with $n = 3$ and $p < 0.05$.

The distribution of differentially regulated genes from the different copepod treatments based on annotations according to KOG groups and categories (eukaryotic orthologous groups; Tatusov et al. 2003) indicate a broad response concerning multiple cellular function (Fig. 4.6). A full list of the regulated genes with annotations can be retrieved from the supplemental material for the *Calanus*, the *Oithona*, and the *Acartia* treatment, respectively (Table S4.1, S4.2, S4.3).

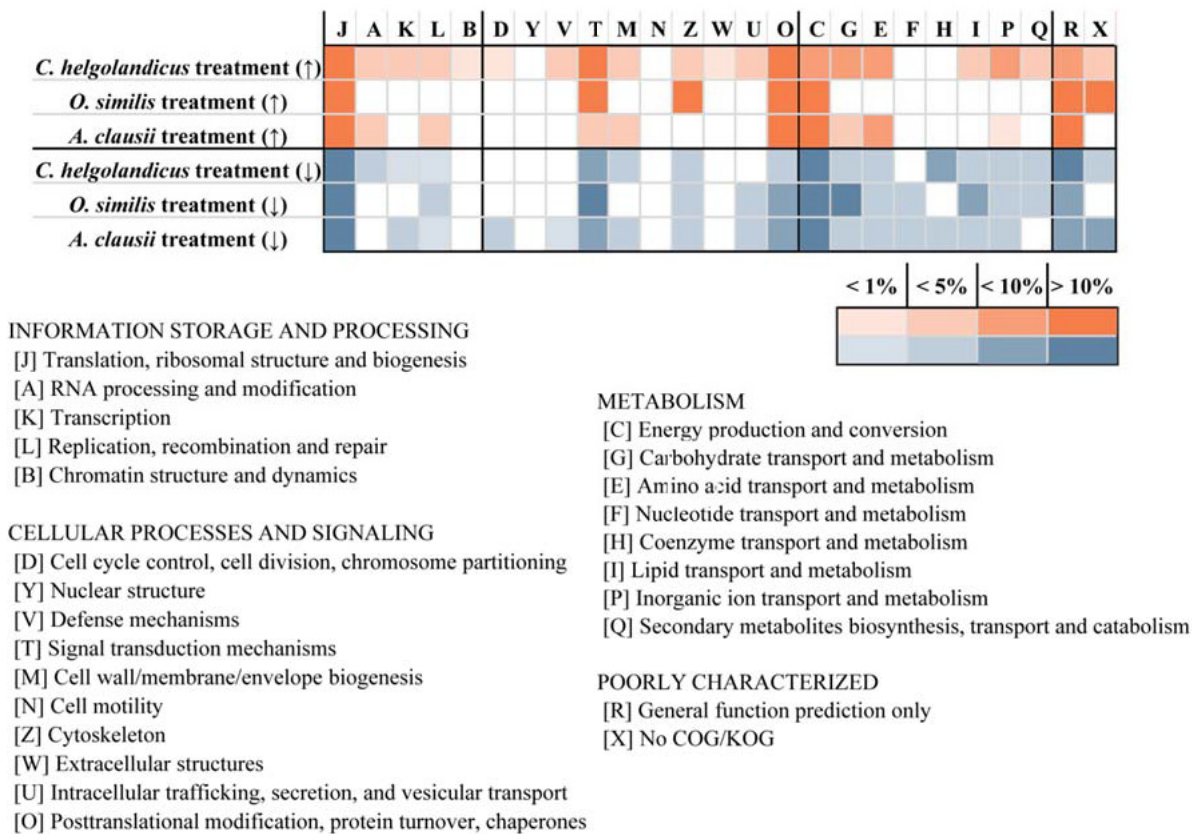


Fig. 4.6: KOG category distributions of differentially expressed ESTs identified through microarray hybridizations. The table displays the KOG category distribution of up- (↑) and down- (↓) regulated ESTs in comparison to a control treatment. The color intensity displays the number of ESTs per group calculated in percent from total ESTs grouped into KOGs with known or general function prediction.

The difference in expression of main signaling proteins, the serine/threonine kinases, as well as further proteins containing calcium-binding domains is highlighted in Table 4.2. Up-regulation of serine/threonine kinases was observed in *Alexandrium* exposed to live *C. helgolandicus*, leading to an increased expression of 8 serine/threonine kinases, 3 of them possessing a calcium-dependent activity.

Down-regulation of serine/threonine kinases was also observed for the *C. helgolandicus* and for the *A. clausii* treatment (Table 4.2). In contrast, the treatment containing *O. similis* individuals resulted in no difference regarding the expression of serine/threonine kinases. Altered expression of transcripts encoding for proteins containing calcium-binding domains were triggered by exposure to all live copepod species investigated in this study.

Table 4.2: Differentially regulated genes coding for proteins with serine/threonine kinase activity and/or calcium binding domains.

Treatment	Regulation	Putative gene product	Serine/threonine kinase	Ca ²⁺ -dependent activity	Fold change		
<i>Exposure to C. helgolandicus</i>	upregulated	<i>Mitogen-activated protein kinase kinase</i>	x		1.9		
		<i>ERK activator kinase</i>	x		3.7		
		<i>Mitogen-activated protein kinase ERK-A</i>	x		1.7		
		<i>Serine/threonine protein kinase</i>	x		1.7		
		<i>Serine/threonine protein kinase</i>	x		1.5		
		<i>Calcium-dependent protein kinase</i>	x	x	1.5		
		<i>Calcium-dependent protein kinase</i>	x	x	3.2		
		<i>Calcium-dependent protein kinase</i>	x	x	1.6		
		<i>Calmodulin</i>		x	1.7		
		<i>C2 domain containing protein</i>		x	2.0		
		<i>C2 domain containing protein</i>		x	1.6		
		<i>Efhand containing protein</i>		x	1.6		
		<i>Efhand containing protein</i>		x	2.2		
		<i>Calcium-dependent protein kinase</i>	x	x	1.6		
		downregulated	<i>Efhand containing protein</i>			x	1.6
			<i>C2 domain containing protein</i>			x	3.2
<i>Calcium dependent ion transporter</i>				x	1.7		
<i>cAMP-dependent protein kinase regulatory subunit</i>	x				1.7		
<i>Exposure to O. similis</i>	upregulated	<i>Efhand containing protein</i>		x	1.5		
	downregulated	<i>C2 domain containing protein</i>		x	1.9		
		<i>Efhand containing protein</i>		x	1.6		
		<i>Calmodulin</i>		x	1.7		
		<i>Efhand containing protein</i>		x	1.7		
upregulated	<i>Protein kinase domain containing protein</i>	x		1.5			
<i>Exposure to A. clausii</i>	downregulated	<i>Calcium-dependent protein kinase</i>	x	x	1.6		
		<i>Calmodulin</i>		x	2.1		
		<i>Calmodulin</i>		x	2.0		
		<i>Calreticulin</i>		x	1.6		
		<i>Efhand containing protein</i>		x	2.0		
		<i>Efhand containing protein</i>		x	2.4		
		<i>Efhand containing protein</i>		x	1.6		
		<i>Efhand containing protein</i>		x	2.1		
<i>Efhand containing protein</i>		x	1.7				

4.5 Discussion

The present study shows that the response of *A. tamarense* towards copepod grazers is species-specific. This species-specific response is evident on the level of PST content of the *Alexandrium* cells as well as on the molecular level as indicated by the gene expression patterns. Furthermore, the toxins produced by *A. tamarense* affected the tested copepod species distinctly and the toxins were retained in different amounts in the copepods.

Alexandrium tamarense showed the highest increase of PST content when exposed to *C. helgolandicus* waterborne cues and/or individuals. Despite this finding, *C. helgolandicus* had the highest ingestion rates among the tested copepods, and the lowest weight-specific internal PST levels. In addition, *C. helgolandicus* was the least affected species of the tested copepods in this study, therefore we interpret that these results indicate a predator-prey relationship in which both, predator and prey show evidence of co-evolutionary processes. The high ingestion rate, low influence on fitness, and low weight-specific internal PST levels of *C. helgolandicus* indicate an adaptation through detoxification mechanisms that lead to decreased sensitivity to PST. Detoxification mechanisms in copepods have been observed in previous studies (Guisande et al. 2002, Kozłowsky-Suzuki et al. 2009). The increased cellular PST level in *A. tamarense* for both, the treatment with live *C. helgolandicus* and only their cues, suggests a magnified defense response towards a predator capable of handling PST and thus posing a potentially higher risk for the prey population. The significantly higher PST content in *A. tamarense* exposed to live *C. helgolandicus* compared to only their cues may be due to continuous release of potential alarm signals from wounded *A. tamarense* cells and chemical compounds from the copepods. Alternatively, the amount of cues that could trigger PST production in the treatment with only waterborne cues will either remain constant or decrease through e.g. degradation processes during the 48 h incubation. Taken together, the magnified defense response of *A. tamarense* against *C. helgolandicus* and the latter's potential ability to detoxify PST indicates that *Calanus* is a historical threat to natural *A. tamarense* populations. High grazing impacts of *Calanus* on *Alexandrium* have previously been found during an *in situ* study (Turner et al. 2000), and support the potential predator-prey co-evolution between the two species.

Alexandrium cellular PST level did not respond to *O. similis* waterborne cues and individuals, indicating the *A. tamarense* did not regard *O. similis* as a potential grazer. This was also found by Turner (Turner et al. 2000) who observed low grazing impact from *O. similis* on *A. tamarense*. Despite no changes in PST production, *O. similis* was still the most affected copepod in our study and had the highest weight-specific internal PST levels. Thus, *O. similis* does not seem to have any resistance or detoxification mechanisms for PST. The grazing by *O. similis* observed in this study might have occurred due to *A. tamarense* offered as the sole diet, since *Oithona* present *in situ* avoids the ingestion of toxic *Alexandrium* (Turner et al. 2000). It is possible that *Oithona* might have evolved a prey avoidance strategy when alternative diets are available, rendering it sensitive to even moderately low PST production. Consequently, there is no need to enhance the PST production in *Alexandrium*. Exposure to *A. clausii* waterborne cues and individuals only slightly raised the internal PST content of *A. tamarense*. Mean ingestion rates, internal PST content as well as the proportion of affected *A. clausii* individuals were intermediate compared to the *C. helgolandicus* and *O. similis* treatments.

Avery and Dam (Avery & Dam 2007) described individual PST resistance in the copepod *Acartia hudsonica*. Their results suggest that naturally occurring populations of *A. hudsonica* possess different resistant phenotypic frequencies. They further suggested that there is a substantial cost involved with resistance for the phenotype involved, and therefore no allele fixation occurs within a single population. If similar mechanisms are present in *A. clausii*, this might explain the high standard deviations regarding the fitness of the individuals. Additionally, different frequencies of resistant copepods in the replicate treatments could account for high variations in the internal PST measurements of *A. tamarense*. Higher numbers of unaffected, grazing copepods could indicate a higher predation risk of *A. tamarense* and, thus, raise internal PST content.

The results of this study support the findings of Selander et al. (Selander et al. 2006), concerning increased toxin content in *A. tamarense* after exposure to waterborne cues from copepods. Similar to the observations for *A. minutum* by Bergkvist et al. (Bergkvist et al. 2008), we observed that increased cellular toxin levels in *A. tamarense* upon exposure to copepods and their cues were target specific. It has been suggested that PST production evolved as a defense towards copepod species which pose a threat to *A. minutum* under natural conditions (Bergkvist et al. 2008). Our findings support this hypothesis, since (1) not all of the tested copepod species triggered an increased toxin production, and (2) toxin

production seems to correlate with both the presence of and cues from copepods that had high grazing impact on *A. tamarense*. The internal toxin content of *A. tamarense* as a defense mechanism seems to be dependent on the potential grazing impact that a given copepod species has on the population of *A. tamarense*.

The continuous production of PST in *A. tamarense* may lead to a permanent basic protection against copepods which did not evolve detoxification abilities and consequently may lead to grazing avoidance strategies. This might also apply for *Oithona* and *Acartia*, which are ambush feeders (Paffenhöfer 1993, Kiørboe et al. 1996) and are therefore able to feed more selectively than *Calanus*, a filter feeder (Boyd 1976).

Necessary for this adaptive response to different copepod species is that *A. tamarense* recognizes a specific copepod as a threat. Although mechanisms controlling adaptive responses in phytoplankton have not been thoroughly studied, Laforsch et al. (2006) proposed that unspecific alarm cues from conspecifics may in fact indicate a predation risk in the cladoceran *Daphnia*, and activate inducible defense. White-dotted mosquito pupae have been shown to associate novel predatory odors, with alarm cues released from injured conspecifics, thus developing a response solely to the predator cues (Ferrari et al. 2008). The ability of phytoplankton to gather specific information through chemical signals concerning abiotic and biotic properties of their current surroundings and to respond appropriately to this information is indeed remarkable (reviewed in i.e. Fink 2007, Pohnert et al. 2007, Pohnert 2010). Even resting cysts are able to recognize the presence of grazers (Hansson 2000). With respect to these sensing capacities, such mechanisms like 'threat sensitive learning' could have evolved the grazer-specific response toward copepod species that present a threat (e.g. *C. helgolandicus*). Hence, the species-specific alarm cues are not activated when non-threatening copepods are present, e.g. *O. similis*.

On a molecular level this means that *Alexandrium* must have receptors transmitting the received signals into the cell where they are processed, and finally induce the expedient response to the received infochemicals. Such receptors comprise fast evolving proteins that are coupled to signal cascades and are capable of adapting to new or changing environmental cues in a relatively short period of time through slight mutations of an ancestor receptor (Rausher 2001). Despite the lack of knowledge regarding these processes of adaptive receptor radiation in phytoplankton, their existence is evidenced by, e.g. the presence of adaptive feeding

receptors (C-type lectins) (Medhekar & Miller 2007), enabling food particle selection in the marine dinoflagellates *Oxyrrhis marina* (Wootton et al. 2007). Thus, the existence of adaptive and therefore tunable receptors could have evolved in *Alexandrium* to integrate waterborne cues from the environment and to respond appropriately.

Chemical signals generated by live copepods and their waterborne cues induced a change in *Alexandrium* gene expression with observed overlapping responsive genes in the respective copepod/copepod-cue treatment for every species tested. Thus, the observed gene expression patterns indicate that the presence of all three tested copepod species and their cues was sensed by *A. tamarense*, yet elicited a different response (Fig. 4.4). Hence, when the ability of *A. tamarense* to recognize the presence of a copepod is solely based on PST level changes in *Alexandrium* it cannot be identified if the lack of increased PST production is due to no recognition of the copepod or if the copepod is recognized but not viewed as a potential risk.

For the interpretation of the results gathered from the microarray analyses we assume that the sum of regulated genes reflects those genes, for that altering the expression rate is required to integrate the external stimuli into the current stable cellular state, i.e. cell cycle progression (Jenster 2004). Thus, grazing from copepods triggers gene regulation for a larger range of genes and not only the genes directly involved in defense. This process is indicated by the distribution of regulated genes in different functional categories observed in this study (Fig. 4.5). Such complex responses are also known from plants being attacked by herbivores. After being attacked, herbivory-specific signals are generated by the plant and further converted to large-scale biochemical and physiological changes through complex networks (Schwachtje & Baldwin 2008, Wu & Baldwin 2009). A very prominent and adaptive signaling event is the phosphorylation of different target proteins through serine/threonine kinases (Downward 2001). The changing number of these kinases over time is often the result of a feedback regulation from the integration of signals stimulating receptors (Downward 2001). Altered transcriptions levels of protein kinases are known to trigger the responses of plants towards herbivore attack (Wu et al. 2007, Wu & Baldwin 2009). Vardi et al. (2002) showed that changes in the activity, abundance, and expression of protein kinases are involved in the competitive interactions between the cyanobacterium strain-specific perception of *Microcystis* and the dinoflagellate *Peridinium gatunense*.

Different expression rates of serine/threonine kinases observed in this study suggest that these serine/threonine kinases are involved in the different responses of *A. tamarensis* to the received copepod-cues (Table 2.2). Three of the serine/threonine kinases that were differentially expressed in the *C. helgolandicus* treatment are grouped (based on annotations) into the class of mitogen-activated protein kinases (MAPKs) and respective downstream kinases (MAPKKs) and four are grouped into the class of calcium dependent protein kinases (CDPKs). In the *A. clausii* treatment, one kinase could be grouped into the class of CDPKs whereas no MAPK or CDPK was among the regulated genes in the *O. similis* treatment. MAPK cascades are known to be an important pathway downstream of receptors regulating cellular responses to several external stimuli and are evidenced to be involved in regulating defensive responses through jasmonic acid formation after wounding in tobacco species (Korth 2003). An activation of CDPKs and MAPKs after attack by a generalist herbivore has been described for *Nicotiana attenuate* and *Solanum nigrum* (Maffei et al. 2007). Additionally, an accumulation of transcripts coding for MAPKs and CDPKs as part of the defensive response in *N. attenuate* is described in Wu et al. (Wu et al. 2007). In the present study, the treatment with *C. helgolandicus* inducing the highest increase in internal PST levels of *A. tamarensis* showed the strongest change in altering expression levels of MAPKs and CDPKs transcripts. A direct involvement of MAPKs and CDPKs in coordinating the response towards copepod grazers in *A. tamarensis* should therefore be considered.

In addition to CDPKs, further transcripts of proteins with a dependency of calcium ions as second messengers have been identified as differently expressed in this study. The regulation of intracellular calcium concentrations and oscillations is a well-described mechanism to transduce environmental changes into the cell (reviewed in Jingwen et al. 2006, Verret et al. 2010). Also in marine phytoplankton, several studies concerning Ca^{2+} signaling highlight their importance in responding to environmental changes (Jingwen et al. 2006; Verret et al. 2010). The transcriptional regulation of genes coding for proteins possessing a calcium dependent activity might be another important trigger in tuning the different responses at the cellular level towards copepod grazers in *A. tamarensis*.

4.6 Conclusions

This study demonstrates that increased PST production in *A. tamarense* is inducible through the presence of copepod grazer in a species-specific manner and that these specific responses are detectable through functional genomic approaches. The role of PST in acting as defense compound should therefore be reconsidered with respect to genotypic variation and phenotypic plasticity of *Alexandrium* species and tested copepod grazers. Since the present study investigated the effect of one clonal strain of *A. tamarense* on the copepod species we cannot exclude different outcomes of the same study when using different strains. The treatments with copepods or copepod-cues resulting in altered gene expression patterns can be considered as an outcome of the ongoing dialog between *Alexandrium* and its environment and the result of integrating and processing the received information into the most appropriate response selected through evolutionary processes. Different abundances of MAPKs together with CDPKs and further Ca²⁺-dependent proteins potentially influence the overall cell response through directing the external stimuli into different intracellular cascades and networks.

5. Fight and flight in dinoflagellates? Kinetics of simultaneous grazer-induced responses in *Alexandrium tamarense*

5.1 Abstract

We monitored the kinetics of grazer-induced responses in the marine dinoflagellate *Alexandrium tamarense*. Chemical cues from each of three calanoid copepods (*Calanus* sp., *Centropages typicus*, and *Acartia tonsa*) induced an increase in intracellular toxin content and suppressed chain formation in *A. tamarense*. Both chemical and morphological responses augmented over 3 d. The toxin content subsequently averaged 299% higher than controls, and average biovolume 24% lower than controls because of suppression of chain formation in grazed treatments. Grazer-induced toxin content returned to control levels after approximately 11 d, equivalent to five cell divisions, and average biovolume returned to control levels within 1 to 4 d (one to two cell divisions). This suggests that dinoflagellates simultaneously reduce grazer encounter rates and increase chemical defense levels in the presence of copepod grazers. Media replacement experiments showed that the inducing cue(s) attenuate rapidly in seawater, which allows *A. tamarense* to adjust resource allocation to grazer-induced responses to follow fluctuations in grazer density. Grazer-induced responses, however, develop too slowly to be accounted for in short-term grazing experiments with laboratory cultures.

5.2 Introduction

Induced behavioral, morphological, chemical, or life history changes triggered by predators or grazers are common and widespread in many groups of organisms (Kats & Dill 1998, Tollrian & Harvell 1999, Toth & Pavia 2007). Induced responses also occur in phytoplankton (Hessen & Van Donk 1993, Jakobsen & Tang 2002, Selander et al. 2006), and can be efficient in reducing losses to consumers (Long et al. 2007). As an example, inducible defenses may prevent population fluctuations in laboratory experiments with bi- and tri-trophic planktonic food chains (Verschoor et al. 2004), and offset trophic cascades in laboratory experiments with chlorophyceans and herbivorous and carnivorous rotifers (Van der Stap et al. 2007).

Reducing losses to consumers can be achieved either by decreasing encounter rates with grazers or by increasing the probability to successfully escape encountered enemies. Phytoplankton adopts both strategies. Freshwater dinoflagellates delay excystment in response to zooplankton cues to avoid grazers (Rengefors et al. 1998), and the marine dinoflagellate *Alexandrium ostenfeldii* forms temporary cysts in response to waterborne cues from parasites (Toth et al. 2004). The related dinoflagellate *Alexandrium tamarense* suppresses chain formation and reduce swimming speed in response to copepod cues to reduce encounter rates with grazers (Selander et al. 2011). In contrast, post-encounter mechanisms to escape grazers in marine protozoans involve escape swimming bursts (Jakobsen 2002) as well as morphological or chemical defense strategies. Both marine (e.g., *Phaeocystis globosa*) and freshwater (e.g., *Scenedesmus*) colony-forming phytoplankton respond to grazers by growing larger or smaller than the size spectrum that can be efficiently handled by inducing grazers by adjusting the number of cells in colonies (Hessen & Van Donk 1993; Long et al. 2007). The freshwater cyanophycean *Microcystis* (Jang et al. 2003) and the marine dinoflagellates *Alexandrium minutum* and *A. tamarense* increase cellular toxin content in response to specific zooplankton grazers (Selander et al. 2006; Bergkvist et al. 2008; Wohlrab et al. 2010).

Despite the mounting evidence that grazer-induced responses are common in phytoplankton, laboratory experiments on zooplankton–phytoplankton interactions are often performed with algae from grazer-free laboratory cultures. Moreover, incubation times in grazing experiments are typically short (days) and grazer induced responses are not likely to develop unless the response time is very short. A better knowledge of the timing of the onset and relaxation of grazer-induced changes is clearly needed to evaluate the relevance of grazer-induced responses for phytoplankton–grazer population dynamics, as well as their implications for experimental plankton ecology, but the kinetics of grazer-induced responses in phytoplankton has received little attention.

In the present study we followed the onset and relaxation of grazer-induced responses in *A. tamarense* in incubation experiments with copepod grazers. We show that the inducing signal is chemical and evaluated the signal duration in media replacement experiments. Finally, we showed that simultaneous changes in morphology and secondary chemistry are general responses to chemical cues from calanoid copepods in *A. tamarense*.

5.3 Material and Methods

Cultures

Cultures of *A. tamarensis* were isolated from the Swedish west coast in 2008. Two strains were used in the current experiment: *A. tamarensis* No. 1 and No. 9, deposited at University of Gothenburg Marine Culture Collection (GUMACC). Cultures were grown in K-medium (Keller et al. 1987) in a temperature- and light-controlled room (salinity 33, 18 °C, 14:10 h light:dark cycles and a photon flux density (PFD) of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Copepods were collected in the Gullmars Fjord on the Swedish west coast by vertical hauls from 20 m to the surface (200- μm mesh-size, work package 2 net mounted with a non-filtering cod end). The copepods were diluted with filtered seawater in the laboratory and fed *Rhodomonas baltica* (deposited at GUMACC) two times a week until the start of the experiment. Both copepods and dinoflagellates were isolated from the same area on the Swedish west coast.

Cage experiment

The grazer cue experiment was conducted in 100 mL glass flasks with cages made from 50-mL polypropylene tubes. The cage bottom consisted of a 10- μm plankton mesh that constrained the organisms to their original compartment (flask or cage), but allowed grazer cues to move between compartments. Each flask received 70 mL *A. tamarensis* No. 1 culture (532 ± 15 cells mL^{-1} , mean \pm SD) in K/10-medium (Keller et al. 1987). Cages were deployed in flasks and filled with 30 mL of the same *Alexandrium* culture. Four replicates were kept as controls without copepods in cages, four received one *Calanus* sp. late copepodite, four received two *Centropages typicus*, and the last four received five adult *Acartia tonsa* females. Different numbers of copepods were added to achieve comparable biovolumes of grazers among treatments. To promote exchange of chemical cues between cages and flasks, the cages were gently moved up and down in the flask every second hour five times daily. After 3 d, the experiments were terminated. The volume and cell concentration in the flasks were determined (Coulter Counter Elzone 5380) and a known volume of *A. tamarensis* culture was filtered onto GF/F filters and stored frozen until toxin analysis. Single cells and chains are enumerated and sized as single particles in the Coulter Counter. To avoid confounding effects from chain

splitting, growth rate and toxin content were calculated from Coulter Counter biovolume concentrations ($\mu\text{m}^3 \text{ mL}^{-1}$). For growth rate the total biovolume in the flask was divided by the initial volume of the flask (70 mL) to adjust for any concentration or dilution effect of the cage design.

Kinetic experiment

To investigate the kinetics of the grazer-induced responses *A. tamarensis* No. 9 ($272 \pm 5 \text{ cells mL}^{-1}$) were exposed to direct grazing from *C. typicus*. The culture was divided between 16 flasks, 500 mL in each flask. Four flasks were kept as controls, without copepods (“control”) and four received five adult *C. typicus* females (“grazed”). The remaining eight flasks (“terminated” and “washed”) also received copepods, but in order to follow the relaxation of induced responses, the copepods were sieved out after 5 d by pouring the culture through a submerged 64- μm plankton sieve. All treatments were subjected to the same filtration treatment to avoid confounding handling effects, but copepods were reintroduced in the grazed treatment. In the washed treatment, the culture volume was reduced by inverse filtration (10 μm mesh size) to one tenth of the original volume and re-diluted with fresh K/10-medium. The procedure was repeated twice to reduce copepod cues in the media by $\sim 99\%$. The terminated and washed treatments were performed to evaluate the persistence of the inducing cues. If the response attenuated more slowly in the terminated compared to the washed treatment that would indicate that the cues remained in solution for a corresponding amount of time. In the grazed treatment copepods were checked for vital signs (motility) and dead copepods were replaced. On Days 1, 3, 5, 6, 9, 10, 13, 14, 16, 17, and 20, a sample ranging from 100 to 250 mL, depending on the sampling interval, was drawn and replaced with freshly prepared K/10-medium. The samples were pre-filtered through a 64- μm nylon mesh to remove eggs and copepods. Cells were counted and sized with a Coulter counter (Elzone 5380) mounted with a 100- μm orifice tube and continuous stirring. Toxin samples were suction filtered onto glass-fiber filters on Days 1, 3, 5, 6, 10, 16, and 20, and stored frozen until toxin extraction and analysis. On one occasion two samples were not mixed before sampling; these samples and the subsequent sample from the same replicates were excluded from growth rate calculations but included in the cell-specific toxin content and average biovolume estimates.

PST analysis

Paralytic shellfish toxin (PST) samples were lyophilized and extracted with 1 mL 0.05 M acetic acid through three consecutive freeze–thaw cycles. The extract was filtered (GF/F) and stored frozen in glass vials until analysis by high-performance liquid chromatography with fluorescence detection (LC-FD). Liquid chromatographic analyses were carried out on a Hitachi-7000 system equipped with a RP8 Column (Vydac Genesis C8, 4 μm , 150 mm, inner diameter 3 mm). PST standards (saxitoxin, neosaxitoxin, and gonyautoxin 1–4) were purchased from the Certified Reference Materials Program, National Research Council, Halifax, Canada. To quantify gonyautoxin 1–4, neosaxitoxin, and saxitoxin in a single run, we modified the method described by Asp and coworkers (2004). The PST samples were hydrolyzed with 0.1 M HCl at 100° C for 10 min to transform the n-sulfocarbamoyl toxins into their corresponding carbamates. Two separate isocratic runs described in this method were combined in a gradient elution that allowed separation of all carbamates in a single run lasting 30 min (Table 5.1).

Table 5.1: Gradient elution for the quantification of saxitoxin, neosaxitoxin and the gonyautoxins 1-4. Eluent A: 2 mM sodiumheptanesulfonate in 10 mM ammonium phosphate buffer (pH 7.1). Eluent B: 2 mM sodiumheptanesulfonate in 20 mM ammonium phosphate buffer (pH 7.1): acetonitrile (96:4) (modified from Asp et al. (2004)).

Time	Eluent A	Eluent B
0 min	100 %	0 %
5 min	100 %	0 %
6 min	0 %	100 %
20 min	0 %	100 %
21 min	100 %	0 %
30 min	100 %	0 %

The eluent with the separated toxins were oxidized with 7 mM periodic acid in 50 mM sodium phosphate buffer (pH 9.0, 0.2 ml min⁻¹) in a PEEK capillary (10 m, 80° C). The oxidation was terminated with 0.5 M acetic acid (0.2 mL min⁻¹) before fluorescent detection (λ_{ex} 330 nm; λ_{em} 390 nm)

Statistical analysis

A single-factor analysis of variance (ANOVA) with the copepod species identity as the main factor was applied to test the effect of waterborne cues from different copepods on *Alexandrium* cell size, toxicity, and growth rate, in the cage experiment. Homogeneity of variances was tested with Cochran's *C* test ($p < 0.01$). When the ANOVA indicated a significant effect, the Student-Newman-Keuls post hoc procedure was used to detect significant differences between means.

Growth rate was calculated from the general formula:

$$\mu_t = \frac{\ln C_t - \ln C_{(t-1)}}{\Delta t}$$

where C_t and $C_{(t-1)}$ are the stop and start concentrations ($\mu\text{m}^3 \text{ mL}^{-1}$), respectively, in the cage experiment and the stop and start concentration for each sampling interval in the kinetic study; Δt is the time between C_t and $C_{(t-1)}$ in days. Intracellular toxin content and average biovolume in the kinetic experiment were evaluated with a repeated measures (RM) ANOVA with four levels (control, washed, terminated, and grazed). Significant effects were followed by the Student-Newman-Keuls post hoc procedure before grazer removal (comparing grazed treatments with the non-grazed control) and after grazer removal (comparing the washed, terminated, and control treatments). To compare the recovery phase for the washed and terminated treatments, the slope of the curve during the first day of recovery (biovolume) or the entire recovery phase (toxin content) was compared with Student's *t*-test.

5.4 Results

Cage experiment

The toxin content of *A. tamarense* exposed to cues from caged copepods increased in response to both *Calanus* sp. and *A. tonsa* ($p < 0.05$; Fig. 5.1a). There was also a tendency towards an increased toxin content in response to *Centropages* ($p = 0.07$), which was confirmed in the kinetic experiment (see below).

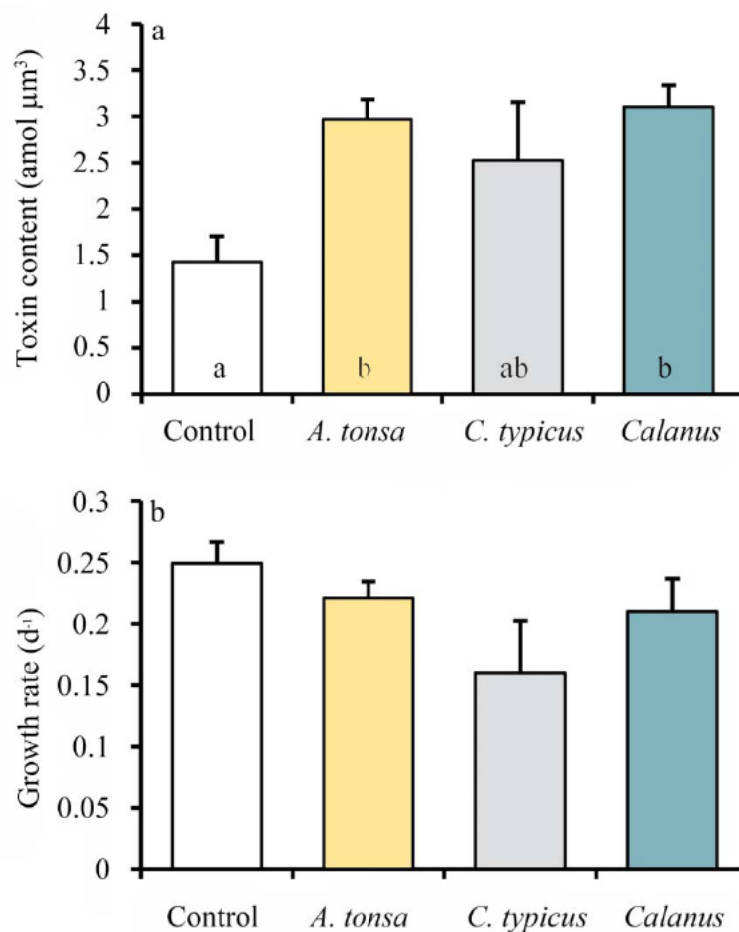


Fig. 5.1: (a) Toxin content per biovolume (amol μm^{-3}) in the indirect grazing experiment, where *Alexandrium tamarense* cells were exposed to waterborne cues from different species of caged calanoid copepods, *Acartia tonsa*, *Centropages typicus*, and *Calanus* sp. Letters indicate statistically homogenous subsets based on the Student-Newman-Keuls post hoc procedure ($p < 0.05$, ANOVA $F_{3,11} = 3.86$ $p = 0.042$). (b) Growth rate (d^{-1}) based on biovolume ($\mu\text{m mL}^{-3}$) outside cages in the cage experiment. Bars represent mean values of four replicates. Error bars show standard deviation; $n = 3$.

In addition, size distribution changed to significantly smaller average biovolume in all treatments exposed to copepod cues ($p < 0.05$; Fig. 5.2a). Microscopic examination of the cultures revealed that the size reduction resulted from higher abundance of two- and four-cell chains in control treatments compared to copepod-exposed treatments, which were dominated by single cells (Fig. 5.2b). The proportion of cells in chains is thus different between treatments, and toxicity and growth rates are based on biovolume concentrations to avoid confounding effects of chain splitting.

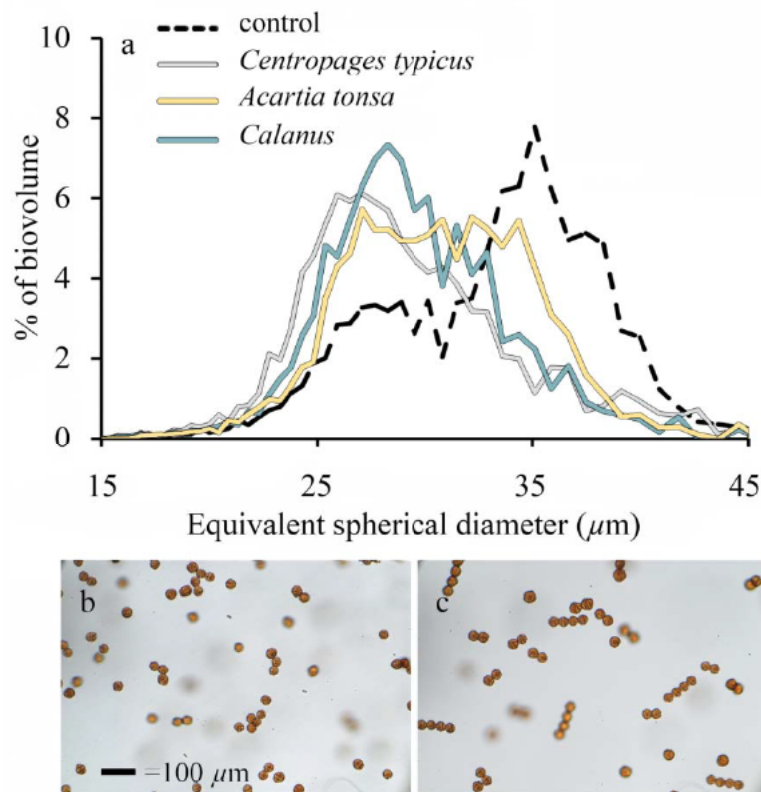


Fig. 5.2: (a) Histograms showing size distribution in *Alexandrium tamarense* cultures exposed to waterborne cues from caged copepods and controls. Average biovolume in control (grazer-free) cultures were significantly larger than all grazer-exposed treatments (ANOVA, $F_{3,12} = 68.1$, $p < 0.001$, Student-Newman-Keuls post hoc procedure at $p < 0.05$). Pictures showing (b) *A. tamarense* cells exposed to direct grazing (Day 17 in the kinetic experiment), observed mainly as single cells or occasionally as two-cell chains. (c) Control (grazer-free) culture from the same occasion. Two- and four-cell chains were more common.

For comparison, the highest toxin content measured in this study, 33.3 amol μm^3 corresponds to 467 fmol PST cell⁻¹ for a 30- μm -diameter *A. tamarense* cell. Growth rates based on biovolume concentration estimates were not significantly

affected by copepod cues (ANOVA, $F_{3,12} = 1.84$, $p = 0.19$, $n = 3$; Fig. 5.1b). Grazers were not in direct contact with the responding cells in the induction experiment, which shows that the difference in size and toxicity is induced by waterborne signals from the caged grazers and is not the result of physical damage or selective grazing.

Kinetic experiment

The results from the kinetic experiment are mostly in congruence with the cage experiments. Only *C. typicus* in direct contact with the responding *Alexandrium* cells was used in this experiment and the toxin content increased in all grazed treatments and leveled off at two- to threefold higher compared to controls after 3 d (Fig. 5.3; $p < 0.05$). Copepods were removed after sampling on Day 5 in the terminated and washed treatments, after which toxicity gradually decreased to control levels in both treatments. In contrast, the cells in the continuous grazed treatment retained high toxin content throughout the experiment (RM ANOVA, $F_{1,6} = 1159$, $p < 0.001$; Fig. 5.3). The difference in size distribution between grazed and control cultures culminated on Day 5, with 40% lower average biovolume in grazed treatments compared to controls ($p < 0.05$). Average biovolume was consistently lower in the continuously grazed treatment (grazed) compared to controls (RM ANOVA, $F_{1,9} = 9.45$, $p < 0.05$; Fig. 5.3). If copepod cues were persistent to degradation, we would have expected a slower recovery to control levels in the terminated compared to the washed treatment, in which dissolved copepod cues were reduced by media replacement after copepod removal. There is, however, no significant difference in the rate of change in average biovolume ($t = -1.19$, $df = 6$, $p = 0.28$) or toxicity ($t = 1.36$, $df = 5$, $p = 0.23$) between the washed and the terminated treatments after grazer removal, which shows that the inducing cue attenuates rapidly after grazer removal.

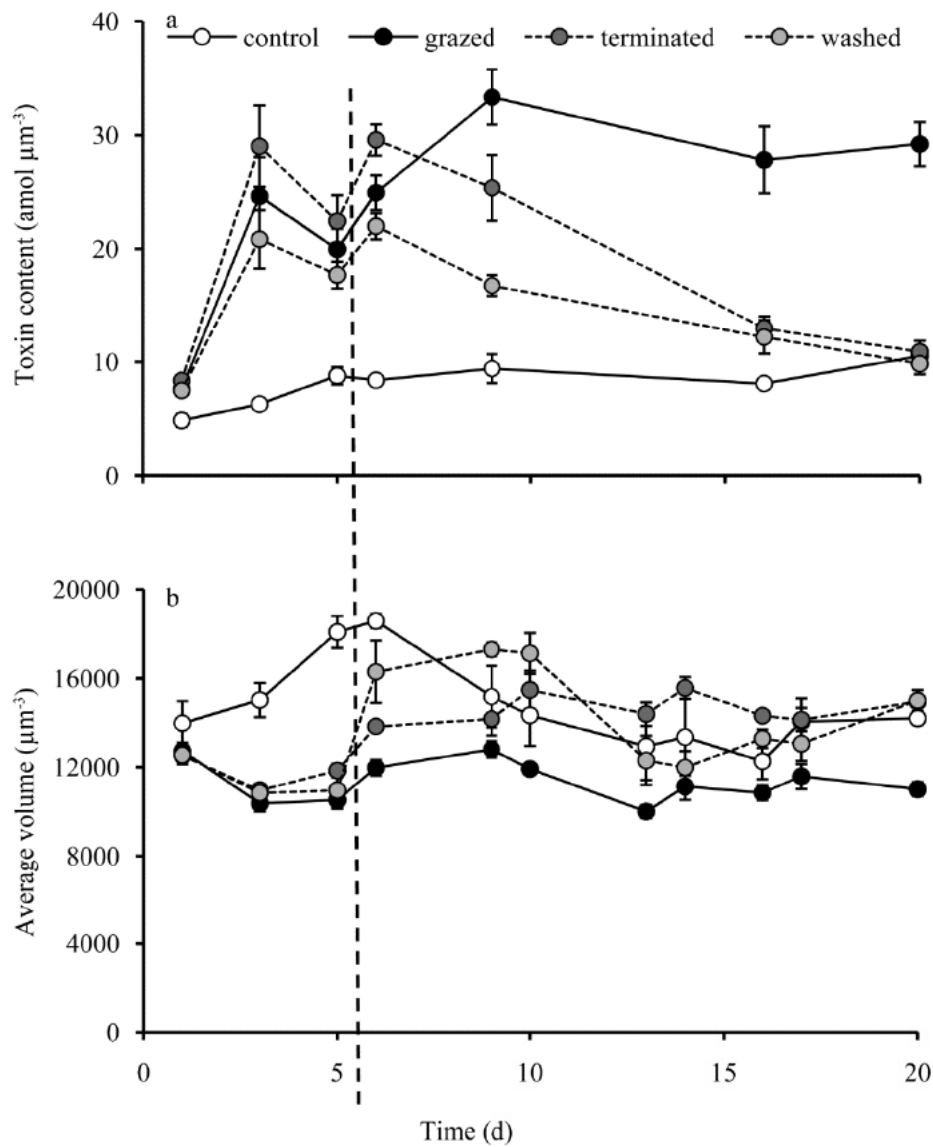


Fig. 5.3: (a) Toxin content per biovolume ($\text{amol } \mu\text{m}^{-3}$) in the ungrazed control treatment (white symbols), the continuously grazed treatment (black symbols), the terminated treatment (dark gray symbols) and the washed treatment (light gray symbols). Copepod grazers were removed in the terminated and washed treatments after 5 d (indicated by dashed vertical line). In addition, the medium was replaced to eliminate $\sim 99\%$ of remaining copepod cues in the washed treatment. (b) Average particle volume in cultures; symbols as above. Symbols represent mean values of four replicates; error bars denote standard deviation.

5.5 Discussion

The present study clearly shows that *A. tamarense* cells grown together with copepod grazers develop a different set of traits compared to grazer-free cultures. The number of cells in chains decreases, and the toxin content increases. Both prey size (Berggreen et al. 1988, Hansen et al. 1994) and toxin content (DeMott 1989, Teegarden & Campbell 2001) are important factors in grazer-phytoplankton interactions. In addition to their larger size, two- and four-cell chains swim faster than single cells (Fraga et al. 1989, Lewis et al. 2006). Larger and faster units are more easily detected by rheotactic predators such as many copepods (Visser 2001). Theoretical predator encounter rates are 2.5- to 5- fold higher for two- and four-cell chains than for single cells of *A. tamarense* (Selander et al. 2011). Thus, there is an immediate reduced risk of encountering grazers for the individual cells splitting up from a chain, whereas the effect on the population level depends on the proportion of chains splitting up. In this case, the 24% lower biovolume in grazed treatments in the kinetic experiment corresponds to 32% of the units splitting up into shorter chains or single cells (or a smaller proportion of four-cell chains splitting up to single cells). Because the relative reduction in encounter rates is comparable between four-cell chain splitting up to two-cell chains and two-cell chains splitting up to single cells (2.1- and 2.2-fold; Selander et al. 2011), the average reduction in copepod encounter rate for the whole population can be estimated as 26% in grazer-induced cultures.

The significance of increased PSTs is less clear. Experiments with *Alexandrium* spp. of different toxicities give mixed support for a grazer deterrent role of PSTs. Some grazers show a strong preference for nontoxic *Alexandrium* cells, with more than 80% lower ingestion rates of toxic *Alexandrium* cells than non-toxic *Alexandrium* cells (Teegarden 1999). Other grazers are seemingly resistant (Teegarden et al. 2001; Colin and Dam 2003). In addition, *Acartia hudsonica* populations from areas frequently exposed to PSTs are more resistant to PST-containing algae than populations with less history of PST exposure (Colin and Dam 2007). Thus the effect of grazer-induced toxicity on grazing rates will depend on grazer abundance and composition. If grazers that discriminate against toxic prey dominate, *Alexandrium* cells may benefit further from reduced losses relative to nontoxic competitors.

No significant costs were associated with the grazer-induced responses observed here. This is in agreement with a previous study where no detectable decrease in growth rate was observed in *A. minutum* exposed to copepod cues under different combinations of nitrate and phosphate limitation (Selander et al. 2008). It is, however, possible that the induced responses are associated with other costs that do not manifest in batch culture experiments. *A. tamarensis* is, e.g., known to perform diurnal vertical migrations to retrieve nutrients from depth. Four-cell chains swim approximately twice as fast as single cells, and are consequently able to access deeper layers and shorten migration time substantially compared to single cells (Selander et al. 2011).

It is not possible to tell if the cue(s) originates from copepods, injured dinoflagellate cells, or a combination of both, but the cues that induce toxicity in *A. minutum* has been characterized as organic compound(s) of intermediate polarity originating solely from the copepods (Selander 2007). Relaxation rates largely agree between the washed and the terminated treatments, which shows that the inducing cue attenuates rapidly. Rapid signal attenuation is crucial for a signal to accurately mirror decreasing grazer densities, as long-lived signals would falsely indicate grazer presence even when the threat is no longer present. The time scale of grazer-induced responses (Fig. 5.3) suggests that they are fast enough to track tightly ambient grazer densities. Both the chemical and the morphological changes developed within 3 to 5 d. Even if the local concentration of copepods may change more rapidly because of dynamic patchy distributions and diurnal migrations, average population density typically does not. Relaxation of grazer-induced toxin content increase is slower compared to the recovery of chain formation. Chain formation demands one to two cell divisions to be fully restored because chains are formed by daughter cells staying attached after division. The slower relaxation of grazer-induced toxin content most likely reflects that several divisions are needed to dilute the internal pool of PSTs. Production rate, and hence the cost of PST production, is probably adjusted more rapidly. The dynamics of chain length plasticity has been examined in greater detail for *Alexandrium monilatum*, where chain length is proportional to growth rate (Aldrich et al. 1967). The shorter chains in grazed treatments could consequently reflect lower growth rate caused by costs associated with toxin production. However, the relationship between growth rate and chain length is present only in control treatments. Suppression of chain formation in grazed treatments is clearly not related to growth rate in grazed treatments (Fig. 5.4).

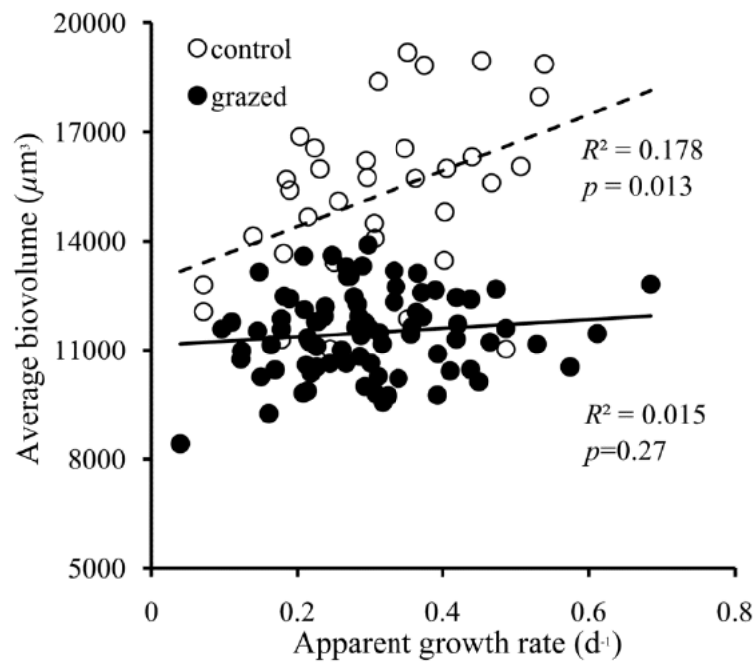


Fig. 5.4: The relationship between the apparent growth rate and average biovolume of cells in both the cage experiment with *Alexandrium tamarens* No. 1 and the kinetic experiment with *A. tamarens* No. 9, excluding the treatments terminated and washed, from which grazers were removed after 5 d. Filled symbols represent grazer-exposed cultures and open symbols control cultures without grazers.

Both grazer-induced toxin content and chain suppression required, two cell divisions to develop fully, corresponding to 2–4 d. Grazing experiments are often incubated for shorter periods, e.g., overnight. Furthermore, experimental studies on zooplankton interactions with PST-producing algae are often performed with laboratory cultures, grown in the absence of grazers. Grazer-free cultures are 2 to > 20 times less toxic than grazed cultures (Selander et al. 2006; Bergkvist et al. 2008; this study) and as a consequence, the effect of PSTs on phytoplankton–zooplankton interaction has likely been underestimated. To control for grazer-induced responses, a treatment with algae acclimatized to grazers for ≥ 2 cell divisions prior to incubations should be included. In this study, we only challenged *A. tamarens* with cues from single copepod grazers. In the field, the dinoflagellate cells will be exposed to more complex chemosensory information, and investigations under more natural conditions are clearly needed.

The results of this study are in agreement with those of other studies showing increased toxicity in *A. tamarense* cultures exposed to *Calanus* copepods (Wohlrab et al. 2010) and also the suppression of chain length for dinoflagellates exposed to *C. typicus* (Selander et al. 2011). The suppression of chain length and the increased toxin content are therefore, probably, general and co-occurring responses to calanoid copepods in *A. tamarense*. *A. tamarense* reacts in a similar way to the three copepod species tested here, suggesting a lower level of specificity compared to that in *A. minutum*, where grazer-induced toxicity is highly grazer-specific (Bergkvist et al. 2008). However, Wohlrab and colleagues (2010) report that the cyclopoid copepod *Oithona similis* does not induce toxin production in *A. tamarense*. The inducing cue hence seems to be more specific than a general excretion product. In contrast to the chain splitting behavior observed here, Jiang and colleagues (2010) report that the dinoflagellate *Cochlodinium polykrikoides* forms longer chains in response to copepod cues or vitamin B additions. Furthermore, copepods ingested fewer four-cell chains compared to two-cell chains and single cells, and these authors proposed that four-cell chains are long enough to create a predator-prey size mismatch. Berggren and colleagues (1988), however, stated that single cells, two-cell chains, and four-cell chains are within the food size spectra of *A. tonsa*, which suggests that there may be additional grazer-induced responses also in this species. Regardless of the mechanism, the study by Jiang and colleagues (2010) shows that the higher encounter rates of longer chains do not translate into higher ingestion rates for this species.

5.6 Conclusions

In conclusion, the simultaneous onset of grazer-induced morphological and chemical responses shows that *A. tamarense* adjust both size and secondary chemistry in response to ambient grazing pressure. Size and toxin content are important factors in grazer-phytoplankton interactions and grazer-induced responses needs to be controlled for in experimental studies. A treatment where algae have been acclimatized to grazers for at least two cell divisions can be included to achieve this. The presence of more than one grazer-induced response suggests that unbiased screening methods like transcriptomics, proteomics, and metabolomics may be useful complements to traditional methods in order to comprehend the full response of phytoplankton to grazer cues.

6. Grazer induced reaction norms - a transcriptomic comparison

5.1 Abstract

Top-down processes such as predation, parasitism, and competition are major forces that structure planktonic species succession and diversity. Hence, grazing exerts a strong selective pressure on phytoplankton and has led to the co-evolution of defensive traits. The toxigenic marine dinoflagellate *Alexandrium tamarense* induces several defensive traits simultaneously in response to waterborne cues of copepod grazers. The high genotypic and phenotypic diversity within natural populations of *A. tamarense* may add another level of complexity to the species range of possible reactions towards grazing. This study compares the defensive response in two different *A. tamarense* strains to reveal potential intra-species variability in grazer induced responses. Our results show that transcriptional changes associated with induced responses towards grazing are not uniform among different representatives of one population of *Alexandrium tamarense*. Several gene classes are differentially expressed upon grazing in the two *A. tamarense* strains, and around 95% of these genes are strain-specifically up- or down regulated. However, both strains are able to induce a defense response. This indicates the existence of different defensive strategies is fixed in the genotypes of a given population. Hence, genetic diversity ensures a broader reaction norm for the population than a clonal population would provide. A wider reaction norm is likely to make populations more resistant to grazers, and less vulnerable to extinction. The different genotypes are therefore a reservoir of phenotypes to ensure the populations' stability in an unpredictable environment.

5.2 Introduction

Marine phytoplankton contributes to nearly half of the global carbon dioxide fixation and is moreover the basis of marine food webs. More than 5000 species of marine phytoplankton take part in these utmost important, but often overlooked global ecosystem services (Hallegraeff 1993, Falkowski et al. 1998). Thus, the proliferation of these species is in most cases beneficial, however mass

proliferations called algal blooms can become harmful. There are at least 300 marine phytoplankton species that are capable of forming harmful algal blooms (HABs), either due to oxygen depletion within the water column or due to the production of toxins that harm invertebrates, fish and/or humans (Hallegraeff 2010). Marine dinoflagellates of the genus *Alexandrium* are one of the most important groups of harmful algal bloom (HAB)-forming taxa because they are ubiquitous, diverse and can potentially have a deep impact on eco-systems (Anderson et al. 2012a). *Alexandrium* species produce three different known families of toxins (saxitoxins, spirolides, and goniodomins). One of these, the saxitoxin family, is responsible for outbreaks of paralytic shellfish poisoning (PSP). PSP is a serious hazard to human health causing intoxications due to the consumption of contaminated shellfish. Further impacts include alterations of ecosystem trophic structure, the loss of wild and cultured seafood resources as well as affects on socio-economic aspects (Anderson et al. 2012a).

Marine dinoflagellates have relatively low maximum growth rates and nutrient uptake rates compared to other dominating marine phytoplankton groups (Smayda 1997). Thus, their ability to form dense and long lasting blooms ('red tides') could appear somewhat puzzling. The traditional view of phytoplankton community ecology defines bottom-up processes (competition for abiotic resources) as the main factors structuring pelagic communities (Hutchinson 1961, Tilman et al. 1982). The combination of bottom-up factors favoring the growth of red tide species is, however, unusual and has been described as a "sort of illness not purged by evolution" (Margalef 1978). 'Dirty tricks' (Thingstad 1998) such as the ability to feed heterotrophically and the ability to migrate in the water column have later been considered to contribute to the dinoflagellates' ecological success (Smayda 1997, Litchman et al. 2007). Mortality and growth rate changes have inverse effects on the population size (Smetacek et al. 2004). Yet, factors regulating mortality rates have long been neglected in these scenarios. Selection for strategies that favor the reduction of loss-factors provides an alternative explanation for the success of marine dinoflagellates that are characterized by slow growth rates (Tillmann et al. 2008).

Grazing is the sole most important loss factor for phytoplankton organisms. On average, more than 80% of marine phytoplankton production is consumed by herbivores (Cyr & Pace 1993, Calbet 2001, Calbet & Landry 2004). Grazing consequently exerts a strong selective pressure on phytoplankton and contributes significantly to the structuring of phytoplankton communities (Verschoor et al.

2004). Intensive grazing favors the evolution of defensive traits and according to the “Red Queen Hypothesis”, involved species will continually co-evolve to survive (Van Valen 1973, Liow et al. 2011). Grazer induced responses are therefore found in diverse taxa of both marine and freshwater phytoplankters (Van Donk et al. 2011). Grazing pressure is, however, variable in the pelagic realm and following the predictions of the optimal defense model, phytoplankton should allocate resources to defensive traits in relation to the level of threat (Rhoades 1979).

The motile chain forming marine dinoflagellate *Alexandrium tamarense* combines colony size plasticity and reduced swimming speed to avoid the encounter with copepod grazers, the main zooplankton grazers in the pelagic ecosystem (Selander et al. 2011). In addition, *A. tamarense* produces more paralytic shellfish toxins (PSTs) in response to the grazer (Wohlrab et al. 2010, Selander et al. 2012) which also applies for the related species *Alexandrium minutum* (Selander et al. 2006; Yang et al. 2011). These grazers' induced defenses have been shown to be mediated through infochemicals present in the water, whereas the strength of the defense response depends directly on the density of copepod grazers (Selander et al. 2006; Selander et al. 2011). Furthermore, *Alexandrium* is able to discriminate between different copepod species based on these infochemicals (Bergkvist et al. 2008; Wohlrab et al. 2010). Sympatric species of copepods induce a stronger response in *A. minutum* supporting a grazer driven evolution of phycotoxins defenses (Bergkvist et al. 2008). For *A. tamarense*, the strength of the induced defensive response is proportional to the level of threat represented by each sympatric copepod species (Wohlrab et al. 2010). The regulation of serine/threonine kinases and proteins with calcium-binding domains seems to have a major influence on regulating these infochemical-based species specific responses (Wohlrab et al. 2010). On the other hand for copepod species, the threat is determined by the ability of the copepod to handle the toxins produced by *A. tamarense* (Wohlrab et al. 2010). Copepod populations that frequently experience toxic algal blooms are more resistant to toxins (Colin & Dam 2005). Copepod grazing on toxic *A. tamarense* cells therefore shows signs of co-evolution and subsequently indicates a strong selective pressure that acts on both sides.

In depth analysis using molecular markers revealed a high degree of intra-population diversity within a natural *A. tamarense* population. Furthermore, all examined genotypes showed phenotypic differences concerning the production of the PSTs and the production of uncharacterized allelochemical compounds that act lytically on co-occurring algae and heterotrophic grazers (Tillmann & John 2002;

Tillmann & Hansen 2009; Alpermann et al. 2010). Such differences in the expression of functional traits within one population are also described for other marine dinoflagellates like *Karlodinium* spp (Bachvaroff et al. 2008, 2009, Place et al. 2012). The simultaneous maintenance of different genotypes expressing different phenotypes may add to the ecological success paradox of marine dinoflagellates. The differential expression of these important ecological traits within such populations most likely ensures their survival due to either clonal selection or genotype dependent plasticity or, potentially, a combination of both strategies. Populations of *A. tamarense* that display genetic variation in secondary metabolite production as well as of the ability to respond plastically to an encountered trait should therefore basally be able to respond accordingly to a suite of top-down processes. Hence, intraspecific variation may add to the complexity of grazer-phytoplankton interactions in *A. tamarense*, and substantially widens the norm of reaction to zooplankton on the species level.

Here we have determined genotypic specific responses towards grazing copepods. We used a transcriptomic approach to obtain a more complete view of grazer induced responses in *Alexandrium tamarense*. We included two strains to achieve a first estimate of intra-specific variability in grazer induced responses in microalgae. We employed a broad experimental design which comprised direct grazing, indirect grazing and indirect exposure to copepods to minimize any misinterpretation of the strain comparison data due to experimental artifacts (Bergkvist et al. 2008; Wohlrab et al. 2010; Selander et al. 2011). In addition to the transcriptomic analysis we monitored the exposed cultures for grazer induced chain size plasticity and PST increase.

5.3 Material and Methods

***Alexandrium tamarens*e strains and zooplankton collection**

The two clonal strains of *Alexandrium tamarens*e were isolated in May 2004 from the North Sea coast of Scotland (Alpermann et al. 2010) and grown in K-medium (Keller et al. 1987) in a temperature and light controlled room (salinity ~33, 18 °C, 14 h:10 h light-dark cycles, ~150 fmol m⁻² s⁻¹). The two strains, described as *Alexandrium tamarens*e strain 2 and *Alexandrium tamarens*e strain 5 (both belong to the North American clade) are both producers of PSTs. *Alexandrium tamarens*e strain 2 is further characterized by producing allelochemically active, unknown lytic compound(s) (Tillmann & Hansen 2009).

Female copepods were collected with vertical WP2 net hauls (200 µm mesh size) from ~20 m depth to the surface in the Gullmars fjord on the Swedish west coast. Females were chosen because they release metabolites that attract males and function as a chemical trail (Bagøien & Kiørboe 2005, Kiørboe et al. 2005) potentially perceived by *A. tamarens*e. The copepods were kept in the laboratory in filtered seawater (0.2 µm; salinity ~33) and fed *Rhodomonas baltica* (deposited at GUMACC) until the start of the experiments.

Direct grazing experiments

For both *A. tamarens*e strains, a direct grazing experiment was set up over a time period of 72 h with three harvesting time points (12 h, 48 h & 72 h). Each *A. tamarens*e strain was incubated in 500 mL glass bottles at a concentration of approximately 5×10⁶ cells L⁻¹ in K/10 medium (Keller et al. 1987). This concentration was necessary to ensure enough material for downstream analysis (PST determination, generation of a cDNA-library and gene expression analysis). In total, 18 bottles were prepared for each strain: 6 bottles for each harvesting time point (12 h, 48 h & 72 h) consisting of three replicates of control treatments or grazed treatments. Grazed treatments received 10 *Centropages typicus* individuals per bottle. Ingestion rates were calculated using the equations of Frost (1972).

Cage experiments

The experiments with caged fed and caged starved *C. typicus* were conducted in 500 mL glass bottles with cages made out of 50 mL polypropylene tubes containing a 10 µm plankton mesh at the bottom. The plankton mesh constrained

the organisms to their compartment (flask or cage) while allowing waterborne cues to diffuse between the compartments. For each *A. tamarensis* strain, 9 bottles were set up consisting of three replicates of control treatments, caged fed treatments and caged starved treatments. Each bottle received 450 mL of *A. tamarensis* culture with approximately 4×10^6 cells L^{-1} in K/10 medium (Keller et al. 1987). Cages were deployed into the flasks and filled with 30 mL of same culture for the control treatments and the caged fed treatments. The flasks for the caged starved treatment received 30 mL of K/10 medium instead. Each cage of the caged fed and caged starved treatments received 10 *C. typicus* individuals. The cages were gently moved up and down 5 times a day in order to promote the exchange of chemical cues between the compartments. The experiments were terminated after 48 h.

Cell harvesting

Bottles were carefully turned over 7 times to ensure equal mixing of the culture and for the direct grazing experiments pre-filtered through a submerged 64 μm nylon mesh to remove copepods and copepods eggs. Afterwards, around 60-70 mL were sub-sampled and weighted, then counted and sized using a coulter counter (Micromeritics, Norcross, USA) mounted with a 100 μm orifice tube and continuous stirring. A known volume of the subsamples was then suction filtered onto glass-fiber filters and stored at $-20\text{ }^{\circ}C$ until toxin extraction and analysis.

The remaining culture was poured through a 10 μm mesh to collect the *A. tamarensis* cells. The 10 μm mesh was washed out with sterile filtered seawater into a 50 mL collection tube and immediately centrifuged at $4\text{ }^{\circ}C$ for 5 min for RNA samples. Excess water was discharged and the pellet was immediately mixed with 1 mL $60\text{ }^{\circ}C$ hot TriReagent (Sigma-Aldrich, Steinheim, Germany) and transferred to a cryovial containing acid washed glass beads. The cryovial was vortexed for 10 s and submerged into liquid nitrogen. The samples were stored at $-80\text{ }^{\circ}C$ until RNA isolation.

PST analysis

Paralytic shellfish toxins samples (PST) were freeze-dried and extracted in 1 ml 0.05 M acetic acid (aq) through three consecutive freeze-thaw cycles. Extracts were filtered (GF/F) and stored frozen in glass vials. Samples were hydrolyzed with 0.1 M HCl at $100\text{ }^{\circ}C$ for 10 minutes to transform any C-toxins into their corresponding carbamates. We used a modified version of the method described by Asp and co-workers (2004) to analyze all carbamates in a single run (see Publication

II for details). Briefly, HPLC analysis was carried out on a Hitachi-7000 system equipped with a Genesis C8 column, (Vymac, 4 μm , 150*3 mm). A gradient between elution with 2 mM L⁻¹ sodiumheptanesulfonate in 10 mM L⁻¹ ammonium-phosphate buffer (pH 7.1) and 2 mM L⁻¹ sodiumheptanesulfonate in 30 mM L⁻¹ ammonium phosphate buffer (pH 7.1): acetonitrile (96:4) was used to separate all carbamates (Selander et al. 2012). After the separation, toxins were oxidized with 7 mM periodic acid in 50 mM sodium phosphate buffer (pH 9.0) in a PEEK capillary (10 m, 80 °C). The oxidation was terminated with 0.5 M acetic acid before fluorescent detection at $\lambda_{\text{ex}} = 330 \text{ nm}$, $\lambda_{\text{em}} = 390 \text{ nm}$. Toxin standards were obtained from the certified reference materials program, National Research Council, Halifax, Canada.

Total RNA isolation

TriReagent fixed and frozen cells were lysed using a Bio101 FastPrep instrument (Thermo Savant Illkirch, France) at maximum speed (6.5 m s⁻¹) for 2 x 45 s. Lysed cells were cooled on ice, 200 μL chloroform was added and vortexed for 20 s. After incubation at room temperature for 5 min, the samples were transferred to a phase lock tube (Eppendorf, Hamburg; Germany) and incubated for another 5 min at room temperature followed by centrifugation for 15 min at 13.000 x g and 4 °C. The upper aqueous phase was transferred to a new tube and mixed with the same volume isopropanol, 1/10 volume of 3 M Na-acetate (pH = 5.5; Ambion by Life Technologies, Carlsbad, California, USA) and 2 μL linear polyacrylamide (Ambion). Total RNA was precipitated for 90 min at -20 °C and collected by centrifugation for 20 min and 13.000 x g at 4 °C. The obtained pellet was washed twice, first with 1 mL 70% EtOH followed by 10 min centrifugation at 13.000 x g and 4 °C. The second wash was done with 1 mL EtOH absolute, followed by 5 min centrifugation at maximum speed. The remaining ethanol was discharged; the RNA pellet was dried for 1 min at 37 °C and resolved in 30 μL RNase free water (Qiagen, Hilden). RNA quality check was performed using a NanoDrop ND-100 spectrometer (PeqLab, Erlangen, Germany) for purity and the RNA Nano Chip Assay with the 2100 Bioanalyzer device (Agilent Technologies, Santa Clara, California, USA) was just to examine the integrity of the extracted RNA. Only high quality RNAs (OD 260/280 > 2 and OD260/230 > 1.8) as well as with intact ribosomal peaks (obtained from the Bioanalyzer readings) were used for further experiments.

EST- Library construction and 454 sequencing

Subsamples of the *A. tamarensis* strain 2 and *A. tamarensis* strain 5 RNAs from the direct grazing experiments and from the cage experiments were pooled and 10 µg of total RNA was used as the starting material for a normalized cDNA library for GS FLX Titanium sequencing. The construction of the cDNA library was done by Vertis Biotechnology AG (Freising-Weihenstephan, Germany). In short, poly(A)⁺ RNA was prepared from the total RNA and 1st-strand cDNA synthesis was primed with random hexamers. Then 454 adaptors were ligated to the 5' and 3' ends of the cDNA. The cDNA was amplified with 19 PCR cycles using a proof reading polymerase. Normalization of the cDNA was carried out by one cycle of denaturation and re-association. The cDNA was passed over a hydroxylapatite column to separate the re-associated cDNA from the ss-cDNA. The ss-cDNA was then amplified with 9 PCR-cycles. cDNAs with a size range between 450 – 650 bp were cut out and eluted from a agarose gel and converted to a 454 Roche titanium sequencing library according to the manufacturer's protocols. The sequencing run and base calling on the 454 NGS machine was conducted with default settings.

In total, 424,240 reads could be assembled into 56,021 contigs from which 14,512 contigs were larger than 500 bp with an average coverage of 13.5 reads per contig.

Microarray design and microarray hybridizations

Reciprocal best blast hit search against a library with 7,649 assembled ESTs derived from the 10,894 public available ESTs from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) and the contigs larger than 500 bp from the EST-Library from the current study (grazer induced library) revealed an intersection of common ESTs sequences of 1,961 occurring in both libraries with a cut-off of e^{-20} . Based on this reciprocal best blast hit search, the potential unique contigs were selected. From these contigs microarray probes (60mers) were designed using Agilent's E-array online platform (<https://earray.chem.agilent.com/earray/>), leading to a total of 4,499 singlets and 15,677 contigs represented through probes on the microarray used in this study.

Microarray hybridizations were carried out by comparing both, RNA from treatments and controls, against a RNA reference pool consisting of pooled RNAs from the control treatments. These comparisons were done separately for each *A. tamarensis* strain. Each labeling reaction was prepared from 500 ng total RNA

starting concentration from every treatment, every control and the reference pools using the Agilent two color low RNA Input Linear Amplification kit (Agilent Technologies, Santa Clara, USA) in addition with the Agilent RNA Spike-In Mix (Agilent). During the labeling reaction, the RNA was first reverse transcribed into cDNA and afterwards amplified with Cyanine 3 – or Cyanine 5-labeled CTPs according to the “Agilent Low RNA Input Linear Amplification Kit” protocol (Agilent). Dye incorporation rates and cRNA concentrations were determined using the NanoDrop ND-100 spectrometer. Hybridizations were performed on 8x60k microarray slides with 300 ng of each, cyanine-3 and cyanine-5 labeled cRNA at 65 °C for 17 h (Agilent). Afterwards, the microarrays were washed with GE wash buffer I and II according to the manufactures instructions (Agilent) followed by immersing them into Agilent’s’ drying and Stabilization solution (Agilent). Microarrays were scanned using an Agilent G2505B scanner. Raw data processing was performed by the Agilent Feature Extraction Software version 9.1.3.1 (FE); quality monitoring was performed with the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07. The array design, raw data and normalized data and the detailed experimental design are MIAME compliant and deposited in a MIAME compliant database (ArrayExpress at the EBI; <http://www.ebi.ac.uk/microarray-as/ae/>; ID: E-MEXP-3422). Differential gene expression was evaluated using the GeneSpring GX software platform version 11 (Agilent). Genes were considered to be differentially expressed when test statistics reply *p*-values were less than 0.05 and fold changes exceeded 1.5.

Expression analysis of the putative *sxtA* gene via RT- qPCR

The quantitative expression of the putative *sxtA* gene in *A. tamarensis* strain 2 was evaluated via RT-qPCR using the method described in Freitag et al. (2011). For this RT-qPCR analysis, we only considered treatments where a significant increase in saxitoxin on the total PST profile compared to control cultures was observed. Prior to cDNA synthesis, 500 ng of total RNA of the samples of interest were spiked with 10 ng of MA mRNA and 1 ng of NSP mRNA (Freitag et al. 2011). The cDNA was synthesized with the Omniscript RT kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions with anchored oligo(dT)²⁰ primers. The synthesized cDNAs were diluted 1:5 and 2 µl were used to analyze the expression of the putative *sxtA* gene, the MA gene and the NSP gene with a SYBRgreen assay according to the manufactures protocol (Applied Biosystems/Life Technologies, Carlsbad, California, USA). Primers for the putative *sxtA* gene fragment had the sequences:

5'GCGAGACCGACGAGAAGTTC'3 and 5'AGCCGCTTGCGCTGAAG'3; primer sequences for the MA and NSP gene are given by Freitag et al. (2011). The qPCR reaction were carried out in a StepOnePlus™ Real-Time PCR System-device (Applied Biosystem by Life Technologies, Carlsbad, California, USA) with the following cycling parameters: 10 min initial denaturation at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was performed at the end of the reactions to verify the formation of a single PCR product in each reaction. Standard curves with 6 points and a serial dilution factor of 1:10 starting with 10 ng PCR product from each gene were also analyzed with the SYBRgreen assay using the same primers and parameters. PCR products for the standard curves for the *sxtA* gene were obtained from the prepared cDNAs and the following primers: 5'CCGCCATATGTGCTTGTTTG'3 and 5'AGCTCCCTGTACACCTCTGC'3. The PCR was performed with the following cycling parameters: 2 min with 94 °C for initial denaturation, followed by 30 cycles of 20 s at 94 °C, 10 s at 55 °C, 1 min at 65 °C and a final elongation step with 10 min at 65 °C. PCR products were cleaned with the Qiagen MinElute PCR Purification Kit (Qiagen, Hilden, Germany) according to the provided manual. Expression ratios were calculated with the REST-2009 software platform (Qiagen, Hilden, Germany) using the equations of Pfaffl (2001):

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control-treatment})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control-treatment})}}$$

where E_{target} is the RT-PCR efficiency (see below) of the target gene transcript; E_{ref} the RT-PCR efficiency of the reference gene transcript; $\Delta CP_{\text{target}}$ is the cycle threshold value deviation of control - treatment of the target gene and ΔCP_{ref} the respective deviation of control - treatment of the reference gene (Pfaffl 2001).

The RT-PCR efficiency (E) was evaluated by the standard curves of the respective gene from the slope of the cycle threshold value (CP or C_t) versus the concentration of the PCR product according to the formula:

$$E = 10^{-1/\text{slope}}$$

5.4 Results

The main results from this study are summarized in Fig. 5.1 to give an overview on the assessed parameters and are described in detail in the following sections.

Experimental design	Direct grazing experiment		Cage experiment							
	Control		Direct grazing		Control		Fed		Starved	
	Strain 2	Strain 5	Strain 2	Strain 5	Strain 2	Strain 5	Strain 2	Strain 5	Strain 2	Strain 5
Estimated % of single cells	-	-	↑	↑	-	-	↑	↑	↑	↑
PST content (amol / μm^2)	-	-	↑	↑	-	-	↑	-	↑	-
# differential expressed genes (vs. control)	-	-	366 172	152 539	-	-	-	-	-	-
PST profile alteration	No	No	Yes	No	No	No	Yes	No	Yes	No

Fig. 5.1: Overview of the experimental design and the main results. The data shown represent the results obtained after 48h; ↑ indicates an increase in the values compared to the respective controls

Cell size distribution

The size distribution of the two strains of *A. tamarensis* shifted in the presence of copepods towards a smaller average diameter (Fig. 5.2). This shift was caused by a different proportion of the culture occurring as single cells and two or four cell chains in the controls and treatments as observed by microscopic examination. The shift is present in both the direct grazing experiments and the waterborne-cue experiments, with caged copepods, showing that the observed responses were triggered by the waterborne cues from the copepods. The response was, however, strongest for *A. tamarensis* strain 2 exposed to direct grazing copepods (Fig. 5.2A). Here, the average diameter decreased by 10% after 48 h and by 16% after 72 h in grazer exposed cultured which corresponds to a 101% increase in the amount of single cells after 48 h and 107% increase after 72 h respectively compared to control cultures at the respective time point. The mean diameter of *A.*

tamarensis strain 2 cells exposed to waterborne cues of caged fed and starved copepods dropped both by ~7%, corresponding to an estimated increase in the amount of single cells of 38% (Fig. 5.2B & Supplemental table S.5.1).

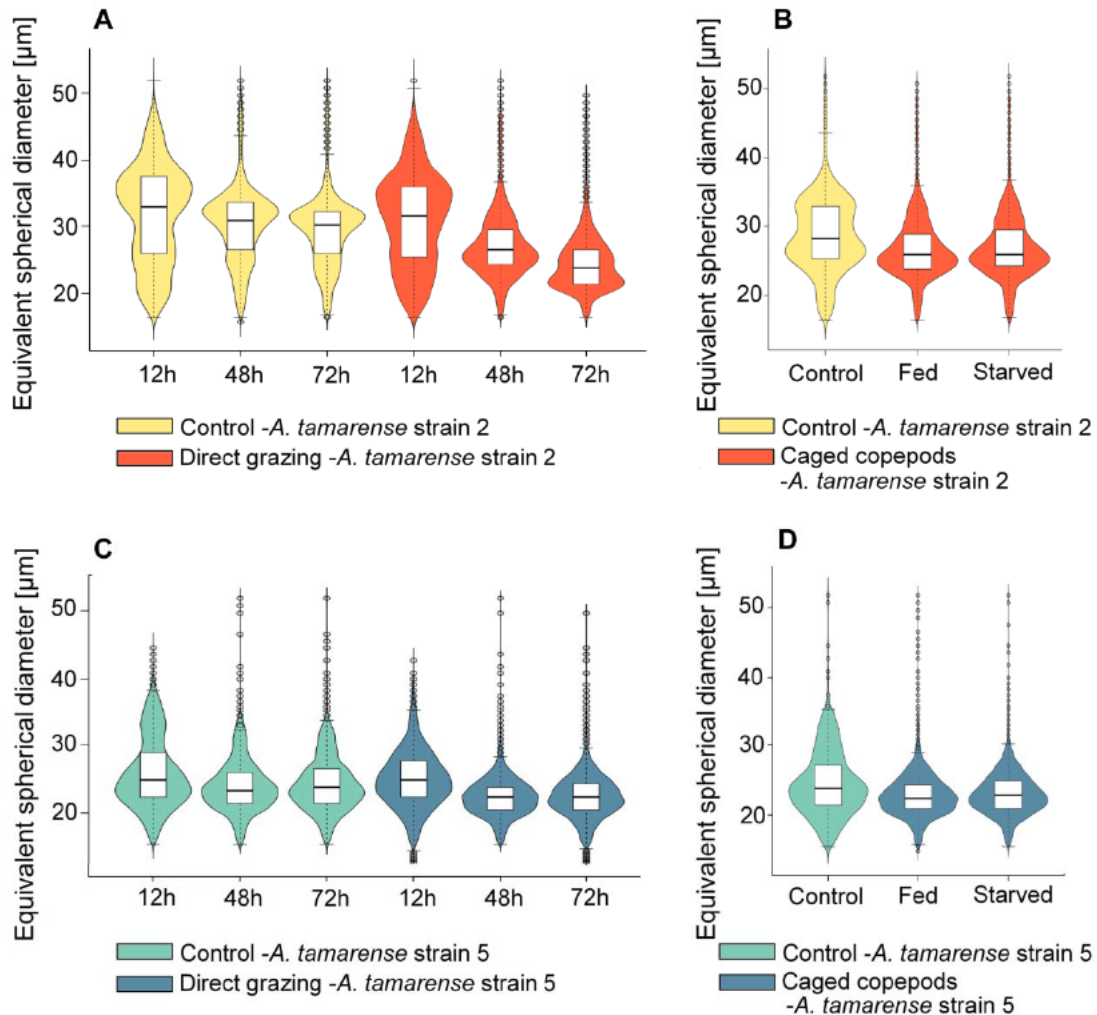


Fig. 5.2: Cell size distribution of the *Alexandrium tamarensis* strains. Cell size distribution of *Alexandrium tamarensis* strain 2 (A & B) and *A. tamarensis* strain 5 (C & D) after exposure to direct grazing *Centropages typicus* (A & C) or after exposure to waterborne-cues from caged fed and caged starved *C. typicus* (B & D). Treatments with waterborne-cues were terminated after 48h. The diameter of the measured particles (equivalent spherical diameter in μm) is plotted as kernel density estimation with the width corresponding to the relative occurrence of a particle in a respective size class. Box plots show the 25th, 50th and 75th percentile; the ends of the whiskers mark the 95% intervals.

A. tamarensis strain 5 was generally less prone to form two-, and four cell chains (Fig. 5.2C & 5.2D). Yet, exposure to both direct grazing and grazer cues resulted in a reduced average size also for this strain, corresponding to a 19% (48 h) or 24% (72 h) increase in the amount of single cells in response to direct grazing or

grazer cues, respectively. In the treatment with waterborne cues from caged fed and caged starved copepods, the cell size means dropped by ~6.5% with a corresponding estimated increase in the amount of single cells of by 24% (Fig. 5.2D).

PST- contents and clearance rates

Exposure of *A. tamarensis* strains to direct grazing *C. typicus* individuals resulted in significantly increased PST contents per biovolume between the controls and the treatments (two-way ANOVA $p < 0.05$, TukeyHSD $p < 0.05$, Fig. 5.3A and 5.3C). Whereas in strain 5 a significant increase in PST content in the grazed treatments could be observed over time (TukeyHSD $p < 0.05$), grazed treatments of *A. tamarensis* strain 2 did not further increase their PST content after 48 h (TukeyHSD $p > 0.05$). Additionally, there was an increase in the average PST content of the controls of strain 5 from 48 h to 72 h (TukeyHSD $p < 0.05$).

The treatment with waterborne cues from caged fed and starved copepods however showed significant differences in the mean PST content for strain 2 only (one-way ANOVA $p < 0.05$, Fig. 5.3B), where PST content increased compared to the control (TukeyHSD $p < 0.05$), and not for strain 5 (one-way ANOVA $p > 0.05$; Fig. 5.3D).

A significant decrease of the clearance rates for copepods grazing on *A. tamarensis* strain 2 cultures could be observed dropping from 1.7 ml per female per hour to 0.4 mL per female per hour (ANOVA $p < 0.05$, Fig. 5.3A). Clearance of copepods grazing on *A. tamarensis* strain 5 cultures, however, was nearly constant over the experimentally observed time with values around 0.4 mL per female per hour (Fig. 5.3C).

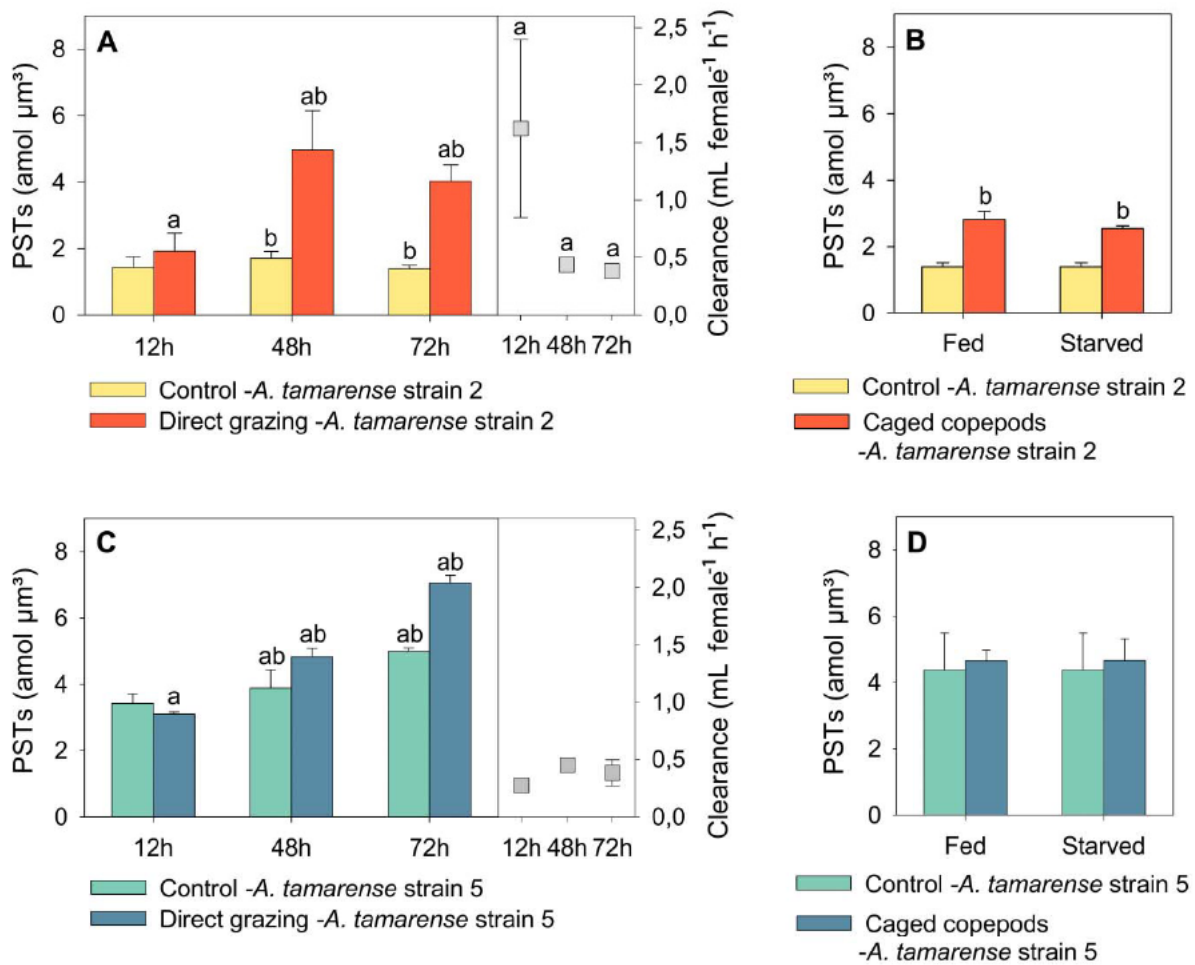


Fig. 5.3: PST contents of the *Alexandrium tamarensis* strains and clearance rates for *Centropages typicus*. PST contents of *Alexandrium tamarensis* strain 2 (A & B) and *A. tamarensis* strain 5 (C & D). PST contents after exposure to direct grazing *Centropages typicus* individuals and respective clearance rates are given in A and C. PST contents after exposure to waterborne-cues from caged fed and caged starved *C. typicus* are given in B and D. Treatments with waterborne-cues were terminated after 48 h. Bars marked with “a” and/or “b” show significant differences compared to either the control at this time point (b) or between the treatments at different time points (a) (ANOVA $p < 0.05$, TukeyHSD $p < 0.05$). Clearance rates for *C. typicus* grazing on *A. tamarensis* strain 2 cultures (A) and *A. tamarensis* strain 5 cultures (B) are shown. Bars marked with “a” indicate significant differences between the clearance rates (ANOVA $p < 0.05$, TukeyHSD $p < 0.05$).

PST profiles after exposure to grazers/grazer cues

The increase in the total PST content in *A. tamarensis* strain 2 cultures after exposure to copepods in the “direct grazing” treatment and after exposure to caged fed and starved copepods was accompanied by a shift in the PST profile (Fig. 5.4).

Yet, this phenomenon was not observed for *A. tamarensis* strain 5. In the *A. tamarensis* strain 2 cultures, the amount of saxitoxin in the grazed treatment increased significantly from a mean of ~26 % of total PSTs towards a mean proportion of 48% at 48 h and further to mean proportion of 52 % at 72 h (ANOVA $p < 0.05$, TukeyHSD $p < 0.05$).

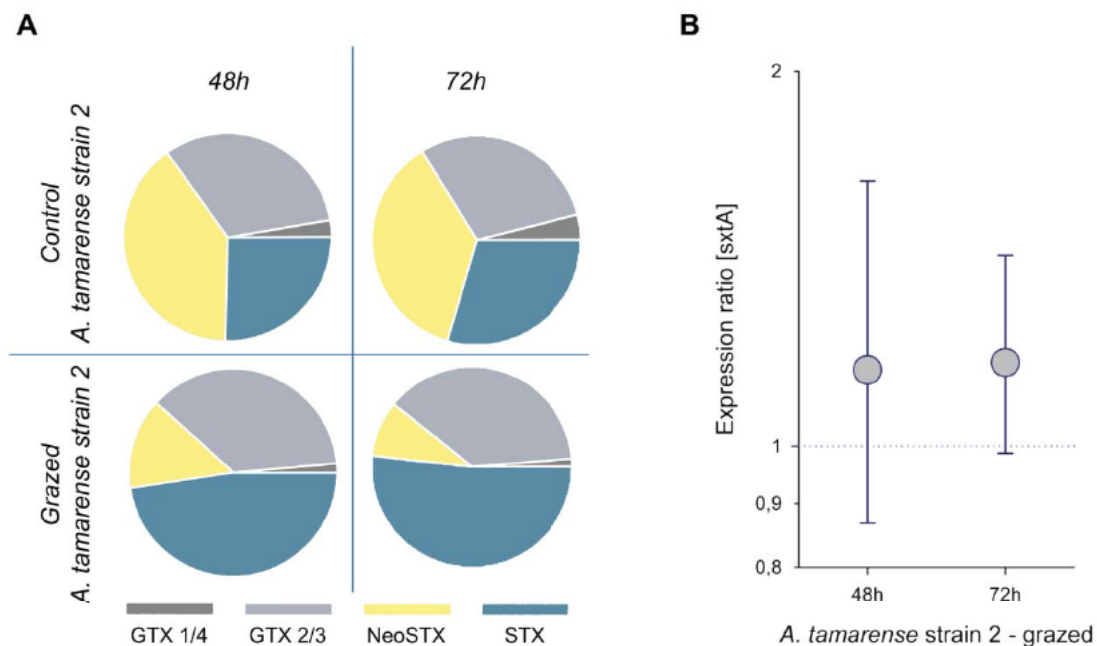


Fig. 5.4: PST profile and expression ratio of the *sxtA* gene fragment. The PST profiles of *Alexandrium tamarensis* strain 2 after 48 h and 72 h exposure to direct grazing *Centropages typicus* and the respective control are shown in (A). The expression ratio for the putative *sxtA* gene fragment (B) as determined for *Alexandrium tamarensis* strain 2 cultures from the direct grazing experiment after 48 h and 72 h. Quantification of the relative expression compared to the control treatments was done using a reference spike-in gene to normalize expression level of controls and treatments.

Expression analysis of the putative *sxtA* gene via RT- qPCR

Since the probe representing the putative *sxtA* gene fragment (Stüken et al. 2011) on the microarray did not pass the quality criteria after raw data processing, the differential expression of the respective gene (contig 05769) was evaluated via RT-qPCR for the 48 h and 72 h time points of the direct grazing experiments with *A. tamarensis* strain 2 using the same RNA as template as for the microarray experiments (Fig. 5.4). After 48 h and 72 h, a mean expression ratio of 1.15 and 1.17,

respectively, was determined compared to the control treatment. Both results do not show a significant increase in expression of the *sxtA* gene.

Gene expression analysis through microarray hybridizations

In order to compare the strain specific responses on a molecular level, differential expression of genes was evaluated for each strain separately after 48 h of exposure to direct grazing in comparison to the respective control treatment (fold change cut-off > 1.5, *t*-test $p < 0.05$). In total, 538 genes were differentially expressed in *A. tamarensis* strain 2 (366 \uparrow /172 \downarrow) and 691 genes were differentially expressed in *A. tamarensis* strain 5 (152 \uparrow /539 \downarrow) (Fig. 5.5).

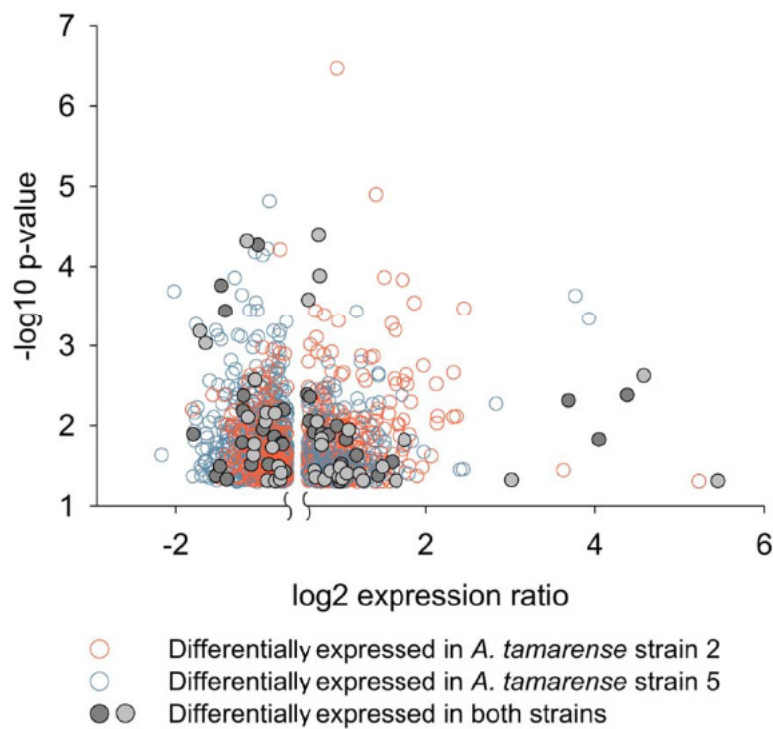


Fig. 5.5: Distribution of significant differentially expressed genes in both *Alexandrium tamarensis* strains. The significant differentially expressed genes of both *Alexandrium tamarensis* strains after 48 h from the direct grazing experiment are plotted together as a volcano plot (fold change cut-off > 1.5; *T*-test $p < 0.05$). Genes that are significant expressed in both strains are filled with grey. Light grey dots represent expression values derived from *A. tamarensis* strain 2; Dark grey dots represent expression values derived from *A. tamarensis* strain 5.

Only 34 genes were differentially expressed in both *A. tamarensis* strains. From these, 14 genes were up-regulated in both strains (Table 5.1), 10 genes were down-regulated in both strains and another 10 genes were regulated in a reversed manner.

Table 5.1: List of in common, up-regulated genes from both *A. tamarensis* strains. List of genes from the direct grazing experiments (48 h) which are up-regulated in both *A. tamarensis* strains compared to control conditions. Assigned KOG categories and calculated fold change (FC) values are given for both strains.

Putative gene product	Sequence ID	FC-value strain 2	FC-value strain 5	KOG
Cytochrome b	contig20144	43.8	18.7	[C]
Translation elongation factor EF-1 alpha/Tu	contig04912	23.9	7.9	[J]
Heme/copper-type cytochrome/quinol oxidase	contig13526	5.1	2.7	[C]
Unknown protein	40757246	3.4	2	[S]
Unknown protein	54437298	2.1	2	[S]
Unknown protein	40756664	2.4	1.5	[S]
Unknown protein	58179891	1.6	2.3	[S]
Unknown protein	contig31652	2	1.9	[S]
Unknown protein	42749055	2	1.7	[S]
Unknown protein	42749396	1.7	2	[S]
Unknown protein	40756089	1.5	2.1	[S]
Unknown protein	tc_00927	1.8	1.7	[S]
Unknown protein	42752322	1.7	1.6	[S]
Protein kinase domain containing protein	contig36762	1.6	1.5	[T]

All genes were assigned to functional categories according to KOG (eukaryotic orthologous groups) (Tatusov et al. 2003). The percentage of differentially expressed genes in each category for each strain is shown in Fig. 5.6. We observed differences between *A. tamarensis* strains in functionally assigned genes that were up- or down regulated. In strain 2 genes were up regulated in the categories of: O) Posttranslational modification, protein turnover, chaperones and T) Signal transduction mechanisms. Further distinct differences were observed in the categories of P) Inorganic ion transport and metabolism and C) Energy production and conversion. Conversely, *A. tamarensis* strain 5 showed a down-regulation in comparison to strain 2 in T) Signal transduction mechanisms, J) Translation,

ribosomal structure and biogenesis, P) Inorganic ion transport and metabolism, O) Posttranslational modification, protein turnover, chaperones, and I) Lipid transport and metabolism.

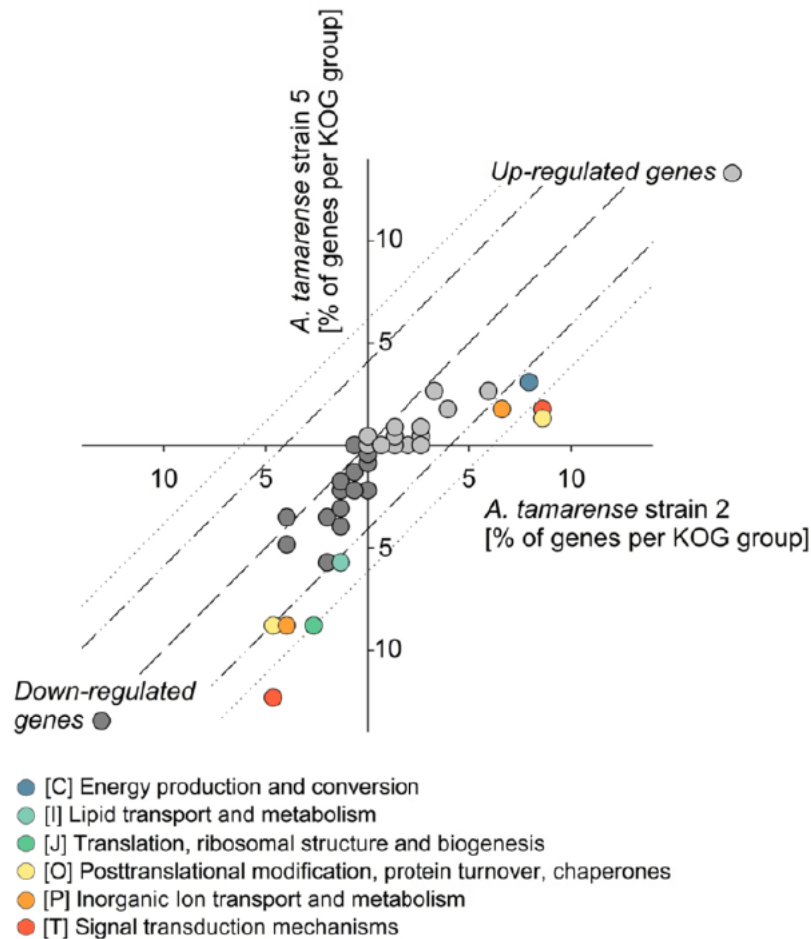


Fig. 5.6: Comparison of significant differentially expressed genes assembled into KOG categories of the *Alexandrium tamarensis* strains. Dots on the dashed line indicate an equal distribution of the relative abundance of the respective KOG category in both strains. Dashed-dotted lines mark the cut-off of $+1\sigma$ calculated from the mean distance of dots from an equal distribution, dotted lines mark the $+2\sigma$ cut-off. Categories represented with colored dots have a greater distance than $+1\sigma$ or $+2\sigma$ from the mean distribution.

To further analyze the expression profile of *A. tamarensis* strain 2 we extended the microarray analysis to 72 h direct grazing exposure. In total 482 (337 \uparrow /145 \downarrow) genes were significantly differentially expressed after 48 h and 293 (220 \uparrow /73 \downarrow) genes were differentially expressed after 72 h (ANOVA $p < 0.05$, fold change cut-off > 1.5). Functional classification of differentially expressed genes into

well characterized KOG categories was possible for 137 genes for the 48 h comparison and 73 genes for the 72 h comparison (Fig. 5.7). KOG categories holding the majority of genes were: C) Energy production and conversion and G) Carbohydrate transport and metabolism (metabolism), T) Signal transduction mechanisms and O) Posttranslational modifications, protein turnover, chaperones (cellular processing and signaling) and the category J) Translation, ribosomal structure and biogenesis (information storage and processing) (Fig. 5.7).

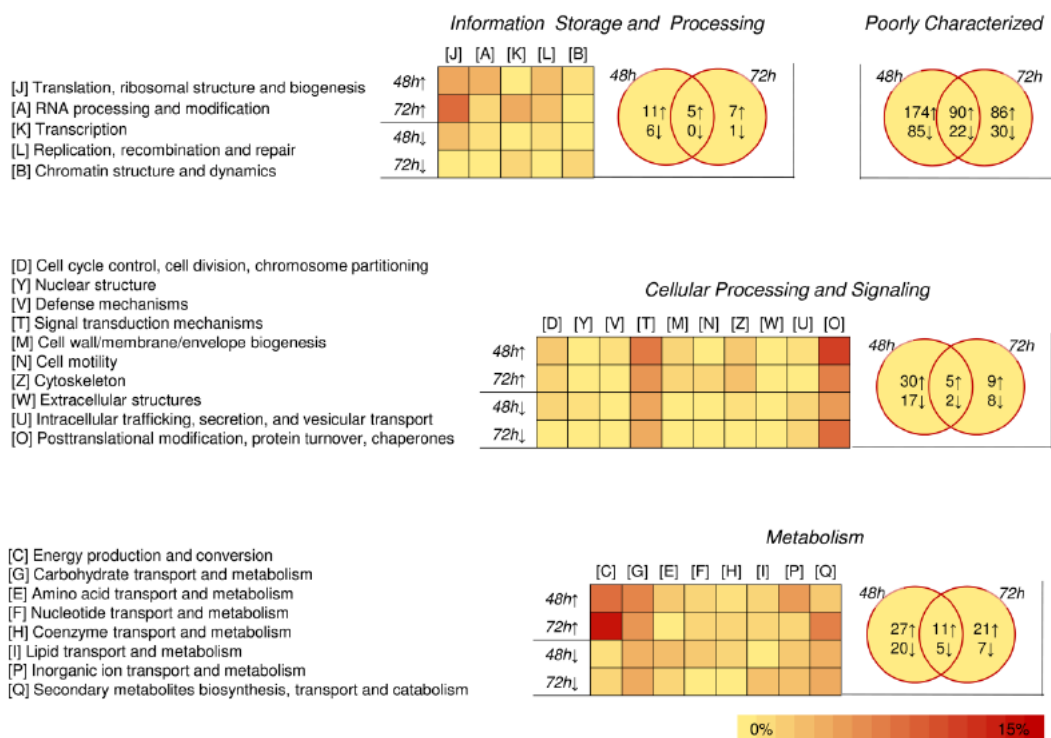


Fig. 5.7: KOG category distributions of differentially expressed genes from the *Alexandrium tamarense* strain 2 time series. The relative abundance of each category per group after 48 h and 72 h from the direct grazing experiment shown as color code. The corresponding Venn diagrams indicate the numbers of in common regulated and condition specific regulated genes for each group. (↑) up-regulated genes; (↓) down-regulated genes.

Both treatments showed an overlap of 140 responsive, differentially regulated genes. Table 5.2 shows the functional annotation of selected putative gene products from these co-expressed genes for which direct effects could be inferred. These genes were selected because they may indicate the existence of costs associated with defense.

Table 5.2: Co-expressed genes from the times series (direct grazing experiment) from *A. tamarensis* strain 2. Selected putative gene products of common regulated genes from the direct grazing experiment with *A. tamarensis* strain 2 and their putative integrated effect on the cells metabolism.

Energy production and conversion & carbohydrate metabolism				
<i>Putative gene product</i>	<i>Sequence ID</i>	<i>Fold change 48h/72h</i>	<i>Pathway</i>	<i>Functional effect</i>
Cytochrome B6-f complex protein	40758296	2.3/1.7	Photosynthesis	
Peridinin-chlorophyll A binding protein	tc_01855	1.5/2.0	Photosynthesis	Increase in glucose, ATP and NADPH.
Photosystem II protein Y (PsbY)	40757585	1.9/1.5	Photosynthesis	
Enolase	contig10680	2.6/1.8	Glycolysis	Increase in 3-phosphoglycerate levels.
Phosphoglycerate kinase	38452464	3.4/1.8	Glycolysis	Increase in 3-phosphoglycerate levels and ATP.
Light-dependent protochlorophyllide oxidoreductase	contig20489	2.0/1.9	Porphyrin and chlorophyll metabolism	Increase in building blocks for photosynthesis and respiration.
Light-dependent protochlorophyllide oxidoreductase	tc_01584	2.6/1.9	Porphyrin and chlorophyll metabolism	
Ribokinase	58179552	1.8/1.5	Pentose phosphate pathway	Increase of phosphorylated ribose for reduction equivalents and secondary messengers.

5.5 Discussion

We observed a large number of differentially regulated genes in response to copepod cues. The majority of those differed between the two strains of *A. tamarense*. This indicates that grazer-induced responses are highly strain specific in *A. tamarense* at the transcriptomic level and in addition, that our understanding of grazer induced responses in phytoplankton is incomplete. The effects of grazer-induced responses on population dynamics are mainly founded on correlations between easily observable traits and consumption rates in relevant grazers. The functional annotation of the regulated genes indicates, however, that they are a part of a wide spectrum of cellular processes. How the alteration of these cellular processes influence the species' performance and thereby trait mediated interactions under natural conditions, remains speculative. Correlations between easily observable traits and consumption rates could be confounded by numerous grazer induced traits correlated to the observed ones. Along with the high level of strain specificity this substantially widens the norm of reactions of the responding algae.

Our results on sizes of cellular associations are in congruence with the findings published by Selander et al. (2011) showing that the separation of cell chains into single cells is a defensive trait expressed by *A. tamarense* to reduce their encounter rate with grazers. Additionally, the previously described induction of PST biosynthesis after exposure to copepod grazers or their waterborne-cues in *Alexandrium* species and strains (Selander et al. 2006; Bergkvist et al. 2008; Wohlrab et al. 2010; Yang et al. 2011) was again verified in this study.

Variations in pre-expressed traits between the two strains did not only comprise the previously known existence of allelochemical compound production and differences in PSTs cell quota (Tillmann & Hansen 2009). Differences on the morphological and behavioral level of the control cultures were also observed in this study: the two *A. tamarense* strains differed in the proportion of single cells and cell chains present in the culture (Fig. 5.2). Clearance rates before any measureable induction of defensive traits (12 h) may reflect this impact (Fig. 5.3). Clearance rates at this time point were around 4 fold higher for *A. tamarense* strain 2 cultures. This result correlates with the above described variations in pre-expressed traits which are a proxy for the ability of *Alexandrium* to defend against copepods (Selander et al. 2006; Selander et al. 2011). Additionally, it indicates that the allelochemicals

produced by *A. tamarensis* strain 2 do not seem to provide any additional benefit with respect to defense against grazing copepods. Differences in the cell size distribution concerning time point 12 h and the following distributions measured at 48 h and 72 h can be explained by the fact that *A. tamarensis* cells did not go through a dark phase after 12 h. The light-dark cycle is a trigger for cell division and hence, just before the onset of the dark cycle cells with a high biovolume, ready to divide, might lead to the scattered size distribution observed after 12 h.

The observed genotype dependant vulnerability towards grazing can explain the further differences in the expressed strength of the defensive traits. The onset of the induced defense response was measurable after 48h in both strains of *A. tamarensis* (Fig. 5.2 and 5.3). The induced toxin production and the splitting of cell chains were, however, more pronounced for *A. tamarensis* strain 2 cultures (Fig. 5.2 and 5.3), whereas these defensive traits were already partly a constitutive phenotypic characteristic of *A. tamarensis* strain 5 cultures. Parallel to these observations are the values of the calculated clearance rates: *A. tamarensis* strain 5 had values that could be denoted for *A. tamarensis* strain 2 just after the expression of the defensive traits (Fig. 5.3A and 5.3B). The intra-strain differences in the direct grazing experiment and the cage design emphasize the importance of a broad experimental set-up. Cue densities in the cage design were most likely too low to induce a defensive response, in terms of induced PST production in *A. tamarensis* strain 5. Furthermore, *A. tamarensis* strain 2 did not respond as strong as in the direct grazing experiments. The signal dampening effect due to the cage design is explicitly shown in Bergkvist et al (2008). However, the cage design demonstrates that nothing except the copepod cues are necessary to induce defense.

The observed initial phenotypic variance and the further induced responses indicate the existence of different defensive strategies towards copepods within natural *A. tamarensis* populations. *A. tamarensis* strain 5 shows a higher constitutive defense that is, however, further inducible in times of high grazing pressure (Fig. 5.3C and Wohlrab et al. (2010)). This strain therefore expresses the defensive traits at a higher level compared to *A. tamarensis* strain 2. *A. tamarensis* strain 2, however, expresses a phenotype that is by its nature less vulnerable to unicellular heterotrophic grazers than *A. tamarensis* strain 5 (Tillmann & Hansen 2009). Upon copepod grazing, *A. tamarensis* strain 2 may therefore be forced to shift its phenotype towards grazing resistance, and hence its phenotype becomes comparable to the phenotype expressed by *A. tamarensis* strain 5. Yet, the fitness gain of this response has to be re-evaluated under natural conditions since this shift

may cause a reduction of this strains' ability to deter unicellular grazers due to a decrease in the allelochemical cloud that surrounds the cell (Jonsson et al. 2009).

The increase in toxicity in *A. tamarensis* strain 2 was accompanied by a shift in the toxin profile (Fig. 5.4) towards a higher proportion of saxitoxin. The toxin profile of *A. tamarensis* is assumed to be a stable phenotypic character (Hall 1982, Cembella et al. 1987, Ogata et al. 1987) which changes only under rather extreme culture conditions (Boczar et al. 1988, Anderson et al. 1990). PST profile changes under grazing pressure have not been reported so far. The history of grazing pressure could therefore be one explanation for why differences in the PST profiles of different populations of *A. tamarensis* have evolved. The presence of grazing copepods seems until now the only approach that can clearly induce a change in cellular PST contents in *Alexandrium* spp. to an extent that indicates *de novo* biosynthesis rather than an accumulation of toxins. However, the higher amount of PST and in particular saxitoxin on the total PST profile of *A. tamarensis* strain 2 did not result in a higher expression of the putative *sxtA* gene (Stüken et al. 2011) as verified via RT-qPCR (Fig. 5.4). Hence, the gene coding for *sxtA* cannot be linked to the phenotypic trait expressed in this study by *A. tamarensis* strain 2. Further research efforts will be necessary in order to characterize other potential *sxt* related-genes in dinoflagellates and to perform expression analysis concerning their role in controlling the PST synthesis rates.

The transcriptomic comparison using microarrays revealed that hundreds of genes are differentially expressed in response to trace amounts of copepod cues. Moreover, the response is highly strain specific with large discrepancies between the two strains of *A. tamarensis* tested here (Fig. 5.5). Yet, a small subset of genes was identified as differentially expressed in both strains. The common up-regulated transcripts from both strains (Table 5.1) contained two genes that were among the three top up-regulated genes. One of them is a homologue of the Translation elongation factor EF-1 alpha/TU (eEF-1 α). Besides translation elongation, the eEF-1 α has acquired additional functions without being expressed as alternative form (Sasikumar et al. 2012). One of those functions is the ability of eEF-1 α to bind actin (Yang et al. 1990) thereby influencing local actin cytoskeleton organizations and probably also local translation events (Sasikumar et al. 2012). Studies in *S cerevisiae* revealed that an over-expression of the eEF-1 α disrupts the organization of the cytoskeleton without appreciable effects on protein synthesis (Munshi et al. 2001). The actin cytoskeleton provides a scaffold for cell adhesion systems through 'adherens junctions' that connect actin filaments via cadherins, and such adherens

junctions probably evolved even before the metazoan divergence (Abedin & King 2008, Sebé-Pedrós & Ruiz-Trillo 2010). Hence, local actin re-organizations might alter the cytoskeleton of *A. tamarensis* cells and suppress the formation of two- or four cell-chains, thus might link the high expression of this gene in both strains and the observed reduction in the number of cells present as cell chains.

Additional genes that are both up-regulated and commonly expressed in both *A. tamarensis* strains comprise the cytochrome b gene, as well as a heme/copper-type cytochrome/quinol oxidase (Table 5.1). Both transcripts encode important proteins for the respiratory chain, and in dinoflagellates are localized on a relict mitochondrial genome (Kamikawa et al. 2009). Retention of these genes by the organelles has previously been proposed to be necessary in order to ensure a fast and accurate delivery of the redox machinery components, as a response to redox poise signaling, in times of high electron transport chain activity (Koumandou et al. 2004). Thus, higher expression of these genes coding for polypeptides within the respiratory chain, may in fact reflect a high respiratory activity and energy demand, in response to grazer pressure and subsequently induced defenses in *A. tamarensis*.

An involvement of serine /threonine kinases and proteins possessing calcium binding domains in triggering the defensive response towards copepods was suggested in a previous study (Wohlrab et al. 2010). One serine/threonine kinase domain containing transcript was also among the common up-regulated genes in both strains in this study (Table 5.1) and further transcripts with serine/threonine kinase and/or calcium binding domains could be identified separately (Supplemental table S.5.2 & S.5.3) (Wohlrab et al. 2010). However, by using a microarray with newly designed probes derived from a new gene library and a wider array of different experimental conditions, including a light-dark cycle, we were not able to identify particular serine/threonine kinases or calcium binding proteins that are assignable to “defense traits”. Hence, we observe again the general pattern of differential regulation of these proteins that highlights their importance in triggering the grazer induced response of *A. tamarensis* towards copepods:

Regarding global transcription patterns, *A. tamarensis* strain 2 is characterized by an induction of gene expression (a high proportion of up-regulated genes), whereas in *A. tamarensis* strain 5 a down-regulation of genes is the response to the grazing copepods. These global expression patterns may reflect the initial differences in the defense strategies against copepods and the resulting alteration of the phenotype. The phenotypic shift of the *A. tamarensis* strain 2 towards a

phenotype less vulnerable to grazing is primarily characterized by the expression of additional genes. Contrarily, *A. tamarensis* strain 5 shows a major down regulation of genes in order to streamline its phenotype as it has a greater constitutive defense against copepods. Hence, *A. tamarensis* strain 2 seems to express new traits to cope with copepods; in contrast, *A. tamarensis* strain 5 shuts down not required functions to stabilize, and thus enhances a trait already previously expressed.

The comparison of regulated gene categories in the two strains further supports these observations (Fig. 5.6). Gene categories overrepresented in *A. tamarensis* strain 2 comprise mainly those categories that modify different kinds of protein interactions. Thus, these categories are involved in molecular processes that are the basis for main cellular modifications like phenotypic changes (Cohen 2000, Aubin-Horth & Renn 2009, Oliveira & Sauer 2012): Signal transduction mechanisms (T) and posttranslational modifications, protein turnover and chaperones (O). Furthermore, differences in inorganic ion transport and metabolism (P) were observed, thus, changing the internal state of ion concentrations and ion fluxes, a process strongly linked to signal transduction mechanisms (Anantharaman et al. 2007). Processes of energy production and conversion are also over-represented in this strain, implying the genetic fingerprint of costs associated with the expression of the defensive traits. This situation is opposed for *A. tamarensis* strain 5: this strain is characterized by a general overrepresentation of gene categories being down-regulated and, by categories that could lead to main cellular changes (T, O, P): Here, the intracellular networks are reduced and thus, imply the further channeling of the culture to the already expressed, “defensive”, phenotype.

The results from the gene category expression comparison of the two strains (Fig. 5.7) are in congruence with earlier findings (Wohlrab et al. 2010), showing that grazing copepods induce the differential expression of genes that can be predominantly assigned to the KOG categories T, O, C, and J. The difference in the number of regulated genes in this study and the study carried out by Yang et al. (2011) is given by the use of a broader microarray carrying nearly 10 times the amount of gene specific probes. Furthermore, these genes are primarily derived from RNAs obtained from grazing experiments than from RNAs derived from abiotic stress treatments as in Yang et al (2011).

The genetic background of induced defense can be further studied through the analysis of the gene expression data from the time series of *A. tamarensis* strain 2 cultures. This analysis revealed a co-expression of in total 140 genes assembled

into different KOG categories within this particular genotype specific interaction (Fig. 5.7). The repeated differential expression of these genes makes them a valuable source for identifying cellular traits that are changed due to grazing and, for functional assignment of so far unknown genes (Ungerer et al. 2007, Shiu & Borevitz 2008). In particular genes that indicate the existence of metabolic costs associated with the induced defense should be highlighted here (Table 5.2). Allocation costs could not be detected so far for *A. tamarensis* grazer induced defense responses and also not for the related species *A. minutum* (Selander et al. 2008; Selander et al. 2012). The transcriptomic analysis however provides evidence that allocation costs may exist and are reflected here by an increase in energy supplying processes and the regeneration of energy rich molecules itself (Table 5.2). By placing these genes in a molecular context, we assume that this higher energy demand might be overcome by an increase in the photosynthetic efficiency (Table 5.2). Callieri et al. (2004) showed that grazing by *Daphnia* spp. on picocyanobacteria significantly increases their photosynthesis rate (P_{max} , 2-fold) and efficiency (α , 3-fold). The apparent gain in energy might be redirected into the up-regulated pathways to allow the induced defense response. Additionally, the expression of genes causes costs itself by the expenditure of cellular energy in the synthesis of RNA and proteins (Perkins & Swain 2009).

A resulting increase in energy rich reduction equivalents might not only occur due to an apparent increase in the photosynthesis, the expression of the phosphoglycerate kinase as an important regulatory step in the glycolytic pathway may as well increase the amount of ATP (Table 5.2). The potential increased levels of 3-phosphoglycerate could be used for the synthesis of building blocks for photosynthesis and respiration via the serine anabolism. Overall, the cell might be fueled with more ATP and NADPH that can be used for energy consuming anabolic reactions or physiological reconstructions. ATP and NADPH have further the potential to regulate signaling pathways and to influence the physiological state of the cell (Pollak et al. 2007). NADPH is also a necessary reduction equivalent for the biosynthesis of saxitoxin (Kellmann et al. 2008).

The potential allocation costs may not manifest themselves in batch culture experiments and thus may be more pronounced in natural, heterogeneous environments (Van Donk et al. 2011). Batch culture experiments provide adequate conditions in which the species' intrinsic growth is the main limiting factor. The amount of photosynthetic active radiation can therefore be assumed to be sufficient in batch culture experiments to gain extra energy on demand and thus, to enable the

above described mechanisms. Under natural conditions however, *A. tamarense* may not be able to induce these energy gaining mechanisms to the same extent and those processes have to be traded-off. However, genotypes burdened by high costs of plasticity could be purged out from natural populations (DeWitt et al. 1998, van Kleunen & Fischer 2007, Xavier 2011).

The analysis of the gene expression data from this time series showed that the majority (81%) of the co-expressed genes are of unknown function (Fig. 5.7). These RNAs lack known protein domains and do not align to any sequence with functional assignment. Genes with functional assignment in non model organisms such as *Alexandrium* spp. belong mostly to common and well studied pathways and processes (Vandenkoornhuyse et al. 2010, Pavey et al. 2012). Genes that lack any functional assignment can therefore be considered important for functions within their specific environment i.e. species-specific traits, niche adaptation and environmental interactions (Peña-Castillo & Hughes 2007, Eads et al. 2008, Maheswari et al. 2010). Transcription analysis of ecological important traits like induced defense in phytoplankton organisms will be one important step towards an 'ecological annotation of genes' and thus, will fill gaps in our knowledge of gene function (Ungerer et al. 2007, Aubin-Horth & Renn 2009, Pavey et al. 2012).

5.6 Conclusions

Interactions with copepod grazers cannot simple be evaluated on the species level, as populations may significantly vary in their responses. In addition, phenotypic plasticity among representatives of one population towards grazing copepods appears to be regulated by differing processes. This seems intuitive when considering that the received information has to be processed and integrated into different genotypes expressing different phenotypic traits. This study further indicates a high potential adaptability towards grazing as a top-down process within natural *A. tamarense* populations known to consist of high genotypic variation, expressing different phenotypic traits. This ability may therefore be one mechanism to maintain the high genotypic variation in the populations. However, it must be further examined if the defense responses observed in this study can be extrapolated to natural communities, where different benefits of inducing a response present an entirely different trade-off based on infochemicals available and costs that explicitly manifest themselves in such situations.

6. Mapping genotypic trait alterations in *A. tamarensis* in response to a protistan grazer

6.1 Abstract

Natural *Alexandrium tamarensis* populations are composed of multiple genotypes that display phenotypic variance. Those phenotypic traits comprise parameters that are known to influence top-down processes such as the ability to lyse co-occurring competitors and protistan grazers. Hence, species' interactions within *A. tamarensis* populations can widely vary, resulting in genotype specific community alterations. Detailed analysis on how such specific interactions provide i.e. biochemical feedback to the different genotypes is however missing. As such, we lack fundamental knowledge about its population impact. In order to obtain initial insights into this topic, we exposed two *A. tamarensis* strains to a protistan grazer and analyzed traits that change during this interaction for the two co-existing genotypes. We show that around 5% of all analyzed genes are differentially expressed in those two strains under control conditions, and that at least 3% of all annotated protein families are involved in the specific response to the co-occurring species interaction. Further, we are able to interpret our transcriptomic dataset, and give valuable insight on traits that are involved and change in each genotype. Our results demonstrate that species' interactions lead to genotype specific re-programming of internal processes, and reveal that such interactions cannot solely be evaluated on a genotypic level. Moreover, genotypes alter specific traits in response to interacting species that may strongly influence future interactions. Additionally, our transcriptomic data indicate that mixotrophy may be a very important albeit understudied nutritional mode in *A. tamarensis*.

6.2. Introduction

Hutchinson's classic paper "The paradox of the plankton" addresses the question of how species can coexist in a relatively isotropic or unstructured environment, competing for the same sorts of resources (Hutchinson 1961). This puzzling situation was based on the traditional view of bottom-up processes (competition for abiotic resources) as a main driving force for shaping communities. The failure to make resource competition solely responsible for the observed situation in the plankton led to an increased appreciation of species' interactions, such as top-down processes, as a potential driving force for annual species succession (Margalef 1978, Smetacek et al. 2004) and resulting co-evolutionary processes for species diversity (Smetacek 2001, Hamm et al. 2003, Smetacek et al. 2004).

This situation becomes even more complex when considering the coexistence of multiple genotypes, exhibiting substantial fitness differences in the lab, within predominantly asexually reproducing populations, a phenomenon subsequently named "The second paradox of the plankton" (Hebert & Crease 1980, Fox et al. 2010). These different genotypes within one population often express traits that comprise parameters such as cell size, cell shape and the production of secondary metabolites: parameters known to influence species' interactions (Van Donk et al. 1999). In depth analysis using molecular markers revealed a high degree of intra-population diversity within dominant phytoplankton groups (Ryneerson & Armbrust 2000, Iglesias-Rodríguez et al. 2006, Alpermann et al. 2010). The analysis of the genotypic diversity within a population of the toxigenic marine red tide dinoflagellate *Alexandrium tamarense* revealed a diversity that exceeds any other study so far on marine phytoplankton populations (Alpermann et al. 2010). Within 77 examined strains, no repeated sampling of any genotype occurred. Furthermore, all genotypes showed phenotypic differences concerning the production of secondary metabolites (Alpermann et al. 2010). The secondary metabolites examined comprise the paralytic shellfish toxins (PSTs), a group of potent neurotoxins responsible for the toxic effects of *A. tamarense* on marine mammals and humans (Cembella 2003) and so far unknown allelochemical compounds that act lytic on co-occurring algae and heterotrophic grazers (Tillmann & John 2002; Tillmann et al. 2008; Tillmann & Hansen 2009).

The paradox of the plankton, as well as the co-existence of several genotypes that differ in their expression of traits within one population, may be one factor that limits our ability to answer basic questions concerning community ecology and the resulting succession patterns within the plankton. Fluctuations in species' abundances are largely driven by species' interactions. Consequently, the predictability of species' abundances decreases with time, leading to chaotic fluctuations generated by those interactions (Benincà et al. 2008). These chaotic fluctuations could partly be caused by the variety of traits that are expressed within interacting populations. On the one hand, species' interactions will be dependent on the *net* traits expressed in the population: On the other hand, internal population variation in traits will result in varying genotype per genotype interactions (Agrawal 2001). These genotype specific interactions therefore may cause a cascading effect with the potential to alter the *net* population traits and subsequently, alter further interactions. Understanding how communities interact and are composed should therefore focus on the interaction of basic components within communities, which is the single genotype. Resolution of different genotype per genotype interactions will therefore contribute to our understanding of plankton community ecology and furthermore, to the evolutionary basis of intra-population diversity.

An example of a well-studied system investigating effects caused by different genotypes is the above mentioned allelochemical potency within *Alexandrium tamarense* populations. Their displayed variation in genotype-dependent production of lytic acting allelochemicals is directly proportional to the growth and survival rate of co-occurring heterotrophic grazers and phototrophic competitors (Tillmann et al. 2008, Tillmann & Hansen 2009). The variation of this trait within one population ranges from lytic, at very low cell concentrations ($EC_{50} < 10 \text{ cells mL}^{-1}$), to no observed lytic effects even at high cell concentrations (Tillmann et al. 2008; Alpermann et al. 2010). Since the allelochemicals affect both competitors and predators, genotype specific species' interactions lead to alterations of competitive and bi-trophic systems. These differences will in turn have a cascading effect on the further development of the composition of the temporal present microbial community structure (Fistarol et al. 2004, Weissbach et al. 2010). In this example, regarding the lytic potency in *A. tamarense*, two members of the same population have the potential to re-structure the local community composition in a genotype-specific manner.

The aim of our study was to resolve species interactions in *A. tamarense* on a genotypic level. We therefore utilized a simple model where a lytic and a non-lytic

strain of *A. tamarensis* are exposed to cues from a protistan grazer grazing on conspecifics. We used a functional genomic approach to (1) assess the differences in the two *A. tamarensis* strains and (2) to screen for traits that change in each genotype during exposure to the heterotrophic grazer. Applying functional genomics to resolve genotype specific traits that change during species interactions served to enhance our understanding of underlying mechanisms in such strain specific responses. Furthermore, screening for transcriptional regulation is a useful complement to traditional methods, which provides us with insights into traits that are selectively induced and may hence be beneficial for the species' ability to succeed under pressure of biotic interactions. Understanding how species' interactions select and alter traits on a genotypic level therefore contributes fundamental knowledge to the field of planktonic ecology.

6.3 Material and Methods

*Alexandrium tamarens*e and *Polykrikos kofoidii* cultures

Two clonal strains of *Alexandrium tamarens*e were isolated in May 2004 from the North Sea coast of Scotland (Alpermann et al. 2010) and grown in K-medium (Keller et al. 1987) in a temperature and light controlled room (salinity ~ 33 , 18°C , 14h:10h light-dark cycle, $\sim 150\text{ fmol m}^{-2}\text{ s}^{-1}$). The two strains, described as *Alexandrium tamarens*e strain A2 and *Alexandrium tamarens*e strain A5, were selected based on differences in their lytic capacity as quantified by a *Rhodomonas* bioassay (Tillmann et al. 2009). *A. tamarens*e strain A2 showed a high lytic capacity in the *Rhodomonas* bioassay, whereas *A. tamarens*e strain A5 showed no lytic effect at all tested cell concentrations (Tillmann et al. 2009).

The culture of *Polykrikos kofoidii* was established from a plankton net tow (20 μm) taken in 2009 in coastal waters of Scotland and grown in K-medium (Keller et al. 1987). The culture was routinely held in 63 mL culture flasks on a slow rotating plankton wheel (1 rpm) at 15°C and low light ($10\text{-}20\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$) and fed using either *Lingulodinium polyedrum* (CCMP 1738) or the non-lytic *A. tamarens*e strain A5. Prior to the experiments, *Polykrikos* was regularly (\sim once a week) fed *Lingulodinium polyedrum* for a period of three month. A dense subculture used for experiment inoculation was starved for ~ 1 day so that no food algae were present but *Polykrikos* did not yet start massive gamete formation.

Experimental design

*Alexandrium tamarens*e cultures were washed three times with sterile filtered seawater (VacuCap 0.2 μm , Pall Life Science) and diluted to a final concentration of $\sim 1000\text{ cells mL}^{-1}$. The washing steps were applied in order to exchange culture media containing lytic substances prior to the experiments. *Polykrikos kofoidii* cultures were diluted to a final concentration of $\sim 40\text{ cells mL}^{-1}$.

For the experimental setup, a cage design was chosen to separate the two *A. tamarens*e strains from mixed *P. kofoidii*/*A. tamarens*e cultures. With this approach, we ensured to only isolate RNA from *A. tamarens*e cultures. This was necessary in order to avoid cross-hybridization on the microarrays since both, *A. tamarens*e and *P. kofoidii* are dinoflagellates having similar gene sequences of core dinoflagellate genes.

The cages were made out of 50 mL polypropylene tubes containing a 10 μm plankton mesh at the bottom and were placed in 250 mL glass bottles. For both *A. tamarensis* strains, 24 bottles were set up consisting of three replicates of controls and 3 replicates of species interaction treatments for each defined harvesting time point (24 h; 48 h; 72 h and 96 h). Each bottle received 125 mL of *A. tamarensis* cultures strain 2 or *A. tamarensis* strain 5 cultures and were diluted with 125 mL K-medium (Keller et al. 1987) to a final concentration of ~ 500 cells mL^{-1} . Cages were deployed and submerged into the flask until they media filled volume of each cage reached 30 mL. Each treatment cage received 1 mL of *P. kofoidii* culture (~ 40 cells) and 1 mL of *A. tamarensis* strain 5 culture (~ 500 cells). Control flasks only received 1 mL of *A. tamarensis* strain 5 cultures within the cage. The cages were gently moved up and down 3 times a day in order to promote the exchange of media and dissolved compounds (allelochemicals) between the two compartments.

Experiments were subsequently terminated after 24 h, 48 h, 72 h and 96 h.

Cell harvesting

After each defined time point, three treatment bottles and three control bottles were harvested for each strain. Cages were removed from the experiment bottles and submerged into separate 50 mL glass bottles filled with sterile filtered seawater until they seawater filled volume of each cage reached 30 mL. The content of the cages was gently mixed and 10 mL of each submerged cage were preserved in acid Lugol's solution for cell counts.

The bottles containing the surrounding *A. tamarensis* cultures were carefully turned 7 times over to ensure equal mixing of the culture and 10 mL of each culture were preserved in acid Lugol's solution for cell counts. Afterwards, the culture was poured over a 10 μm nylon mesh to sample the *A. tamarensis* cells. The 10 μm nylon mesh was washed out into a 50 mL collection tube and immediately centrifuged at 4° C for 5 min for RNA samples.

Obtained cell pellets were immediately mixed with 1 mL 60° C hot TriReagent (Sigma-Aldrich, Steinheim, Germany), transferred to a 2 mL cryovial containing acid washed glass beads, vortexed for 1 min to break the cells and subsequent frozen in liquid nitrogen. The cell pellets were at -80° C until further use.

Total RNA isolation

Frozen cell pellets fixed with TriReagent were lysed using a Bio101 FastPrep instrument (Thermo Savant Illkirch, France) at maximum speed (6.5 m s^{-1}) for 2×45 s. Lysed cells were cooled on ice for 5 min, 200 μL chloroform was added and vortexed for 20 s. The mixture was incubated at room temperature for 5 min, transferred to a phase lock tube (Eppendorf, Hamburg; Germany) and incubated for another 5 min at room temperature followed by centrifugation for 15 min at $13.000 \times g$ and 4° C . The upper aqueous phase was transferred to a new tube and the same volume of isopropanol, 1/10 volume of 3M Na-acetate (pH = 5.5; Ambion by Life Technologies, Carlsbad, California, USA) and 2 μL linear polyacrylamide (Ambion) was added to each tube. Total RNA was precipitated for 90 min at -20° C and afterwards collected by centrifugation for 20 min and $13.000 \times g$ at 4° C . Obtained pellet were washed twice, first with 1 mL 70% EtOH followed by 10 min centrifugation at $13.000 \times g$ and 4° C , then with 1 mL EtOH absolute, followed by 5 min centrifugation at maximum speed. The remaining EtOH was discharged and the RNA pellet was dried for 1 min at 37° C . The pellet was resolved in 30 μL RNase free water (Qiagen, Hilden). RNA quality was checked with the RNA Nano Chip Assay on a 2100 Bioanalyzer device (Agilent Technologies, Santa Clara, California, USA) and with a NanoDrop ND-100 spectrometer (PepLab, Erlangen, Germany) for assessing the yield, concentration, and purity of the RNA.

EST- Library construction and 454 sequencing

Subsamples of the *A. tamarensis* strain 2 and *A. tamarensis* strain 5 RNAs from treatments were pooled and 10 μg of total RNA was used as the starting material for a normalized cDNA library for GS FLX Titanium sequencing. The cDNA library construction was carried out by the Vertis Biotechnology AG (Freising-Weihestephan, Germany, for details see Publication V).

In total, 521,578 reads could be assembled into 19,107 contigs longer than 500 bp with an average coverage of ~ 27 reads per contig. The contigs obtained from the current study were compared with an already existing gene library from *A. tamarensis* (Publication V) by reciprocal best BLAST hit search. This resulted in a final selection of 29,224 unique contigs.

Microarray design and microarray hybridizations

Microarray probes (60mers) were designed from these contigs using Agilent's eArray online Platform (<https://earray.chem.agilent.com/earray/>).

The microarray hybridizations were designed so as to compare both, RNA from treatments and RNA from controls by using a reference pool of RNAs. The RNA reference pool consisted of an equal amount of subsamples of all RNAs obtained from the control treatments.

Labeling reactions were prepared from 200 ng total RNA from every RNA sample and the reference pool using the Agilent two-color Low Input Quick Amp Labeling kit (Agilent Technologies, Santa Clara, USA) in addition with the Agilent RNA Spike-In Mix (Agilent). In short, RNA was first reverse transcribed into cDNA using T(7) promoter primer to select for Poly(A)-RNAs and afterwards labeled fluorescently by linear amplification of cDNAs into complementary RNA (cRNA) with Cyanine-3-CTPs or Cyanine-5-CTPs according to the manufactures' protocol.

Dye incorporation rates and cRNA concentrations were determined with a NanoDrop ND-100 spectrometer (PeqLab). Hybridizations were carried out onto 8x60k microarray slides with 300ng of each, cyanine-3 and cyanine-5 labeled cRNA at 65° C for 17 h (Agilent) and afterwards washed according to the manufactures' protocols. Microarrays were scanned with an Agilent G2565AA scanner.

Raw data processing was performed by the Agilent Feature Extraction Software version 9.1.3.1 (FE); quality monitoring was performed with the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07. Testing for differentially expressed genes was done with the GeneSpring GX software platform version 12 (Agilent). A two-way ANOVA was used in order to separate genes that only respond to the treatment from genes that response in the control conditions to the factor "time". Screening for differentially expressed genes in both genotypes under control conditions was done in the same way. Genes were considered as differentially expressed when test statistics indicated *p*-values less than 0.05 and fold changes that exceeded 1.5.

Gene set enrichment analysis

All sequences represented on the microarray were subject to a hidden markov model (HMM) based search for Pfam families (Finn et al. 2008). Sequences were first *in silico* translated (Wernersson 2006) and subsequent online batch

processed to screen for Pfam families (<http://pfam.sanger.ac.uk/>). At the background of all Pfam families, Pfam families of up-regulated genes were tested for their significant enrichment by calculating p -values from a hypergeometric distribution (Subramanian et al. 2005). Pfam families were considered as significantly enriched for a given experimental time point when test statistics replied a p -value < 0.05 .

6.4 Results

Differentially expressed genes between the two genotypes

Variability in gene expression patterns in the two *A. tamarensis* genotypes were assessed by comparing the gene expression profiles of the control cultures. This comparison of the microarray hybridizations between the control cultures of *A. tamarensis* strain A2 and *A. tamarensis* strain A5 revealed that ~ 5% of all genes represented on the microarray are differentially expressed at all time points. From those, 923 genes were always higher expressed in *A. tamarensis* strain A2 cultures, and 584 in *A. tamarensis* strain A5 cultures respectively. Fold changes (FC) of those differentially expressed genes reached a mean maximum of 385 FC for *A. tamarensis* strain A2 and 977 FC for *A. tamarensis* strain A5 (Fig. 6.1).

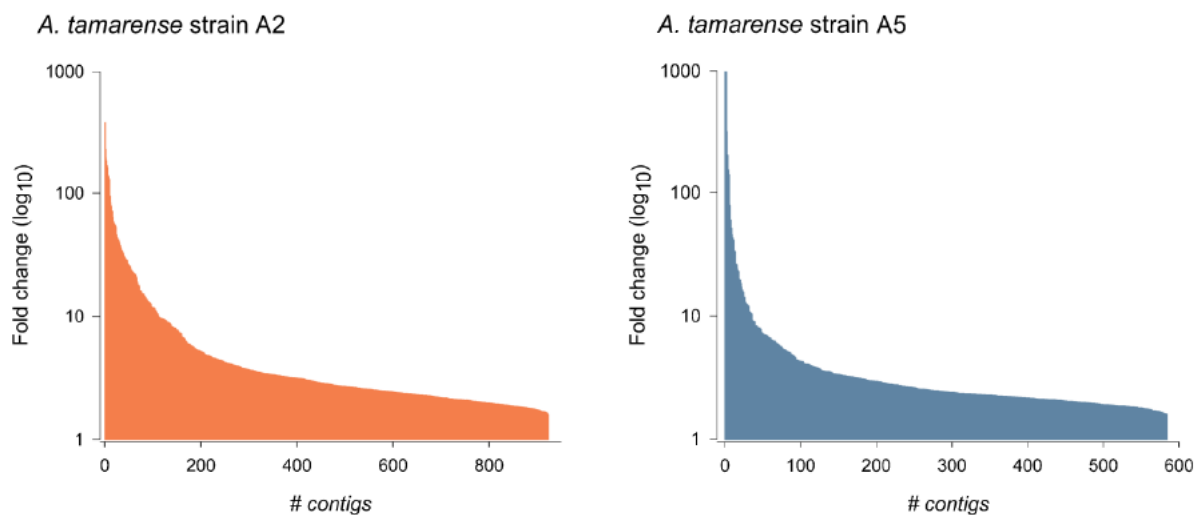


Fig. 6.1: Differentially expressed genes between control cultures of *A. tamarensis* strain A2 and *A. tamarensis* strain A5. The graphs indicate the fold changes and total numbers of differentially expressed genes (contigs) in the two *A. tamarensis* control strains.

The top 10 over-expressed genes for each strain along with their assigned Pfam protein family are listed in Table 6.1 (for strain A2) and Table 6.2 (for strain A5).

Table 6.1: List of the top 10 over-expressed genes in *A. tamarensis* strain A2 control cultures and over-expressed genes coding for proteins potentially involved in secondary metabolism. The table includes calculated fold change (FC) values and the assigned Pfam families of the respective gene as well as the attributed function of each annotatable gene.

Contig	Mean FC value	Pfam family name	Attributed function
Top 10 over-expressed genes in <i>A. tamarensis</i> strain A2			
contig11508	385.70	-	-
contig47146	232.12	-	-
Atam01000	199.42	-	-
contig01736	188.40	Ion channel	Ion flux control
Atam27888	172.62	-	-
Atam09090	164.73	-	-
contig48538	144.33	-	-
contig14000	141.05	-	-
contig54109	140.65	-	-
Atam19896	133.97	-	-
Prominent secondary metabolite protein coding genes over-expressed in <i>A. tamarensis</i> strain A2			
contig46061	28.56	Glycosyl transferase family 8	Transfer of glycosyl groups
contig27559	9.43	ABC transporter transmembrane region	Transport
Atam22087	5.87	FAD binding domain	Redox reactions
contig50076	5.44	ABC-2 type transporter	Transport
contig53666	5.41	Glycosyl transferase family 8	Transfer of glycosyl groups
contig03340	4.17	Glycosyl transferase family 8	Transfer of glycosyl groups
contig30821	3.81	Major Facilitator Superfamily	Transport
contig33504	3.79	Major Facilitator Superfamily	Transport
Atam04504	2.93	Aldo/keto reductase family	Redox reactions
contig29413	2.90	Aldo/keto reductase family	Redox reactions
contig47324	2.82	Taurine catabolism dioxygenase TauD, TfdA family	Redox reactions
contig51994	2.69	Aminotransferase class-III	Special, transaminase activity
contig05529	2.68	Zinc-binding dehydrogenase	Redox reactions
contig06697	2.66	Acyl-CoA dehydrogenase, middle domain	Redox reactions
Atam18919	2.55	Glycosyl transferase family 8	Transfer of glycosyl groups
contig08880	2.52	ABC-2 type transporter	Transport
Atam25893	2.49	ABC transporter	Transport
contig02047	2.47	SnoaL-like polyketide cyclase	Polyketide synthase
contig18051	2.22	ABC transporter transmembrane region	Transport
Atam05470	2.19	Cytochrome P450	Redox reactions
contig21528	2.17	Methyltransferase domain (2)	Methyl group transfer
contig14651	2.09	Beta-ketoacyl synthase, C- & N-terminal domain	Polyketide synthase
contig40268	2.09	Oxidoreductase family, C-terminal alpha/beta domain	Redox reactions
contig41426	1.82	Glycosyl transferase family 8	Transfer of glycosyl groups

Since the ability of allelochemical, lytic compound production is either present (strain A2) or absent (strain A5) in those two genotypes, obtained differentially expressed genes were analyzed for the occurrence of Pfam protein family annotations with a known involvement in secondary metabolite production. A file containing Pfam domains and families associated with secondary metabolite

synthesis was kindly provided by Takano and Medema who constructed the antiSMASH antibiotics and Secondary Metabolite Analysis Shell (Medema et al. 2011).

In total, *A. tamarensis* strain A2 showed a higher expression of 24 genes coding for such protein families (Table 6.1). For *A. tamarensis* strain A5, a higher expression of 13 genes coding for proteins with the potential to participate in secondary metabolism was found (Table 6.2).

Table 6.2: List of the 10 uppermost over-expressed genes in *A. tamarensis* strain A5 control cultures and over-expressed genes coding for proteins potentially involved in secondary metabolism. The table includes calculated fold change (FC) values and the assigned Pfam families of the respective gene as well as the attributed function of each annotatable gene.

Contig	Mean FC value	Pfam family name	Assessed function
Top 10 over-expressed genes in <i>A. tamarensis</i> strain A5			
contig52451	977.12	Fatty acid desaturase	Unsaturated fatty acid synthesis
contig10253	318.80	-	-
contig22596	205.62	-	-
contig22292	156.37	-	-
contig03283	143.74	-	-
contig14894	78.45	-	-
58180097	59.28	-	-
contig05597	51.09	EF-hand domain pair (2)	Calcium-ion binding
contig22152	45.17	-	-
Atam02515	42.39	-	-
Prominent secondary metabolite protein coding genes over-expressed in <i>A. tamarensis</i> strain A5			
contig48765	10.39	Methyltransferase domain	Methyl group transfer
Atam13914	5.12	Methyltransferase domain	Methyl group transfer
Atam35211	4.65	EamA-like transporter family	Transport
Atam25791	3.31	Acetyltransferase (GNAT) family	Acetyl group transfer
Atam23762	2.94	Cytochrome P450	Redox reactions
contig33556	2.86	Aldo/keto reductase family	Redox reactions
contig34741	2.04	ABC-2 type transporter	Transport
contig01330	1.99	Taurine catabolism dioxygenase TauD, TfdA family	Redox reactions
Atam01694	1.88	NAD-dependent epimerase/dehydratase family	Redox reactions
contig06638	1.84	EamA-like transporter family	Transport
contig16321	1.80	ABC-2 type transporter	Transport
contig37655	1.79	Periplasmic binding protein	Transport
Atam12516	1.76	Acyltransferase family	Acyl group transfer

Cage experiments

Both genotypes of *A. tamarensis* were exposed to the protistan grazer *P. kofoidii* grazing on conspecifics. When the mixture of *P. kofoidii* and non-lytic *A. tamarensis* strain A5 cultures was surrounded by lytic *A. tamarensis* strain A2 cultures, numbers of *A. tamarensis* cells inside the cage significantly increased over time (Fig. 6.2, ANOVA $p = 0.015$). The *P. kofoidii* cell density inside the cage in this treatment did not increase over time (ANOVA $p = 0.196$). *P. kofoidii* pseudocolonies with engulfed *A. tamarensis* cells were present at all time points.

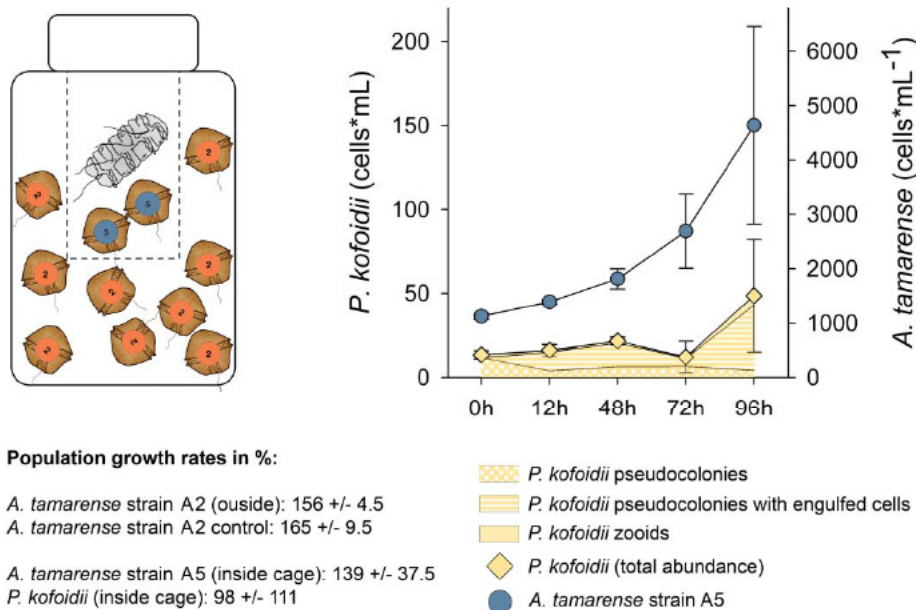


Fig. 6.2: Cell numbers of *A. tamarensis* strain A5 and *P. kofoidii* cells inside the cage, surrounded by *A. tamarensis* strain A2 cultures. The abundance of *P. kofoidii* morphologies and *P. kofoidii* with ingested *A. tamarensis* cells are represented by area fills. The experimental setup and the growth rate of *A. tamarensis* strain A2 and strain A5 cultures as well as for the *P. kofoidii* cultures are given as percentage population increase over 4 days.

Exposure of the *P. kofoidii*/non-lytic *A. tamarensis* mixture to non-lytic *A. tamarensis* strain A5 cultures resulted in a significant increase of *P. kofoidii* cells (Fig. 6.3, ANOVA $p = < 0.001$). In this treatment, the abundance of *A. tamarensis* cell numbers inside the cage did not change significantly over time (ANOVA $p = 0.793$).

Growth rates of *A. tamarensis* cultures surrounding the cage in both treatments did not differ significantly from growth rates of the control cultures (Students *t*-test $p > 0.05$)

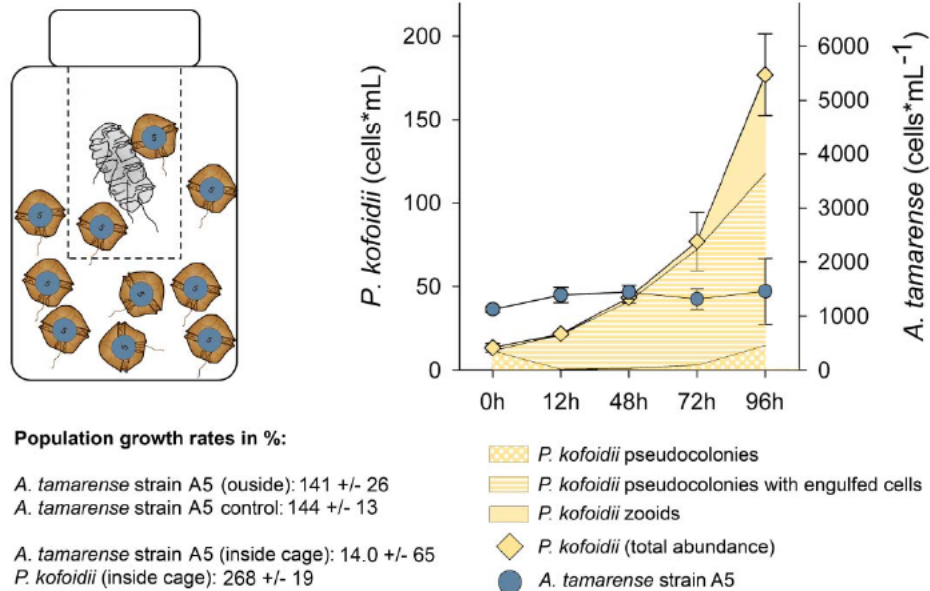


Fig. 6.3: Cell numbers of *A. tamarensis* strain A5 and *P. kofoidii* cells inside the cage, surrounded by *A. tamarensis* strain A5 cultures. The abundance of *P. kofoidii* morphologies and *P. kofoidii* with ingested *A. tamarensis* cells are represented by area fills. The experimental setup and the growth rate of *A. tamarensis* strain A5 cultures as well as for the *P. kofoidii* cultures are given as percentage population increase over 4 days.

Gene expression analysis through microarray hybridizations

In order to screen for traits that changed in each genotype due to waterborne cues of interacting species, *A. tamarensis* cultures surrounding the cage were analyzed for gene expression changes. Numbers of up-regulated genes increased over 9 fold in the lytic *A. tamarensis* strain 2 during the exposure time frame (Table 6.3). In the non-lytic *A. tamarensis* strain 5 cultures, the number of up-regulated genes doubled over time (Table 6.3).

Table 6.3: Results from the treatment gene expression analysis for each *A. tamarensis* strain. The table displays the numbers of responsive, up-genes for every time-wise comparison after exposure of each *A. tamarensis* strain to the cage containing interacting *P. kofoidii* cells and conspecifics. The last column gives the number of genes that are up-regulated at every time point.

Investigated genotype	24h ← vs. → 48h	24h ← vs. → 72h	24h ← vs. → 96h	# regulated at all time points
<i>A. tamarensis</i> strain A2	604	959	5724	244
<i>A. tamarensis</i> strain A5	496	1177	909	61

Quantities of significantly enriched Pfam families showed a time specific amount of enriched families (Fig. 6.4). In average, three percent of all specific families identified on the microarray were significantly enriched at every time point.

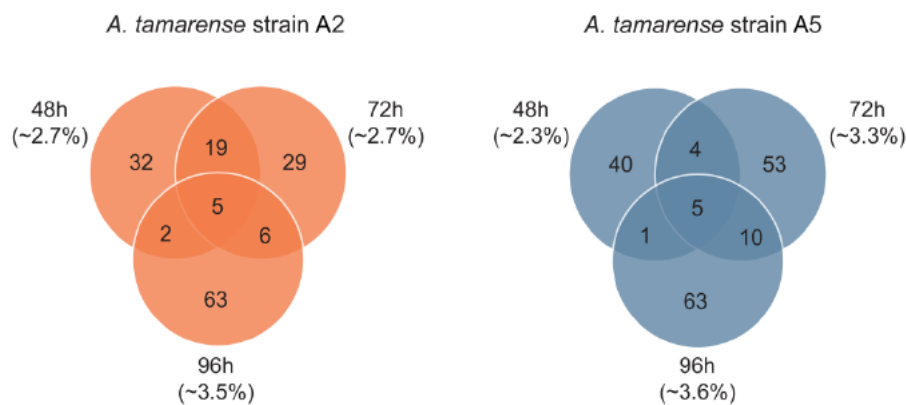


Fig. 6.4: Numbers of significant enriched Pfam families. The Venn diagram summarizes the distribution of significant enriched Pfam families ($p < 0.05$) for each *A. tamarensis* genotype and each investigated time point. Percentages denoted in brackets indicate the amount of significantly enriched families in the background of all protein families that could be identified in the gene library.

These significantly enriched families were manually grouped into categories that assemble different processes. Category A clusters processes involved in cell cycle control and genetic information storage and processing. Category B clusters intracellular vesicle associated processes. Category C includes processes that mediate, translate and modify different kinds of signals in the cell. The numbers of genes that code for Pfam families assembled into these categories are displayed as area circles in Fig. 6.5.

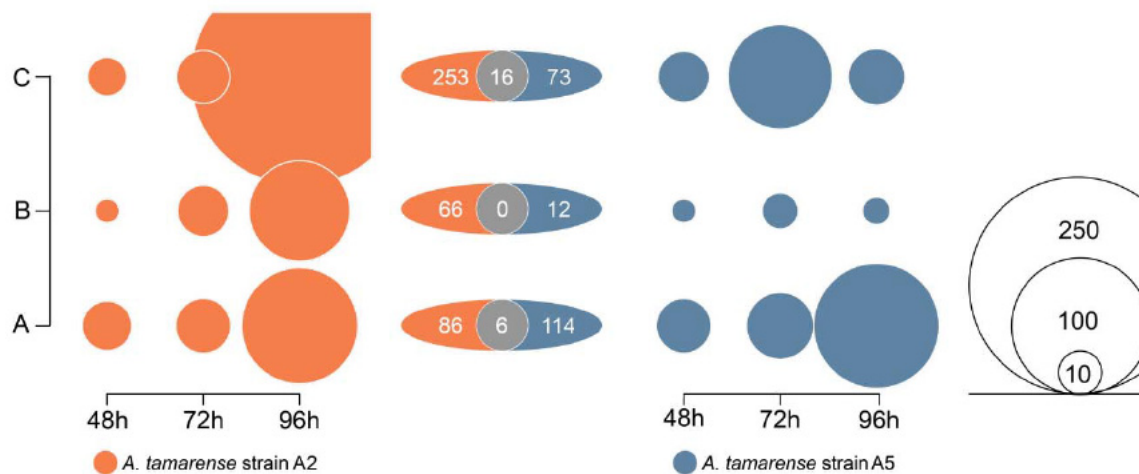


Fig. 6.5: Selected differentially expressed genes from *A. tamarensis* cells outside the cage. Selected differentially expressed genes are grouped into the categories A - C. The area of the circles represents the amount of genes per category and per time point. The Venn diagrams in the middle display the amount of genes per category that are differentially expressed in both strains.

Category A clusters processes involved in cell cycle control and information storage and processing. **Category B** clusters intracellular vesicle associated processes. **Category C** includes processes that mediate, translate and modify different kinds of signals in the cell.

Genes assembled into category A showed a similar increase over time in transcript abundance, however, expressed genes within this group differ notably by their Pfam family annotations (Fig. 6.5; Supplemental table S.6.1 & S.6.2).

Categories B and C showed differences in their time-wise development for both strains, here, transcript abundance and gene products develop differentially over time.

6.5 Discussion

The results from this study show that species' interactions cause gene expression changes in a genotype specific manner. Indeed, these genotype-specific responses might be influenced by the differences in the allelochemical properties of both strains as only one is lytic (strain A2). Hence, this lytic strain may construct a niche different from the conditions that evolved in the experimental set-up with the non-lytic strain A5.

Differences in the transcriptome between the two *A. tamarensis* strains under control conditions accounted for ~5% of all tested genes. Additionally, the comparison of the transcriptomic difference between the two *A. tamarensis* strains revealed surprising high fold change values (Fig. 6.1). Whereas detected fold changes in the treatments reached maxima values of ~ 15 (for strain A2) and ~77 (for strain A5) respectively, fold changes in the strain comparison display maxima values of ~385 (strain A2) and ~977 (strain A5). Dinoflagellates have the tendency to expand particular gene abundances by tandem repeats of those genes in the DNA. Furthermore, high numbers of certain transcripts are correlated to the genomic abundance of those genes (Bachvaroff & Place 2008). Whether the differences in the observed transcriptional activity are caused by such expansions within the genomic DNA has further to be tested. If so, different traits could arise by the selective duplication of particular genes in dinoflagellates. Suggested mechanisms of mRNA recycling proposed for dinoflagellates (Slamovits & Keeling 2008) could contribute by manifestation and diversification of traits in the genome via gene duplication events. Irrespective of the underlying mechanisms responsible for those transcriptomic differences, the results clearly show that different genetic programs are active in both strains. This might contribute to the differences in their phenotype which is at least partly reflected by their allelochemical properties. Hence, differences in the expression of genes that participate in secondary metabolism (Table 6.1) should be further investigated for their role in lytic compound production in *Alexandrium tamarensis*. Polyketide synthases (PKS), for example are involved in the biosynthesis of the majority of known dinoflagellate toxins (Kellmann et al. 2010) and the expression level of PKS genes positively correlates with lytic compound production in the haptophyte *Prymnesium parvum* (Freitag et al 2011).

Differences in the allelochemical properties of the two *A. tamarensis* strains are known to influence the survival rate of co-occurring unicellular heterotrophs (Tillmann et al. 2008). This is in congruence with our observations, as a result the final composition of species' abundances differs in the two treatments. The lytic strain A2 facilitates the growth of the conspecific *A. tamarensis* strain inside the cage by constraining an increase in the abundance of *P. kofoidii*. Contrarily, the presence of the non-lytic strain A5 allows an increase of active grazing *P. kofoidii* cells, resulting in a steady abundance of conspecifics inside the cage.

Both observed internal differences in gene expression as well as their specific influence on species' interactions led to a subsequent alteration of genes expressed in the two strains (Table 6.3). In general ca. ~3 % of all identified protein families were considered significantly enriched at every time point and for both strains (Fig. 6.4), however, they differ remarkably for both strains (Fig. 6.5, Supplemental table S.6.3) Considering all possible life history events that a marine dinoflagellate may face during one generation, a remarkably high number of protein families seems to be involved in those species interaction processes, hence emphasizing the selective strengths of such interactions (Cembella 2003, Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008).

In the lytic strain A2, intracellular vesicle associated processes (category A) increased over time whereas in the non-lytic strain A5 these processes were merely slightly enriched without any time effect (Fig. 6.5). Changes in dissolved and particulate organic matter (DOM & POM) might have caused these differences in genes that cluster into category A processes. The lytic effects of strain A2 will increase levels of DOM and POM during the experimental time frame which could be used as additional nutrient source for *A. tamarensis* (Weissbach et al. 2010). Molecular genetic investigations of underlying processes that take up DOM/POM via endocytosis in marine protists are reduced to the description of two endocytosis related genes obtained within a metatranscriptomic study (Lin et al. 2010, Lin 2011). Within our dataset we found several genes associated with vesicle transport processes that are significant enriched in treatments where cells get lysed. Genes coding for proteins necessary for the formation of clathrin coated vesicles are significant enriched at every time point in the lytic strain A2. Clathrin coated vesicle sample solutes or receptor bound molecules from the surrounding and subsequent develop into lysosomes, digestive compartments with an acid pH value (Alberts et al. 2008). Based on the gene expression data, proton-translocating inorganic pyrophosphatases may play an important role in acidifying these digestive

compartments. These proton pumps were considered to be restricted to higher plants and some primitive photosynthetic bacteria, however they could subsequently be found in many protists and bacteria (Pérez-Castiñeira et al. 2001). Inorganic pyrophosphates are proton pumps distinct from F-, P- and V-ATPases that utilize inorganic pyrophosphate (PPi) hydrolysis as driving force for H⁺-movement across the membrane (Rea & Poole 1993). PPi is produced by an anabolic reaction through the hydrolysis of ATP and the further hydrolysis of PPi is essential to replenish the cellular orthophosphate pool. PPi is rather considered to be waste product of the anabolism (Pérez-Castiñeira et al. 2001). The use of PPi rather than ATP to acidify the lysosomes would therefore be an efficient and astute recycling process in *Alexandrium* cells in order to gather nutrients derived from endocytosis.

Prior investigations of endocytosis in marine heterotrophic protists have revealed that protein kinases (Hartz et al. 2008) and lectins (Wootton et al. 2007) can be directly linked to phagocytosis. Hence, genes that cluster into the category C include lectins that are significantly enriched after 48 h and 96 h. In addition, further adhesive (MAM) receptors were enriched at those time points and may contribute to endocytotic processes. Ion channels and protein kinases make up the majority of genes clustered into category C after 96 h. Such an increase in the expression of ion channels thus can be placed in direct connection to endocytosis as a necessary mechanism to keep the intracellular ion homeostasis if accompanied by a probable inevitable uptake of culture media. Ion channels and protein kinases also led to an increased abundance of genes assembled into category C (72 h) for the non-lytic strain A5. However, the enrichment of protein families involved in endocytotic processes in this strain was not as strongly developed as for the lytic strain A2 (Fig. 6.5). Strain A5 cultures may however utilize compounds excreted from the heterotrophic grazer.

During the time course of experimental, the expression of motor proteins, kinesins and predominately dyneins including associated motor units, increased. These data strongly indicate an establishment of intracellular trafficking routes in *A. tamarense* strain A2, which could be directly associated with enhanced vesicular transport, endosome and lysosome organization and maintenance. Furthermore, after 96h, the expression of acidifying V-ATPases in addition to inorganic pyrophosphates was found to be significant enriched. Endocytotic processes thus seem to increase over time in the lytic strain A2.

Protein families clustered in the category C for strain A2 and to a lower extent for strain A5, further include several types of ligand binding receptors which may be connected to receptor mediated endocytosis of particles, as well as proteins involved in glycoprotein and glycolipid modification that can subsequently alter the properties of the outer glycosylated membrane surface. The biochemical compositional change of the glycosylated membrane surface determines important traits such as self-self recognition, non-self discrimination as well as “cell taste” (Gahmberg 1981, Wolfe 2000), thus could have a strong cascading effect on further interactions.

Significantly enriched protein families involved in signaling processes emphasize signal transduction via G-protein coupled receptors, as proposed by Hartz et al. (2008). Cascades induced by ligand binding on G-protein coupled receptors lead to increased levels of diacylglycerol (DAG), an important intracellular second messenger (Alberts et al. 2008). Our data show that diacylglycerol kinase (DAGK) accessory domains are significantly enriched in both strains after 48 h and additionally after 72 h in the lytic strain A2. DAGK drives the conversion of DAG into phosphatidic acid (PA), thus serving as a switch by terminating the signaling of one lipid while simultaneously activating signaling by another (Arisz et al. 2009). PA signaling can be associated with a wide range of biotic (i.e. pathogens) and abiotic stress responses in plants (Arisz et al. 2009). PA has therefore emerged as an important lipid second messenger with direct effects on vesicular trafficking and membrane surface charge: traits that become altered according to the transcriptomic data in our study. The involvement of PA signaling in biotic interactions between marine protists might therefore be an important link between the activation of G-protein coupled receptors and subsequent responses. Our expression data indicate that the conversion of DAG into PA takes place in both *A. tamarensis* strains, underpinning the importance of G-protein coupled receptors and DAG/PA signaling as an intracellular response inducer, in the biotic interactions investigated in through this study.

PA signaling could furthermore be directly coupled to reactive oxygen species (ROS) signaling in plants. Genes coding for ROS detoxifying enzymes (glutathione peroxidases) were also found to be significantly enriched after 96 h in strain A2 cultures. ROS have interestingly been claimed to be involved in the lytic properties of *A. tamarensis* (Flores et al. 2012), hence, the expression of glutathione peroxidases could prevent self-toxicity by those compounds, as the external application of these enzymes reduces the lytic potential (Flores et al. 2012).

However, lytic compounds are thought to be basally expressed rather than increasable (Zhu & Tillmann 2012), and therefore the expression of glutathione peroxidases, if involved in preventing self toxicity should also be induced in control treatments. Despite these observations, these enzymes may be part of a general stress response in this *A. tamarensis* strain.

The number of significantly enriched genes with protein families that are involved in information storage and processing and/or cell cycle control increased in both strains over time (Fig. 6.5). For *A. tamarensis* strain A2, this category primarily comprised protein families that act on several levels of transcriptional and translation control. The increase after 96 h is predominantly caused by an increase in RNA-binding proteins which act on post-transcriptional regulation of gene expression. Additionally, several genes with protein families that possess transcription factor properties are enriched. A co-occurring expression pattern at all time points and for one time point in strain A5 is represented by an up-regulation of genes with protein families involved in DNA m⁶adenine methylation and the primary translation initiation factor If4e that binds the cap structure of mRNAs. M⁶adenine methylation is an epigenetic regulation process found in prokaryotes and lower eukaryotes that differs in its impact from cytosine methylation, e.g. activating transcription vs. suppressing transcription (Hattman 2005). Adenine methylation is also a prominent epigenetic modification in the transcriptionally active macronucleus of ciliates, however it is absent in the transcriptionally inactive micronucleus (Gutiérrez et al. 2000). Adenine methylation is therefore believed to facilitate transcription through local denaturation of the promoter region and by reducing the DNA duplex stability (Engel & Von Hippel 1978, Gutiérrez et al. 2000). Given the low abundance of histones in the dinoflagellate genome (Lin 2011) and their importance in transcription control through opening the chromatin structure (Zhang & Reinberg 2001), m⁶adenine methylation could be a convergent mechanism to histone acetylation in dinoflagellates and could potentially have profound effects on transcriptional regulation.

Protein families clustered in the category “A” for the non-lytic strain A5 indicated an influence on cellular growth in *A. tamarensis*, due to cues originating either from heterotrophic protists, the conspecifics (cage), or from cues due to biotic interactions. In this category, protein families of genes involved in cell-cycle control processes were significantly enriched and were followed by an accumulation of ribosomal protein gene up-regulation (*RP*-genes), leading to the observed increase in the gene abundance after 96 h in this category (A). In microorganisms, the

synthesis of ribosomal proteins is linked to nutrient availability and stress-related signals (Powers et al. 2004). Model studies in yeast have revealed that the *RP*-gene regulon is activated via the protein kinase A (PKA) pathway (Jorgensen et al. 2004) which regulates a range of growth related processes. In our transcriptomic enrichment data, a PKA activated transcription regulator is expressed after 48 h, concurrent with the expression of a cyclin domain containing gene. This pattern is followed by genes coding for the Proliferation Cell Nuclear Antigen (PCNA) after 72 h along with several DNA replication, modification and repair proteins. Whereas, finally after 96 h the transcriptional accumulation of *RP*-genes surfaces. Investment into new ribosomes provides a platform for faster growth, if all other essentials will be available (Warner 1999). PKA mediated transcriptional regulation along with *RP*-gene transcription and cell cycle gene expression exemplify how cellular growth may be internally regulated on a molecular basis in *A. tamarensis*, yet at the same time demonstrates how external cues derived from biotic interaction (of whatever nature) have the potential to restructure the internal growth rhythm.

6.6 Conclusions

Our transcriptional analysis indicates a change in several traits for each *A. tamarensis* strain, due to the presence of a heterotrophic protistan grazer. The higher expression of genes involved in endocytotic processes, particularly in the lytic strain A2, suggests that mixotrophy seems to be inducible, even under laboratory conditions where ample nutrients and light are available. Lytic compound production thus not only provides a competitive advantage and grazing protection, it might also have a positive feedback on an under-investigated trophic mode in *A. tamarensis*.

Judging from the gene expression data, further traits that are changed in a strain-specific manner include properties of the outer glycosylated membrane. Thus traits that are altered through these species' interactions have the potential to influence subsequent interactions. Strain specific alterations of traits indicate a complex interplay within community ecological processes. It should therefore be further evaluated how specific genotypes alter species' interactions, including the analysis of the outcomes of past and future interactions. Interactions at the population level may in fact be dependent on historically experienced interactions, thus shaping the phenotypic distribution within a certain population.

7. Assembly and functional annotation of a comprehensive expression based gene library to study biotic interactions in the marine dinoflagellate *Alexandrium tamarense*

7.1 Abstract

Dinoflagellates possess unusually large genomes with a high content of repetitive sequences and genes occurring in multiple copies. Transcription-based sequence-analyses are therefore the method of choice to gain insights into the dinoflagellate genome. The here performed assembly of three transcription based gene libraries of the toxic red tide dinoflagellate *A. tamarense* and their annotation provides insights into secondary metabolism, signal transduction and conserved cellular processes this organism. Core metabolic and cellular processes often account for only a small amount of all protein coding gene in an organism. Thus, to increase the library coverage, two of the three gene libraries were obtained from experiments where *A. tamarense* interacts with grazers (copepods: *Centropages typicus*; protists: *Polykrikos kofoidii*). The results from the annotation of the final gene library showed a high coverage of basic metabolic processes, allowing to identify several genes involved in signal transduction, posttranslational regulation, and secondary metabolite synthesis. Further, the results from the annotation allowed to propose 10 putative paralytic shellfish poisoning toxin biosynthesis gene candidates. Overall, the established gene library provides a valuable source for further functional genomic studies.

7.2 Introduction

Dinoflagellates belong to the eukaryote crown group of Alveolata and are a phylum of aquatic unicellular eukaryotes (~10-200 μm in size) (Lin 2011). They have evolved diverse lifestyles ranging from unicellular planktonic and benthic species to

symbiotic and parasitic species that live within the tissue, cytoplasm or nucleoplasm of other organisms. Most dinoflagellate species are heterotrophic or mixotrophic and pure autotrophy does not seem to be common among them (Taylor 1980, Hackett et al., 2004, Jeong et al. 2005, 2010). Yet, they are the second most important eukaryotic primary producers in the ocean and contribute significantly to the global carbon cycle as well as to the marine food web (Lalli & Parsons 1997). However, some dinoflagellates are able to form so called “Red Tides” and/or harmful algal blooms (HABs) that affect virtually every coastal region of the world (Anderson et al. 2012b). The harmful effect of these blooms is mainly caused by the dinoflagellate’s ability to produce a wide variety of secondary metabolites that can be toxic to other aquatic organisms, hence can have a strong impact on the ecosystem they inhabit as well as human health (Hallegraeff 2010, Wisecaver & Hackett 2011).

Marine dinoflagellates belonging to the genus *Alexandrium* spp. are among the most important HAB species in terms of their severity of impact, diversity and global distribution. Three different families of toxins (saxitoxins, spirolides and goniodomins) are known to be produced by this genus, with saxitoxin being the most significant in terms of its impact (Anderson et al. 2012a). Saxitoxin is highly neurotoxic and its accumulation in the food web is responsible for paralytic shellfish poisoning (PSP) outbreaks. Such outbreaks can impair human health due to the consumption of contaminated seafood, leading to losses of wild and cultured shellfish or fish resources. Further impacts include alterations of the marine trophic structure as well as death of marine mammals, fish and seabirds (Anderson et al. 2012a).

On account of this, scientific effort has been invested and lead to major findings in our understanding of the biogeography, toxinology, physiology, ecology and management of *Alexandrium* (Anderson et al. 2012b). There is already ample evidence on the abiotic conditions that favor a bloom development, and an increasing awareness about the importance of allelopathic interactions and antipredator adaptations contributing to this (Smayda 1997, Tillmann et al. 2008). Increased saxitoxin levels in *Alexandrium minutum* and *Alexandrium tamarense* have recently been shown to be associated with such antipredator adaptations, shedding light on the ecological function of the toxins (Selander et al. 2006, Bergkvist et al. 2008, Wohlrab et al. 2010, Yang et al. 2011). The subsequent implementation of advanced genomic technologies led to the identification of saxitoxin biosynthesis genes (Stüken et al. 2011) as well as underlying molecular

processes of the antipredator responses (Wohlrab et al. 2010, Yang et al. 2011). As of yet, the application genomic technologies is still in its infancy even though they can provide valuable information about fundamental processes related to HAB dynamics.

Due to still ongoing technological developments, which have led and lead to a reduction in sequencing costs, genomic programs increased exponentially and involve now organisms in all domains of life (Bertin et al. 2008). Until now, more than 3700 species from various domains of life have been fully sequenced (<http://genomesonline.org>) yet no dinoflagellate genome is among them. The large haploid genome size (up to ~245 GB) of dinoflagellates in addition to several unusual features, like large tandem gene arrays (Liu & Hastings 2006, Bachvaroff & Place 2008) and long stretches of repetitive sequences (Jaeckisch et al. 2011) still impede such attempts. Sequencing of Expressed Sequence Tags (ESTs) is until now the method of choice to unravel the mysteries of such genomes and is therefore an alternative to complete genome sequencing (Rudd 2003). Such data create a resource for molecular studies, gene discovery and microarray-based expression analysis (Rudd 2003). Until now EST-libraries of 13 dinoflagellate species are publically available (<http://www.ncbi.nlm.nih.gov>) and further sequencing projects are ongoing (Lin 2011). A primary aim of this thesis was to compile a comprehensive expression based gene library for *Alexandrium tamarense*, one of the most widely dispersed *Alexandrium* species. Given the emerging importance of biotic interactions in the field of HAB research, mRNA derived for this gene library was recovered from laboratory induced biotic interactions experiments. The finally assembled EST-library will hence contain a comparable high proportion of genes that are involved interactions of *A. tamarense* with it's the biotic environment.

7.3 Material and Methods

cDNA- library construction and analysis

A set of 10,885 ESTs for *A. tamarensis*, publicly available at the National Center for Biotechnology Information (NCBI) were assembled into 5,794 contigs and 1856 unique ESTs with the "Sequence Analysis and Management System" (SAMS, Center for Biotechnology, University of Bielefeld). Further EST libraries were constructed externally by Vertis Biotechnologie AG (<http://www.vertis-biotech.com/>) using RNA from my experiments (Publication III & IV) to extend the coverage of ESTs from *A. tamarensis*. The procedure for EST library preparation and sequencing is described in brief here. Total RNA isolated from the experimental set-ups was pooled for the copepod-induced experiments (Publication III) and the protistan grazer-induced experiments (Publication IV). Out of those total RNA pools poly(A)⁺RNA was prepared. To increase the probability of obtaining and/or assembling full length cDNAs with 5'ends, 1st-strand cDNA synthesis of the poly(A)⁺ selected RNA was primed with random hexamers. The 454-sequencing adaptors were then ligated to 5' and 3' ends of the cDNA and the cDNA was amplified with 19 PCR cycles using a proof-reading polymerase. The amplified cDNA was normalized by one cycle of denaturation and re-annealing. Single stranded cDNA was separated from renatured cDNA by passing the cDNA over a hydroxylapatite column. Obtained ss-cDNA was amplified with 9 PCR cycles and cDNA with a size range of 450 - 650 bp was cut out and eluted from an agarose gel. The 454 Roche titanium sequencing library was built according to the manufacturer's protocols (Roche, Basel, Switzerland). Derived EST-sequences were assembled into contigs with the *Newbler Assembler (Roche)*. The obtained 454-libraires were assembled into a final *A. tamarensis* gene-library by best reciprocal BLAST search. This was done by applying the BLAST algorithm on two libraries to obtain best BLAST hits, i.e. library A against library B. The best BLAST hits from library B were then used as query to obtain best hits in library A. If A1 had a hit B1 and vice versa with an e-value cut-off of e^{-20} , the redundant sequences were eliminated from the dataset. This step was repeated including the third assembled EST-library.

General gene annotation workflow

The annotation of the assembled contigs was done by comparing the sequences with several publically available sequence and motif databases to obtain a more complete functional insight into the *A. tamarensis* transcriptome (Fig. 7.1).

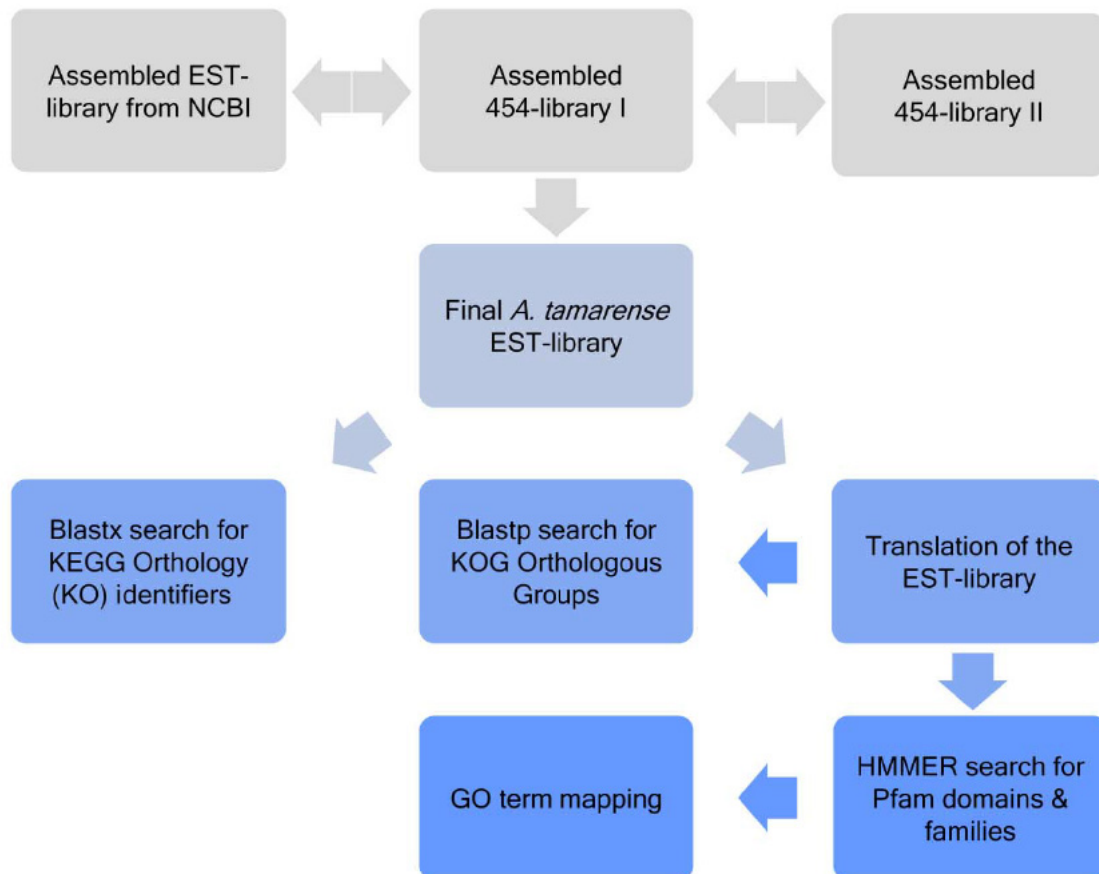


Fig. 7.1: Overview of the generation of the *A. tamarensis* transcriptomic resources database and the gene annotation workflow. The “Final *A. tamarensis* EST-library” is a non-redundant reference library.

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database is an integrated resource consisting of three types of databases for i.e. genomic, chemical and network information. The implemented pathway database provides KEGG reference pathways maps for several networks of functional significance (Kanehisa et al. 2004). KEGG Orthology (KO) identifiers are used to map genes to these reference pathways. For the final *A. tamarensis* contigs, a web-based server called KAAS (KEGG Automatic Annotation server <http://www.genome.jp/kegg/kaas/>)

(Moriya et al. 2007) was used to automatically assign KO identifiers to the contigs. This server allows the direct use of nucleotide sequences (blastX) for the identification of KEGG orthologies. The following KAAS server settings were applied: assignment method: single-directional best hit; gene data set: customized organism selection (mbr, ddi, ehi, edi, pfa, pyo, pkn, tan, tpv, bbo, cpv, cho, tgo, tet, ptm, tbr, tcr, lma, lif, gla, tva, pti, tps, hsa, xla, dre, dme, cel, nve, ath, olu, ota, cme, sce, ncr, ssl, ani, spo, cne, ecu); BLAST bit score cut-off > 60.

For other web-based servers used for functional annotation of *A. tamarensis* genes it was first necessary to translate them into amino acid sequences. This was done with the Virtual Ribosome (<http://www.cbs.dtu.dk/services/VirtualRibosome/>), a translation tool that can rapidly process large datasets as obtained from 454-sequencing (Wernersson 2006). The batch web CD-search tool (<http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) (Marchler-Bauer et al. 2011) was used (e-value < e^{-07}) to assign eukaryotic orthologous groups (KOGs) and their higher categories to the sequences (Tatusov et al. 2003). The publically available KOG set consists of 4852 clusters of orthologs grouped into 25 higher functional categories (Tatusov et al. 2003). The translated sequences were additionally screened for the occurrence of protein domains and families. The Pfam database (<http://pfam.sanger.ac.uk/>) is a widely used database for such approaches and contains 13,672 high quality, manually curated families and domains. Pfam families and domains represent functional units of proteins which are expected to share a common evolutionary history (Punta et al. 2012). Screening sequences of Pfam families therefore gives information about the basic units that will build a functional protein. Such units can also be detected in comparably short sequences and therefore often extend the number of genes for which functional annotations can be retrieved. Finally, Gene Ontology (GO) terms (<http://www.geneontology.org>) were mapped to the identified Pfam domains and families. Gene Ontology terms are provided by the GO Consortium (<http://www.geneontology.org/GO.consortiumlist.shtml>) and comprise a three structured controlled vocabulary (ontologies) that describes gene products in terms of their associated biological process, cellular component and molecular function.

Screening for genes involved in saxitoxin biosynthesis

The next generation sequencing technologies considerably increased the numbers of ESTs available for *A. tamarensis*. These ESTs are a solid basis for screening for homologues of the cyanobacterial *sxt* cluster and the recently discovered *sxtA* gene from *A. fundyense*. The screening for putative *sxt* genes in *A. tamarensis* was done with the commercially available CLC Main Workbench (<http://www.clcbio.com>), which enables the simultaneous analysis of a large number of DNA, RNA, and protein sequence analysis. *Sxt* genes were retrieved from public databases (National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>) and the Universal Protein Resource (Uniprot, <http://www.uniprot.org/>) and Pfam families were assigned to those genes. Afterwards, they were used to retrieve best BLAST hits from the final assembled *A. tamarensis* EST-library. These best BLAST hits were subsequently blasted against the non-redundant protein database at NCBI. Best BLAST hits from *A. tamarensis* and best BLAST hits from the non-redundant protein database were both screened for Pfam families.

7.4 Results and Discussion

Generation and analysis of the gene libraries

The 454-libraries generated in this study lead subsequently to a comprehensive transcriptomic resource for *A. tamarense* (Fig. 7.2). The first sequenced 454-library (454-library I, copepod-induced) increased the number of unique contigs (assembled ESTs) by ~ 64% (+ 12,552 contigs); the second sequenced 454-library (454-library II, protistan grazer-induced) added another 9234 unique contigs.

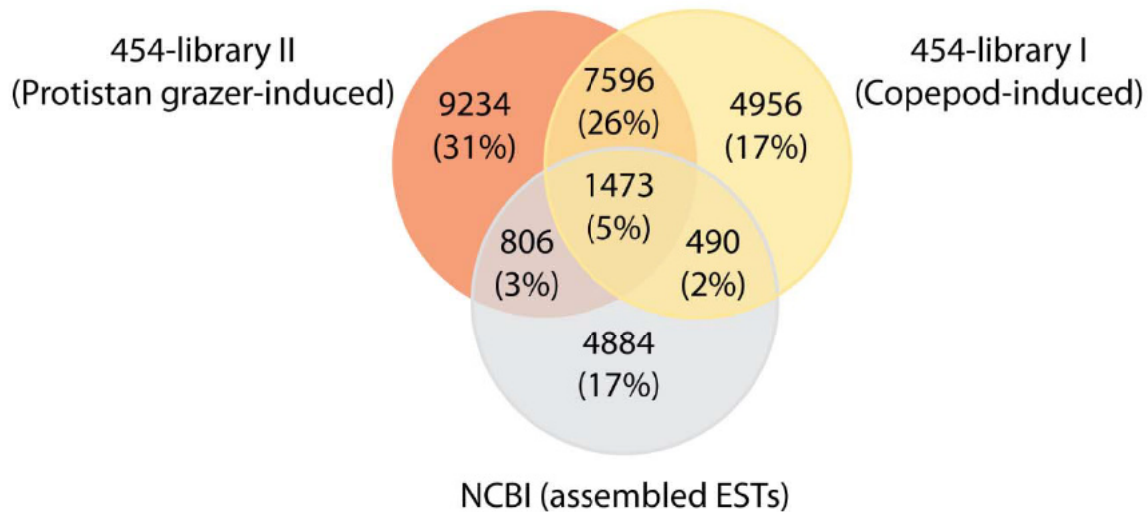


Fig. 7.2: Venn diagram showing the distribution of contigs obtained from the assemblies of ESTs from three different treatments/sequencing runs. Numbers in the circles denote the raw number of contigs and their percentage of the total number of contigs analyzed are given in brackets.

The final number of 29,439 contigs obtained in this study is comparable to the findings of Chan et al. (2012); 32,110 contigs for *A. tamarense*) and covers ~ 73% of the estimated number of ~40,000 genes for *Alexandrium tamarense* (Moustafa et al. 2010). The gene content of dinoflagellates is estimated to lie between 38,000 – 88,000 (Hou & Lin 2009). The high number of identical gene copies arranged in tandem arrays probably adds to the total number of estimated genes, thereby reducing the contingent of unique genes (Hou & Lin 2009). Such arrays are assumed to cluster together in one contig in the dataset presented here. However, redundancy might be present due to short contigs that may actually cover the same

functional gene without any overlap. Regardless of this, the results demonstrate how advances in sequencing technologies make it possible to investigate a large part or even almost the whole transcriptome of a dinoflagellate and give a reasonable number of unique genes in comparison to what is expected for a typical unicellular eukaryote (Hou & Lin 2009, Stüken et al. 2011).

The final assembled EST library was mapped against several databases for functional annotation. KEGG Orthology (KO) identifiers could be assigned to ~20% of the assembled ESTs. In total, 5975 sequences were grouped into 1947 different KEGG Orthologies. Figure 7.3 shows an overview of in 481 KO identifiers that could be mapped to the global metabolic pathway map using the KEGGMapper (<http://www.genome.jp/kegg/mapper.html>).

The amount of genes participating in basic metabolic processes (Fig. 7.3) implies a high functional coverage of the investigated *A. tamarensis* transcriptome. Core metabolic processes (i.e. for energy, amino acids, nucleotides, fatty acids) are in general better covered than prominent secondary metabolite pathways (Fig. 7.3). Secondary metabolite production is however widespread in dinoflagellates (Kellmann 2010) indicating substantial sequence and/or pathway divergence in *A. tamarensis* for genes involved in secondary metabolite pathways. Such divergence is often the result of species- and environment-specific selection pressure for new secondary metabolite pathways. Given the high coverage of basic metabolic processes, genes without functional characterization that are highly divergent from functionally categorized genes in model-species, can be assumed to be sufficiently represented in the present dataset. Such genes are generally considered to be involved in serving species-specific ecological purposes, i.e. interactions with the biotic environment (Jackson et al. 2002, Pavey et al. 2012). Taken together, the compiled gene library for *A. tamarensis* seems well suited to identify genes involved in species interactions through subsequent high-throughput functional genomic studies (e.g. microarrays or RNA-sequencing). Additionally, transcription-based alterations of conserved processes in species interactions can be studied based on the available annotation of genes involved in such basic cellular processes.

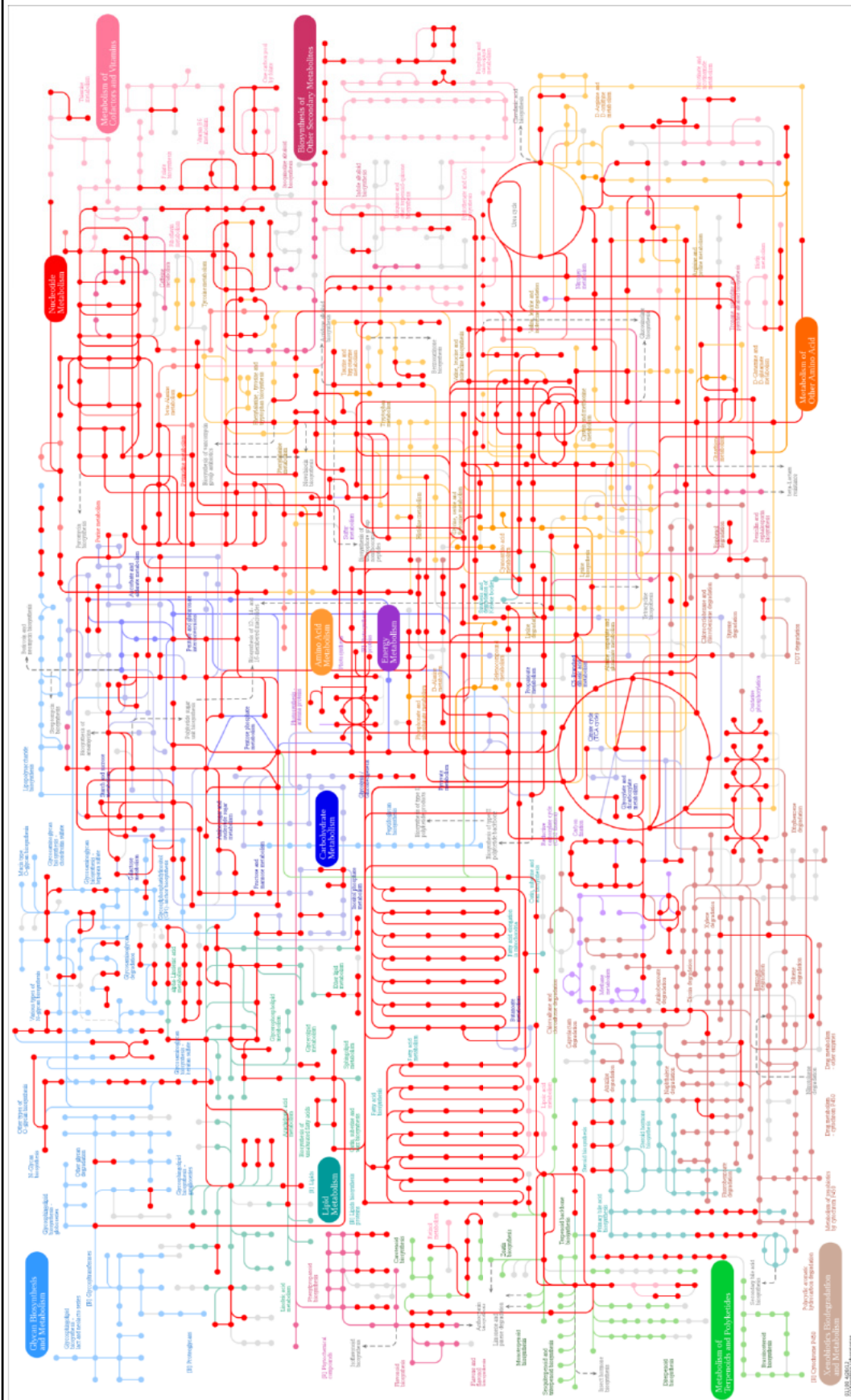


Fig. 7.3: Overview of in total 481 KeggOrthology (KO) identifiers mapped to the global metabolic pathway map. Red lines indicate the identified enzymes from the *A. tamarensis* gene library; faded background colors represent the respective pathways.

The assignment of KOGs to the final assembled EST library resulted in 5058 (~17%) functional annotations into 1130 different clusters of orthologs. The distribution of these 5058 different KOGs into the 25 higher functional categories is depicted in Fig. 7.4. The break-down of annotated genes in 25 KOG categories gives a summarized overview of the functional distribution of annotated genes into different processes. The high abundance of genes that fall into the group “O” (Posttranslational modification, protein turnover, chaperones) well represents the general view of widespread posttranslational regulation processes in dinoflagellates (Lin 2011).

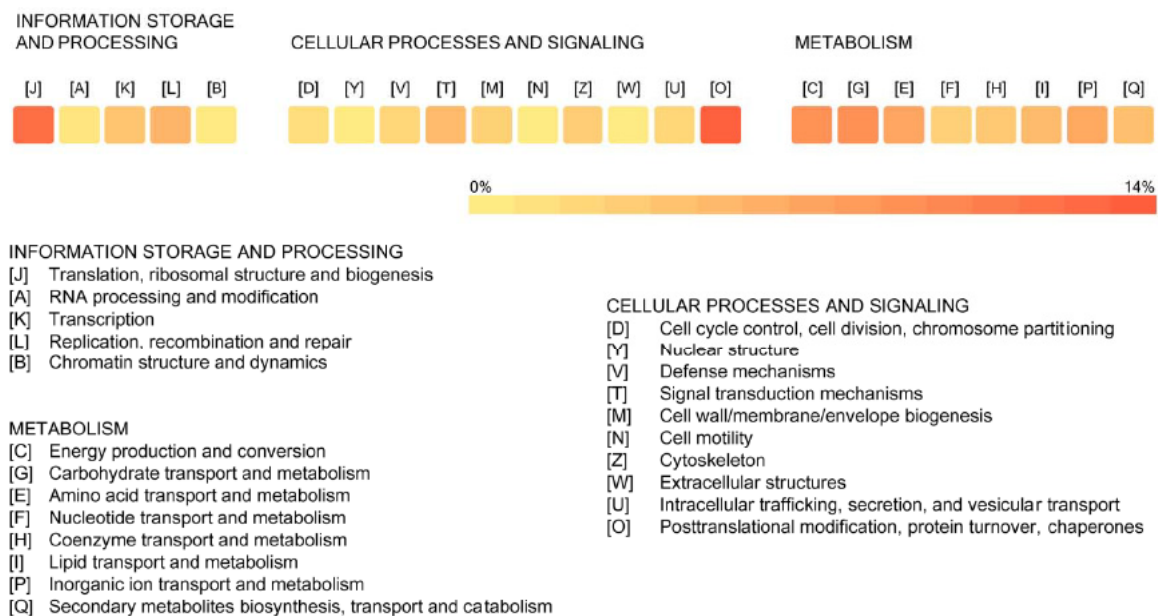


Fig. 7.4: Distribution of the 5058 mapped gene models according to KOG into their corresponding functional category.

Screening for Pfam domains and families led to 6760 Pfam domains identified in 5655 sequences and 6509 Pfam families identified in 5813 sequences. Thus, in total, in ~38% (11,107) of all assembled ESTs at least one Pfam domain or family could be detected. Screening for Pfam families hence almost doubled the number of sequences with a functional assignment (KEGG: 20%, KOG 17%). Functional domains are present in several sequences that did not match any homologues by the prior performed BLAST-comparisons. Conserved gene functions are therefore likely embedded in otherwise highly diverged sequences, indicating functional diversification of several protein families. The 50 most abundant Pfam

domains are shown in Fig. 7.5. Among those most abundant protein families are protein kinases, calcium binding domains (Ef-hand) and cyclic nucleotide (cNMP)-binding domains, all of which are involved in signal transduction processes. Paired with the high abundance of ion transporters, these results imply that besides protein phosphorylation as the mainstay of eukaryotic signal transduction, intracellular cyclic nucleotide-regulated ion fluxes might play a major role in signal transduction, as observed for ciliates (Anantharaman et al. 2003) (Fig. 7.5). So far, just 11 proteins of the Bestrophin family which represents a new class of chloride channels have been described in the Alveolates in the Pfam database. *A. tamarensis* adds another 70 protein coding genes. The role of such an apparent high radiation of the Bestrophin family in *A. tamarensis* should be analyzed in more detail later. In summary, the high amount of protein domains and families involved in signal transduction mechanisms reflects the demands of a free-living lifestyle (Anantharaman et al. 2003) and highlights the potential of this dinoflagellate to respond to its biotic and abiotic environment.

The transcriptome further holds a huge repertoire of proteins that are known to participate in secondary metabolism through duplication and diversification (Pichersky & Gang 2000). This includes proteins belonging to the polyketide synthases with the ketoacyl synthetase as a main building block, the family of methyl-(and acyl)transferases and 2OG-Fe(III) oxygenases, all found in high abundance in this gene library (Fig. 7.5). Viviplantae possess a comparable gene content to dinoflagellates and it was suggested that 15-25% of their genes encode enzymes for secondary metabolism (Somerville 1999, Pichersky & Gang 2000). Structurally characterized secondary metabolites for *A. tamarensis* comprise saxitoxin and its derivatives and mycosporine-like amino acids (Callone et al. 2006). Allelochemical properties as well as a membrane lytic activity have been demonstrated for some *A. tamarensis* strains (Eschbach et al. 2001, Tillmann & John 2002) indicating the existence of at least one more group of bioactive secondary metabolites. However the structure of the allelochemical substance(s) has not yet been elucidated (Ma et al. 2009, 2011, Yamasaki et al. 2009) and thus it is possible that more than one compound or even a macromolecular complex is responsible for these properties. The described abundance of Pfam domains and families supports the hypothesis that *A. tamarensis* may synthesize a higher repertoire of secondary metabolites than currently known.



Fig. 7.5: Graphical summary of the top 25 Pfam domains and families identified in the final assembled *A. tamarensis* EST-library. The area of the circles corresponds to the abundance of the respective Pfam domains/families.

The mapping of GO terms to the identified Pfam domains and families resulted in a total of 888 GO terms that could be assigned 20,332 times. In summary, 313 Biological Process terms were assigned 6850 times, 473 different Molecular function terms were assigned 10,393 times and 102 Cellular Component terms were assigned 3090 times. The 50 most abundant GO terms of the respective group (Biological Process, Molecular Function, and Cellular Component) are shown as word clouds in Fig. 7.6.

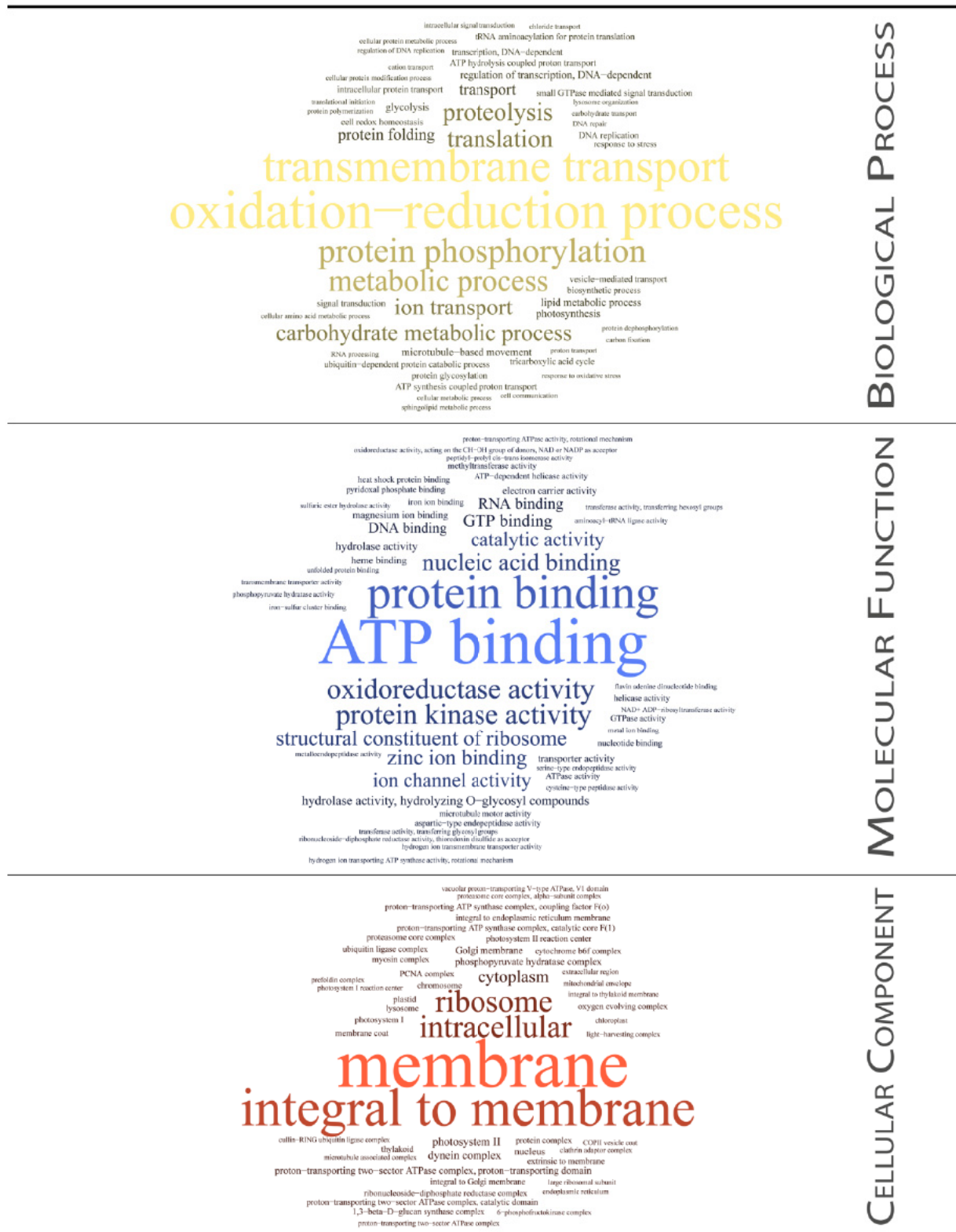


Fig. 7.6: Graphical overview of the top 50 occurring Gene Ontology (GO) terms in the *A. tamarense* gene library. The size of the phrases corresponds to the abundance of the member term in its category.

High-throughput comparative analysis of genomes and transcriptomes often yields a dataset from which the extraction of biological insights by manual reviewing annotations can be daunting and challenging. Gene Set Enrichment Analysis (GESA) is often used to overcome this problem (Subramanian et al. 2005). This method focuses on groups of genes that share a common biological function and tests for their significant enrichment based on all gene information available in the subjects that are compared (Subramanian et al. 2005). GO terms are often used to calculate such enrichments between two datasets, i.e. to elucidate processes that are over-represented in one condition compared to another condition or to the overall background distribution. The here performed attachment of GO terms based on Pfam annotations provides the basis for fast comparisons of significant enriched processes without an over-interpretation of the associated GO processes. Such an over-interpretation can occur if GO-terms are inferred from simple homology-based BLAST comparisons between i.e. model- and non-model organisms.

Screening for genes involved in paralytic shellfish poisoning toxin biosynthesis

Screening the final assembled gene library for homologues for the *sxtA* gene from *A. fundyense* and the cyanobacterial *sxt*-gene cluster resulted in a total of 10 sequences that showed similarities with a blast e-value cut-off $< e^{07}$. The best supported homologue could be retrieved for the *sxtA* gene from *A. fundyense*. *A. fundyense* belongs to the *A. tamarensis/fundyense/catenella* species complex, a phylogenetically unresolved cluster of different morphospecies that might be conspecifics. The *A. tamarensis* gene fragment (contig05769) showed a 99% percent similarity to the *sxtA* gene from *A. fundyense* and contains the same functional domain (Table 7.1).

The putative *sxtG* sequence from *A. tamarensis* was the only *sxt*-gene candidate that was more similar to the cyanobacterial *sxtG* sequence than to any other sequence from the NCBI non-redundant protein database. The similarity is however low (only 26%) but both sequences (from *A. tamarensis* and *Aphanizomenon* sp.) share the same functional Pfam domain. Together with the recent studies from Stüken et al. (2011) and Hackett et al. (2012) there is reinforced evidence for another putative *sxt* gene (*sxtG*) homologue in dinoflagellates.

Table 7.1: Overview of results gained from a similarity search between the cyanobacterial *sxt* genes and the *A. tamarensis* gene library.

<i>sxt</i> gene	Blast db	Query sequence	Hit sequence	Description hit	E-value blastp	%Identity	Pfam domain(s) of hit	E-value domain hit	Origin/habitat
sxtA	Custom	ADY62525	contig05769	<i>sxt gene candidate A. tamarensis</i>	4.49e-167	99	Aminotran_1_2	2.5e-12	● ~
	NCBI	contig05769	ADY62525	SxtA long isoform precursor [Alexandrium fundyense].	1.40e-167	99	PP-binding Aminotran_1_2	4e-05 0.2	
sxtG	Custom	ACG63813	contig33000	<i>sxt gene candidate A. tamarensis</i>	1.76e-11	26	AmidinoTransf	0.0068	● ~
	Custom	contig33000	ACG63813	SxtG [Aphanizomenon sp. NH-5]	8.79e-12	26	AmidinoTransf	2.1e-06	
	NCBI	contig33000	ZP_06887679	Glycine amidinotransferase [Methylophilus trichosporium OB3b]	3.33e-10	42	AmidinoTransf	1.1e-05	
sxtH	Custom	ACF94649	Atam17991	<i>sxt gene candidate A. tamarensis</i>	2.31e-18	31	Rieske	4.1e-19	● ~
	Custom	Atam17991	ACF94649	Dioxygenase [Anabaena circinalis AWQC131C]	1.10e-18	31	Rieske	6.1e-16	
	NCBI	Atam17991	XP_001421195	Predicted protein [Ostreococcus lucimarinus CCE9901]	9.63e-54	44	PaO Rieske	1.9e-22 1.2e-19	
sxtI	Custom	ABI75099	contig46883	<i>sxt gene candidate A. tamarensis</i>	2.70e-14	30	Cmch_NodU	4.4e-13	● ~
	Custom	contig46883	ABI75099	Carbamoyltransferase [Cylindrospermopsis raciborskii T3]	2.24e-14	29	Cmch_NodU	4.2e-117	
	NCBI	contig46883	ZP_09685053	Carbamoyltransferase [Mycobacterium tusciae JS617]	1.99e-45	54	Cmch_NodU	1.1e-57	
sxtN	Custom	ABI75104	Atam20042	<i>sxt gene candidate A. tamarensis</i>	1.98e-16	24	Sulfotransfer_3	2.9e-06	● ~
	Custom	Atam20042	ABI75104	Sulfotransferase [Cylindrospermopsis raciborskii T3]	1.98e-16	24	Sulfotransfer_3	0.0073	
	NCBI	Atam20042	YP_003270111	Unnamed protein product [Haliangium ochraceum DSM 14365]	2.04e-47	38	Sulfotransfer_3	0.0073	
sxtO	Custom	ABI75115	contig10686	<i>sxt gene candidate A. tamarensis</i>	3.35e-29	48	APS_kinase	5e-36	● ~
	Custom	contig10686	ABI75115	Adenylylsulfate kinase [Cylindrospermopsis raciborskii T3]	3.06e-29	48	APS_kinase	1.6e-66	
	NCBI	contig10686	EGX50239	Hypothetical protein [Arthrotrichy oligospora ATCC 24927]	1.12e-39	57	APS_kinase	4.8e-67	
sxtT	Custom	ACG63810	Atam17991	<i>sxt gene candidate A. tamarensis</i>	8.77e-19	30	Rieske	4.1e-19	● ~
	Custom	Atam17991	ACG63810	SxtT [Aphanizomenon sp. NH-5]	1.04e-18	30	Rieske	8.2e-17	
	NCBI	Atam17991	XP_001421195	Predicted protein [Ostreococcus lucimarinus CCE9901]	9.63e-54	44	PaO Rieske	1.9e-22 1.2e-19	
sxtU	Custom	ACG63811	contig51880	<i>sxt gene candidate A. tamarensis</i>	2.20E-21	30	adh_short	1.7e-30	● ~
	Custom	contig51880	ACG63811	SxtU [Aphanizomenon sp. NH-5].	6.90E-21	33	adh_short	1.9e-35	
	NCBI	contig51880	XP_001745589	Hypothetical protein [Monosiga brevicollis MX1]	3.05E-39	52	adh_short	1e-22	
sxtW	Custom	ACZ26230	42752265	<i>sxt gene candidate A. tamarensis</i>	3.91E-11	32	Fer4_7	1.1e-10	● ~
	Custom	42752265	ACZ26230	SxtW [Lyngbya wollei]	3.75E-11	32	Fer4_10	7.4e-09	
	NCBI	42752265	YP_004769680	Photosystem I subunit VII [Spirodela polyrhiza]	3.41E-34	76	Fer4_7	7.9e-10	
sxtX	Custom	ACG63803	Atam07929	<i>sxt gene candidate A. tamarensis</i>	1.76E-15	26	Cmcl	6.5e-39	● ~
	Custom	Atam07929	ACG63803	SxtX [Aphanizomenon sp. NH-5]	3.46E-13	29	Cmcl	6.1e-38	
	NCBI	Atam07929	YP_632587	Cephalosporin hydroxylase [Myxococcus xanthus DK 1622]	2.76E-21	34	Cmcl	1.2e-63	

Origin: ● Cyanobacterial origin ● Prokaryotic origin ● Eukaryotic origin
Habitat: ~ Marine ~ Limnic

The remaining eight sequences that were discovered as best BLAST hits with the cyanobacterial *sxt* genes as search query all showed a higher similarity to other sequences in the NCBI non-redundant protein database than to the cyanobacterial *sxt* genes. In addition, the *A. tamarensis* query sequences matched sequences of non-cyanobacterial origin. Four of them were closer to eukaryotic sequences than to prokaryotic sequences. However, all cyanobacterial *sxt*-genes and obtained best hits in *A. tamarensis* harbor the same functional Pfam domain (Table 7.1).

Since this is the third study based upon a comprehensive transcriptomic dataset derived from 454-sequencing platforms it becomes less probable that the missing *sxt*-genes are too low expressed to be detected. The saxitoxin gene cluster, as it exists in cyanobacteria, does not seem to be present in the same form in dinoflagellates. Further core genes of the *sxt*-gene cluster might therefore never

have been transferred into the dinoflagellates, or alternatively their sequences are highly diverged, making it impossible for similarity search approaches to detect them. The putative *Alexandrium* homologues *sxtA* and *sxtG* discovered herein are involved in the first two steps of saxitoxin biosynthesis in cyanobacteria (Kellmann et al. 2008). The following biosynthetic steps could have been fulfilled by other proteins from i.e. already existing enzymes that catalyze chemically similar reactions on a different substrate (Weng et al. 2012). Like in plants, genes might have diverged repeatedly and independently from a common ancestor so that the newly described gene cannot be assigned solely on the degree of sequence homology. For example, limonene synthase genes in gymnosperms and angiosperms evolved more than once from a member of the terpene synthase gene family, hence the limonene synthase genes are more similar to those of other terpene synthases within their lineages than to each other (Pichersky & Gang 2000). Until now, the gene cluster that is putatively responsible for saxitoxin biosynthesis has been identified from five species of cyanobacteria (Kellmann et al. 2008, Mihali et al. 2009, Moustafa et al. 2009, Stucken et al. 2010, Murray et al. 2011). The comparative gene analysis of three of those clusters done by Mihali et al. (2009) indicated that accessory genes responsible for tailoring reactions may be complemented by other gene homologues. The core genes for the biosynthesis of saxitoxin seem therefore to be mostly conserved in cyanobacteria, whereas the accessory genes responsible for the formation of analogues differ among the species. The genes responsible for the formation of the several analogues of saxitoxin hence do not seem to have a common ancestor even in cyanobacteria, indicating well the process of radiation of genes involved in secondary metabolite synthesis.

7.5 Conclusions

The assembly and analysis of the here presented gene library for *A. tamarense* demonstrated that EST-driven approaches are an effective tool to gain insights into the genomic capability of non-model organisms. In addition, the assembly of a high-coverage EST-library is a first essential step for further of functional genomic analysis. The high coverage of the transcriptome that includes processes related to the biotic environment makes this gene library especially suited for species interaction studies. Furthermore, the data provided an important resource for the ongoing search for genes related to saxitoxin- and saxitoxin analogue-biosynthesis in dinoflagellates.

8. Synthesis

This thesis illustrates that environmental cues that result from the presence of a species can change several traits in another species. As a consequence, the expressed phenotype becomes altered (Fig. 8.1). Within this common context of environmentally-cued responses in species interactions, two concepts were investigated here:

- I. induced defense, with emphasis on waterborne-cues, their specificity and genotypic responses (Publication I-III) and
- II. genotype-specific feedback responses to interacting species within an environment, shaped by each genotype (Publication IV).

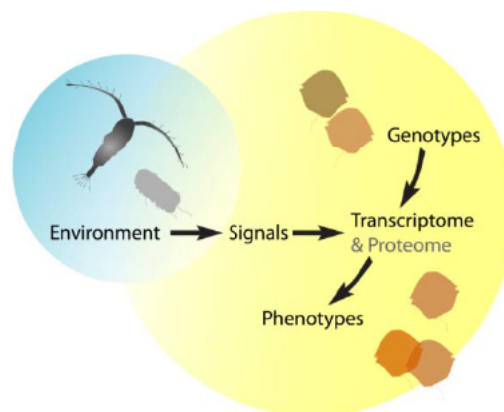


Fig. 8.1: Schematic overview of the central perspective concerning environmental cued changes in phenotypes.

Gene expression changes are a plausible basis for alterations of the phenotype due to environmental cues, however, the impact of gene expression changes on the phenotype is largely unknown for marine plankton (Schlichting & Smith 2002, Aubin-Horth & Renn 2009). The studies described in Publication I, III and IV included functional genomic approaches to elucidate such impacts. The results provided valuable mechanistic insights concerning:

- how the response is realized on a molecular level, and
- which genes and processes are involved in grazer induced responses

and thereby an initial estimation on how species interactions can shape the expression of traits on a molecular level. The analysis and annotation of the cDNA-library performed in Publication V furthermore provides additional insights into the transcriptome of *A. tamarense* and allows for a broader view on the genomic background of the observed gene expression changes.

Cumulatively, the present work illustrates the ability of the unicellular marine dinoflagellate *Alexandrium tamarense* to:

- discriminate among co-occurring grazer species (Publication I);
- simultaneously change traits at different hierarchical levels (Publications I-III);
- express an optimal phenotype from even different genotypes (Publication III);
- dynamically restructure the internal cellular environment as a response to cues from species interactions, which are in turn a result of the genotype (Publication IV).

8.1 Induced defense against copepods

An important goal of the studies presented in this thesis was to characterize the interaction of *A. tamarense* and copepod-grazers and to elucidate its complexity. The results presented in the Publication I-III not only, for the first time, demonstrate that an increase in PST production occurs in *A. tamarense*, but also reveal a complex response of *A. tamarense* towards copepod grazers driven by co-evolutionary processes. These responses comprise copepod-specific PST level alterations, the simultaneous onset of grazer-induced defenses as well as disparities in the responses depending on the *A. tamarense* strain used. Conversely, the copepods themselves appear to have different tolerance levels regarding their ingestion rate of toxic cells. This makes clear that the outcome of the grazer induced defense response in *A. tamarense* is dependent on several variables (see also Teegarden et al. 2001). Furthermore, the inducing cue(s), though unknown in nature, seem to be able to indicate the level of threat emanating from the copepod species and the induced defense response translates into different transcriptomic responses in the two *A. tamarense* strains (A2 and A5) examined. Within the following sections the grazer induced responses are dissected and discussed in a broader context to highlight how *A. tamarense* evolved to reduce losses that occur from top-down processes.

8.1.1 Signal specificity and induced defense

The results presented in this thesis show that *A. tamarensis* has evolved the ability to process and to correctly “assess” the significance of cues that emanate from different copepod species. Sympatry (the overlapping distribution of species in evolutionary history) as a possible premise of the grazer induced PST increase (Bergkvist et al. 2008) was given in all studies here. That the inducing cue is of chemical nature could be proven in the Publications I-III. This chemical cue has been shown to be well suited to indicate the level of threat (Publication I) and attenuates rapidly after grazer removal (Publication II). Hence the defense response is reversible (Publication II) and well-adjusted towards a reliable cue. However, it cannot be ruled out that the physical presence of a grazer increases the defensive response due to mechanical or tactile cues in addition to chemical signals. Yet, selective feeding on less defended cells may result in an average higher defense level in the remaining (and measured) cells (Publications I-III). Chemical cues can accumulate in the water and are thus perfect for indicating the level of threat based on threshold and density dependent responses (Tollrian & Harvell 1999). In addition, threshold dependent responses can be synchronized within a population thus amplifying their effects (Matz & Kjelleberg 2005). Receptors that track such cues could be stimulated by the structure, concentration and/or composition. Variable cue concentrations due to species specific production rates could therefore be an alternative explanation for the observed species specific responses (Publication I). Hence, *A. tamarensis* may use an intrinsic cue production rate of the respective copepod species as a proxy for the potential threat level. Impairment of the predator could further play a limiting role in species specific production rates, thus more closely resembled the conditions in direct grazing experiments with *A. clausii* and *O. similis* to the waterborne-cue experiments (Publication I). Density dependent responses could also be the potential basis for differences in the caged and direct grazing experiments described in Publication III. Besides species specific production rates, the inducing cue could be mix of several molecules with different profiles among species. Receptors tracking such cues could therefore be stimulated with different strengths depending on the cue composition. Whether or not the inducing cue is truly species specific or if its density or blend is used primarily as a proxy for the species and threat must be investigated further. The observation that cues and the physical presence of copepods elicit a response on the transcriptomic level, even when no measurable PST induction occurs, implies that this “change in external conditions” is indeed recognized and evaluated similarly by regulating an

overlapping set of genes (Publication I). This response may either indicate that the cells are primed for a possible future attack or occur due to changes in other traits (see below) that were not examined at that point.

8.1.2 Grazer induced defense responses and trade-offs

The grazer induced defense response comprises changes on several hierarchical levels (morphological, behavioral & physiological) (Publication II, III and Selander et al. 2011). The simultaneous onset of these responses indeed questions the function of PSTs as defense compounds. If PSTs are chemical weapons against copepods, why should there then be additional responses induced? After what was observed in this thesis (Publication II & III) and by Selander et al. (2011) it becomes obvious that the morphological and behavioral responses result in a “stealth behavior” (Selander et al. 2011), and thus they are preventative. PSTs, however, are only preventative when grazers would be able to discriminate between toxic and non-toxic cells before killing them, or, as a result, avoid *Alexandrium* spp. completely as food source. Both abilities (discrimination and avoidance) are described for copepods but with inconsistent results (e.g., Turner & Tester 1997, Teegarden 1999), most likely due to specific counter-adaptations where the outcome of the interaction is dependent on the inter- and intra-species variability. In addition, intoxication of the grazer is only preventative for the rest of the population and also for other co-occurring species that are potential competitors and grazers. It is doubtful that paying the bill for killing the grazer in a way that benefits undefended species equally will favor the evolution of toxin production. A recent meta-analysis that aimed to detangle the effects of secondary metabolites on herbivore resistance in plants presumed that traits other than secondary metabolites are more effective in defense (Carmona et al. 2011). These authors showed that morphology, life history, phenology as well as primary chemistry and physiology were most strongly related to herbivore susceptibility (Carmona et al. 2011). Morphological changes in *A. tamarense* (Publication II & III) are also induced when no measurable increase in toxin content occurred (Publication III). Morphological changes might therefore be more strongly correlated to the induced defense response under the given conditions. Together with behavioral changes (Selander et al. 2011) these traits might have a larger effect on conferring grazing resistance than intracellular PST levels. This, however, reverses the aforesaid question: why should the toxin content be increased if other traits already confer resistance? A major hypothesis about induced defense responses is that costs

connected to the response can offset the benefits of the defense; accordingly, trade-offs hamper the fixation of those traits in the genome (Harvell & Tollrian 1999). For example, reduced swimming activity may constrain nutrient retrieval migrations in *A. tamarensis* (Selander et al. 2011). The splitting of chains into single cells may dilute the allelochemical cloud that surrounds the cells and weakens their lytic capacity (Jonsson et al. 2009). Both mechanisms (reduced swimming activity and weakened allelochemical cloud) can result in ecological costs. The transcriptomic data point even further towards an increase in energy supplying processes associated with the induced defense response indicating allocation and infrastructure costs (Publication III). Finally, the ability to increase intracellular toxin content could be offset in situations of low nitrogen availability as shown for the close relative *A. minutum* (Selander et al. 2008). Costs of the induced defense response can therefore occur dependent on the environment. Due to these costs, the induced defense response might be reduced at the different levels (morphological, behavioral or physiological), a hypothesis that remains to be tested. The ability to induce different levels of defense, each bearing their own trade-offs can therefore reduce the probability of attack over a wide range of environmental conditions while minimizing costs. Secondary metabolites hence may have evolved as important defenses not because of their huge effect on grazers, rather because evolutionary constraints acting on those traits are relatively weak compared to other traits (Carmona et al. 2011). In other words, changes in the morphology and behavior are probably constrained by more variables than changes in secondary metabolite synthesis. Additionally, morphology and behavior can only be changed to a certain extent as they can be assumed to be associated with a wide range of vital functions.

8.1.3 PSTs as defense compounds

Counter-adaptations of copepods are probably the most striking argument for PSTs acting as defense compounds. However, they are as much driven by trial and error as any other evolutionary processes. Different populations of the same species can therefore manifest different evolutionary solutions towards the same problem, as in the case of point-mutations in sodium channel gene of copepods and shellfish (Bricelj et al. 2005, Chen 2010). Yet counter-adaptations seem to occur in copepod populations and may have driven the selection towards individuals that are able to increase their PST-content. While the basic level of PST production may affect copepod species (*Oithona similis*), which normally avoid *Alexandrium* spp. as prey item, PST production has to be increased when threatening copepods are

present, which in turn are more resistant to the toxins and require higher doses to be defeated (Publication I). This observation additionally indicates that an increase in PSTs production is associated with trade-offs that constrain a higher production rate and that the synthesis of PSTs creates an arms race that drives evolution in *A. tamarense* and copepod populations. Yet, since the extent of counter-adaptations to PSTs in copepods is largely unknown having been investigated only recently, the current state of the defensive role remains speculative (Colin & Dam 2002, 2003, 2005, Avery & Dam 2007, Chen 2010). In addition, we lack basic knowledge about all selective forces that act on this group of secondary metabolites in *Alexandrium*. Besides defense, further functions that have been proposed for PSTs are ion homeostasis (Pomati et al. 2004, Soto-Liebe et al. 2012), nitrogen storage (Cembella 1998), pheromone activity (Wyatt & Jenkinson 1997), and selection for associated bacteria (Jasti et al. 2005). Besides allocation costs, unknown ecological costs might therefore be one reason why PST production is not permanently increased. Such additional functions may also explain why saxitoxin, even though the most potent PST, is not produced exclusively but rather also other less potent analogues. Van de Waal et al. (submitted) show that the toxin profile of PSTs in an *A. tamarense* strain shifts towards a higher amount of less toxic, sulfated analogues. The results in this thesis (Publication III) show that the presence of grazing copepods shifts the toxin profile towards a higher amount of saxitoxin and therefore not only increase the total amount of PSTs. Hence, the co-occurrences of *A. tamarense* genotypes with different PST profiles may reflect the variability in selection acting on different PST analogues. Given the ambiguous nature of the genes coding for saxitoxin biosynthesis enzymes in dinoflagellates (Publication V), substrate permissiveness may add pleiotropic costs due to the production of multiple metabolites by one enzyme and hence hamper a permanent higher PST toxin production in *Alexandrium*. In turn, such pleiotropic effects can also be the cause for inducible PST production as part of the defensive response in *Alexandrium*, rendering increased PST production as an indirect, yet beneficial effect caused by changes in other physiological processes (as further discussed below).

8.1.4 Transcriptomic and strain-specific responses towards copepod grazers

The study described in Publication I & III clearly shows how the implementation of a functional genomic approach broadens the types of information we can gather from grazing experiments. Genes involved in signal transduction processes experienced strong diversification in *Alexandrium*

tamarensis (Publication V) and are involved in the responses towards copepods (Publication I & III). Their diversification within the genome might therefore be one premise to specifically react to the presence of copepods/environmental cues. In addition, genes with a strong influence on the activity of the proteome are differentially expressed between the defended and undefended phenotypes. Genome-wide association studies that attempt to link genes to phenotypes indicate that complex traits are governed by many loci, thus, genes involved in plastic responses are most likely global regulators of gene products (Flint & Mackay 2009, Simon et al. 2011). Global acting processes therefore seem to be essential for enabling phenotypic plasticity. While further differentially expressed genes could indicate the genetic fingerprints of costs associated with the induced defense response and could be assigned to a putative molecular mechanism involved in morphological changes, the majority of regulated genes remain without any functional assignment (Publication I & III). The analysis of gene expression patterns in response to copepods therefore provided a valuable source for an “ecological annotation” of genes (Publication III). These unknown genes might also be involved in protein-protein interactions. Such gene products can indeed harbor very diverse amino acid motifs, as long as they match the interacting partner, hence giving rise to the evolution of new connections within cellular protein networks readily exposed to selection (Lynch 2007b). Thus, protein interactions can be mediated by the presence of prior functionally unassigned regions that in turn lack a functional assignment. The recent discovery of a so far unknown protein motif at the N-terminal region of polyketide synthase genes in *Alexandrium* spp. indicates the emergence and fixation of such motifs in an environmentally important group of genes (Eichholz et al. 2012). The phenotypic characteristics of the strains before the induction of the defense response indicate that strain A5 expresses the “defended” phenotype already to a large extent, whereas in strain A2 major morphological and physiological changes must be induced (Publication III). In combination with the results from the KOG category analysis and the general expression patterns (numbers of up- and down-regulated genes that may result in different infrastructure costs) the induced defense seems to be based upon different backgrounds: The strain A5 seems to streamline its variability of the formerly expressed phenotype towards the defended phenotype. The strain A2 however has to shift its prior phenotype towards traits that were as such, rarely represented in their characteristics. KOG categories with a high discrepancy in the strain specific gene expression are exactly those that act globally and can affect many target proteins within the cell by regulating the activity of the proteome (KOG categories O

(Posttranslational modification, protein turnover, chaperones), P (Inorganic ion transport and metabolism) & T (Signal transduction mechanisms)).

The KOG categories O, P & T contribute to a large extent to the up-regulated genes in strain A2, in turn, they contribute to a large extent to the down-regulated genes in strain A5. Hence, there is a strong potential for strain A2 to restructure and expand intracellular networks, whereas this potential is decreased for the strain A5. The strain specific global expression changes indicate further differences in the costs that each genotype has to overcome to induce the appropriate phenotype. The expression of additional genes requires the expenditure of cellular energy into the synthesis of RNA and proteins and has to be balanced in the light of the expected benefits (Perkins & Swain 2009). Considering the ratio of up- to down-regulated genes i.e. is 1:0.47 for the strain A2 and 1:3.55 for the strain A5, those infrastructure costs are only emerging for the strain A2. Both genotypes are however able to express the “defended phenotype” (higher PST content and reduced chain lengths) by strain-specific gene regulations, hence with different efforts for each genotype (Publication III). This is also indicated by the differences in the expression of genes for energy production and conversion related processes which exhibit a higher gene expression induction in strain A2. The absolute number of genes within this category even increases after 72 h exposure to the copepods. As a result, both strains express a phenotype with similar characteristics. This highlights the selective pressure that emanates from grazing and subsequent lead to the evolution of the ability to produce a common phenotype from different genotypes, involving different molecular mechanisms. In addition, this ability may shield genetic variability from selection and can be one reason for the maintenance of a high genotypic diversity in natural *A. tamarense* populations.

Despite the large differences in the genes that are differentially expressed in the two strains, a small subset of genes (14) was identified to be up-regulated in both strains (Publication III). Those genes might be the strongest candidates for being the inducer of the phenotypic changes towards similar characteristics, whereas the strain-specific regulated genes can be seen as rather a consequence of implementing and expressing the appropriate response. Indeed, even though the response is highly strain specific, some transcriptional change should be associated with being the catalyst that starts and/or sustains subsequent genotype specific changes for inducing the defense response. In congruence with the observed strength of changes of the phenotypes, 11 out of those 14 genes are higher

expressed in the strain A2. In addition, two of the common up-regulated genes belong to the top three induced genes in terms of fold change values.

The induction of the PSTs in both strains as a response towards *C. typicus* differs in its physiological characteristics: the strain A5 induced the overall amount of PSTs whereas in the strain A2 an additional shift in the PST profile occurred towards a higher amount of saxitoxin (Publication III). The comparable higher amount of saxitoxin on the overall PST profile did however not correlate with the expression of the *sxtA* gene that initiates the saxitoxin biosynthesis pathway (Publication III, Kellmann et al. 2008). The biosynthesis of saxitoxin hence might be regulated in other ways e.g. by the provision of precursors or reduction equivalents. Also, the reaction performed by *sxtA* may not be the limiting step and an increase in saxitoxin biosynthesis might be achieved due to an up-regulation of other enzymes participating in this pathway. Hence, the saxitoxin biosynthesis pathway could be convoluted and several routes could lead to an increase in intracellular PST content. The increase in PSTs could therefore as well be associated with changes in other traits. If any of those trait associations have been proven to be beneficial, species bearing such trait associations would have a selective advantage. This might contribute to the existence of differences in the PST profile in natural *A. tamarensis* populations.

8.2 Genotype specific feedback responses towards a protistan grazer

The results within Publication IV of this thesis show that the interaction between a protistan grazer (*Polykrikos kofoidii*) and *A. tamarensis* at the transcriptomic level is even more multifaceted than the previously discussed responses towards copepod grazers (Publication I & III). This study differs from the above investigated induced defense towards copepods in the sense that the associated *A. tamarensis* strains are able to modify the interactions in a genotypic specific manner. Hence, the presence of the lytic *A. tamarensis* strain A2 has an influence on the fitness of heterospecifics and conspecifics, which, in turn, feeds back to influence the phenotype expressed by this strain. This feedback mechanism was not given for the second investigated genotype of *A. tamarensis* (A5) since this phenotype lacks the ability to produce lytic compounds. The lytic strain A2 therefore has the potential to alter its niche, a behavior that in turn influences the traits expressed by this genotype

8.2.1 Genotype specific gene expression patterns

A highly intraspecific variation in gene expression in control conditions between the two strains of *A. tamarensis* was observed, comprising 5% of all genes on the microarray. The variability in traits expressed by these two strains hence might go beyond the described changes in secondary chemistry (PSTs and lytic compound production). However, it cannot be excluded that the presence of the caged strain A5 caused those changes in the strain A2, at least to some extent. However, the fold change values are unusually high (max. 900 FC) and such values were never observed in response to any treatment tested so far in *Alexandrium tamarensis* (Publication I; III & IV), supporting the idea that intraspecific variation in gene expression generated those observed gene expression patterns. This conclusion is further supported by literature data, showing that i.e. intraspecific variation in gene expression in *Arabidopsis* spp. is a greater source of variance than treatment effects (Van Leeuwen et al. 2007, Kliebenstein 2008) The correlation of transcript copy number and genomic abundance of those genes in dinoflagellates (Bachvaroff & Place 2008) renders gene duplication events as a source of such observed transcriptional differences between strains. Such correlations between gene duplication and increased gene expression diversity within species have also been found for many other species (i.e. *Arabidopsis thaliana*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*) (Gu et al. 2004, Landry et al. 2006, Kliebenstein 2008) and are considered to be important for shaping specific phenotypes as well as for species differentiation (Gu et al. 2004).

The observation of the occurrence of high intraspecific variation in gene expression can therefore be a valuable source for our understanding of the evolution and variance of specific traits not only in *A. tamarensis* for several reasons:

- I) In general, gene duplication events are a major source for the evolution of new traits and thereby facilitate adaptation to a dynamic environment.
- II) Gene duplication and expression noise might decrease with increasing constraints acting on the respective genes' function, which, in turn develops over time (Kliebenstein 2008). The highest rate of intraspecific gene expression variation can therefore be expected to occur in recent duplicated genes and those genes can indicate which

traits are currently under selection. The ability of dinoflagellates to reintegrate mRNA into the genome could furthermore significantly contribute to the expression and diversification of environmentally important traits.

- III) Comparing intraspecific variations in gene expression patterns between different populations could enable tracing of traits responsible for local adaptations.

8.2.2 Genotype specific trait alterations inferred from functional genomics

The results in Publication IV showed that a large range of the genome's function of *A. tamarense* is involved in shaping the phenotype due to the presence of a protistan grazer. Based on functional annotations of Pfam families covering all genes on the microarray (Publication V), around 3% of those specific Pfam families were involved in the response to the respective biotic interaction. In addition to the integration of the response into different genotypes, the strain specific alteration of the co-occurring species interactions might have a major influence on the observed changes in the gene expression patterns (Publication IV). The production of allelochemical compounds in the strain A2 with a lytic effect on co-occurring heterospecifics (Tillmann & Hansen 2009), may indeed construct an environment different from the one that the strain A5 experiences under the otherwise comparable experimental conditions. The genotype specific modification of the environment might therefore be the cause of a comparable increased expenditure of cellular resources to build up endocytotic processes in the strain A2 than in the strain A5. As changes in the phenotype are aimed to increase fitness under changed conditions, it would seem mixotrophy increases fitness in *A. tamarense* even under optimized laboratory conditions. However, neither increased growth rates nor the presence of food vacuoles could be observed for the two *A. tamarense* strains (Tillmann & Hansen 2009), but are described for other *A. tamarense* strains (Jeong et al. 2005, 2010). Yet, the occurrence of food vacuoles might depend on the strains and culture conditions used. Batch cultures with ample nutrients might already thrive at maximum growth rates and benefits could therefore be hardly measureable. Other parameters such as nutrient stoichiometry or an increase in storage compounds might be better suited for identifying beneficial effects of mixotrophy. In addition, non-formation of food vacuoles large enough to be observed with light microscopy does not exclude the existence of endocytotic

processes *per se*. In any case, lytic compound production in *A. tamarensis* seems not only to benefit the population by reducing grazing pressure from protistan grazers.

The recognition and translation of the cues emitted from the environment into different genotypes should have a common transcriptional catalyst that is specific to each interaction, as already discussed for the strain specific response towards copepod grazers. Hence, in the case of *Polykrikos kofoidii* grazing on conspecifics, the cues elicit transcriptional changes in both strains for genes that drive the conversion of diacylglycerol (DAG) into phosphatidic acid (PA), indicating that G-protein coupled receptors and DAG/PA signaling maybe the underlying intracellular response inducer (Publication IV). In addition, both strains respond with transcriptional changes that outnumber the observed gene expression changes in the copepod interactions (Publication I & III). Both strains further induce the expression of genes involved in DNA m⁶adenine methylation, implying that a high transcriptional activity is largely driven due to a reduction in DNA duplex stability. Given that dinoflagellates lack or have a low abundance of histones (Lin et al. 2010, Lin 2011, Wisecaver & Hackett 2011) m⁶adenine methylation could be a convergent mechanism to histone acetylation that accelerates or even enables large transcriptional changes.

The differentially expressed genes induced by cues emanating from co-occurring species interactions further point towards changes in the outer glycosylated membrane surface. The outer membrane is the first contact area between an organism and its external environment, and therefore represents the bridge between the environment and associated intrinsic cellular processes and responses. Hence, the outer glycosylated membrane surface determines important traits such as self-self recognition, non-self discrimination as well as “cell taste” (Gahmberg 1981, Wolfe 2000). Changes in its characteristics can subsequently influence further biotic interactions. Thus, the observed ranges of responses towards a protistan grazer provide strong implications that such feedback responses can shape community ecology processes in an unpredictable manner. In other words, if the presence of a protistan grazer modifies the niche of *A. tamarensis* (due to cell lysis) and expressed traits (setting-up of endocytosis and changes in membrane glycosylation), the outcome of interactions with a further species can differ from the interaction that would have occurred if the protistan grazer would not have been present. Consequences of species interaction therefore have the potential to scale up and might be one reason for the challenge of formulating general principles that can explain community ecology.

8.3 Future perspectives

The work in this thesis indicates that the responses of *A. tamarensis* towards co-occurring grazers are more complex and multifaceted than previously recognized, even though the experiments performed on pair-wise interactions in the laboratory were indeed reductionist in approach. However, fundamental processes can be studied only by defining conditions and minimizing variability, particularly when molecular mechanisms such as transcriptomic changes are analyzed. Future investigations on the adaptive value of the observed range of trait changes should be performed with different abiotic limitations to better characterize associated costs. In addition, exposing *A. tamarensis* to a multi-predator environment will further enhance our understanding of trade-offs associated with the induced defense response. For example, investigations could focus on the question of whether or not the lytic *A. tamarensis* strain A2 will respond to copepods by reducing chain length if additional protistan grazers are present. Given the observed differences on how the induced defense response is realized in the two *A. tamarensis* strains demands further analysis: Does this range of observed differences holds true or even expand, when analyzing the response of further genotypes? What are the consequences of the induced defense response for each genotype under nutrient- and/or light-limited conditions and in multipredator environments? How does a mix of *A. tamarensis* strains in i.e. multipredator environments perform? Answering those questions would also deepen our understanding concerning the cause of the high genotypic and phenotypic diversity observed in natural *A. tamarensis* populations. In general it remains open if the expression of different target defended genotypes is the result of diffuse selection acting on individuals, or if selection acts on the traits expressed by the whole population and if individuals are able to support each other (group selection). Group selection can be explained by Hamilton's inclusive fitness theory where an individual gains inclusive fitness through their impact on their own reproduction (direct fitness effects) as well as through their impact on the reproduction of related individuals (indirect fitness effects) (Hamilton 1964). However, such cooperation is vulnerable to "cheaters" who do not cooperate but obtain the benefit of individuals that cooperate (Czárán & Hoekstra 2009). In the case of the production of allelochemicals, it is arguable that non-producers are protected in the proximity of the producers to a certain extent. Are these non-producers cheaters, as observed with several bacterial populations, or do such non-producers benefit the population

with other traits? Cooperation and hence group selection is in prokaryotic microorganisms often regulated by quorum sensing, a communication system to coordinate behaviors at the population level (Shapiro 1998, Henke & Bassler 2004). Yet, intra-specific communication and cooperative behavior is poorly studied in phytoplankton. With respect to the ecological success of *A. tamarensis* such questions are indeed essential in order to emphasize the focus: clonal selection and individual fitness or population traits and performance. Further investigations on this topic that resample natural environments, as well as quasi-natural systems such as microcosms and mesocosms, are therefore needed to better characterize the adaptive value of observed responses in this study. In addition, the performance of a mix of *A. tamarensis* strains versus the performance of a single strain should be tested in such investigations.

Combining such studies with transcriptomic investigations can definitely broaden our knowledge about how species interactions contribute to traits and to the genetic make-up of an organism. For example, by quantifying 3D movements and population distribution of the toxic phytoplankton species, *Heterosigma akashiwo* in response to a ciliate predator and its cues in a mesocosm, Harvey & Menden-Deuer (2012) were able to show an induced flight behavior in *H. akashiwo*. Their results indicated that surface aggregation in low salinity regimes above the halocline (the typical HAB niche of *H. akashiwo*) may be an escape response to avoid predation. Hence, not only bloom formation could be driven by grazer cues, also the euryhaline character of *H. akashiwo* might be imposed by top-down selection. Consequently, top-down selection could have fostered a genetic make-up that shapes the physiology towards a high salinity tolerance. Analyzing if predator cues alone are able to induce the expression of genes necessary to thrive in a low salinity environment could indicate a genetically fixed association of both traits and therefore shed light on the selective agent. Transcriptomic associations between cause and consequence are, for example, known in the bacterium *Escherichia coli*: a sudden increase in temperature leads to an expression of not only genes necessary to acclimate to the new condition, but also genes necessary to survive in anoxic conditions (Tagkopoulos et al. 2008). In this case, the cell infers from the temperature shift that it has entered a host, expecting anoxia (Tagkopoulos et al. 2008). In addition, applying functional genomic studies that investigate species interactions for a broader range of dinoflagellates and other phytoplankton will contribute to exploration of the function of the vast amount of genes that lack a functional homology. However, this demands a uniform classification system that

explicitly describes and classifies the environmental context in which a gene is expressed (Pavey et al. 2012).

The grazer interactions addressed in this thesis further showed that cues emanating from a grazer have the power to change several phenotypic traits, as observed on the transcriptomic level. The broad distribution of these regulated genes into various physiological processes might be associated with a shift in the realized niche under grazing pressure. Nevertheless, the impact of changes in the expression of genes that code for global-acting proteins on the physiology remains unresolved. Metabolomic and/or proteomic studies could therefore be combined with transcriptional analysis in order to obtain a better understanding about changes in the physiology. Therefore, a change in abundance of selected metabolites and proteins indicated by a transcriptomic analysis could be verified with chromatographic, mass spectrometric or antibody-binding based methods. In addition, comparative metabolomics studies between grazed and control conditions can give an idea about the extent of physiological changes in *A. tamarense* evoked by grazing and hence an estimation about the impact of unknown gene products on the physiology. Such an analysis is also necessary to reveal the true impact of the observed gene expression changes, since not all transcripts are necessarily translated and not all translated proteins are functionally active. Hence, combining different “-omics” approaches can also be used to identify potential processes that are genetically associated with the grazer induced response, but not further implemented until the appropriate environmental stimulus initiates their integration to change cellular processes.

The assigned function of the differentially expressed genes in response to the protistan grazer indicated that *A. tamarense* may alter traits that can lead to additional modifications of further interactions. Such changes have the power to scale up and influence community structure and dynamics. Such indirect effects have emerged as strong agents to alter the community composition in terrestrial and aquatic ecosystems (Werner & Peacor 2003, Preisser et al. 2005, Walsh 2013). Analyzing if and how cues from a grazer modify further interactions is therefore another aspect that should be considered in future research concerning harmful algal bloom formation and persistence for *A. tamarense*. Altered traits and behaviors as a result of species interactions are generally poorly investigated in the phytoplankton. A better characterization of indirect effects may however be helpful for our understanding of population dynamics and species' compositions. Performing experiments with a defined community existing of e.g. micro- and

mesozooplankton grazers and competitors for light and nutrient with and without *A. tamarensis* can reveal the impact that the presence of *A. tamarensis* has on the community. Further, it can be tested if the prior exposure of *A. tamarensis* towards e.g. a protistan grazer changes the observed impact on the community

In addition to these broader questions that have emerged from the results of this thesis, there are more concrete topics that need further research. These include the identification and characterization of additional genes involved in PST biosynthesis in *Alexandrium* spp. as well as further investigations on the regulation of the PST biosynthesis pathway. Results from such studies will help in the identification of cellular processes that lead to increased PST levels in *A. tamarensis*. The analysis of the nature of the waterborne-cue that elicits the induced defense response is another open question. Identifying the chemical structure will be the first step in elucidating whether or not this cue is really species-specific, and hence will give us further insights into the ability of *A. tamarensis* to sense and respond to its' biotic environment. In addition, we would be able to better assess how specific the grazer induced response is evolved in *A. tamarensis*, adding to the general knowledge of the capability of planktonic species to co-evolve.

Given that the herein presented interactions are mediated by cues in the water and the expected ocean acidification scenario, changes in the pH could alter the signature of the cues and hence, the induced interaction. The analysis of grazer cue induced changes in *A. tamarensis* should therefore be performed in a comparative manner with media containing elevated partial pressures of CO₂. To avoid confounding results, either the chemical cues direct or the spent media of the grazers/grazer-interaction has to be used.

In summary, the work performed in this thesis showed that we are still far from a detailed description of the autecological capacity of *A. tamarensis* and therefore an evolutionarily grounded understanding of this species' success and ability to form harmful algal blooms. Yet the results of this thesis project have successfully demonstrated that interdisciplinary approaches can greatly contribute to our understanding of processes that enables *A. tamarensis* to thrive in its environment. Until now, the temporal and spatial distribution of species within the plankton often remains enigmatic. This especially holds true for the commonly unpredictable formation of various toxic dinoflagellate blooms. The contribution of species interaction and co-evolutionary processes to a species' success within the phytoplankton hence have to be considered as important driving forces and have to

receive more attention. Interdisciplinary approaches that combine molecular genomic and ecological knowledge are an effective way to analyze species' interactions and can greatly contribute to our understanding of processes that shape the various phytoplanktonic genomes and hence their evolutionary success.

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Supplemental material