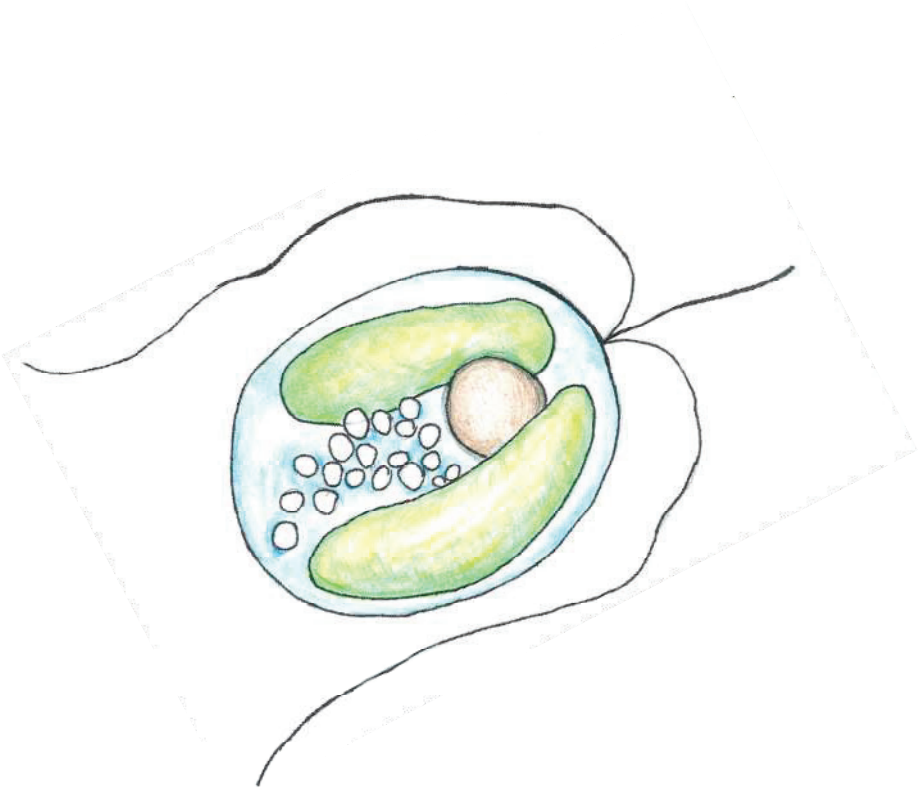
Functional genomic insights into cellular processes related to harmful bloom formation in ichthyotoxic prymnesiophytes

Dissertation



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Sára Beszteri

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1. Gutachter: **Prof. Dr. Allan Cembella**

Alfred-Wegener-Institut für Polar- und Meeresforschung

Bremerhaven und Universität Bremen

2. Gutachter: **Prof. Dr. Kai Bischof**

Leibniz-Zentrum für Marine Tropenökologie

Universität Bremen

Marcinak és Tádénak

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I. Acknowledgements

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II. Summary

Not much information is available about the genetic background of growth and toxicity-related processes in toxic Haptophyta species. The aim of my thesis was to contribute to better understanding of these issues using functional and comparative genomic approaches with the ichthyotoxic prymnesiophytes *Chrysochromulina polylepis* and *Prymnesium parvum*. In particular, I explored different gene-expression profiling methods in order to monitor the transcriptomic responses in these species to different environmental conditions.

Through the sequencing of a cDNA library, a transcriptomic database (Expressed Sequence Tag library) was established for both prymnesiophyte species. Approximately 2900 and 6300 contigs were found in the *Chrysochromulina polylepis* and *Prymnesium parvum* datasets, respectively. The sequences were annotated and compared to similar data sets available from other Haptophyta species (*Pavlova lutherii*, *Isochrysis galbana* and *Emiliania huxleyi*). This analysis revealed a `core set` of approx. 1500 genes which were found in all Haptophyta species investigated in this study. Moreover, 67 and 362 genes were present only in *C. polylepis* and *P. parvum*, respectively.

The physiological background and cellular regulation of synthesis and liberation of *Chrysochromulina* and *Prymnesium* toxin(s) is still poorly understood, but the involvement of PKS genes in the biosynthesis of certain compounds is likely. The presence of the conserved ketosynthase (KS) domains - an obligatory part of PKS genes – were shown in both species, represented by fourteen and four copies in *C. polylepis* and *P. parvum*, respectively.

In order to indirectly test the hypothesis invoking a role of PKS genes in toxin biosynthesis, the correlation between toxicity and PKS gene expression was monitored in both species. The observed positive correlation strengthens the hypothesis on the involvement of PKS genes in toxin production *C. polylepis* as well as in *P. parvum*.

A gene expression microarray was generated based on the EST data originating from *P. parvum*, and this tool was used to monitor gene-expression changes during growth in nutrient replete and phosphorus (P)- or nitrogen (N)-deprived *P. parvum* cells. In accord with previously published data, elevated intracellular toxicity was observed in P-deprived cells, whereas it did not change in N-depleted or nutrient replete cells. As a response to P limitation, the upregulation of different genes related to transport and acquisition of phosphate could be observed. On the other hand, N limitation did not lead to such a clear effect on the gene expression level, since most genes likely involved in the uptake, storage and transport of N sources were not upregulated.

Utilizing the tools of ecophysiology and functional genomics we identified gene-expression patterns indicative of physiological (nutrient, toxicity) and growth status of *C. polylepis* and *P. parvum*. With reference to this data set, knowledge about cellular processes in toxic Prymnesiophyceae species was expanded considerably, and pointed the way forward for incorporation of functional genomic approaches to determining regulatory factors involved in prymnesiophyte bloom dynamics through gene expression studies.

III. Zusammenfassung

Bei toxischen Haptophyten ist der genetische Hintergrund von Wachstum und von Prozessen, die mit Toxizität in Verbindung stehen, weitgehend unbekannt. Ziel meiner Arbeit war, durch funktionelle und vergleichende Analysen in *Chrysochromulina polylepis* und *Prymnesium parvum* zum besseren Verständnis dieser Fragestellungen beizutragen. Im Besonderen habe ich unterschiedliche Methoden getestet und verglichen, mit denen Unterschiede in Genexpression-Mustern als Reaktion auf unterschiedliche Umwelteinflüsse analysiert werden können.

Für beide Haptophyten wurde zunächst eine EST (expressed sequence tag) Bank hergestellt. Etwa 2900 Gene von *C. polylepis* und 6300 von *P. parvum* konnten identifiziert werden. Diesen Sequenzen wurden vermutliche Genfunktionen zugeordnet, und der Datensatz wurde mit weiteren Datensätzen von anderen Pavlovophyceae und Prymnesiophyceae Arten (*Pavlova lutherii, Isochrysis galbana* und *Emiliania huxleyi*) verglichen. Durch diese Analyse konnte eine Gruppe von circa 1500 Genen identifiziert werden, die in allen Haptophyten vorkommen. Die weitere Analyse ergab darüber hinaus 67 und 360 Gene, die ausschließlich in *C. polylepis* und *P. parvum* zu finden waren.

Obwohl der physiologische Hintergrund und die zelluläre Regulationen von Toxinsynthese in *Chrysochromulina* und *Prymnesium* noch weitgehend unbekannt sind, ist eine Beteiligung von PKS (Polyketide Synthase) Genen sehr wahrscheinlich. Die Anwesenheit der KS Domäne – ein unerlässlicher Teil der PKS Genes – konnte für beide Arten bestätigt werden, und zwar mit 14 bezw. 4 Kopien in *C. polylepis* und *P. parvum*. Um zu testen, ob diese Genes in der Biosynthese der Toxine eine Rolle spielen, wurde der Zusammenhang zwischen Toxizität und Genexpression der PKS Gene in *C. polylepis* und *P. parvum* untersucht. Die beobachtete positive Korrelation unterstützte die Hypothese, dass

sowohl bei *C. polylepis* als auch bei *P. parvum* PKS Gene an der Toxinproduktion beteiligt sind.

Ein Microarray zur Quantifizierung der Genexpression wurde anhand der *P. parvum* EST Sequenzen konzipiert, um Veränderungen in Genexpressionsmustern während des Wachstums, sowohl unter ausreichender Nährstoffversorgung, als auch bei Phosphor- oder Stickstoffmangel, zu untersuchen. Dabei wurde eine erhöhte intrazelluläre Toxizität unter P Mangel, aber keine Veränderungen unter N Mangel, oder im normalen Medium festgestellt. Darüber hinaus wurde unter P-Mangel eine erhöhte Expression von P-Transport und P-Aufnahme Genen beobachtet. Im Gegensatz dazu wurde bei Stickstoff-Mangel keine eindeutige Reaktion auf Genexpressionsebene ausgelöst, eine Reihe bekannter Gene, die bei der Aufnahme, Speicherung und beim Transport von Stickstoff eine Rolle spielen, waren entgegen der Erwartung nicht höher exprimiert.

Durch die Anwendung ökophysiologischer und funktionell genomischer Methoden konnten wir Genexpressionsmusters identifizieren, die Hinweise auf den physiologischen und/oder wachstumsbedingten Zustand der Zellen liefern. Dieser Datensatz bildet einen wesentlicher Beitrag zum aktuellen Kenntnisstand zellulärer Prozesse in toxischen Haptophyten, und ermöglicht so einen weiteren Schritt auf dem Weg der Entwicklung von neuen, molekularbiologisch basierenden Systemen, zur Überwachung von toxischen Algenblüten.

IV. Abbreviations

AT	acyltransferase
ACP	acyl carrier protein
cDNA	complementary deoxyribonucleic acid
EC_{50}	half maximal effective concentration
ELA	erythrocyte lysis assay
EST	expressed sequence tag
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HAB	harmful algal bloom
KOG	clusters of eukaryotic orthologous groups
KS	ketosynthase
MA	major allergen
NSP	nitrile specifier protein
PKS	polyketide synthase
qPCR	quantitative real-time polymerase chain reaction
RT-PCR	reverse-transcription polymerase chain reaction
SAGE	serial analysis of gene expression

V. Introduction

5.1 Harmful algal blooms

The term harmful algal blooms (HABs) covers a diverse set of events which share two common features: they are caused by mass development and/or rapid growth of particular algae and they have a negative impact on the environment and human activities (Zingone and Enevoldsen, 2000). These blooms occur worldwide with a general tendency to increase in frequency and geographic distribution in recent years (Hallegraeff, 2003; Zingone and Enevoldsen, 2000). Three main factors are involved in this acceleration: increased utilization of coastal waters for aquaculture, human-induced eutrophication and stimulation of algal blooms by climatological conditions. Moreover, an increased scientific awareness of toxic species, and the establishment of routine monitoring probably also contributes to the apparent increase of HAB frequencies (Riegman, 1998; Smayda, 2008).

The formation, stabilization and senescence of HABs are associated with a number of parameters and processes.

The most important abiotic factors involved in the promotion of HABs represent favorable meteorological conditions and limited nutrient availability. On the one hand sunny weather enhances primary production, whereas low wind reduces mixing of water masses and thus dispersion of cells, and represents stable conditions with low turbulence and shearing. On the other hand high N:P ratios and in some cases the lack of micronutrients have been reported to play an important role in the formation of harmful algal blooms (Tillmann and Rick, 2003).

Bloom formation and stabilization happens through processes which cause an expansion of the algal population. An increase in cell numbers is achieved by hatching of resting cysts, through physical accumulation of algal cells or by enhanced cell division. However, grazing, sedimentation and dispersing processes counteract bloom formation/stabilization processes to

a certain degree. Horizontal diffusion caused by inflow or vertical mixing can result in dispersion of algal blooms (Roelke et al., 2010a).

Although almost all HAB taxa were initially described as phototrophic, increasing evidence shows that the majority of them exhibit strategies to supplement autotrophic growth through mixotrophy or osmotrophy (Stoecker et al., 2006). The ability to produce toxins and mixotrophic nutrition strategies clearly provide advantage by allowing for mass proliferation thus bloom development under low available nutrient conditions (Smayda, 1997).

In the collapse of a harmful algal bloom many processes are involved. Here again, horizontal advection, vertical mixing due to sudden changes in weather conditions could be important factors in bloom breakdown. Further factors can be biologically driven effects on physiological response, e.g. nutrient depletion, grazing, self-shading and possibly autotoxicity/autoinhibition. Moreover, the involvement of viruses or bacteria in the termination of algal blooms has been documented in many cases (Lawrence et al., 2001; Schroeder et al., 2003).

Harmful algal blooms can be sorted into three categories based on their impact on humans and the environment (Hallegraeff, 2003):

1. Blooms with no toxic properties

The causative species do not produce toxins, but due to high biomass water discoloration can be observed. These blooms occasionally reach densities which lead to oxygen depletion and consequently to non-specific fish/invertebrate kills.

2. Blooms caused by species able to produce potent toxins which accumulate in the food chain

The produced substances accumulate for example in bivalves, finally reaching humans, thus causing gastrointestinal and/or neurological illnesses.

3. Blooms caused by species able to produce potent toxins, which do not accumulate in the food chain

The produced substances do not have direct impact on human health but may kill fish and invertebrates by high bioactivity, thus causing severe financial losses to fisheries and aquaculture. *Prymnesium parvum* and *Chrysochromulina polylepis* belong to this HAB category.

5.2 Bloom dynamics of C. polylepis *Manton et Parke and* P. parvum *Carter*

Based on data collected from documented blooms, it is accepted that high temperature, calm weather, stratified, low salinity water, and imbalanced nutrient ratios promote bloom formation both in *Chrysochromulina polylepis* and *Prymnesium parvum* (Aure and Rey, 1992; Dahl et al., 1989; Dundas et al., 1989; Guo et al., 1996; Kaartvedt et al., 1991a; Maestrini and Granéli, 1991).

Not much is known about the termination of HABs of these species. Schwierzke-Wade (2010) documented the role of hydraulic dilution on bloom termination in case of *P. parvum* simply by dispersing the cells. Viruses are thought to play a role in the collapse of the algal blooms as well, and indeed Sandaa and co-workers (2001) isolated a lytic virus from *C. ericinea*. The involvement of viruses in the termination of *P. parvum* blooms has not been observed.

Molecular biological methods have been used in field monitoring approaches, to forecast HABs of these species (Töbe et al., 2006). The identification and monitoring of gene expression patterns linked to growth or nutrient limitation could be a first step in the use of

gene expression approaches in bloom forecast, by indicating the beginning of growth limitation in field studies.

5.2.1 Prymnesium parvum *blooms*

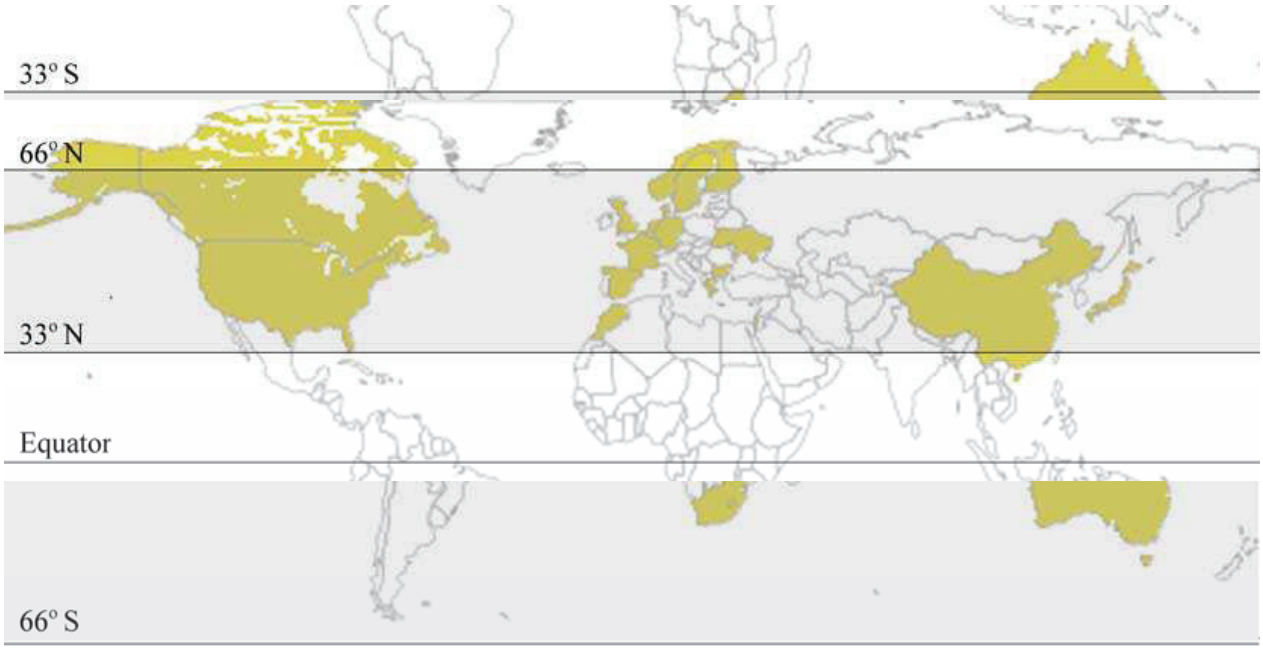

Figure 1. Global distribution of *P. parvum* populations (adapted from Manning and La Claire, 2010)

Prymnesium parvum blooms are reported from low salinity and inshore waters. First recorded fish killing *P. parvum* bloom happened in the Netherlands (Liebert & Deerns, 1920), followed by other incidents in Palestine and Israel (Reich and Aschner, 1947). More recent incidents were recorded in Norway (Kaartvedt et al., 1991a), Finland (Lindholm et al., 1999; Lindholm and Virtanen, 1992), Greece (Michaloudi et al., 2009), Germany (Dietrich and Hesse, 1990), Texas (Baker et al., 2007; James and De La Cruz, 1989), and China (Guo et al., 1996) (Figure 1.). The water bodies where the blooms occur are usually eutrophic due to fish farming, land use, sewage runoff (Edvardsen & Paasche, 1998). The cell density in a

Prymnesium bloom can reach up to 2 x10^8 cells l^{-1} (Reich and Aschner, 1947, Michaloudi et al. 2009, Lindholm and Virtanen 1992). Depending on the density of the blooms, the pH of the water can be very high (9.2-9.5) (Michaloudi et al. 2009, Lindholm & Virtanen 1992).

5.2.2 Bloom dynamics Chrysochromulina polylepis

Before the extraordinary *Chrysochromulina polylepis* bloom in 1988 (Granéli et al., 1993; Skjoldal and Dundas, 1991) this species was not regarded as potentially harmful to the environment. In this toxic event, *C. polylepis* achieved high cell concentration and in the late stage the bloom covered approximately 75,000 km^2 (Granéli et al., 1993). *Chrysochromulina polylepis* is also eurythermal and euryhaline and so ecologically flexible. All such blooms have been reported from coastal waters in the North Atlantic (Edvardsen & Paasche 1998); and are usually initiated by freshwater runoff, sunny and calm weather conditions at high N:P (Dahl et l, 2005, Edvardsen & Paasche, 1998). However, the factors triggering or governing bloom formation in this species are not yet fully characterized.

5.3 Morphology

Chrysochromulina polylepis and *Prymnesium parvum* are unicellular, motile, photosynthetic, nanoplanktonic species, with two yellowish parietal chloroplasts, containing chlorophyll a & c and fucoxanthin (Moestrup and Thomsen 2003). They have two isochont flagella and an appendix positioned between them called the haptonema (Edvardsen and Imai, 2006) (Figure 2, 3). In case of *Prymnesium*, the haptonema is stiff and short, whereas that of *Chrysochromulina* is usually longer and flexible. Cells of these species are covered with organic scales, the function of which is unknown.

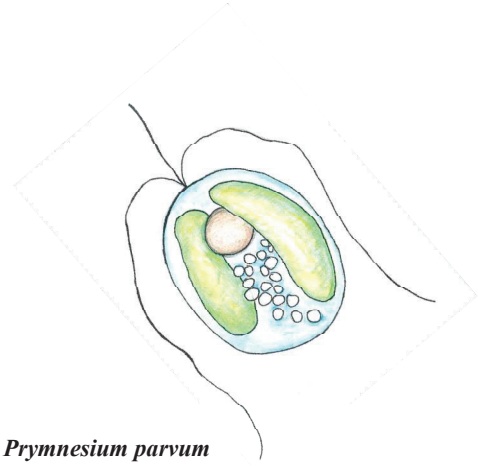

Figure 2. ***Prymnesium parvum***

5.4 Reproduction and life cycle strategies (putative)

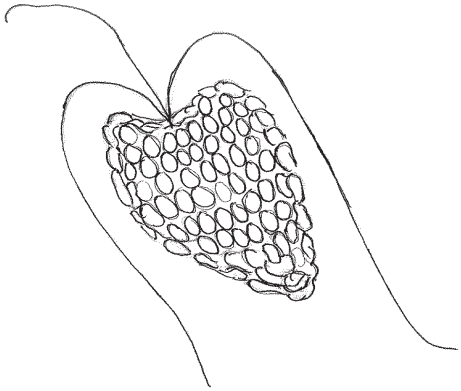

Figure 3. ***Chrysochromulina polylepis***

The reproduction of these species occurs usually by longitudinal fission (Moestrup and Thomsen 2003); both species divide about once a day (Larsen et al., 1993). Although sexual reproduction has not been observed in *C. polylepis* nor in *P. parvum*; a haplo-diplontic life cycle was proposed for both species based on experimental data (see below) (Edvardsen and Vaulot, 1996; Larsen and Edvardsen, 1998).

Edvardsen and Vaulot (1996) suggested the presence of an alternate haplo-diplontic life cycle for *C. polylepis*, which includes different cell forms: two morphologically distinct haploid cell types (authentic and alternate), one diploid (alternate) cell type and possibly a non-motile cell stage (Edvardsen and Vaulot, 1996) (Figure 4.). Transition from alternate to authentic cell types was observed in clonal cultures, (Edvardsen and Paasche, 1992), but transition from authentic to alternate cells was observed only once (Edvardsen and Paasche, 1992). The haploid alternate and authentic cell types may function as gametes representing different mating types, the diploid alternate cells being the result of syngamy.

Similarly, Larsen and Medlin (1997) documented that *P. parvum* and *P. patelliferum* are one species as conferred by molecular phylogenetic analyses. Flow cytometric analyses have shown that *P. parvum* cells are either diploid or haploid, and *P. patelliferum* is always haploid (Larsen and Edvardsen, 1998). The same researchers observed no transition from one species to the other over time in separate cultures. Mating experiments were conducted with *P. parvum* (both haploid and diploid) and *P. patelliferum* cells in different combinations. When *P. patelliferum* cells were mixed, no diploid cells (and no *P. parvum*) were observed. Unexpectedly, a mixture of two diploid *P. parvum* strains produced haploid cells. Moreover,

the same mixture and mixture of haploid and diploid *P. parvum* cells resulted in the formation of *P. patelliferum* cells (Larsen and Edvardsen, 1998). These findings led to the hypothesis that *P. parvum*/*patelliferum* are different cell types involved in a haplo-diplontic cell cycle.

The role of a haplo-diplontic life cycle is not elucidated yet. The development of different life cycle forms would possibly allow the species to occupy different niches. This hypothesis is supported by the fact that the authentic and alternate forms of *C. polylepis* exhibit different physiological optima (Edvardsen & Vaulot, 1996).

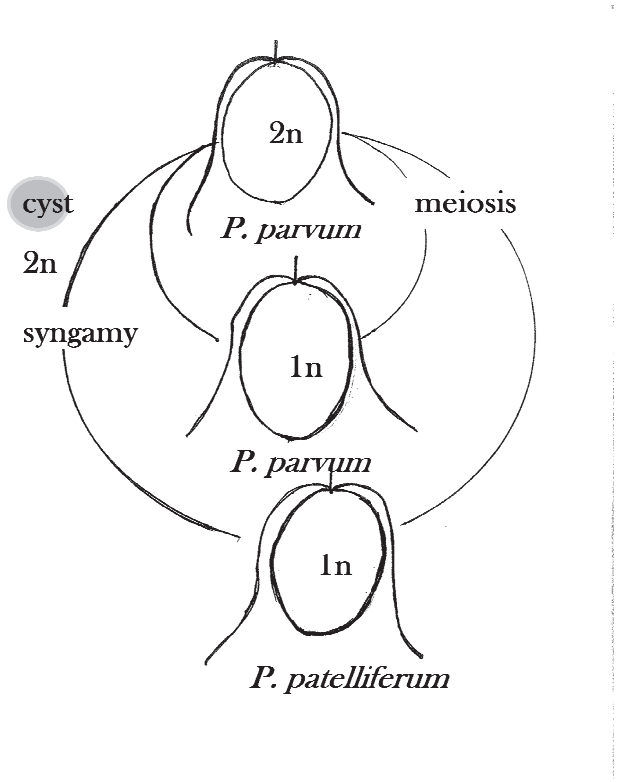

Figure 4. Proposed life cycle of *P. parvum* (redrawn from Larsen and Edvardsen, 1998)

5.5 Taxonomy and Phylogeny

The genus *Prymnesium* and *Chrysochromulina* are closely related prymnesiophyte species, which belong to the division Haptophyta (Green and Leadbeater, 1994), Class Prymnesiophyceae, order Prymnesiales, family Prymnesiaceae. The Prymnesiaceae comprises

four genera, *Prymnesium*, *Chrysochromulina*, *Platychrysis* and *Corymbellus*. At present about 60 species of *Chrysochromulina* and six of *Prymnesium* have been described (Edvardsen and Imai, 2006), of which two (*C. polylepis*, *C. leadbeateri*) (Edvardsen and Imai, 2006) and four are potentially harmful species (*P. parvum*, *P. calathiferum*, *P. faveolatum* and *P. zebrinum*) (Lundholm and Moestrup, 2006).

Edvardsen et al. (2000) investigated the phylogenetic relationships of prymnesiophyte species based on 18S ribosomal DNA (rDNA) data. Eight *Chrysochromulina* and four *Prymnesium* species were analyzed along with other haptophyte species. The analysis splits the Haptophyta into two classes: Prymnesiophyceae and Pavlovophyceae. Based on the genetic marker of 18S rDNA, the former class can be divided into three clades (Figure 1). The first clade contains *Phaeocystis* spp., the second and third clade consists of nonmineralized taxa (*Prymnesium*, *Chrysochromulina*, *Imantonia*), mineralized genera (for example *Emiliania*) (Figure 5.) and *Isochrysis* respectively. The clade that contains *Prymnesium* and *Chrysochromulina* species was subdivided into two groups, namely B1 & B2 (Edvardsen et al., 2000). The first group (B1) contains *Imantonia*, all three *Prymnesium* species investigated and three *Chrysochromulina* species. *Prymnesium parvum* and *C. polylepis* with all the toxic species investigated in this study cluster together in the first clade, whereas the other seven *Chrysochromulina* species constitute clade B2. This suggests that the genus *Chrysochromulina* is paraphyletic.

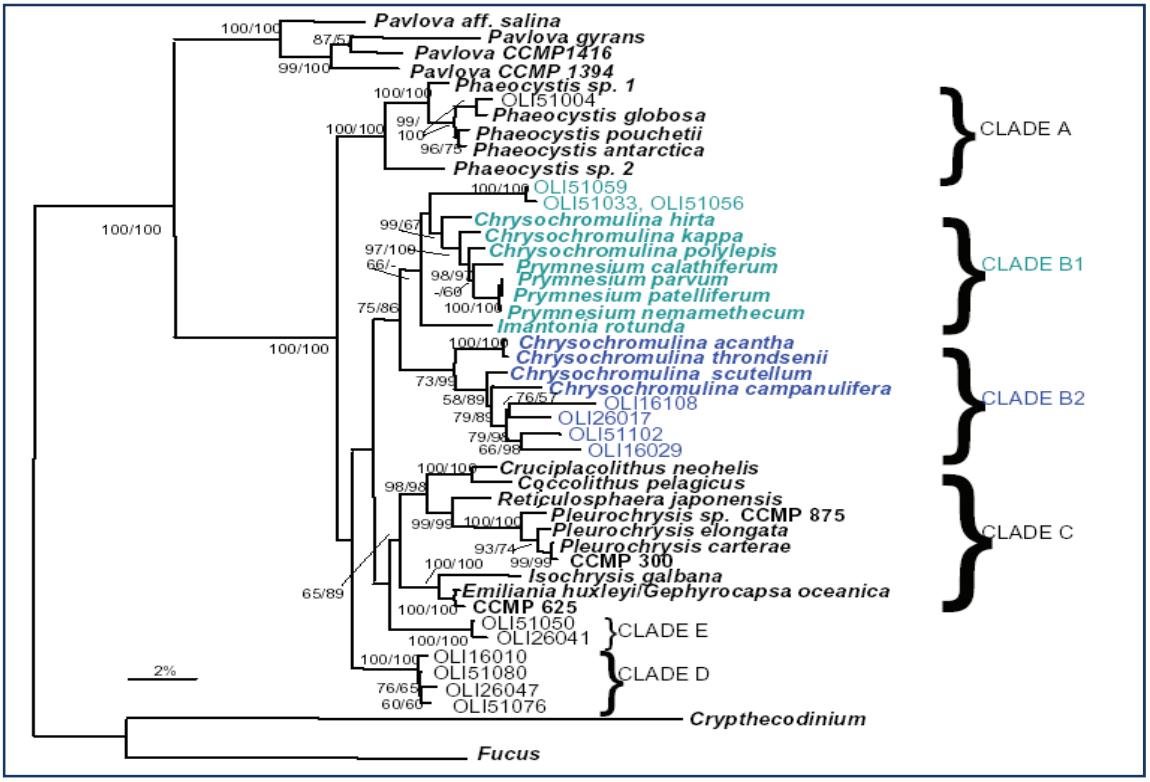

Figure 5. Phylogenetic tree based upon maximum likelihood analysis indicating the relationships of the Haptophyta. Tree is based on 18S ribosomal DNA sequence data. (Edvardsen et al., 2000)

5.6 Feeding strategies

The mixed form of nutrition (photosynthesis and phagotrophy), mixotrophy has been observed both in *C. polylepis* and *P. parvum*. Apart from photosynthetic growth bacterivory has been documented in both species (Nygaard and Tobiesen, 1993). Moreover, Tillmann (1998) described phagotrophy in senescent *P. parvum* cultures. *Prymnesium* cells were incubated with different motile prey organisms, of which a number has lost their motility (presumably due to toxin action). *Prymnesium* cells formed pseudopodia and when in contact with a prey cell, the food particle was enclosed by the pseudopodium and taken up into the cell (Tillmann, 1998) (Figure 7.).

On the contrary, in case of *Chrysochromulina hirta* and *C. spinifera* the haptonema was shown to be involved in particle uptake (Kawachi et al., 1991) (Figure 6.) It is believed that nutrient availability, light and prey abundance triggers phagotrophy (Granéli and Carlsson, 1998) to obtain macro and micronutrients when dissolved organic and inorganic nutrients are limiting. Indeed, phagotrophy has been shown to contribute significantly to nitrogen (N) and phosphorus (P) uptake in *P. parvum* (Carvalho and Granéli, 2010). These authors found that even nutrient sufficient *Prymnesium* cells gain almost 50% of their N and P from organic sources. Apart from phagotrophy, *Prymnesium* developed another ways to gain macronutrients from the environment, namely by means of cell-surface L-amino acid

Figure 6. The haptonema is involved in particle uptake by *C. polylepis*. Redrawn after Kawachi et al. 1991

oxidases, and organic phosphatases. These enzymes act on organic matter, liberating ammonium (Palenik and Morel, 1991) and organic phosphates (Ricketts 1966), which is taken up by the cells for further metabolism. Since the dissolved organic N amount in marine waters is substantially larger than available inorganic N in most stratified waters (Granéli et al., 1999), species able to use dissolved organic matter as food source have an advantage over strictly autotrophic organisms.

5.7 Toxicity/toxins

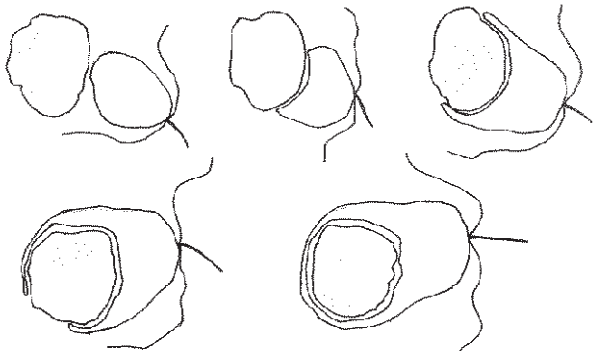

Figure 7. Heterotrophic feeding in *P. parvum*, note the involvement of pseudopodia. Redrawn from Tillmann 2003.

Many of the bloom forming taxa produce potent allelochemicals/toxins. These compounds can have diverse ecological functions, but probably neither of these functions requires toxicity against vertebrates or shellfish. These - from a human perspective - most harmful effects are only side effects of chemical compounds synthesized by the algae. Toxicity has multiple potential ecological roles: it does not only deter grazers, but also inhibits the growth of sympatric species (thus potentially eliminating or reducing competition for nutrients) (Mitra and Flynn, 2006; Smayda, 1997; Smayda, 2008); and due to lytic activity, provides organic matter which promotes growth of the toxic species (Edvardsen, 1996; Larsen, 1998; Tillmann, 2003).

Chrysochromulina polylepis and *P. parvum* are ichthyotoxic and produce compounds which negatively affect eukaryotic cells (Tillmann, 1998, 2003, Granéli & Johansson, 2003). The toxins are a mixture of compounds, but the nature and composition of these is under debate. The active substances of *Chrysochromulina* have been characterized as 1-acyl-3-digalactosylglycerol and octadecapentaenoic acid (Yasumoto et al., 1990). However, when John and co-workers (2002) compared the composition of fatty acid and lipid classes of toxic and non-toxic *C. polylepis* strains, no toxin-relevant difference could be detected (John et al., 2002). In case of the *Prymnesium* toxins there is also some contradiction. We find alternative evidence in the literature that these toxins may be proteolipid (Dafni et al., 1972; Paster, 1968; Ulitzur and Shilo, 1970) lipopolysaccharide (Paster, 1968), galactoglycerolipid (Kozakai et al., 1982) or polyether (Igarashi et al., 1995; Igarashi et al., 1996) in structure. In this latter

publication the authors describe two linear polyether compounds, prymnesin 1 and 2 (PRM 1 & 2) which show similar biological (lytic) activity. Using a bioassay guided analytical approach, Henrikson and co-workers (2010) compared the toxin profiles of laboratory grown algal cultures and field samples collected during a bloom. On the one hand, in the laboratory grown cultures, a group of potent ichthyotoxic fatty acids was detected, and highly toxic uncharacterized compound(s) were also present. In the field samples, on the other hand, the amount of fatty acids or that of the uncharacterized metabolite was not enough to produce the devastating effect that was observed, and more importantly no PRM1 or 2 was found. Therefore, Henrikson and co-workers (2010) claim that PRM1 and PRM2 are not the primary toxins of *P. parvum* (Henrikson et al., 2010).

Valenti and co-workers (2010) observed pH dependent toxicity in *P. parvum*. Higher pH caused high mortality of juvenile fish (fathead minnow, *Pimephales promelas*), but if the pH of the water sample was titrated to seven or below, the toxicity decreased markedly. They speculated that the toxins are weak bases which are unionized at higher pH and thus are responsible for higher toxicity (Valenti Jr et al., 2010).

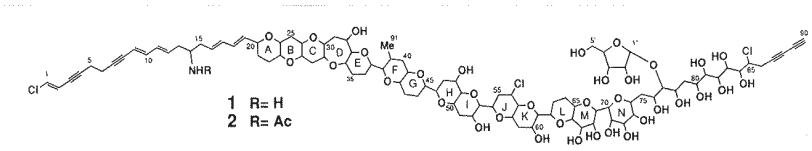

Figure 8. The structure of prymnesin 2

Owing to the fact that the lytic compounds are unknown and so cannot be measured with analytical methods, bioassays are commonly used to get insights into the nature and extent of toxicity. For example, to assess the rate of intracellular toxicity, the erythrocyte lysis assay (ELA) is often employed to monitor the effect of crude *Prymnesium/Chrysochromulina* cell extracts on blood cell membranes (Graneli and Johansson, 2003a; Johansson and Granéli,

1999a; Johansson and Granéli, 1999b). The higher the number of lysed blood cells, the higher is the intracellular toxicity.

On the other hand, *Artemia salina* larvae (Larsen and Bryant, 1998; Larsen et al., 1993), *Rhodomonas salina* cells (Barreiro et al., 2005; Skovgaard and Hansen, 2003) or fish (Baker et al., 2007) have been incubated in the supernatant of *Prymnesium* cultures to assess the allelopathic capacity, e.g. the effect of compounds secreted into the medium by *Prymnesium* cells. In this approach, the EC_{50} or probit is calculated e.g. the concentration of toxic cells needed to lyse 50% of prey cells.

The toxicity of both prymnesiophyte species is strain-specific and reacts to environmental changes. Many researchers have investigated the effects of abiotic (light, salinity, temperature etc.) and biotic (presence of grazer/prey) factors on the toxicity of *Prymnesium parvum* (Baker et al., 2007; Larsen and Bryant, 1998; Larsen et al., 1993) and *Chrysochromulina polylepis* (Eschbach et al., 2005; Johansson and Graneli, 1999a). Exponentially growing, nutrient replete *P. parvum* cultures show only low or no lytic effects (Tillmann, 2003). However, in senescent (Tillmann, 1998) or N- or P-starved cells (Johansson and Graneli, 1999b) the toxicity increases. Moreover, when *P. parvum* is toxic, predators do not graze upon it (Tillmann, 2003). At highest toxicity the grazers turn into victims, the toxin lyses the cells and *P. parvum* eats them (Tillmann, 2003).

Cell cycle dependent differences in toxicity have been demonstrated for *C. polylepis* (Eschbach et al, 2005).The toxicity increased at the transition from the dark to the light phase in synchronized cells, and the hemolytic activity dropped in the middle of the dark phase. When these changes were correlated to cell cycle events, the decrease in toxicity corresponded to cell division, whereas the increase in toxicity coincided with the start of the G1 phase, where the daughter cells produce and accumulate the active compounds. In comparison, nothing is known about the regulation of toxin production in *P. parvum*.

Chrysochromulina polylepis and *P. parvum* produce toxins with ichthyotoxic, neurotoxic and allelopathic activity (Shilo, 1971). To date, the only bioactive compounds described from *P. parvum* are ladder-frame polyether compounds, named prymnesins (Igarashi et al., 1996). Based on the mode of action and the phylogenetic relationship, similar compounds have been suggested to be responsible for the toxicity of *C. polylepis* as well. The toxins act nonspecifically causing membrane disruption and leakage of organic matter. Factors found to increase toxicity are macronutrient (N or P) deficiency and high light intensity (Eschbach et al., 2005; Shilo, 1971).

5.8 Polyketide synthase (PKS) genes

The bioactive compounds (prymnesin 1 & 2) described from *P. parvum* to date exhibit a ladder-frame polyether structure, which structurally resemble other polyether toxins produced by microalgae such as brevetoxins or maitotoxin (Cembella and John, 2006; Wright and Cembella, 1998). Biosynthetic evidence suggests that most, if not all polyether phycotoxins are produced via polyketide pathways (Cembella and John, 2006). Polyketides are a structurally diverse class of natural products derived from the polymerization of acetyl and propionyl subunits in a process similar to fatty acid synthesis. The toxins of *P. parvum* have been proposed to be derived from polyketide metabolism; if so, the synthesis of these substances would be achieved via polyketide synthase (PKS) genes. Polyketide synthase genes are common to all organisms. Currently three types of PKS genes are known, PKS I, II and III. PKS I genes are highly modular proteins, which were described initially from bacteria and fungi, but they have been found in protists as well (John et al., 2008; Monroe and Van Dolah, 2008). Types II PKS are aggregates of monofunctional proteins. Genes for Type II PKS are found exclusively in bacteria, and they are responsible for the production of aromatic

polyketides. The Type III PKS genes work iteratively, and were thought to be unique to streptophyte species, including land plants and several orders of brown algae, but their presence has later been shown in bacteria as well (Cock et al., 2010). These PKS genes share the set of obligatory modules of ketoacyl synthase (KS), acyl transferase (AT), ketoacyl reductase (KR), dehydratase (DH), enoyl reductase (ER), acyl carrier protein (ACP) and thioesterase (TE) domains. Microalgal polyketide toxins are (presumably) synthesized by Type I PKS genes (Weissman & Staunton, 2001), but assignation has not been straightforward. Polyketide synthase genes found so far in microalgae are of modular structure (John et al 2008, John et al 2004)(Monroe et al., 2010; Monroe and Van Dolah, 2008), consisting of at least a ketosynthase (KS) and acyltransferase (AT) domains and an acyl carrier protein (ACP) (Cane and Walsh, 1999). These genes are not conserved evolutionarily; therefore their similarity based identification is difficult.

The characterization and gene expression analysis of PKS genes correlated with observed toxicity could provide further insights into the regulation and cellular background of toxin synthesis in these prymnesiophyte species.

5.9 Functional and comparative genomics of Haptophyta

The identification of genes involved in biosynthetic pathways for toxins associated with HAB species has recently become an important issue. However, sequence information is mandatory for the identification/annotation of genes, as well as for quantitative analysis of their expression. Although genome sequencing of prokaryotes is well established and can now be routinely performed in small-scale projects, e.g. for toxic cyanobacteria (Stucken et al., 2010)), this is still not the case for eukaryotic algae, due to their large genome sizes (Rudd, 2003). A less expensive alternative method for gene discovery from large genomes is based on the construction of transcriptomic databases. The main limitation of this approach is that it

only captures genes expressed at the time of library construction. Such transcriptomic databases can be derived from sequencing of cDNA libraries. Here, mRNA fragments present in the sample are transcribed to cDNA, cloned and randomly picked fragments are sequenced, resulting in what is often referred to as an expressed sequence tag (EST) database. Second generation sequencing technologies also allow direct sequencing (i.e., no cloning step necessary) of the cDNA fragments after transcription. To improve the transcript coverage of transcriptomic databases, RNA samples originating from different treatments are often pooled and normalization procedures aimed at reducing the dominance of the most abundant transcripts are performed (Rudd, 2003).

The functional annotation of putative coding fragments is based on *in silico* methods, primarily similarity searches on nucleotide and predominantly on protein level. Orthologues to known genes are identified through alignment with available sequences in reference databases (in most cases public sources such as GenBank non-redundant database, Swissprot, TrEMBL etc.). Functional characterization on the amino acid level is also possible through identification of conserved domains. These analyses give a probability value (E-value), which describe the random background noise, thus the lower the E-value, or the closer it is to zero, the more "significant" the match. However, the drawback of this method is that only rather evolutionarily conserved genes can be identified this way. Furthermore, in non-model organisms only general function prediction can be assigned to the majority of the identified tentative coding sequences, or no corresponding annotation can be found. These fragments could represent Orthologues of genes in which evolutionary processes affected the DNA sequence and therefore they cannot be identified based on similarity; or else, they could be candidates for enzymes involved in specific but to date uncharacterized pathways. The understanding and investigation of adaptation processes can thus only be completed by the

assessment of unknown genes. Comparative approaches and gene expression studies open new dimensions in the characterization of these fragments.

A more comprehensive understanding of the interconnection between physiology and genetic potential is needed. Approaches aiming at connecting genetic responses to observed physiological changes are possible, since gene expression studies allow for the identification of the genetic potential. Recent developments of different new techniques allow for large scale transcriptomic investigations or `gene mining´ e.g., gene expression studies of single genes. Limited genomic studies (based upon EST libraries) of members of the Haptophyta have been published for the non-toxic species *Isochrysis galbana* and *Pavlova lutheri* (Patron et al., 2006) and more importantly for the toxic *P. parvum* (La Claire, 2006). As a reference organism the genome of the closely related coccolithophorid *Emiliania huxleyi* (http://genome.jgi-psf.org/Emihu1/Emihu1.home.html) is available.

Different techniques, including Northern blot and quantitative real-time PCR (qPCR), have been used to monitor the expression of single genes in haptophyte algae (Bruhn et al., 2010; Kang et al., 2007). Via the Northern blot approach, estimation of the gene expression level is obtained through visualizing the mRNA abundance in the sample. This method is time consuming, requires a large amount of RNA and allows for only qualitative or semi-quantitative quantification of the transcripts (Bustin, 2000). The qPCR method enables the accurate detection and relative quantification of expressed genes in a small amount of sample (Bustin et al., 2005).

In large scale approaches, the expression analysis of up to the whole genome is possible (with microarrays, long SAGE, RNA sequencing, etc.) but an essential prerequisite of all these methods is the availability of a reference genome or at least a reference database. With these methods the identification of regulation and expression patterns is possible but the approaches have different foci and advantages. On the one hand, the sequencing approaches

are less laborious (but expensive) and require relatively high computing skills and capacity, but allow for a more precise quantification. Moreover, direct sequencing approaches can only be conducted for a relatively simple experimental matrix. The microarray analyses on the other hand are less expensive and the data processing is relatively easy. This approach allows for analysis of complex experimental matrices. Large scale gene expression studies have proven useful in detecting responses to different ecophysiological treatments in a number of Haptophyta species, including toxic Prymnesiophyceae species (Dyhrman et al., 2006; Freitag, 2011; La Claire, 2006).

Gene expression studies can provide further insights into cellular backgrounds of toxicity and bloom formation. The identification of genes involved in regulation of toxicity or in the biosynthesis and liberation of the toxins can be achieved through comparative functional genomic approaches. These methods allow for identification of gene expression differences between for example toxic and non-toxic strains.

VI. Outline of the thesis

Experiments linking ecophysiological observations with gene expression studies deliver valuable results about regulatory and cellular processes; but these approaches have seldom been accessed for toxic Prymnesiophyceaen species. The general objective of my thesis was the identification of bloom relevant cellular processes and responses, related to allelopathy, toxicity, nutrient limitation and/or growth, in the two prymnesiophytes *C. polylepis* and *P. parvum*. Applying the tools of functional genomics the genomic potential of *C. polylepis* and *P. parvum* was identified and gene expression studies were conducted for both species.

The specific aims of my thesis were:

- Construction of transcriptomic databases for gene discovery for *C. polylepis* and *P. parvum*;
- Investigation of the growth and/or toxicity of both toxic prymnesiophyte species as a response to different changes in abiotic parameters of their environment;
- Identification of cellular processes potentially involved in the development of toxic blooms of *P. parvum* by gene expression profiling under P- and N-limitation.

The thesis comprises three experimental studies written up as separate research articles and included in the respective chapters.

6.1 Functional genomics of toxic Prymnesiophyceae species

Investigations of gene expression at the transcript level rely on the availability of sequence information for the gene/transcript of interest. In fact, availability of target specific hybridization probes is sufficient for some methods, and this is feasible without knowledge of sequence information, but possibilities of interpretation are rather limited in the latter case. In

non-model organisms without available genome sequences, this sequence information can be generated in a cost-effective manner by sequencing cDNA libraries. In the case of Haptophyta species, the only currently available genome sequence is that of the coccolithophorid *Emiliania huxleyi*, but no such resource is available for other species of this group.

Gene inventories, whether exhaustive (at least for the strain sequenced) as when a genome sequence is available, or more limited, as in the case of transcript sequencing, provide insights into the genetic and physiological capacity of the organism of interest and also potentially about the evolutionary history of individual genes of functional relevance. The set of genes encoded in a genome is the blueprint of the proteins, and, eventually, the phenotype an organism is capable of expressing. Thus, it is tempting to think that comparing gene complements of organisms expressing a particular phenotypic feature with those of organisms lacking this feature might highlight candidate genes which could be encoding the traits of interest (Kudela et al., 2010). For instance, one could expect that comparing the gene complements of toxin-producing species with those of non-toxic ones could reveal candidate genes putatively associated with toxin biosynthesis. A major underlying assumption is of course that the traits of interest are homologous in the compared species, i.e., are derived from a common evolutionary ancestor or gained by lateral gene transfer from the same donor. Indeed, a comparative study of two closely related cyanobacteria has been conducted in order to reveal the genetic background of the differences in their morphology and physiology (Stucken et al., 2010). Alternative candidates could be genes sharing common functions in the adaptation to low nutrient levels for example through mixotrophy.

With these aims in mind, a comparative genomic and gene expression analyses of Prymnesiophyceaen species was conducted for both *C. polylepis* and *P. parvum* (Chapters I and III), based upon construction and annotation of a normalized cDNA library for each species. In order to enhance the complexity of the databases, RNA samples originating from

different treatments and/or physiological phases were pooled. The set of transcripts identified were compared to genes/transcripts available from other Haptophyta species. Applying bioinformatic tools, we aimed at the identification of genes which are present only in toxic species, assuming that these may be related to bloom formation (Chapter III).

6.2 Toxicity and PKS gene expression

Identification of the cellular background of toxin biosynthesis from the perspective of molecular physiology and genetics has been pursued in the field of HAB research for some time (reviewed by Cembella and John 2006). In algae, where toxin synthesis can be induced by abiotic or biotic factors (an example is *P. parvum*), comparative gene expression analyses between weakly and highly toxic conditions provide reliable experimental setups for identification of candidate genes involved in toxin synthesis (Yang et al., 2010). However, species for which toxin production cannot be triggered by environmental factors often also exhibit fluctuations in toxicity. This was the case in *C. polylepis,* for which cell cycle linked toxicity could be observed in synchronized cells (Eschbach et al., 2005).

This experimental system allowed for the first time to indirectly test the hypothesis invoking a role of PKS genes in toxin biosynthesis in *C. polylepis*. For this test, we identified PKS gene fragments in the transcriptomic library of *C. polylepis,* and monitored the expression of one fragment throughout the cell cycle in highly synchronized cells with a semi-quantitative reverse transcription PCR assay (Chapter I).

In contrast to *C. polylepis*, *P. parvum* exhibits inducible toxicity in response to environmental cues (Larsen and Bryant, 1998). *Prymnesium parvum* is an ecologically rather flexible species, with wide tolerance ranges for temperature, salinity, and nutrient status of the aqueous medium. Short term abiotic `shock´ treatments were applied, and the expression of three PKS gene fragments obtained from a transcriptomic database (La Claire 2006) was

monitored (Chapter II). The degree of intracellular toxicity and allelopathic capacity was correlated to the PKS gene expression data, to validate the hypothesis invoking a role of PKS genes in the toxicity of *P. parvum*.

The qPCR method enables the detection and relative quantification of expressed genes in a small amount of sample with high accuracy. However, as with other gene expression investigation methods, qPCR procedures involve numerous steps (notably, reverse transcription, and PCR amplification), the efficacy of which is highly dependent on sample quality. Any variation in RNA integrity or quality, cDNA synthesis efficiency differences or cDNA sample loading variation (Stahlberg, 2003, 2004a, 2004b) can cause large variation in magnitude of the eventually observed signal. To correct for inconsistencies introduced at any of the measuring steps, it is necessary to normalize the data when the measurement is completed. In order to improve existing methods, we established a "spike in" method, where we applied foreign RNA for normalization.

6.3 The effect of P and N starvation on gene expression level in P. parvum

Macro- or micronutrient starvation has long been recognized as a major factor affecting bloom formation and dynamics. Imbalanced nutrient levels (usually high Redfield ratios) have been observed in almost all documented *P. parvum* blooms (Kaartvedt et al., 1991a; Michaloudi et al., 2009). Many eco-physiological studies dealt with the toxic responses of *P. parvum* to N or P deprivation and confirmed the enhanced intracellular toxicity per cell under macronutrient limitation (Carvalho and Granéli, 2010; Johansson and Granéli, 1999b). Tillmann's (2003) experiments highlighted the probable ecological meaning of this elevated toxicity, as part of a strategy involving the switch to mixotrophic nutrition

under low inorganic nutrient concentrations. To date, however, little is known about the details of the cellular responses of *P. parvum* to low nutrient levels and the invoked associated nutrition mode shift. Chapter III addresses this gap by comparative gene expression analysis of cultures growing under nutrient replete vs. N- or P-limited conditions.

We aimed at monitoring physiological responses to nutrient starvation in terms of growth, toxicity, and nutrient uptake (Chapter III). For this purpose *P. parvum* was grown under N or P deprivation, and the intracellular and extracellular toxicity was assessed with bioassays. The EST library constructed served as basis for the development of a gene expression microarray and was applied to observe the effects of nutrient starvation on transcript level in *P. parvum* (Chapter III). Through comparative approaches, we aimed at the identification of growth (and thus bloom formation) relevant genes and focused on P- or N-starvation related expression patterns in the integrated data-set. A special focus was the identification of genes that can be used later as proxies in bloom forecast or control.

VII. Chapters/Manuscripts

The chapters of my theses correspond to separate research articles of which two are already published and the third one is under review.

7.1 Chapters/Manuscripts

I. Genomic characterization of the ichthyotoxic prymnesiophyte *Chrysochromulina polylepis,* and the expression of PKS genes in synchronized cultures

Uwe John, Sára Beszteri, Gernot Glöckner, Rama Singh, Linda Medlin and Allan D. Cembella

European Journal of Phycology (2010) **45**(3): 215-229

The author of this thesis together with U. John performed the experiments, analyzed the data and written and corrected the manuscript.

II. Effect of physiological shock treatments on toxicity and polyketide synthase gene expression in *Prymnesium parvum* (Prymnesiophyceae)

Michael Freitag, Sára Beszteri, Heiko Vogel and Uwe John

European Journal of Phycology (2011) **46**(3): 193-201

The author of this thesis together with M. Freitag and U. John developed the conceptual frame of this study. The candidate established the central methodological part of the study (spike-in real time qPCR). Experiments were designed and performed and the manuscript was written and corrected by the candidate and M. Freitag.

III. Transcriptomic response of the toxic prymnesiophyte *Prymnesium parvum* (N. Carter) to phosphorus and nitrogen starvation

Sára Beszteri, Ines Yang, Nina Jaeckisch, Urban Tillmann, Stephan Frickenhaus, Gernot Glöckner, Allan Cembella, Uwe John

Submitted to Harmful Algae

The author of this thesis elaborated the concept of this study together with U. John. The experimental setup was planned with U. Tillmann and U. John and performed by the candidate. The data analysis and interpretation was done by the candidate who then drafted the manuscript in discussion and consultation with all the co-authors.

CHAPTER I

Genomic characterization of the ichthyotoxic prymnesiophyte *Chrysochromulina polylepis,* and the expression of PKS genes in synchronized cultures

ABSTRACT

The widely distributed prymnesiophyte species *Chrysochromulina polylepis* is prominent and well known for occasional formation of ichthyotoxic blooms. The chemical structure of the *C. polylepis* toxin(s) has not yet been elucidated, but the associated hemolytic activity, potent membrane disruption interactions and toxicity to finfish and protists has led to the suggestion that they may be similar to the prymnesins of *Prymnesium parvum*. Such polyether toxins are presumably formed partially or completely via polyketide biosynthetic pathways. In this genetic study of *C. polylepis*, we generated and analyzed a genomic DNA and a normalized cDNA library. We estimated a genome size of approximately 230 mbp based upon analysis of >1000 genomic library clones. Of the cDNA library, 3839 clones were partially sequenced and annotated, representing approximately 2900 unique contigs. We detected several genes putatively related to toxin synthesis. Thirteen putative polyketide synthase (PKS) related gene sequences were identified and phylogenetic analysis identified two of these as containing ketoacyl domains of the modular type I PKS. Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) was used to follow the expression of PKS genes over the light/dark cycle of synchronized *C. polylepis* cultures. This is the first study showing the expression of PKS genes in marine microalgae, in this case in the toxigenic *C. polylepis.*

KEY WORDS:

cell cycle, *Chrysochromulina,* expressed sequence tags (ESTs), gene expression, genomic characterization, ichthyotoxins, polyketide synthase (PKS), prymnesiophyte

1. INTRODUCTION

Harmful algal blooms (HABs) occur worldwide, with an apparent increase in frequency, intensity, and environmental impact and geographic distribution in recent years (Hallegraeff 2003). Among HAB taxa, the widely distributed prymnesiophyte genus *Chrysochromulina* from the class Prymnesiophyceae within the algal division Haptophyta (Jordan and Green, 1994, Edvardsen et al., 2000) is prominent and well known for occasional formation of ichthyotoxic blooms. The genus *Chrysochromulina* has close morphological and molecular phylogenetic relationships with the ichthyotoxic prymnesiophycean *Prymnesium* and shares the capacity to form HABs in coastal and brackish waters (reviewed in Edvardsen, 1996). Although several of over 50 described *Chrysochromulina* species are reported to be potentially toxic, most attention has been focused on *C. polylepis* Manton et Parke*,* the source of a devastating toxic bloom that occurred in the Kattegat and Skagerrak region near the Norwegian coast in the late 1980s (Dahl et al., 1989); (Granéli et al., 1993). This bloom resulted in extensive fish kills and caused severe ecological damage to wild biota with high economic losses at fish farms along the Norwegian and Swedish coasts (Gjøsæter et al., 2000); (Nielsen et al., 1990); (Skjoldal and Dundas, 1991).

The toxicity of *Chrysochromulina* in culture is highly species- and even strain-specific and has only been demonstrated in bioassays. For *C. polylepis*, there is evidence of allelochemical effects against other microalgae (Myklestad, 1995; Schmidt and Hansen, 2001), as well as related grazing inhibition (John et al., 2002). Certain zooplankton exposed to *C. polylepis* experienced reduced rates of growth and reproduction and enhanced mortalities (Nielson et al., 1990).

The mechanisms involved in the expression of toxicity in *C. polylepis* are still poorly understood. Several reports have indicated the expression of toxicity (calculated as change of toxicity per cell) triggered by external factors, such as phosphorus deficiency (Edvardsen et al., 1990; Johansson and Graneli, 1999) and increased salinity (Edvardsen et al., 1996) but this could also be attributable to indirect effects on growth rate. However, maximal cell toxicity has been claimed to occur during exponential growth (Edvardsen et al., 1996; Schmidt and Hansen, 2001). Cell cycle analysis of synchronized cultures of *C. polylepis* showed that toxicity of cell extracts was discontinuous during the cell cycle, with maximal toxicity occurring during the light phase of the photoperiod (Eschbach et al., 2005).

The mode of action of the C. *polylepis* toxin(s) is apparently non-selective, but is associated with hemolytic activity, potent membrane disruption and toxicity to finfish and protists (Skjoldal and Dundas, 1991; Gjøsæter et al., 2000; Edvardsen et al., 1996). The observations of similar ichthyotoxicity and cytolytic effects on cells and tissues caused by exposure to other prymnesiophytes, such as *P. parvum*, led to speculations that they may be caused by similar toxins (John et al., 2002). Among the prymnesiophytes, the prymnesins (PRM 1 and PRM 2) isolated from *P. parvum* are the only structurally described toxins (Igarashi et al., 1998). The prymnesins are potent hemolytic components and are ichthyotoxic, particularly with Ca^{++} ion as supplement, but it is not clear whether or not prymnesins alone can account for all toxic and allelochemical effects because non-toxic species can also release compounds that can lyse cells (Eschbach et al., 2005).

Prymnesins are mixed linear polyether compounds and thus share some structural similarity to the polyether toxins produced by marine dinoflagellates, although the mode of action may not be analogous (Wright and Cembella, 1998). Based upon the results of stable isotope labeling studies of other polyether toxins, such as spirolides (MacKinnon et al., 2006), we assume that prymnesins are derived by similar polyketide biosynthetic pathways.

Organisms known to produce polyketides using polyketide synthases (PKSs) include bacteria, fungi, sponges, microalgae and higher plants (e.g. Proksch et al., 2002; Dittmann and Wiegand, 2006, John et al., 2008). A common element of all polyketide biosynthesis is the strictly ordered regulated generation by PKS (Staunton and Weissman, 2001; Moore and Hertweck 2002; Cembella and John, 2006; John et al., 2008; Moor and Hertweck, 2002). The PKSs are large multi-domain enzymes or enzyme complexes closely related to fatty acid synthases (FASs). They are composed of the identical ancestral set of functional modules: ketoacyl synthase (KS), acyl transferase (AT), ketoacyl reductase (KR), dehydratase (DH), enoyl reductase (ER), acyl carrier protein (ACP) [or phosphopantetheine attachment site (PP)], and thioesterase (TE) domains. Whereas all units are needed for fatty acid production by FASs, the minimal structure of PKSs requires only ACP, KS and AT for the condensation reaction of acetate units. The other domains, if present, can catalyze the stepwise reduction of the initial carbonyl units (for details see Staunton and Weissman, 2001; Moore and Hertweck, 2002; Kusebauch et al., 2009)

The PKSs are involved not only in phycotoxin biosynthesis, but also in synthesis of other compounds with a diverse spectrum of functions in nature, ranging from chemical defense to complex cell-to-cell communication (Ikeda and Omura, 1997; Borejsza-Wysocki and Hrazdina, 1996; Börner and Dittmann, 2005). Starter units of PKSs can be short-chain (branched) fatty acids, different alicyclic and aromatic acids, and amino acids. Additionally, post-PKS tailoring events such as glycosylation, acylation, alkylation and oxidation further add to polyketide structural and functional diversity (Moore and Herweck, 2002).

Degenerate primer sequences and heterologous probes for PKS genes for fungi and bacteria have been published and can be used effectively for the isolation of gene sequences in these organisms (Ayuso-Sacido and Genilloud, 2005; Nicholson et al., 2001; Lee et al., 2001, Schumann and Hertweck, 2006). Yet these primers are specifically designed for certain

phylogenetic groups and are not generally usable on a wider range of species. Thus, the isolation of PKS genes from species for which only limited genome information is available is still a challenge.

No whole genome sequences are available for the target genus *Chrysochromulina*, therefore we adopted a limited genomic approach based upon "expressed sequence tag" (EST) surveys targeting transcribed coding regions. ESTs are partial cDNA sequences that derive from single-pass sequencing. They provide a rich source of information that has been used for the identification of novel genes, gene mapping, comparative genomics and functional characterization of gene products (Rudd, 2003). After construction and sequencing of a cDNA library, an EST database was analyzed, compared with other Prymnesiophyceae, and screened for the presence of putative PKS genes.

The aim of the present work was to enhance the genetic and molecular characterization of *C. polylepis* and haptophytes in general. The focus was to: i) obtain insights into the genomic structure of this member of the Prymnesiophyceae, ii) identify putative PKS genes that might be involved in synthesis of toxins, and iii) determine whether or not the expression of PKS genes is regulated in a cell cycle-dependent manner, in correlation with the toxicity. We demonstrated for the first time the expression of PKS genes in Haptophyta and provided one of the most detailed genomic studies on an ichthyotoxic prymnesiophyte to date.

2. MATERIALS AND METHODS

2.1 Synchronization conditions and sampling procedure

Experiments were conducted with a toxic, haploid strain (B1511) of *Chrysochromulina polylepis* Manton & Parke. The strain was isolated by Bente Edvardsen, University of Oslo, from the Oslofjord, Norway (59°00' N, 10°45' E). *Chrysochromulina polylepis* was grown in

enriched seawater medium IMR 1/2 (Eppley et al., 1967) supplemented with 10 nM selenite in batch cultures at 15 °C under cool-white fluorescent light with a photon flux density of 45 µmol s^{-1} m^{-2}, applied over a 14:10 h light-dark regime. Unialgal cultures in exponential growth phase were scaled up for the synchronization experiments by inoculation of ca. 1 x 10^4 cells ml^{-1} in sequence into 0.05, 0.5, 1, 5 and finally into 10 l flasks to reach a final concentration of 1.5 x 10^5 cells ml^{-1} before each transfer. Five and 10 l cultures were gently aerated with sterile-filtered air to provide CO_2 and to achieve a homogeneous cell distribution. For the synchronization experiments, three parallel cultures were sampled during exponential growth over 24 h at 2 h intervals, at a starting cell concentration of about 4 x 10^4 cells ml^{-1}. Samples were collected from 10 l cultures via a silicone-rubber tube with an inner diameter of 3 mm by gently applying a vacuum created by drawing on a 50 ml syringe. Samples were immediately stored on ice and after determination of the cell numbers, processed for RNA extraction. Sample collection during the dark period was under a red darkroom safety light.

2.2 Determination of cell concentration

Culture samples (2 ml) were diluted in 18 ml sterile-filtered seawater pre-cooled to 15 °C. Cells were counted with a Multisizer II particle counter (Coulter Electronics, Krefeld, Germany) equipped with a 100 µm aperture, within a size-window of 5 to 12 µm, which excluded most background particles. Average cell size with standard deviation (SD) was calculated with the Coulter Multisizer Software. Calculation of specific growth rate (µ, unit per day) was performed by the formula:

$$\mu\ (d^{-1}) = (\ln(C_1) - \ln(C_0))/\ 1$$

where C_1 is the cell concentration at time 1 and C_0 is the cell concentration at time 0.

2.3 Cell cycle analysis

Samples (20 ml) of *C. polylepis* culture were fixed with 0.25% glutaraldehyde, stained with 5 µM SytoxGreen (Molecular Probes, Leyden, The Netherlands) and subsequently analyzed for relative DNA content using a FACS Vantage flow cytometer (Becton-Dickinson, San Jose, California) equipped with an Innova Enterprise II 621 laser. This procedure, including analysis of at least 1 x 10^4 cells per sample at 1 psi, was essentially as previously described (Eschbach et al., 2001). Dot plots and histograms were created with the WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, California). Cell cycle analysis was achieved with the Multicycle software (Phoenix Flow Systems, San Diego, California). The number of cells in a certain cell cycle phase was expressed as the percentage of the total cell number in the sample. Duration of a single cell cycle phase was determined using an algorithm for synchronized cell cultures (Beck, 1978).

2.4 RNA extraction

Samples of 10-15 x 10^6 cells taken at 2 h intervals during a 24 h kinetic study were harvested by centrifugation at 5000 x *g* for 15 min at 4 °C. The cell pellets were resuspended in 500 µl RLT buffer (Qiagen, Hilden, Germany) and immediately frozen in liquid nitrogen. The resuspended pellets were stored at –80 °C until use. Before RNA extraction the cells were mechanically disrupted with a Tissue Lyser (Qiagen, Hilden, Germany) for 30 s, at 30 Hz frequency. Homogenates were processed according to the manufacturer's instructions (RNeasy Plant Mini kit, Qiagen, Hilden, Germany) with few modifications. Briefly, the samples were loaded on a QIAshredder column (Qiagen, Hilden, Germany), and centrifuged for 10 min at 8000 x *g*. A second purification step with the Qiagen RNeasy Cleanup kit was conducted, including the on-column DNA digestion with RNAse-free DNAse (Qiagen, Hilden, Germany). The extracted total RNA was checked for integrity using gel

electrophoresis and quantified by spectrophotometry. The extracted RNA was stored at –80°C until use.

2.5 cDNA library generation

The cDNA synthesis was generated by Vertis biotechnology (Freising-Weihenstephan, Germany) from 2.4 µg total RNA. In brief, oligonucleotide primers were attached to the 5'- and 3'-ends of the cDNA to allow PCR-amplification and directional cloning of the cDNA as well. The *Not* I/*Asc* I-sites of the plasmid vector pFDX3840 (supplied by Prof. Dr. Ralf Reski, Freiburg) were used for directional cloning. All PCR amplification steps were performed with a long and accurate (LA) PCR system as described by (Baskaran et al., 1996).

Normalization of the cDNA was performed according to Ko (1990) with several modifications. Specifically, the cDNA for normalization was not sheared but rather was used full length. Normalization was achieved by two consecutive cycles of denaturation and reassociation of the cDNA, resulting in N1- and N2-cDNA. With the N2-cDNA, a Cot-value of approximately 90 was achieved. Reassociated double stranded-cDNA was separated from the remaining single stranded-cDNA (normalized cDNA) by passing the mixture over a hydroxylapatite column (Ausubel et al., 1987). The hydroxylapatite-purified single-stranded (ss)-cDNA was amplified with 12 (N1) and 14 (N2) LA-PCR cycles and size-fractionated on 1% agarose gels. The fractions >750 bp were cut out from the gel and isolated by electroelution. The eluted fragments were cloned directionally into the vector pFDX3840 bearing ampicillin antibiotic resistance. The plasmids were transformed into *E. coli* TOP 10F` (Invitrogen, Karlsruhe, Germany).

2.6 EST sequencing

A total of 3839 clones from a normalized *C. polylepis* cDNA library were picked using a Q-pix (Genetix, Hampshire, UK) colony picker. Following overnight growth of picked clones

at 37 °C in LB medium containing 50 µg ml^{-1} ampicillin, stocks were made by adding 7.5% glycerol. The stocks were frozen immediately in liquid nitrogen and stored at –80 °C. The template for sequencing was generated directly from glycerol-stock cells with the Templiphi technology (GE Healthcare, Sunnyvale, CA, USA). Sequencing reactions were performed with ET-terminator chemistry (GE Healthcare, Sunnyvale, CA, USA) and M13 forward or M13 reverse primer (Table 1). Cleaned sequencing products were analyzed on MegaBACE 1000 or 4000 sequencers (GE Healthcare, Sunnyvale, CA, USA).

2.7 EST Annotation

The scf traces generated by the MegaBACE 1000/4000 were processed with the pre-gap and gap modules in the Staden software package (http://staden.sourceforge.net/). Vector sequences were removed and the traces were clipped for quality using pre-gap. The Gap4 module of the Staden package was used to assemble overlapping reads and generate sequence files in fasta format. The fasta files were blasted using blastx (Altschul et al., 1997) against the Genbank non-redundant protein database (nr) with an e-value threshold of 0.001. Contigs that did not report a significant hit were blasted using PSI-BLAST against the SwissProt (Bairoch et al., 2005) database. Expectation value threshold was set at 0.1 and three rounds were allowed.

ESTs with e-values below 0.001 were examined manually; those with an identity of 30-50% over a region of similarity of at least 50 amino acids were analyzed further in the SwissProt and non-redundant (nr) databases of NCBI – BLASTX. The sequences were grouped according their functional categories (http://www.ncbi.nlm.nih.gov/COG/, http://www.ebi.ac.uk/interpro/).

2.8 Blast analysis

The contigs from the assembly were compared to custom made and publicly available databases. We retrieved the coding sequences (CDS) of the plant *Arabidopsis thaliana* from TAIR (http://www.arabidopsis.org/), the Prymnesiophyceae *Emiliania huxleyi* CDS from the JGI website (http://www.jgi.doe.gov/), and the CDS of the diatoms *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, of the moss *Physcomitrella patens*, and of the red alga *Cyanidioschyzon merolae* from the respective NCBI web sites (http://www.ncbi.nlm.nih.gov/) in August 2009. Additionally, we used the databases Swissprot (swissprot version 54) and the complete refseq set from the NCBI database (version 33). Comparisons between EST sequences were done as tblastx searches; the protein databases were searched using blastx.

Blast parameters used (WUBlast2.0):

B 120 maximum number of database sequences for which *any* alignments will be reported

E 1E-15 the expectation threshold for reporting database hits

W 11 seed word length for the ungapped BLAST algorithm.

2.9 Genomic library

DNA of *C. polylepis* was extracted using the PLANT DNA extraction Kit following the instructions of the manufacturer (Qiagen, Hilden, Germany). Genomic libraries with a target insert size of 1.5 kb were constructed from total DNA as described previously (Glöckner et al., 2004). Colonies containing bacterial vectors with cDNA inserts were grown in LB medium. The Qiagen magnetic bead protocol was used for the plasmid preparation.

Custom primers and the cycle sequencing method were used for sequencing. The sequencing reaction products were separated on ABI3700 96 capillary machines. Quality clipping was done with phred (Ewing and Green, 1998) and vector removal with phrap (http://www.phrap.org).

2.10 Genomic data analysis

The phrap (http://www.phrap.org/) assembler enabled us to obtain a minimal contig set, mainly by joining paired end reads. Previous results have shown that an automated assembly alone does not join all possible overlaps caused by accumulating sequencing errors at the end region of the sequences. This is particularly the case for contigs with low coverage. Thus, a final manual curation was done on the automatically generated contig set.

In addition to the above mentioned global Blast databases swissprot and refseq we used organism specific databases to pinpoint the potential phylogenetic distribution of blast matches. Databases were generated using predicted proteins from *Cyanidioschizon*, *Chlamydomonas*, *Physcomitrella*, and *Arabidopsis*. Blast hits occurring against bacterial proteins only were assumed to be derived from culture contaminations. However, in many eukaryote genomes “bacterial” genes have been identified, which might result from recent lateral gene transfer. Potential chloroplast- and mitochondria-specific genes were assigned according to their respective best hit to organelle-specific genes.

We estimate that most bacteria-derived clones could be detected, because a diverse set of bacterial genomes has already been completely determined. However, we cannot rule out additional culture contaminations (viruses, other eukaryotes, etc.) because available data are scarce.

Genome size estimation was done under the primary assumption that protist genomes generally code for around 10,000 protein-coding genes (Armbrust et al., 2004; Derelle et al., 2006). The average 'gene space' was determined by dividing the number of sequenced prymnesiophyte nuclear contigs by the number of genes found. This number was then multiplied by the expected number of genes corrected for potential unidentified unknown genes. This unidentifiable, species-specific number of genes has been estimated to be about 40% (e.g. Marsden et al., 2006; Eichinger et al., 2005), if no genome of the same taxon is completely determined. Species-specific gene family extensions would lead to a slight underestimation of genome size. Thus, the number given is the lower threshold value.

2.11 Analysis of candidates

In total 13 putative PKS sequences were identified, based on similarity to known PKS genes among the *C. polylepis* EST dataset. These sequences were further analyzed with the NRPS-PKS software tools (Ansari et al., 2004) for the prediction of the domain organization. For most putative PKS ESTs, a ClustalX (Thompson et al., 1997) alignment was generated using sequences from the BLASTX database. However, the KS domain of PKS4 (930 bp) and PKS7 (1509 bp) was analyzed with the alignment from John et al. (2008). The resulting sequence dataset was aligned with Kalign (Lassmann & Sonnhammer, 2005) and PHYML (Guindon and Gascuel, 2003; see John et al., 2008) was used for Maximum Likelihood phylogenetic analysis with 1000 bootstrap runs. GAPDH and cytochrome f were also identified and analyzed as described herein and served as controls in the gene expression studies.

2.12 Gene expression

In the semi-quantitative RT-PCR approach the genes PKS7, GAPDH, and cytochrome f were analyzed. PCR primers were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 1).

Primer name	Sequence 5`-3`
M13 forward	GTT TTC CCA GTC ACG ACG TTG
M13 reverse	TGA GCG GAT AAC AAT TTC ACA CAG
GAPDH forward	TCA ACG ACG CCA AAT ACA ATG
GAPDH reverse	ACC CTT CGT GAT GCC GTA GT
Cytochrome forward	ATG GCC ACC ACG AAA TCC T
Cytochrome reverse	ATA CCT CGC CTC TGA ATG CAA
PKS7 forward	GGT GTT CAA GCT GCT GAT GC
PKS7 reverse	TGC CTG CAT ACC CAA ATG AG

Table 1. Primer sequences for the semi-quantitative RT-PCR procedure

To obtain reliable data using the semi-quantitative RT-PCR approach it is crucial to identify the proper amount of total RNA used for reverse transcription, the dilution factor, the amount of cDNA template for the PCR reaction and the optimal number of PCR cycles. Therefore a step-by-step optimization was performed before running the final set of samples. Finally, reverse transcription (RT) was performed with the Omniscript RT-PCR Kit (Qiagen, Hilden, Germany) with some modifications. One µg of DNAse-treated (Qiagen, Hilden, Germany) total RNA was reverse-transcribed at 42 °C, with 50 pmol oligo-VNdT_{18} primer and 1 mM of dATP, dGTP, dCTP and dTTP each in a total volume of 20 µl. After reverse transcription the 20 µl reaction was diluted 1:5 with low TE buffer (10 mmol Tris-HCL, 10 mM EDTA pH 8). A 2 µl aliquot was used for PCR amplification by the Hot-MasterTaq procedure (Eppendorf, Hamburg, Germany) with 10 mmol dNTPs. The PCR cycle conditions were 1 min at 94 °C denaturation and different numbers of cycles consisting of 30 s at 94 °C denaturation, 30 s at 60 °C annealing, and 45 s at 72 °C elongation. As the final settings, 30 cycles for PKS7, 24 cycles for GAPDH, and 20 cycles for cytochrome f were run. For the

semi-quantitative PCR reaction, equal aliquots of each PCR reaction (10 µl) were separated on 2% agarose gels using Tris–borate buffer containing 1.0 M Tris, 0.9 M boric acid and 0.01 M EDTA and photographed after ethidium bromide staining. Gels were analyzed with 1D Image analysis software (Kodak Digital Science, Jahnsdorf, Germany). Analyses were made semi-quantitatively on the basis of net band intensities.

3. RESULTS

3.1 Genome characteristics

In total 1056 clones, containing genomic DNA fragments, were sequenced, yielding 781.5 kb of assembled data (from 950 kb raw data). Of these, 761,952 bp of genomic DNA were counted as of *C. polylepis* origin. However, 0.83% of this sequence data appears to belong to the chloroplast genome and 0.08% to the mitochondrial genome, leaving 754,991 bp, which are part of the *C. polylepis* nuclear genome. Using Blast we were able to identify putative genes on 3.26 % of the sequence contigs, resulting in a gene space (basepairs/identifiable gene) of 45580 bp and a minimum estimate of genome size of approximately 230 MB.

bacterial contamination	**2.52%**
net *Chrysochromulina* (including chloroplast +mitochondria)	**761952**
chloroplast	**0.83%**
mitochondrial	**0.08%**
transposon	**1.01%**
virus	**0.59%**
gene hit	**3.26%**
nuclear genome	**754991**
nuclear hits	**3.29%**
gene space (bp/identifiable gene)	**45580**
genome size (MB; 5000 identifiable genes)	**227.9**

Table 2. Genome survey of sequencing results for *C. polylepis*.

Also 2.5% potential bacterial and 0.59% viral potential contamination was identified (all summarized in Table 2).

3.2 EST database

From the normalized cDNA library of clones that were 5'-end sequenced, 2927 sequences passed the quality control as successful reads. Assembly of the resulting ESTs yielded 476 contigs and 1724 singletons. The sequences reached an average length of 684 bp and exhibited an average GC content of 57.27%. Highly significant matches were most frequently obtained with sequences from unicellular eukaryotes, animals, fungi, and plants. However, significant matches to sequences from prokaryotes were also observed.

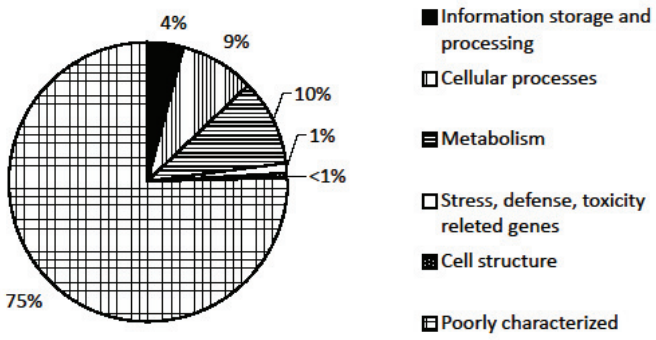

Figure 1. Functional characterization of 2200 annotated cDNA contig sequences of *Chrysochromulina polylepis*

Grouping of the ESTs according to the KOG categories for putative cellular function assigned about 75% (1679) of the ESTs into the category 'poorly characterized', which includes the genes with no hit (1526 sequences) and genes with unknown functions (153

sequences) (Figure 1). The relatively high number of ESTs without assignable function may be attributable to the fact that only partial sequence information was available for *Chrysochromulina* and only a few haptophyte genome sequences are represented in gene data banks. Furthermore, some EST sequences may be derived from non-coding transcribed parts of the genes, thereby obscuring their function.

The percentage distribution of sequences falling into different functional categories (521 genes) is plotted in Figure 2.

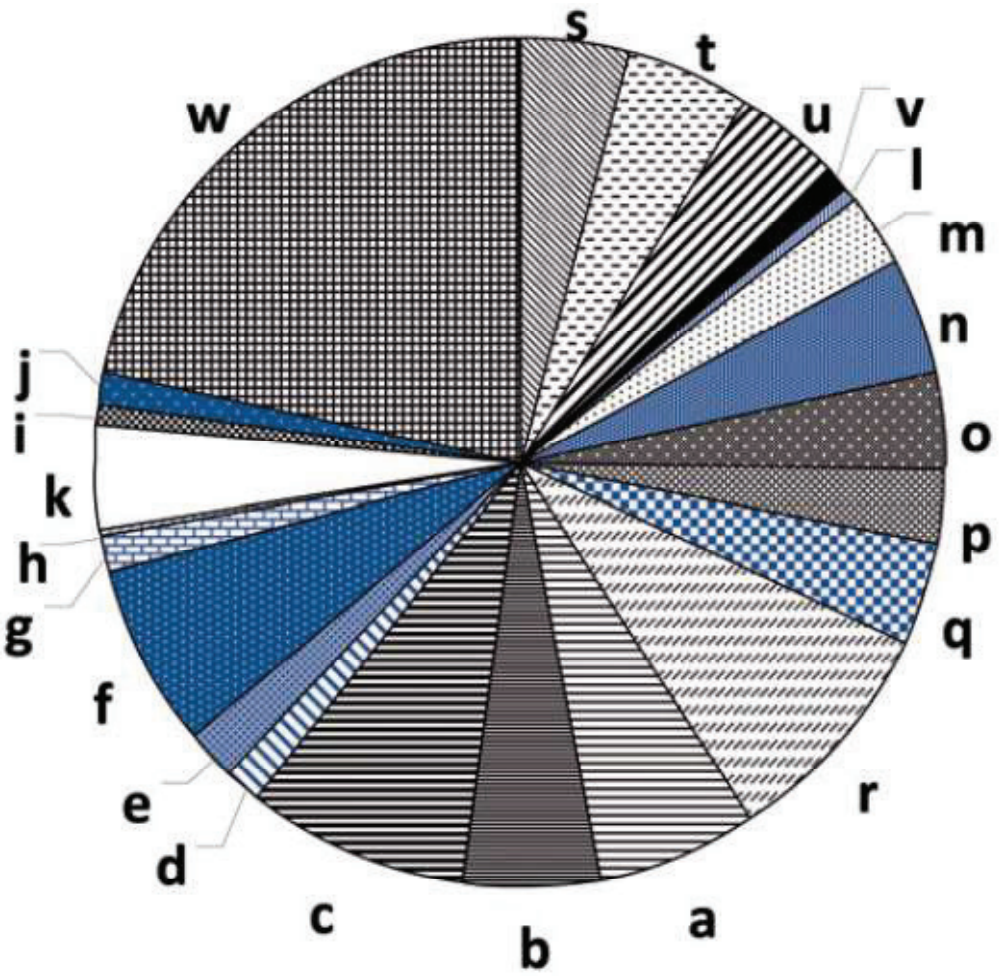

Figure 2. Distribution of contigs of the normalized cDNA library by eukaryotic categories of orthologous groups (KOG) classes

Ten % (224) of the sequences encode proteins involved in metabolism, nine % (191) are involved in cellular processes, 4% (98) are related to information storage and processing and

less than 1% (15) are predicted to encode cell structure proteins. One % (27) of the represented sequences encoded proteins that are apparently involved in cell defense and toxicity. Table 3 and Figure 2 show a more detailed view of the contig distribution among the functional categories.

Category	Metabolism	N° of contigs
g	Carbohydrate metabolism	43
c	Energy production and conversion	37
e	Amino acid transport and metabolism	59
f	Nucleotide transport and metabolism	11
h	Coenzyme transport and metabolism	14
i	Lipid metabolism	48
q	Secondary metabolites biosynthesis, transport and catabolism	10
u	Intracellular trafficking	2
	ESTs in total	**224**
	Cell structure	
w	Extracellular structures	6
z	Cytoskeleton	9
	ESTs in total	**15**
	Stress, defense, and toxicity	
?	Stress, defense and toxicity related genes	27
	ESTs in total	**27**
	Cellular Processes	
b	Chromatin structure and dynamics	5
d	Cell division and chromosome partitioning	18
o	Posttranslational modification, protein turnover, chaperones	33
m	Cell wall, membrane, envelope biogenesis	24
n	Cell motility	21
p	Inorganic ion transport and metabolism	28
t	Signal transduction	62
	ESTs in total	**191**
	Information storage and processing	
k	Transcription	30
j	Translation, ribosomal structure and biogenesis	33
l	DNA replication, recombination and repair	30
a	RNA processing	5
	ESTs in total	**98**
	Poorly characterized	
r	General function prediction only	1679

Table 3. Eukaryotic orthologous gene group (KOG) classes and respective numbers of *C. polylepis* unigenes

3.3 EST library comparison

We performed BLAST-based comparisons to the two EST sets of *P. parvum* and *Isochrysis galbana*, respectively, together with the whole coding potential of *Emiliania huxleyi*. This shows that approximately 100 genes shared between *C. polylepis* and *P. parvum* are not present in the non-toxigenic *I. galbana* data set (see Figure 3 A).

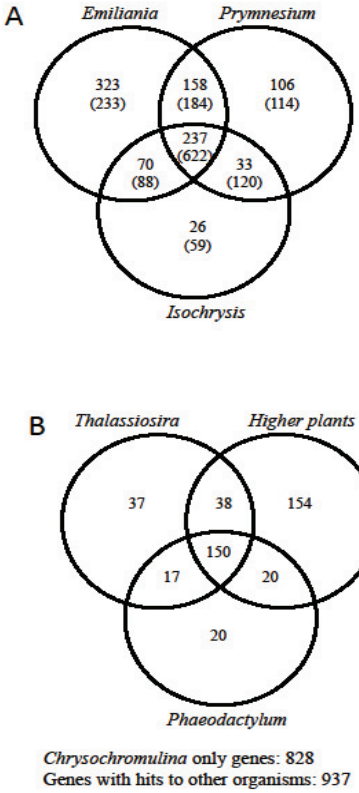

Figure 3. Venn diagrams depicting matches of *C. polylepis* ESTs to different databases. A: Matches to *Prymnesium parvum*, *Emiliania huxleyi*, and *Isochrysis galbana* with score thresholds of 120 and 100 (in brackets). B: Matches (score threshold 100) to diatoms *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, and higher plants. C. Matches (score threshold 100) to *Physcomitrella patens*, *Arabidopsis thaliana*, and *Cyanidioschyzon merolae.*

Depending on the threshold used, 781 (threshold 100) or 1248 (threshold 120) EST contigs had no counterpart in other genomes. We then analyzed which part of the data set is

common between different, only distantly related species. First we compared our data set to complete genomes of diatoms (*Thalassiosira pseudonana* and *Phaeodactylum tricornutum*) and to proteins from higher plants.

This analysis revealed that a considerable part of the shared genes is present in higher plants and not in diatoms despite a comparable evolutionary history (secondary endosymbiosis; Figure 3B). Furthermore, 937 contigs appear to have similarities to genes in other genomes as we found by comparing them to the complete NCBI protein reference set currently available (not shown). A third comparison to individual complete genomes of photosynthetic species (*Cyanidioschyzon merolae*, the moss *Physcomitrella patens*, and *Arabidopsis thaliana*) confirms that most of the detectable similarities of our data set are found with land plants and not to unrelated algal species (Figure 3C).

3.4 Kinetics of synchronous growth

Synchronous growth of *C. polylepis* clone B1511 was achieved after reaching early exponential phase within 24 h in the 10 l cultures (Figure 4a). Maximum cell concentration (~1.4 x 10^5 cells ml^{-1}) was attained within 6 days after inoculation. *Chrysochromulina polylepis* cultures exhibited a stepwise increase of cell number during the 24 h sampling period on Day 4 (Figure 4b). The cell number increased from the middle of dark period until early light period. The mean growth rate of *C. polylepis* over the 6 days was $\mu = 0.57$ and was similar over the 24 h sampling period ($\mu = 0.53$).

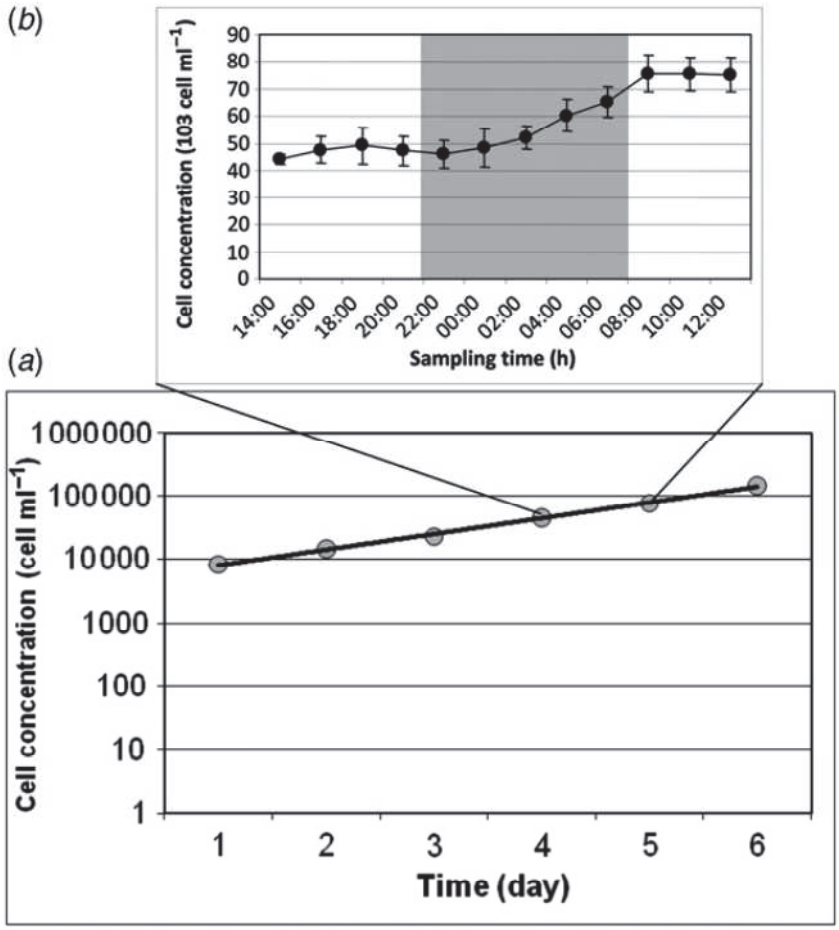

Figure 4. *C. polylepis* cell concentrations. (a) Mean ± SD (*n*=3) cell concentrations over a 6-day period in synchronized batch cultures (b) Mean ± SD (*n*=3) over the sampling period of 24 h. Shaded area indicates dark phase over the 24 h light-dark cycle.

3.5 Cell cycle analysis

Flow cytometric determination of the relative DNA content of *C. polylepis* nuclei revealed successive cell cycle phases typical of eukaryotic cells (Figure 5). Single distinct peaks for G1, S and G_2+M phases were obtained, respectively. The G_2 and M phases cannot be resolved by flow cytometry since cells in these two phases contain the same amount of DNA. As expected, *C. polylepis* cell division proceeded through the typical transitions of the eukaryotic cell cycle: DNA synthesis (S) began two hours before the beginning of the dark period and was completed two hours before the end of the dark period (Figure 5). Cell division (G_2+M)

started and was completed during the dark period. An increase in the number of cells in S and G_2+M phases was always synchronous with a decrease in the number of cells in G_1 phase.

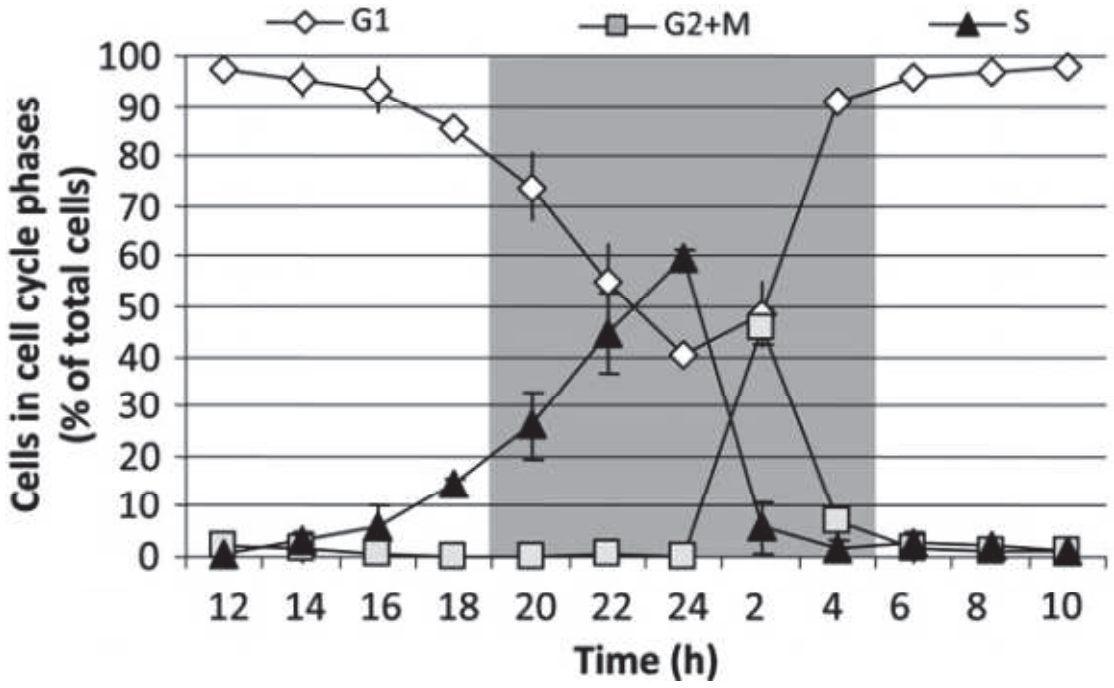

Figure 5. DNA concentration from three synchronized *C. polylepis* batch cultures. Percentage of cells in Gap 1 (G1), DNA synthesis (S), and Gap 2 and mitosis (G2+M) phases of the cell cycle. Data points are mean ± SD (n=3), shaded area: 10 h dark period.

3.6 PKS candidate genes and gene expression

A total of 13 potential PKS sequences were identified from the EST dataset. After analysis with the NRPS-PKS software, we identified seven fragments (PKS1, PKS2, PKS3, PKS4, PKS5, PKS6, PKS7) as parts of a KS domain, three fragments (PKS8, PKS9, PKS10) that code for a KR domain, and three fragments (PKS11, PKS12, PKS13) as fragments from an AT domain. To prove their origin the sequences were compared with known PKS sequences of several organisms. Confirmation of their domain function (data not shown) was achieved via analysis with the PhyloGena software (Hanekamp et al., 2007) for most ESTs of potential PKS. In particular, the PKS4 and PKS7 fragments were identified as nearly full length KS domains. The phylogeny of these sequences was analyzed with the data set of John *et al*. (2008) containing all available eukaryotic (PKS Type I) KS sequences. This analysis showed

that PKS4 and PKS7 fall into the haptophyte PKS gene clade. This topology is well supported by bootstrap analysis among PKS sequences of the haptophyte *Emiliania huxleyi* (Figure 6).

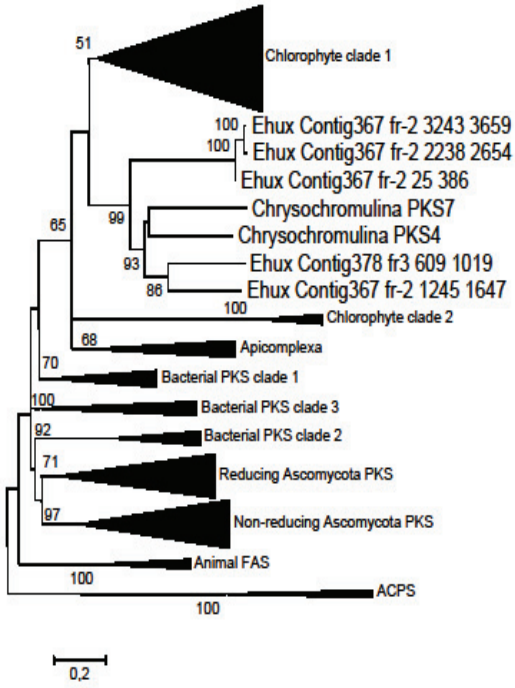

Figure 6. Maximum likelihood phylogenetic analysis of the ketoacyl synthase (KS) domain from the Type I polyketide synthases. Corresponding taxon names can be taken from John et al, 2008. Numbers on the branches indicate bootstrap values, scale bar represents corrected evolutionary divergence.

We chose PKS7 and the control genes GAPDH and cytochrome f for the gene expression studies, the latter two identified based on similarity among the ESTs (Table 4 panel a and b).

After demonstrating that PKS7 was a PKS fragment of *C. polylepis* origin, its expression profile over 24 h was determined by semi-quantitative PCR (Figure 7 and Table 4 panel c). Whereas GAPDH was mainly expressed during the light period (Figure 7b), on the contrary

PKS7 exhibited stronger expression towards the end of the dark period (Figure 7a). The expression of cytochome f remained constant over the light/dark cycle of the synchronized culture (data not shown).

Panel a				
GAPDH	**Species**	**Score**	**e-value**	**Accession**
GAPDH	*Zea mays*	304	3.00E-89	CAA33620.1
GAPDH	*Phaeodactylum tricornutum*	302	1.00E-87	AAU81889.1
GAPDH	*Odontella sinensis*	301	1.00E-88	AAU81890.1
Panel b				
cytochrome f	**Species**	**Score**	**e-value**	**Accession**
cytochrome f	*Emiliania huxleyi*	340	3.00E-92	YP_277330.1
cytochrome f	*Porphyra yezoensis*	283	5.00E-75	YP_536946.1
apocytochrome f	*Guillardia theta*	280	4.00E-74	AAC35685.1
Panel c				
PKS type I (PKS7)	**Species**	**Score**	**e-value**	**Accession**
polyketide synthase	*Microcystis aeruginosa*	276	1.00E-72	BAB12210.1
type I fatty acid synthase	*Cryptosporidium parvum*	275	3.00E-72	AAC99407.1
polyketide synthase	*Cryptosporidium parvum*	254	5.00E-66	AAN60755.1

Table 4. Analysis for orthologoues of GAPDH (panel a), cytochrome f (panel b), and polyketide synthase (panel c) of *C. polylepis* deduced from amino acid sequences.

4. DISCUSSION

4.1 Genome Characterization

Modern high-throughput sequencing projects have yielded a large number of fully sequenced genomes of organisms ranging from prokaryotes to humans, providing many new exciting insights into phylogenetic relationships and genetic diversity. A few whole genome projects on eukaryotic unicellular organisms (protists) have also been completed, e.g. for the prasinophytes *Ostreococcus tauri* (Derelle et al., 2006) and *Micromonas* (Worden et al., 2008), the marine diatoms *Thalassosira pseudonona* (Armbrust et al., 2004) and *Phaeodactylum tricornutum* (Bowler et al., 2008), *Emiliania huxleyi* (by Betsy Read, conducted by the US department of energy Joint Genome Institute (JGI, www.jgi.doe.gov) in

collaboration with the user community), and the slime-mould *Dictyostelium discoideum* (Eichinger et al., 2005).

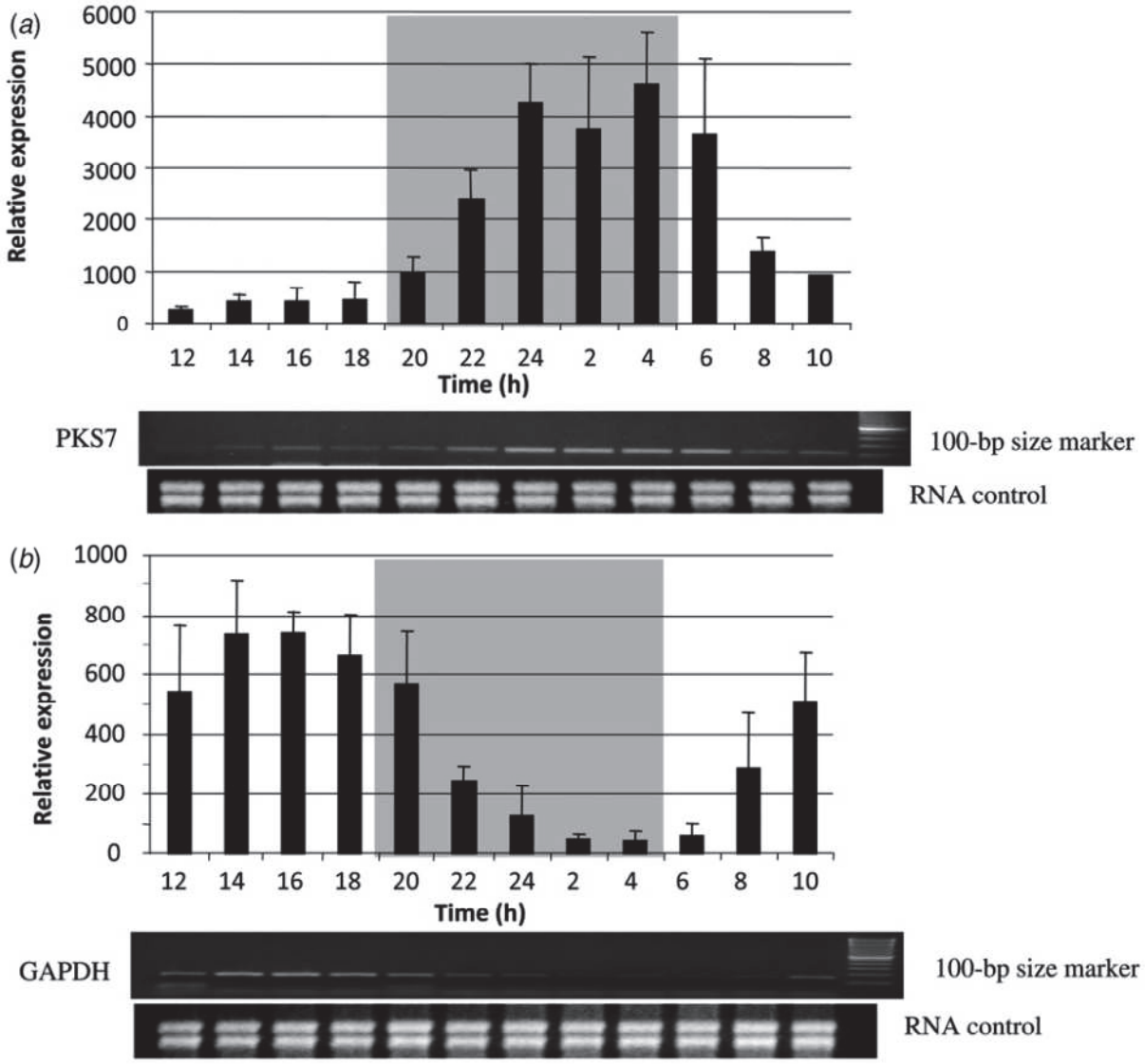

Figure 7. Mean ±SD of relative gene expression of *C. polylepis* in synchronized batch cultures for (a) PKS 7 (b) GAPDH. 18S and 28S rRNA bands used as control to demonstrate equal RNA concentration and quality. Relative gene expression was deduced from band intensities of semi-quantitative RT-PCR amp icons.

Other protist genome sequencing projects are in progress and several are close to completion. However, even with ever increasing sequencing capacities, not all organisms can be easily fully sequenced, because of limitations caused by large genome size and/or high

DNA repetitiveness. A further constraint is that the size, the DNA content, and the organizational structure of the genome are unknown for most protists.

EST data are probably the most extensively produced genetic data at present, providing a huge dataset from phylogenetically and evolutionary diverse organisms (e.g. Rudd 2003). Several EST datasets are available, mostly from diatoms and dinoflagellates, e.g. *Alexandrium fundyense* (Hackett et al., 2005), *Alexandrium ostenfeldii* (Jaeckisch et al., 2008), *Phaeodactylum tricornutum* (Scala et al., 2002), *Karenia brevis* (Lidie et al., 2005), *Fragilariopsis cylindrus* (Mock et al., 2006), *Emiliania huxleyi* (Wahlund et al., 2004), *Isochrysis* (Pereira et al., 2004), *Galdieria sulphuraria* (Weber et al., 2004), and *Prymnesium parvum* (La Claire, 2006), but the generalization of many more is in progress.

Knowledge about the genomic structure and organization of photoautotroph protists in general is scarce, with the exception of species with completed genome projects [*Thalassiosira pseudonana* (Armbrust et al., 2004), *Phaeodactylum tricornutum* (Bowler et al., 2008), *Ostreococcus tauri* (Derelle et al., 2006), *Micromonas* (Worden et al., 2008), *Cyanidioschyzon merolae* (Matsuzaki et al., 2004)]. The genomic data published for haptophytes comprises the genome of *Emiliania huxleyi* (predicted to be 150-200 Mb) http://bioinfo.csusm.edu/Coccolithophorids/Emiliana-huxleyi/ http://genome.jgi-psf.org/ and EST libraries from *Isochrysis* (Pereira et al., 2004) and from *Prymnesium parvum* (La Claire, 2006). In this study the predicted genome size of about 230 Mb for *Chrysochromulina polylepis* is much larger than that of *Arabidopsis thaliana* (125 Mb), *Paramecium tetraurelia* (150 Mb), *Chlamydomonas reinhardii* (120 Mb), *Phaeodactylum tricornutum* (27 Mb), *Thalassiosira pseudonana* (32 Mb), *Ostreococcus tauri* (12 Mb), and *Cryptosporidium parvum* (9 Mb). Yet, it is many times smaller than genomes of *Spirogyra* (1969 Mb), *Euglena* (1300 Mb) and *Fucus* (529 Mb), making it a possible candidate for whole genome sequencing. Moreover, since the closest relative so far characterized (*E. huxleyi)* has a

genome in the same size range, a comparative genomics approach seems to be not only feasible but also desirable. One possible reason for its relatively large genome size might be based on the heteromorphic haploid-diploid life cycle in the Class *Prymnesiophyceae*, whereas in its sister class *Pavlovophyceae* such as life cycle has not been documented to date and has a smaller genome size (Nosenko et al., 2007). Differences in genome size between the two classes are also mirrored in their plastid size (Sáez et al., 2001) and in the structure of mitochondrial genome in the two classes (Sánchez-Puerta et al., 2004).

The cell size of microalgae has significant impacts on their ecological success (summarized in von Dassow et al., 2008). There is current debate about the correlation between cell size and genome size (Gregory, 2001, Cavalier-Smith, 2005). This correlation apparently seems to fit dinoflagellates (LaJeunesse *et al*., 2005), diatoms (von Dassow et al., 2008) and cryptomonads (Beaton and Cavalier-Smith, 1999), but with its cell size of approximately 7 µm *C. polylepis* is smaller or in the same size range as the two diatoms (the centric *Thalassiosira pseudonana,* centric, 2.3-5.5 µm; pennate *Phaeodactylum tricornutum,* 3-25 µm) and the chlorophyte *Chlamydomonas* (~10 µm) (Merchant et al., 2007). Thus, a positive correlation between genome size and cell size is not always observed. Here we compared the EST data sets from *Isochrysis, Prymnesium*, *Emiliania* and *Chrysochromulina* to identify potential ESTs common between these haptophytes. Among many potential applications of ESTs, they can be used in reconstruction of phylogenetic relationships. In this approach, gene fragments are obtained via sequencing of randomly selected cDNA clones. Overlapping sequences are clustered, and Orthologues to known genes are determined by means of sequence similarity. These sequences are then employed to construct phylogenetic trees; for instance Bapteste et al. (2002) used ESTs to study the phylogenetic affinities of amoebans and related taxa. However, we used this comparative approach in order to identify additional candidate genes that might be involved in the biosynthesis and/or regulation of

toxins and their respective toxicity. We found that 303 ESTs were common to *C. polylepis* and *P. parvum*, many of which genes had unknown function but included the PKS genes of both species. Comparative genomic approaches can help to elucidate candidate genes among a large set of ESTs particularly if the majority is of unknown function and the biochemical pathways of interest are at least partially undiscovered and described. We are aware that we compared only a limited EST dataset of only three species and in future more ESTs per species and more species in our comparison will increase significantly the potential success of our approach. Nevertheless, this is a good starting point when exploring non-model organisms and unknown biochemical pathways and their regulation.

In our genomic analysis we placed particular emphasis on the identification and characterization of putative polyketide synthase (PKS) in *Chrysochromulina* because of the relatively common occurrence of polyether toxins among toxigenic protists, particularly in dinoflagellates, and which are likely derived via polyketide biosynthetic pathways. Recent studies have shown that apicomplexans, close relatives of the dinoflagellates, have the modular Type I PKS (Zhu et al., 2002) and phylogenomic analysis indicates that this modular type is typical of protists (John et al., 2008). However, with non-axenic cultures, it is difficult to prove whether or not sequences generated are indeed of protist origin and not from potential bacterial contamination (Snyder et al., 2003, 2005; Cembella and John 2006). This is true in particular for PKS genes from protists because they have been shown to belong exclusively to the modular PKS I Type (John et al., 2008).

Working with degenerate primers, Snyder et al., (2003) managed to amplify fragments from non-axenically grown *Karenia* brevis cultures, showing homology to putative Type I or II PKS genes. Yet, whether this gene was derived from the dinoflagellates or associated bacteria was unclear. Nevertheless, the presence of at least one PKS gene in the toxic dinoflagellate *Karenia brevis* was proven by the same author (Snyder et al., 2003). To

circumvent the problems associated with the use of degenerate primers for PCR approaches, Monroe and van Dolah (2007) managed to isolate full-length sequences of single catalytic domains from PKS genes from the same organism, by screening cDNA libraries. They claim that the sequence is most similar to Type I modular PKS, but that the structure is most similar to Type II (for review see Hertweck et al., 2007). Fragments isolated from the dinoflagellate *Amphidinium* sp., which produces the polyketide amphidinolide, also showed similarity to β-ketoacyl synthase (KS), acyl transferase (AT), dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP) and thioesterase (TE) in known Type I PKS (Kubota and Kobayashi, 2006).

Dinoflagellate genes are known to contain a unique trans-splicing leader sequence, which can now be used to discriminate a dinoflagellate-specific gene from a mixed culture (Zhang et al., 2007; Zhang and Lin 2008; Lidie and van Dolah, 2007; Monroe and van Dolah 2008). Whether a similar mechanism is present in haptophytes such as *Chrysochromulina* is unknown. In any case, phylogenetic analysis of the ketoacyl (KS) domain based upon the sequences of PKS 4 and PKS7 from *Chrysochromulina* showed definitive phylogenetic associations. Both sequences clustered among those of *E. huxleyi* (John et al., 2008) and therefore formed a monophyletic haptophyte clade (Figure 6). The PKS genes of *C. polylepis* belong to the Type I group, as is the case for *K. brevis*, but whereas the structure of the dinoflagellate PKS, with its discrete catalytic domains, suggests a novel Type I-like PKS gene, *C. polylepis* seems to exhibit the conventional modular Type I structure. We cannot totally exclude the possibility of recent horizontal gene transfer of the PKS genes into *Chrysochromulina*, but given their phylogenetic clustering within the haptophyte clade this is unlikely.

4.2 Gene expression and regulation in synchronous growing cultures

Knowledge of the genetic regulation of toxin synthesis may help us to understand the environmental conditions favoring toxicity and the ecological relevance of many of those substances. We studied gene expression in synchronized cultures, which are useful in order to enhance the signal from induced toxin production (Pan et al., 1999; John et al., 2001; Eschbach et al., 2005) and provide insights into regulation because the induction of a given biosynthetic pathway is phased to the cell division cycle and essentially occurs at the same time point for all cells in a culture. A high degree of synchronization is necessary to allow for clear identification of cell cycle stages in following temporal changes in gene expression throughout a 24 h sampling period. In practice, the induction of synchronous (as opposed to merely phased) cell division by manipulation of the photoperiod is only possible when the length of the cell division cycle closely approximates the length of the photoperiod (1 division per day). Partial synchronization in *C. polylepis* was obtained by the sequential inoculation of increasingly larger culture volumes of cells from early exponential growth phase preconditioned to a 14:10 h light-dark regime.

The cell cycle of *C. polylepis* has previously been analyzed by flow cytometry (Edvardsen, 1996, Eschbach et al., 2005). In our experiments, cell cycle analysis was accomplished by quantifying the amount of DNA in fixed cells (Grey et al., 1990), but we used an improved method based on glutaraldehyde fixation (Eschbach et al., 2001). At the beginning of our experiments, *C. polylepis* cultures were in quasi-steady state (roughly balanced growth) and the cells passed through S and G2+M phases during the dark period (Figure 6). Mitotic division in the dark is typical for many microalgae (Taroncher-Oldenburg et al., 1997; John et al., 2001; Farinas, et al., 2006), although there are exceptions to this rule, particularly for shade-adapted benthic species (Pan et al., 1998). In a previous study it was shown that during the light period, all *C. polylepis* cells accumulated in G1 phase during which growth and other

metabolic functions, such as chlorophyll and toxin biosynthesis, were carried out (Eschbach et al., 2005).

The vast majority of phycotoxins are polyethers that are most likely derived via polyketide synthases (Wright and Cembella 1998). As described above, ichthyotoxicity and cytolytic effects on cells and tissues caused by exposure to *P. parvum* led to the speculation that they may be caused by similar toxins (John *et al*., 2002). Among the prymnesiophytes, the prymnesins (PRM1 1 and PRM 2) isolated from *P. parvum* are the only structurally described toxins (Igarashi et al., 1998). In most cases thus far the heterologous expression of PKS genes has been studied for drug discovery or production (Schümann and Hertweck 2006), or for investigation of differential expression patterns in different tissues or species (Lopez-Erraquin et al., 2007, Karppinen and Hohtola 2008). We expect that there are studies currently underway to examine the expression of PKS genes under various environmental stimuli in both lower eukaryotes and bacteria, but nothing is published to our knowledge. The only related work we are aware of is on the biosynthesis of the cyclic heptapeptide regulated by a peptide-PKS system via the *mcy* gene cluster in the cyanobacterium *Microcystis aeruginosa* (Kaebernick et al., 2000). In the cyanobacterium the *mcy* mRNA levels were shown to increase during early and mid-exponential growth phase in a light dependent manner.

We previously showed that the toxicity of *C. polylepis* increased at the transition from dark to the light phase (Eschbach et al., 2005). Here we demonstrated that the PKS genes in *C. polylepis* expressed increased transcript levels in the dark phase. These two observations correlated nicely and suggested that PKS genes may indeed be linked to toxicity in this species. We caution, however, that this cannot be causally demonstrated because the chemical structures of the *C. polylepis* toxins are unknown and toxicity was not measured within this study. Based upon bioassay responses and conjecture regarding their mode of action, these toxins may be analogous or homologous to the mixed polyether prymnesins found in the

related species *Prymnesium parvum*, but this remains to be established. Furthermore, we found several copies of different putative PKS genes, of which at least two are encoded in the *C. polylepis* genome. Thus, it is not clear which (if any) particular PKS gene products are responsible for toxicity. Moreover, the toxin cell quota could be regulated at several steps, involving transcription, mRNA stability, translation, and protein activity, therefore the increase in transcript levels is not necessarily directly linked to an increase in toxin levels (Cembella and John, 2006). In future studies, detailed analyses combining toxin/toxicity measurements (analytical or via bioassay) and molecular genetic approaches will further elucidate insights into the expression and regulation of *C. polylepis* toxin production and its underlying processes.

Compared to the well-studied Opisthokonta and higher plant clade, molecular analysis in protists is to date still limited. Particularly for prymnesiophytes, molecular and physiological data are scarce. Therefore, the molecular approach we present here is a first step which gives insights into genes involved in toxicity and growth control and is a starting point for elucidating genome properties and the complex life cycle of *C. polylepis* and probably other prymnesiophytes. Beside the fact that this is the first study of PKS gene expression in microalgae, we generated interesting insights into the characteristics of the *C. polylepis* genome. With its approx. 230 MB genome size and its evolutionary relationship to *Emiliania huxleyi,* it is a perfect candidate for a future comparative genomics approach.

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CHAPTER II

Effects of physiological shock treatments on toxicity and polyketide synthase gene expression in *Prymnesium parvum* (Prymnesiophyceae)

ABSTRACT

The toxic prymnesiophyte *Prymnesium parvum* has been linked to massive fish kills worldwide. The toxic blooms have been shown to be derived from a seed population, abiotic conditions such as temperature, salinity and water turbulence having encouraged massive growth of the *Prymnesium* population. Within its highly fluctuating coastal marine niche, *P. parvum* must acclimate rapidly to changing conditions. Although studies on the physiology and toxicity of *P. parvum* exist, examination of gene expression in such analyses is novel. In this study we investigate (1) relative toxicity (intracellular vs. extracellular) and (2) differential gene expression of three polyketide synthase (PKS) transcripts via real-time PCR (qPCR). It was hypothesized that these genes play a role in the production of the toxic compounds prymnesin-1 and -2 produced by *P. parvum*. We found that low salinity shock and high irradiation shock increase different aspects of *Prymnesium*'s toxicity (intra- vs. extracellular). Furthermore, we found that these two physiological shock treatments induced higher copy numbers in selected polyketide synthase genes (PKS) genes, suggesting a connection between toxicity and the PKS biosynthetic pathway. Our results demonstrate that PKS genes are likely to play an important role in the toxicity of *P. parvum* and deserve further investigation in populations exposed to changing environmental conditions.

1. INTRODUCTION

The toxigenic haptophyte *Prymnesium parvum* is a mixotrophic species that occurs worldwide (Moestrup, 1994). Blooms of *P. parvum* are associated with massive fish-kills (Edvardsen & Paasche, 1998): the cells produce substances that are directly associated with ichthyotoxicity (lysis of gill cell-membranes) and also lyse both prokaryotic and eukaryotic single-celled organisms (Yariv and Hestrin, 1961; Tillmann, 2003). The effects of both abiotic and biotic factors on the toxicity of *P. parvum* have been extensively studied. Biotic effects include an increase in lytic activity towards the small cryptophyte *Rhodomonas salina* in the presence of the dinoflagellate *Oxyhrris marina* (Tillmann, 2003), while abiotic effects have been shown to cause variations in observed toxicity, suggesting that several compounds may be responsible (Shilo, 1967; Granéli et al., 1998, 2008; Larsen and Bryant, 1998; Baker et al., 2007). Blooms of *P. parvum* are often found in coastal or brackish water areas, where salinity and nutrient availability tend to fluctuate and similar changes may play a role in the variations in toxicity observed in laboratory experiments (Baker et al., 2007). *Prymnesium parvum* can inhabit a wide range of environments and this flexibility may provide a competitive advantage over other microalgal species and lead to the wide distribution of reported *P. parvum* fish kills.

In an effort to characterize the compounds involved in the toxicity of *P. parvum*, Igarashi et al., (1999) revealed the existence of two polyether compounds, prymnesin-1 and prymnesin-2, which possess similar biological activities. Their description prompted interest in polyketide synthase (PKS) enzymatic pathways and their potential role(s) in toxic processes, in both *P. parvum* (John et al., 2008; La Claire, 2008) and other protists (John et al., 2008, 2010; Kellmann et al., 2010). Polyketides are a family of secondary metabolites whose carbon skeleton is formed through sequential condensation reactions of acyl-coenzyme

A (coA); their biosynthesis is therefore related to that of fatty acid compounds (Staunton and Weissmann, 2001; Crawford et al., 2006). Of the known protist PKS enzymes, many have been shown to belong to the same class of biosynthetic pathways, and most marine microalgal species studied so far exhibit two or more functionally different PKS genes (La Claire, 2006; John et al., 2008; Worden and Lee, 2009; Monroe et al., 2010). As PKS biosynthetic pathways have previously been shown to be involved in brevetoxin (Monroe et al., 2010) and spirolide production (MacKinnon et al., 2006), it seems likely that they may also play a role in the biosynthesis of toxic compounds in *P. parvum*. However, no structural information is available concerning the extracellular substances involved in the allelopathic activity of *P. parvum*.

Physiological 'shock' responses have been demonstrated in many species of bacteria in response to a wide variety of extreme or changing environmental conditions (Grzadkowska and Griffiths, 2001). In marine microalgae, hypo-osmotic stress has been shown to induce responses primarily related to impaired photosynthetic capacity (Kirst, 1989). However, other cellular processes may respond differently to disruptions in cellular equilibrium. Our experiments were designed to simulate rapidly changing environmental conditions through a series of 'shock' experiments, in which we monitored the response of *P. parvum* in terms of changes in gene expression and altered cell physiology. Such studies are crucial in explaining how cells cope with stress (Vilaprinyo et al., 2006). Our specific objective was to study the effect of short term 'shock' treatments on the toxicity of *P. parvum* and on the differential expression of three PKS genes obtained from a non-normalized cDNA library constructed by La Claire (2006). We use 'toxicity' to refer to both extracellular/secreted lytic compounds (assessed via a *Rhodomonas baltica* assay) and intracellular lytic compounds (using an erythrocyte lysis assay).

2. MATERIALS AND METHODS

2.1 Culture conditions and experimental set-up

We used a toxic clone of *P. parvum*, RL10, isolated in 1993 by Aud Larsen in the Sandsfjord, Norway (Larsen and Bryant, 1998). It was grown in 5 -l stock cultures in IMR medium (Eppley et al., 1967) at a salinity of 26 PSU, which were gently aerated with filter-sterilized air. Cultures were grown to a concentration of 4.61×10^3 cells ml^{-1}, at a constant temperature of 20°C and a light:dark photocycle of 14:10 h. The photon flux density, which was measured inside the flask by a QSL-100 Quantum Scalar Irradiance Meter (Biospherical Instruments, San Diego, USA), was kept at 90 mmol photons m^{-2} s^{-1}. Cell concentrations were determined daily using a CASY cell counter (Innovatis, Reutlingen, Germany) to confirm that the cells were in the exponential growth phase. From the initial stock culture, 400-ml cultures were inoculated at starting concentrations of $1.5 \times 10^3 \pm 123$ cells ml^{-1} (mean ± SD) and grown under identical conditions as the stock culture (except that smaller batch cultures were not aerated) to a concentration of $3.75 \times 10^4 \pm 1325$ cells ml^{-1} (mean±SD).

Treatment	Description
control	20°C, 90 µmol photons m^{-2} s^{-1}, 26 PSU
25°C	25°C, 90 µmol photons m^{-2} s^{-1}, 26 PSU
5°C	5°C, 90 µmol photons m^{-2} s^{-1}, 26 PSU
turbulence	aeration, 20°C, 90 µmol photons m^{-2} s^{-1}, 26 PSU
16 PSU	20°C, 90 µmol photons m^{-2} s^{-1}, 16 PSU
high light	20°C, 700 µmol photons m^{-2} s^{-1}, 26 PSU
dark	20°C, 0 µmol photons m^{-2} s^{-1}, 26 PSU

Table 1. Control and physiological shock conditions for replicate 400 ml batch cultures.

This cell concentration was crucial, because we could be sure that (1) the cells were still exponentially growing and (2) there would be sufficient material for downstream analysis. At this point, 400-ml batch cultures were separated and 'shocked' for 2 h: a summary of the physiological 'shock' conditions is given in Table 1.

All experiments were carried out in parallel in triplicate, with a single control for all samples. Shock parameters were chosen based on the known tolerances of *P. parvum* (from Edvardsen and Paasche, 1998; Granéli et al., 1998, 2008; Larsen and Bryant, 1998; La Claire, 2006).

2.2 Intracellular toxicity/biosynthesis of lytic compounds: erythrocyte lysis assay

All erythrocyte lysis assays were performed with biological triplicates as previously described by Eschbach et al. (2001). For each flask of the biological triplicates, technical triplicates were tested to achieve a reliable measurement. $1.0x10^7$ *P. parvum* cells from each treatment were harvested via centrifugation (as described for the RNA extraction), and the resulting pellet resuspended in 1ml of assay buffer (150mM NaCl, 3.2mM KCl, 1.25mM MgSO4, 3.75mM CaCl2 and 12.2mM TRIS base, the pH being adjusted to 7.4 with HCl: Eschbach et al., 2001). The resuspended pellets (each containing $1.0x10^7$ *P. parvum* cells) were then sonicated with a Sonoplus HD70 disintegrator equipped with a MS73 sonotrode (Bandelin Electronics, Berlin, Germany) using the following settings: 50% pulse cycle, 70% amplitude for 1 min on ice. During the whole preparation procedure, algal extracts were kept on ice in the dark. Lytic activity was calculated in saponin equivalents, utilizing the standard saponin from higher plants as an indicator of relative lytic capacity.

2.3 Extracellular and/or secreted toxicity: Rhodomonas baltica bioassay

Rhodomonas baltica is a cryptophyte that is commonly used for measurement of the lytic capacity of structurally unconfirmed compounds, such as those produced by *P. parvum*. A dose–response curve is typically established, and an EC50 value is calculated, indicating the concentration of *P. parvum* at which 50% of all *Rhodomonas* cells are lysed within the experimental system. This assay was performed in this study for both biological and technical triplicates, as described by Tillmann et al. (2008). In brief, 4 ml portions of mixtures of *P. parvum* (final cell concentrations in decreasing order: $3.75x10^4$ ml^{-1}, $2.34x10^4$ ml^{-1}, $9.38x10^3$ ml^{-1} and $4.69x10^3$ ml^{-1}) and *R. salina* (final cell concentration $1.0x10^4ml^{-1}$) were incubated in glass scintillation vials at 15°C for 24 h in darkness. Vials were then gently mixed by rotation and 1ml of mixture pipetted into an Utermöhl cell-sedimentation chamber and the cells fixed with glutaraldehyde (2.5% final concentration). After settling, cells were viewed via epifluorescence microscopy (Zeiss Axiovert 2 Plus, Carl Zeiss AG, Göttingen, Germany) at a total magnification of 64x using the Zeiss filter-set 14. Lysed cells were easily distinguishable from non-lysed cells by pigment autofluorescence characteristics (*Prymnesium* autofluoresces red, *Rhodomonas* orange). Control *Rhodomonas* samples in triplicate represented 0% lysis, and lytic capacity for all samples incubated with *Prymnesium* were calculated based on this control value, as percentage *Rhodomonas* cells lysed.

2.4 Statistical significance and standard deviation

For single data points originating from both bioassays as well as between treatments in the gene expression portion, a student's t-test was used with a cut off of $P < 0.05$ to identify significant differences in observed toxicity and transcriptional regulation between physiological treatments and the control. The error bars in Figures 1, 3–5 represent the standard deviation between biological, not technical, replicates.

2.5 Total RNA isolation

Physiologically shocked biological triplicate cultures were centrifuged at 3000x*g* for 15 min at 20°C. The supernatant was decanted, and the remaining cell pellet resuspended in 350 ml of RLT lysis buffer containing β-mercaptoethanol (Qiagen, Hilden, Germany) and subsequently flash-frozen in liquid nitrogen. Samples were then stored at -70°C to minimize activity of potential RNase and prevent degradation. Total RNA was isolated from all samples using Qiagen Plant RNeasy extraction kits (Qiagen, Hilden, Germany), according to the manufacturer's protocol. An additional in-tube DNAse treatment was included, to facilitate downstream qPCR processing of samples. RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany) and the purity estimated by the 260/280 and 260/230nm absorption ratio (all ratios $1.9 < x < 2.1$). RNA integrity was verified using the lab-on-a-chip Bioanalyzer 2100 system (Agilent Technologies, Böblingen, Germany). RNA of an appropriate quality was obtained for all samples, with the exception of the dark treatment.

2.6 In vitro transcription and cDNA synthesis

Complementary to the gene expression analysis, we compared three typically used housekeeping genes (ubiquitin, GAPDH and actin) with two genes from the butterfly *Pieris rapae* (the 'small cabbage white'), namely major allergen (MA: GenBank accession EU265818) and nitrile specifier protein (NSP: GenBank accession EU265817). These two genes show no sequence similarity to any accession outside the Lepidoptera (Fischer et al., 2008) and therefore functioned to (1) normalize cDNA synthesis reaction efficiency and (2) provide a baseline expression value, similar to the function of traditional housekeeping genes. Plasmid vectors (pDNR-Lib) containing full-length cDNAs of both MA and NSP genes approximately 1.9 kb in size were constructed using an EST database and cDNA library

(Fischer et al., 2008) and served as template in PCR reactions to obtain the corresponding DNA fragments. PKS gene sequences were obtained from a non-normalized cDNA library constructed for P. parvum (La Claire, 2006). The sequences originate from expressed sequence tags (ESTs) and there is low homology amongst them. The GenBank accession numbers are DV101611 (for PKS1 6t3), DV099189 (for PKS 2 7t3) and DV101662 (for PKS 3 81t3). All primers used in this study were designed using Primer Express version 2.0 (http:// www.appliedbiosystems.com), using the default settings, and synthesized by MWG biotechnologies (Germany).

In vitro transcription was performed using a T7 RNA polymerase (Invitrogen, Paisley, UK), according to the manufacturer's protocol, to obtain mRNA for both MA and NSP. MA was added at a final concentration of 116 pg μl^{-1} and NSP at 10 fg μl^{-1}. cDNA was synthesized from 500 ng total RNA of all samples with the Omniscript RT kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, using anchored oligoVN(dT)20 primer (Invitrogen, Paisley, UK) at a final concentration of 25 ng μl^{-1}.

2.7 Target gene selection and qPCR

For normalization of the three target PKS genes, we used the two 'foreign' internal reference genes and the three commonly accepted housekeeping genes already mentioned: target genes are listed in Table 2.

The ratio of the amount of target gene mRNA to the amount of housekeeping gene mRNA was analyzed via a SYBRgreen qPCR reaction, designed according to manufacturer's protocol (Applied Biosystems, Darmstadt, Germany) and using 2 µl of 10-fold diluted cDNA. qPCR reaction details are given in Table 3.

Gene	Sequence F(5´-3´)	Sequence R(5´-3´)	Gene name	Amplicon
MA	AAGAGTGGCCAGCACAGTAGACA	AGCTGCCTCCTTGGAAGCATA	major allergen	100 bp
NSP	TTGACCACTACCCACGGATGA	ACGATCAATCCAGTATGCAACAA	nitrile specific protein	100 bp
GAPDH	GTTGAGGCCGCAGCAATTAC	ATTCGCGTCTTTTCTCCCATAC	GAPDH	150 bp
Ubiquitin	CTCAATGTTCGGGTCAGCAA	CTGGACGGCAAAGTCTGCAT	Ubiquitin	150 bp
Actin	GCTGATGTTCGAGACGTTCCA	ATCGCATAGCACTCGT	Actin encoding	150 bp
PKS 1	CGGAAGCTATCCTTCGTTTCA	TGCGCTGGACACGAAGTC	PKS 1	150 bp
PKS 2	GCTCGGAAGCTATCCTTCGTT	GCGCTGGACACGAAGTCAA	PKS 2	150 bp
PKS 3	CGATCACACCGCTTTCCTTT	TCGTTGTACTGCGAGCACATG	PKS 3	150 bp

Table 2. Target genes investigated in this study, primer sequence, and gene name and amplicon or product length

Cycle parameters included an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 59°C for 1 min. A product–primer dissociation step was utilized to verify formation of a single unique product and the absence of potential primer dimerization. All reactions were performed with the same ABI Prism 7000 cycler (Applied Biosystems, Darmstadt, Germany). Amplification efficiency of all qPCR reactions was analyzed through linear regression of standard curves, with six cDNA (originating from the control culture) serial dilution points ($1.0x10^{-3}$ to $1.0x10^{-8}$). Amplification efficiency (E) was calculated from the slope of the threshold cycle (Ct) vs. concentration [cDNA] curve according to the following equation:

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

All amplification efficiencies were 98.88% > x > 92.31% 1.91, all R^2 were > 0.94 (Table 4). All samples were run in biological triplicates (independent cultures) and technical replicates were measured for each biological sample. Means and standard deviation were calculated between biological replicates, after averaging of the technical replicates. An expression ratio

was calculated using the $\Delta\Delta C_t$ as described by Pfaffl (2001), incorporating individual reaction efficiencies as correction factors, according to equation (II)

Expression Ratio = E_{target}^(ΔC_t target(control- sample))/E_{MA}x^($\Delta C_{t\ housekeeping}$(control-sample))

Component	Volume
Primer F (10 µM concentration)	0.25 µl
Primer R (10 µM concentration)	0.25 µl
Power SYBR green PCR master mix*	10 µl
Water	7.5 µl
cDNA template (diluted 1:5 [v:v])	2 µl
Total volume:	**20 µl**

Table 3. Components of qPCR reactions for all samples.

We chose this method of quantification in order to minimize intra- and inter-assay variability, and to aid in a robust comparison between normalization (housekeeping) genes, as well as internal reference genes tested. All calculations were performed using the REST-2009 software platform (Qiagen, Hilden, Germany). An additional Log_2 transformation of the data was also performed, which can account for the negative values displayed in Figures 3–5.

Gene	*%* efficiency	r^2
MA	98.88	0.9823
NSP	98.31	0.9991
GAPDH	95.41	0.9994
Ubiquitin	92.31	0.9998
Actin	96.49	0.9961
PKS 1 6t3	95.83	0.9987
PKS 2 7t3	94.84	0.9992
PKS 3 81t3	95.53	0.9996

Table 4. Efficiency of individual primer pairs in qPCR reaction, as derived from standard curves using PCR products as template.

2.8 Evaluation of reference gene stability via geNorm and NormFinder

To determine differences in stability (variation) between internal reference (MA & NSP) and housekeeping genes, we utilized two previously described algorithms: geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). geNorm uses a pairwise correlative approach. NormFinder is an algorithm that attempts to find the optimum reference genes out of a group of candidate genes. It can also, in contrast to geNorm, take information about groupings of samples into account. The result is an optimal reference gene or pair of genes. The resulting pair might have compensating expression, so that one gene is slightly overexpressed in one group, but the other gene is correspondingly under-expressed in the same group (Andersen et al., 2004). Applying differential ranking approaches, we deemed these two separate algorithms comparable and suitable for our study due to the fact that reference/housekeeping genes should display nondifferential expression across treatments.

3. RESULTS AND DISCUSSION

3.1 Intra- and extracellular toxicity

Prymnesium parvum toxins or toxicity cannot be measured with commonly used phycotoxin analytical chemical detection methods, such as chromatography or mass spectrometry, due to the unconfirmed nature of the compounds involved. We therefore applied two separate bioassays in order to be able to follow changes in toxicity of *P. parvum* under different shock treatments.

a

Treatment *P. parvum* strain RL10	EC_{50} *Rhodomonas baltica*
25° C	$4.1x10^4$ cells ml^{-1} ± 2045
5° C	$9.2x10^4$ cells ml^{-1}± 4732
control	$3.9x10^4$ cells ml^{-1}± 1854
turbulence	$6.8x10^4$ cells ml^{-1}± 2989
16 PSU	$1.3x10^4$ cells ml^{-1}± 789
high light	$8.0x10^4$ cells ml^{-1}± 3689
dark	$5.8x10^4$ cells ml^{-1}± 3125

b

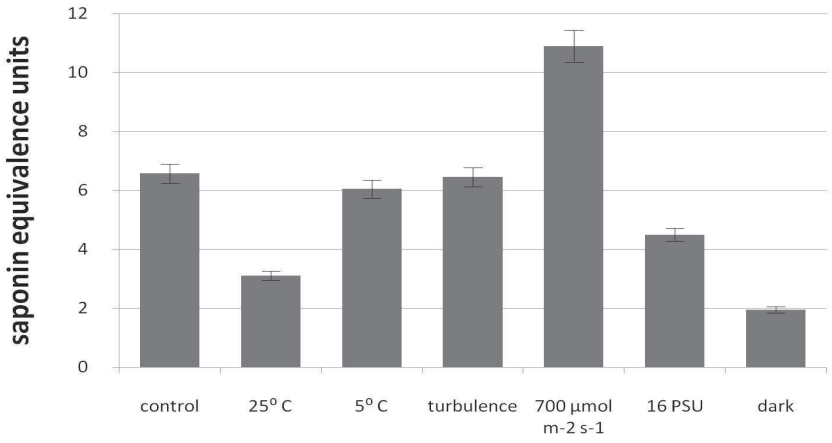

Figure 1a. Results of the Rhodomonas baltica assay, shown as EC50 values, higher numbers mean lower toxicity. 16 PSU shock caused largest increase in extracellular toxicity. b Results of the erythrocyte lysis assay. Light shock treatment (700 µmol $m^{-2}s^{-1}$) showed the highest lytic effect on erythrocytes. Mechanical stress showed the same effect as the control culture, while the remaining treatments show a decrease in lytic capacity on erythrocytes. All shock treatments were performed for a duration of two hours.

Using an erythrocyte lysis assay (ELA) as a measure of intracellular toxicity, we found high light to induce the largest significant (*t*-test, $P < 0.05$) increase in lytic capacity relative to the control culture Figure 1a). 25°C and dark shocks induced significant (*t*-test, $P < 0.05$) decreases in lytic capacity, to 50% or less of that in the control (Figure 1a). 5°C and turbulence shocks did not induce significant changes in lytic capacity (*t*-test, $P < 0.05$) relative

to the control. 16-PSU shock induced a slight yet significant decrease in lytic capacity (*t*-test, $P < 0.05$). It has previously been demonstrated that light increases the cell quota or rate of production of 'icthyotoxin' in *P. parvum* (Shilo and Aschner, 1953). Another study examined activity of extracted toxic substances from *P. parvum* following exposure to light (Parnas et al., 1962), showing that the activity of toxin extracts (both intra- and extracellular) decreased over time. Despite these findings, Parnas et al. (1962) were unable to attribute the observed results to a specific cause. Photosynthesis is probably necessary as an energy source in the production of secondary metabolites in *P. parvum*, and therefore the experimental treatment time of two hours in Parnas et al.'s study might have been sufficient to allow some degree of acclimation to high light (Pan et al., 1996). Furthermore, the toxins may be energetically costly to synthesize, as is the case for many secondary metabolites. For many toxigenic algal species, the effect of light exposure has been linked to variations in toxin production, e.g. in *Alexandrium catenella* (Proctor et al., 1975), *Pseudo-nitzschia multiseries* (Bates et al., 1991) and, more relevantly, also in *Prymnesium parvum* (Shilo et al., 1971).

Using a *Rhodomonas baltica* assay, we measured the extracellular or secreted toxicity, which may or may not be related to the internal toxicity. Prymnesins have been described to interact directly with exposed cell membranes, compromising integrity and permitting ion leakage through selective permeation (Manning and La Claire, 2010). Furthermore, prymnesin toxicity is known to be dose-dependent, and to respond in a linear manner when analyzing changes in membrane conductance after exposure to these compounds (Manning and La Claire, 2010). The mechanism by which these compounds are secreted, however, has yet to be described. The effects observed in the *R. baltica* assay are those that will have an impact on allelochemical interactions, since potential grazers and/or competitors can be affected.

In general the cryptophyte *R. baltica* responded differently to *P. parvum* cells from different shock treatments, indicating changes in extracellular toxin secretion by *P. parvum*. The light-shock treatment showed approximately 50% less toxicity (*t*-test, $P < 0.05$) when compared with the control cultures (Figure 1b). The dark-shock treatment also showed a significant decrease (*t*-test, $P < 0.05$) in lytic capacity (EC_{50} $5.8x10^4$ cells ml^{-1}) compared with the control. On the other hand, low-salinity shock increased the secretion of *P. parvum* toxin, resulting in high allelopathic effect towards *R. baltica*. However, no significant increase of the intracellular toxicity of *P. parvum* was observed as compared with the control culture (Figure 1a). These results are consistent with the findings of Parnas et al. (1962), who claimed that the ichthyotoxicity of *P. parvum* is inversely proportional to salt concentrations. Furthermore, Ulitzer and Shilo (1964) found that uptake of trypan blue (indicating cell permeability and hence toxicity) in the gills of fish was decreased after exposure to increased saline conditions.

	ELA (intracellular)	Rhodomonas test (extracellular)
Treatment	↓	↓
25°C	↓	↓
5°C	↓	↓
Turbulence	↑↓	↓
16 PSU	↓	↑
High light	↑	↓
Dark	↓	↓

Table 5. Overview of changes in observed toxicity, as compared with control. arrows indicate increase (↑) or decrease (↓) in toxic effects. (↑↓) indicates no significant change from the control

To our knowledge, this is the first work where intracellular and extracellular compounds have been measured at the same time. We think it is vital to differentiate between the two types of toxicity, intracellular and extracellular, because differences in the two bioassays (highlighted in Table 5) strongly suggest differences in the biosynthesis and secretion of toxin(s), which may be linked to such cellular processes as chemical signaling or recognition of signaling molecules. Thus, for example, a short intense light treatment

increased the intracellular toxicity of *P. parvum* cells, whereas a low salinity shock treatment increased the amount of extracellular secreted toxin (Table 5). It is possible that intracellular-produced toxin loses activity after reaching the cell surface, due to degradation by exposure to light. This would correspond with the results obtained by Parnas et al. (1962). The other shock treatments showed changes in toxicity as well (Table 5), but could not be correlated with changes in gene expression, rendering these results less conclusive in discussions of the involvement of PKS genes in toxin production in *P. parvum*. It must also be considered, however, that a decrease in extracellular salinity may lead to compromised cell membranes, leading to toxin leakage. The difference between active secretion and leakage through a compromised membrane has yet to be established in *P. parvum*.

3.2 Polyketide synthase gene expression analysis

The evaluation of differences in gene expression using a real time PCR approach (qPCR) required data normalization, which is a crucial step for gene transcript quantification analysis (Pfaffl, 2001; Bustin, 2002). The reliability of any relative qPCR experiment can be improved by including an invariant internal control (reference gene) in the assay to correct for sample to sample variations in qPCR efficiency and errors in sample quantification (Siebert and Larrick, 1992; Bustin, 2000). qPCR-specific errors in the quantification of mRNA transcripts are compounded by any variation in the amount of starting material between the samples, e.g. caused by sample-to-sample variation, variation in RNA integrity, cDNA synthesis efficiency differences or cDNA sample loading variation (Stahlberg, 2003, 2004a, 2004b). The use of housekeeping genes as endogenous controls can be problematic due to differential expression across treatments, hindering an intercomparison. We therefore incorporated mRNA from foreign spike genes into our samples to ensure stable copy numbers for endogenous

normalization across all samples. After analyzing the stability of the candidate reference genes (where the lower the 'M' variability value, the more stable the gene is), we determined both MA and NSP to be generally more stable than the housekeeping genes analyzed (Figure 2). NSP was the more stable, with a Normfinder M-value of 0.004, compared with MA with a Normfinder M-value of 0.016 (Figure 2). The geNorm and NormFinder algorithms gave similar M-value rankings for the genes investigated.

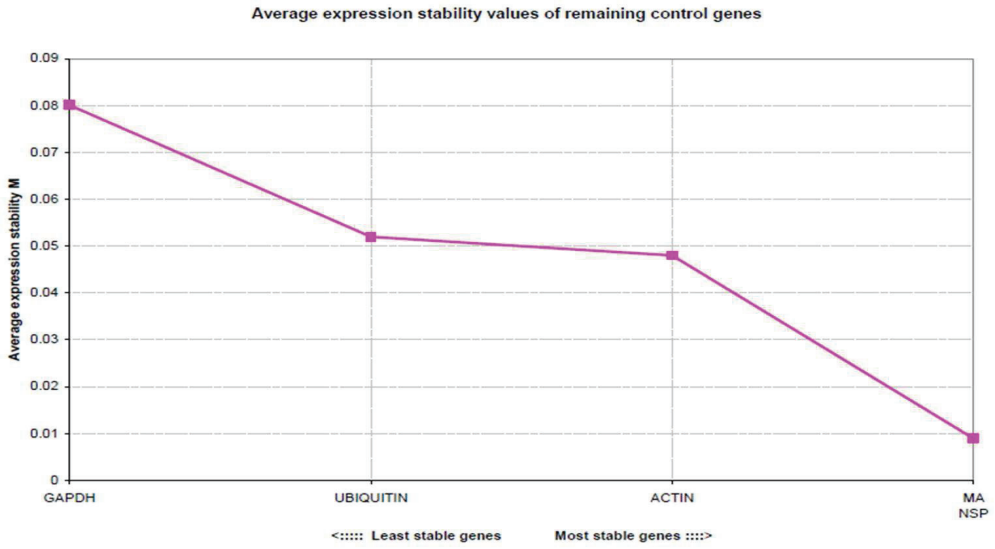

Figure 2. Stability (M) values for the housekeeping genes and endogen controls tested, as compared by Normfinder software. Most stable genes have the lowest M values.

The three housekeeping genes investigated (GAPDH, actin and ubiquitin) demonstrated varying levels of copy numbers across all treated samples. Thus, the calculated expression fold changes in mRNA copy numbers for PKS 6t3, 7t3 and 81t3 differed depending on the gene used for normalization. In contrast, the spike gene MA was detected in all samples at a C_t value of 15.75±0.28 (n=18) and the second spike gene NSP at a C_t of 26.4±0.29 (n=18) (data not shown). This indicates a consistent reverse transcription reaction

efficiency for high copy number (MA) and low copy number (NSP) genes across all samples. Fold change was calculated according to equation (II) (see material and methods).

Data shown in Figures 3–5 are expression ratios, not fold changes, and have been Log_2 transformed, thus accounting for the negative numbers displayed, particularly for the 5°C shock treatment. Fold changes between treatments were calculated from the expression ratios (data not shown). All three PKS transcripts investigated displayed differential expression, depending on the physiological shock treatment applied to *P. parvum* (Figures 3–5).

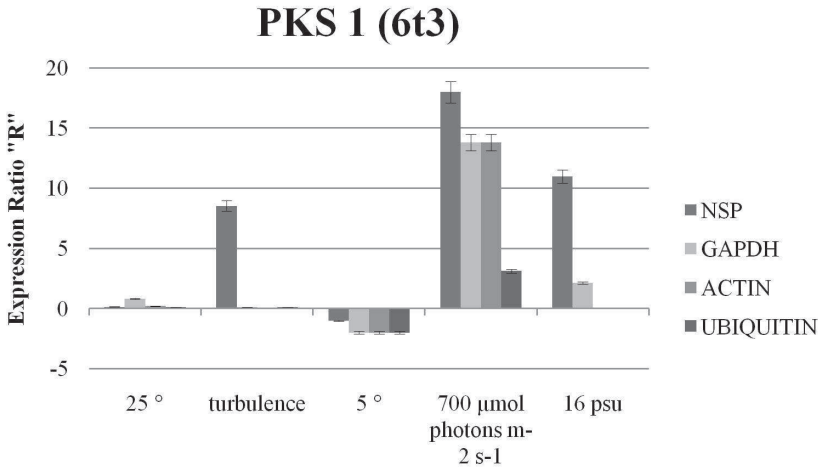

Figure 3. qPCR results for the PKS 1 6t3 gene. Four separate normalizations of the same gene expression data are shown using the artificial endogen NSP, GAPDH, actin and ubiquitin. The data shown have been normalized against the control treatment, indicating the change in gene expression for treatments when compared with the control. .

Variability was also noted, depending on which housekeeping gene was used for normalization. It is important to note that, although several regulation trends are similar, the data are extremely variable depending on which housekeeping gene was used for normalization. This shows that an internal method of normalization is indeed necessary, in order to accurately quantify the changes in relative gene expression. The observed variability among housekeeping genes decreased the confidence interval of a data set relying on these genes for normalization, and thus renders the data open to doubt. We therefore base all discussion as to up- or down-regulation on normalization using the spike gene NSP.

PKS 1 (6t3) was significantly upregulated compared with the control (*t*-test, $P < 0.05$) after turbulence, high-light and 16 PSU physiological shocks (Figure 3). Interestingly, high-light shock showed nearly 500x upregulation of turbulence (based on fold change equation applied), perhaps indicating a correlation with the increase in intracellular toxicity also observed for this same treatment (Figure 1).

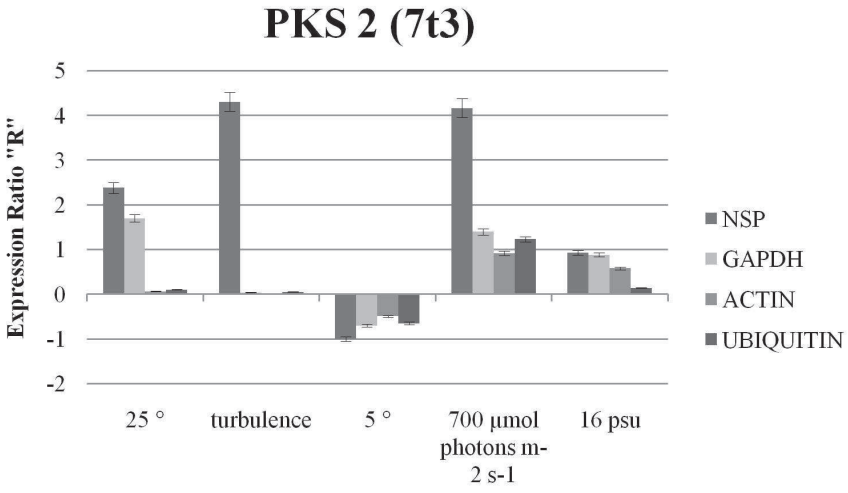

Figure 4. qPCR results for the PKS 2 (7t3) gene. Four separate normalizations of the same gene expression data are shown, see Figure 2.

This upregulation was the highest among all the samples analyzed. PKS 1 (6t3) was insignificantly downregulated following 5°C shock (*t*-test, $P < 0.05$), and minimally significantly upregulated following 25°C shock treatment (*t*-test, $P < 0.05$). PKS 2 (7t3) was significantly upregulated (*t*-test for all, $P < 0.05$) following 25°C shock, turbulence and high light (Figure 4), suggesting a differential biochemical role for this PKS transcript in toxic processes. Perhaps PKS 2 plays a role in extracellular toxicity, because the *R. salina* results indicate that, following this shock, the lytic capacity of *P. parvum* increased the most among all shock treatments (Figure 1b). Gene expression analysis of this transcript following turbulence shock produced significant (*t*-test, $P < 0.01$) variability (Figure 4). PKS 2 (7t3) was slightly upregulated following 16 PSU shock, but insignificantly (*t*-test, $P > 0.05$). Following

the trend observed for PKS 1 (6t3), gene expression was downregulated following 5°C shock, but in contrast to 6t3 this downregulation was significant (*t*-test, $P < 0.05$). PKS 3 (81t3) was upregulated following 25°C, turbulence and high-light shocks (Figure 5).

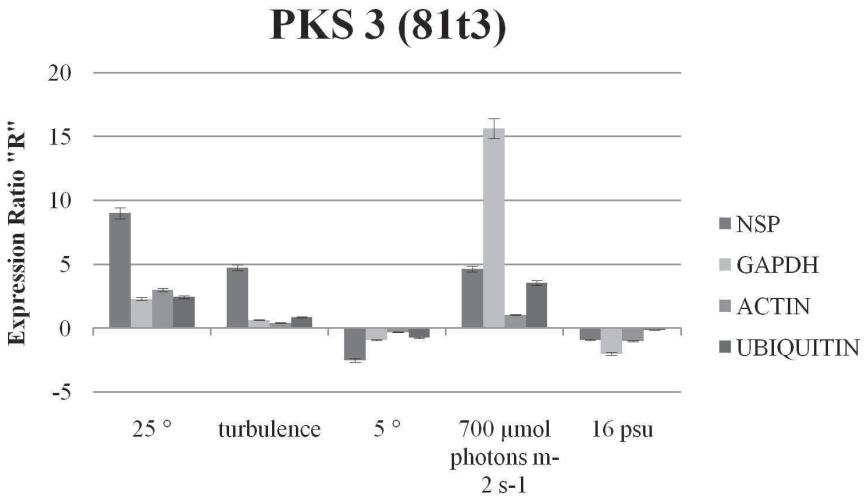

Figure 5. qPCR results for the PKS 3 (81t3) gene. Four separate normalizations of the same gene expression data are shown see Fig. 2.

Finally, it was observed that PKS 3 (81t3) was downregulated following 16 PSU shock and 5°C shock. It is possible that, with further characterization of PKS genes in *P. parvum*, specific sequential and thus structural based traits may be found that will associate a transcript with a particular process, e.g. biosynthesis or transport and/or secretion. Further investigations are necessary, however, to clarify the potential differential roles that PKS 1 (6t3), PKS 2 (7t3) and PKS 3 (81t3) transcripts may play in toxic processes in *P. parvum*.

3.3 Dark treatment difficulties in RNA isolation

As previously described, of dark treatment samples only RNA with high polysaccharide content in was obtained. This can be attributed to degradation of starch within the algal cells, in the absence of light, as has been previously described for *Gracilariopsis lemaneiformis*

(Rincones et al., 1993). The high polysaccharide content of these samples made cDNA formation and subsequent downstream qPCR analysis extremely difficult, and thus they were left out of the gene expression portion of the study.

4. CONCLUSIONS

The impact of short-term (shock treatments) environmental changes on the toxicity and PKS gene expression of *P. parvum* was investigated. The environmental acclimation of this species is of interest as *P. parvum* is known to be competitive in several niches (Edvardsen and Paasche, 1998). We found high light and low salinity to be the stresses with most influence on the induction of intracellular and extracellular toxicity (based upon bioassay results: Figure 1a, b) as well as on differential gene expression of PKS genes (Figures 3–5). The majority of shock treatments induced some level of increase in expression in PKS, suggesting that these gene pathways are of general importance in the stress responses of *P. parvum*. General transcriptional regulation in PKS-related pathways in *P. parvum* following short-term acclimation stress supports the hypothesis that this biosynthetic pathway is involved in the production and/or secretion of toxic substances.

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Chapter III

Transcriptomic response of the toxic prymnesiophyte *Prymnesium parvum* (N. Carter) to phosphorus and nitrogen starvation

Key words: microarray, golden alga, Prymnesiophyceae, ichthyotoxic, harmful algal bloom, nitrogen, phosphorus starvation,

Abbreviations: TUG (tentative unigene), EST (expressed sequence tag), PUFA (polyunsaturated fatty acid)

Abstract

The ichthyotoxic and mixotrophic prymnesiophyte *Prymnesium parvum* is known to produce dense virtually monospecific blooms in marine coastal, brackish, and inshore waters. Fish-killing *Prymnesium* blooms are often associated with macronutrient imbalanced conditions based upon shifts in ambient nitrogen (N): phosphorus (P) ratios. We therefore investigated nutrient-dependent cellular acclimation mechanisms of this microalga, by constructing a normalized expressed sequence tag (EST) library. We then profiled the transcriptome of *P. parvum* under nutrient-replete conditions as well as under nitrogen (N) and phosphorus (P) limitation via microarray analyses. Twenty three genes putatively involved in acclimatization to low nutrient levels were identified, among them three phosphate transporters, which were highly upregulated under P-starvation. In contrast, the expression of genes involved in transportation and acquisition of ammonium or nitrate/nitrite was unaltered in N-starved cells. We propose that genes upregulated under P- or N-starvation

lend themselves as potential tools to monitor nutrient limitation and indirectly the potential for initiation of toxic blooms in *P. parvum*.

1. Introduction

The toxic prymnesiophyte *Prymnesium parvum* develops massive monospecific harmful blooms worldwide, which often cause heavy economic losses through fish mortality and other types of ecosystem damage (Edvardsen and Paasche, 1998; Fistarol et al., 2003; Moestrup, 1994). These blooms are observed primarily in coastal and brackish waters, although more recently, they are frequently occurring in inland waters as well (Michaloudi et al., 2009), (Baker et al., 2007; Roelke et al., 2010b).

Prymnesium parvum is a physiologically rather flexible cosmopolitan species, with a wide tolerance range of salinity and temperature (Edvardsen & Imai, 2006 and references therein). Although primarily photosynthetic, this species is regarded as a mixotrophic because it is able to ingest immobilized bacteria (Nygaard and Tobiesen, 1993), and also takes up dissolved organic matter (Carvalho and Granéli, 2010) . The toxin(s) of *P. parvum* act(s) nonspecifically, and the mode of action is largely undefined, but the ichthyotoxic effect is caused by increasing the permeability of gill membranes. The chemical nature of the toxins also remains controversial. Igarashi and co-workers (1999) first isolated two polycyclic ether compounds (prymnesin 1and 2) from *P. parvum*, both of which showed potent hemolytic activity (Igarashi et al., 1999). However, Henrikson and co-workers (Henrikson et al., 2010) recently obtained a mixture of highly potent ichthyotoxic fatty acids from cultured *P. parvum* cells. Moreover, these authors could not detect prymnesins in either cultured *P. parvum* cells, nor in field collected water samples during a bloom with high *P. parvum* cell concentrations accompanied by fish mortalities (Henrikson et al., 2010). They therefore concluded that

uncharacterized compounds are responsible for the toxic effect of *P. parvum* rather than prymnesin 1 or 2.

In the closely related prymnesiophyte species *Chrysochromulina polylepis* polyunsaturated fatty acids have also been described to be responsible for lytic activity (Yasumoto et al., 1990). However, when John and co-workers (2002) compared the composition of fatty acid and lipid classes of toxic and non-toxic *C. polylepis* strains, no toxin-relevant difference could be detected (John et al., 2002).

Toxin production in *P. parvum* is probably intricately linked with mixotrophic nutrient acquisition strategies. The organism produces and releases toxic substances even under nutrient-replete conditions, but toxicity is increased under abiotic or biotic stress conditions including inorganic nutrient (nitrogen [N], and especially phosphorus [P]) limitation (Freitag et al., 2011; Granéli and Salomon, 2010). *Prymnesium parvum* is not able to feed on motile prey (Skovgaard and Hansen, 2003); it is supposed therefore, that the role of the induced allelopathic / toxic compounds is to immobilize and lyse competing and prey algal species (Tillmann, 1998) and potential grazers (Tillmann, 2003). This strategy to kill (and then eat) its enemies by means of toxic compounds, besides its relatively high growth rate, is thought to substantially contribute to the ability of *P. parvum* to form dense and persistent toxic blooms. In accord with this scenario, the ambient nutrient status was found to be imbalanced (high N:P ratio) during all studied *P. parvum* blooms (Kaartvedt et al., 1991b; Michaloudi et al., 2009), (Lindholm et al., 1999). This observation led to the assumption that the lack of available P in the water triggers enhanced toxicity, mixotrophy and toxic bloom formation (Lindholm et al., 1999). The effect of P- and N- limitation on the exhibited toxicity of *P. Parvum* has been shown in several laboratory experiments to include elevation of both intracellular, (Barreiro et al., 2005; Carvalho and Granéli, 2010; Johansson and Granéli, 1999b; Uronen et al., 2005) and extracellular (Graneli and Johansson, 2003b; Tillmann, 2003; Uronen et al., 2007; Uronen

et al., 2005) toxicity. Details of the cellular response to low inorganic nutrient conditions are, however, still poorly understood in this species.

The biosynthetic and regulatory mechanisms associated with toxicity in *P. parvum* and nutrient-dependency of toxigenesis at the molecular level remains to be elucidated. Although no whole genome sequence is available for *P. parvum*, a normalized expressed sequence tag (EST) library has been published (La Claire, 2006). Moreover, among related prymnesiophytes, considerable comparative genomic data are now available: the complete genome of *Emiliania huxleyi* has been sequenced (http://bioinfo.csusm.edu/Coccolithophorids/Emiliana-huxleyi), and EST libraries have been generated from *E. huxleyi* (von Dassow et al., 2009), *Chrysochromulina polylepis* (John et al., 2010); *Pavlova lutheri* und *Isochrysis galbana* (Patron et al., 2006).

The recent development of high throughput transcriptomic methodologies has led to numerous investigations of the gene expressional response to nutrient limitation in various algal groups. Examples include the chlorophyte *Chlamydomonas* (Moseley et al., 2006; Wykoff et al., 1999), the prasinophyte *Micromonas* (McDonald et al., 2010), the dinoflagellate *Alexandrium minutum* (Yang et al 2011 in press), various diatoms (Brown et al., 2009; Parker and Armbrust, 2005), as well as the prymnesiophyte *E. huxleyi* (Bruhn et al., 2010), (Riegman et al., 2000) (Dyhrman et al., 2006).

In this current study, we conducted physiological experiments on growth and toxin induction and applied cDNA sequencing and microarray technology to study gene expression under N- and P- starvation in *P. parvum*.

The objectives of our study were to: 1) determine physiological responses to nutrient starvation in terms of growth, toxicity, and nutrient uptake; 2) monitor the effects of nutrient starvation on *P. parvum* at the transcriptomic level and identify genes involved in the cellular response to P- and N-limitation. We investigated the transcriptomic status of the toxic

prymnesiophyte *P. parvum* under nutrient deficient conditions by constructing a normalized EST library. The sequences identified served as the gene base for microarray design. With this tool, we investigated the gene expression changes at three different growth phases of nutrient-starved and replete *P. parvum* cells.

2. Materials and methods

2.1 Culturing of algal strains

Prymnesium parvum strain RL10, isolated from Sandsfjord, Norway (Larsen and Bryant, 1998) was the subject of this study. The cryptomonad *Rhodomonas salina* (KAC 30, Kalmar Algal Collection, Kalmar, Sweden) served as a test organism in the toxicity bioassay. Cultures were grown on filter- sterilized IMR medium consisting of North Sea seawater (salinity 25 psu) enriched with macro- and micronutrients and vitamins (Eppley et al., 1967) in a controlled growth chamber at 20°C. Illumination was provided by daylight fluorescent lamps at a photon flux density (PFD) of 200 µmol m^{-2} s^{-1} on a 16:8 light:dark photoperiod.

For cDNA library construction, *P. parvum* strain RL10 was grown in 800 ml batch cultures in 1l Erlenmeyer flasks supplied with a mixture of antibiotics (50 µg ml^{-1} penicillin, 0.025 µg ml^{-1} streptomycin sulfate, 10 µg ml^{-1} ciprofloxacin) to obtain axenic cultures. One flask was sampled daily to determine cell concentration with a Casy cell counter (Innovatis AG, Reutlingen, Germany), whereas the other three parallel cultures were harvested at the treatment-specific time point without being previously opened during the experiment. A variety of growth conditions (Table 1) was applied, to obtain a high diversity of expressed genes. Salinity was adjusted either by dilution of the seawater by double distilled water or by addition of NaCl to achieve the desired salinity of 5 and 50 psu, respectively. To generate P- and N- depleted cultures, KH_2PO_4 and KNO_3, respectively were omitted from the growth medium.

Starvation experiments were carried out to study the effect of nutrient limitation on growth, toxicity and gene expression of *P. parvum*. Pre-inoculum cultures were kept in exponential growth phase by repeated sub-culturing and were treated twice for four days with an antibiotic cocktail consisting of penicillin (50 µg ml^{-1}), streptomycin sulfate (0.025 µg $^{-1}$) and ciprofloxacin (10 µg ml^{-1}). Before inoculating the experimental cultures, 1 ml of each inoculum stock culture was stained with Acridine orange and checked for bacterial contamination (Hobbie et al., 1977). Only cultures without evidence of bacteria were used as inoculum. Experimental cultures were grown under standard conditions as stated above in 5 l screw-cap glass bottles under gentle aeration and were sampled with a sterile tube-vacuum system (Eschbach et al., 2005).

A 15 ml subsample was taken daily for pH measurements and cell concentration was determined with a Casy cell counter (Innovatis AG, Reutlingen, Germany). At three time points along the growth curve, in early exponential (*ca*. $5x10^5$ cells ml^{-1}), mid- exponential or early stationary (*ca*. around 2 x 10^6 cells ml^{-1}) and in the stationary phase, samples were taken for toxicity analyses, RNA extraction for microarray, and N/P measurements (particulate and dissolved). Specific growth rates (µ) were calculated as:

$$\mu = (\log Nt_2 - \log Nt_1)\,(t_2 - t_1)^{-1}$$

with N = cells ml^{-1} and t = sampling day.

On the last sampling day (stationary phase), two aliquots of 50 ml each of all treatments and all replicates were transferred into Erlenmeyer flasks to serve as run-on cultures for further monitoring of cell growth. To confirm nutrient limitation, one of the two aliquots per nutrient-limited culture was supplemented with the originally omitted nutrient, the other served as a follow-up control culture.

2.2 Nutrient analysis

Samples (30 ml) of filtered growth medium for dissolved nutrient analysis were preserved by adding 3 µl 3.5% (w/w) $HgCl_2$ per ml sample and stored at 4°C until analysis. Dissolved nutrients were determined by continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany). For total dissolved P and N, the analysis was preceded by digestion with peroxodisulphate in an autoclave according their standard protocol.

Samples for particulate nutrient analysis were filtered onto pre-combusted glass fibre filters (GF/F, Whatman, Omnilab, Bremen, Germany) and stored at -20 °C. Filters for C/N measurements were dried at 60 °C and encapsulated into chloroform washed tin containers. Samples were analyzed on an NA 1500 C/N Analyzer (Carlo Erba Instrumentazione, Milan, Italy). Particulate phosphate was measured photometrically by continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany) after digestion with peroxide and sulfuric acid (Kattner and Brockmann, 1980).

2.3 Allelopathic capacity assay

Allelochemical activity was determined by co-incubation of *P. parvum* cells with cultured cells of *Rhodomonas salina* as previously described in detail (Tillmann et al., 2008). In brief, 4 ml of a mixture of *P. parvum* cells at different dilutions (2 x 10^3 ml^{-1}, 5 x 10^3 ml^{-1}, 1 x 10^4 ml^{-1}, 2,5 x 10^4 ml^{-1}, 5 x 10^4 ml^{-1}, 1 x 10^5 ml^{-1}, 2,5 x 10^5 ml^{-1}, 5 x 10^5 ml^{-1}, 1 x 10^6 ml^{-1} and undiluted) and *R. salina* (final cell concentration 10^4 ml^{-1}) were incubated in glass scintillation vials at 20° C for 24 h in darkness. Vials were then gently mixed by rotating, and 1 ml of the mixture was pipetted into an Utermöhl cell sedimentation chamber and fixed with gluteraldehyde (1% final concentration). After settling, cells were viewed via epifluorescence microscopy (Zeiss Axiovet 2 Plus, Carl Zeiss AG, Göttingen, Germany) with Zeiss filter-set 14 at 400x magnification and intact *R. salina* cells were counted. Control *R. salina* samples in

triplicate represented 0% lysis, and the effective concentration that causes 50% lysis (EC_{50}) for all samples incubated with *P. parvum* was calculated based on this control value, as percentage of *R. salina* cells lysed. The EC_{50} was estimated in a Bayesian statistical framework by fitting the following model to the cell count data using the general purpose Markov Chain Monte Carlo (MCMC) sampler OpenBUGS (Lunn et al., 2009):

$N_{final} \sim \text{binomial}(p, N_{total})$

$N_{total} \sim \text{poisson}(N_{real})$

$N_{control} \sim \text{poisson}(N_{real})$

$p = 1 / (1 + (x/\log EC_{50})^h)$

where N_{final} = *R. salina* cell concentration after incubation with *P. parvum* cells, $N_{control}$ = *R. salina* cell concentration after incubation without *P. parvum* cells, x = log-transformed cell concentration of *P. parvum*; N_{total} = the (unknown) total number of *Rhodomonas* cells in the experiment; N_{real} = the (unknown) expectation for the latter. In a hierarchical fashion, the $\log EC_{50}$ and h values for the three replicate cultures were assumed to have been drawn from a common distribution (normal in the case of h, and log-normal for $\log EC_{50}$). We used uninformative normal hyperpriors on the means of these distributions, and a flat gamma (0.001, 0.001) for their precision (= the inverse of the variance). The prior on N_{real} was a flat (precision = 0.001) normal distribution centered on $N_{control}$. The model was fit separately to each sampling/treatment combination (i.e., three replicates each) using three chains with overdispersed random starting values. The initial 30,000 MCMC iterations were discarded as burn-in, and 1,000 further samples were recorded every 20^{th} iteration for posterior inference. Convergence was checked visually as well as using the Gelman-Rubin statistic and by comparing posterior inferences from multiple runs. Results are expressed as the posterior median of EC_{50} and their 95% highest posterior density intervals.

2.4 Intracellular toxicity measurement

Measurements of intracellular toxicity, defined as due components normally retained within *Prymnesium* cells, were carried out as detailed in (Eschbach et al., 2001). In brief, an aliquot containing 10^7 cells from each culture was centrifuged at 4,000 x *g* for 10 min at 20 °C. The cell pellet was resuspended in 1 ml of the assay buffer (150 mM NaCl, 3.2 mM KCl, 1.25 mM $MgSO_4$, 3.75 mM $CaCl_2$ and 12.2 mM TRIS base, pH adjusted to 7.4 with HCl). Cells were then ultrasonicated for 1 min (50% pulse, cycle; 70% amplitude) with a sonicator (Bandelin Sonopuls, Bandelin Electronic, Berlin, Germany). 100 µl of the lysed cell suspension was incubated with an equal volume of fish erythrocytes (5 x 10^6 cells). After 24 h incubation, hemolytic activity was determined by measuring absorbance at 540 nm in an Ultrospec III UV/Visible photometer with Wavescan Application Software (Pharmacia LKB Biotechnology, Uppsala, Sweden). A standard hemolytic curve was prepared from a saponin dilution series in the assay buffer. Results are expressed as ng saponin equivalents per cell (ng SnE $cell^{-1)}$ utilizing the standard saponin from higher plants (Sigma Aldrich, Hamburg, Germany) as an indicator of relative lytic capacity.

2.5 RNA extraction

Prymnesium parvum cultures (50ml) were harvested in a swinging-bucket rotor centrifuge (Eppendorf 5810, Hamburg, Germany) by centrifugation for 8 min at 3000 x *g* at 20°C. The supernatant was discarded, the cell pellet resuspended in 500 µl lysis buffer (buffer RLT, RNeasy Plant mini Kit, Qiagen, Hilden, Germany) and immediately frozen in liquid N. Pellets were stored at -80°C until RNA extraction. Directly before RNA extraction, the resuspended cells were lysed twice for 30 s each with a TissueLyser II (Qiagen, Hilden, Germany). Total RNA extraction was performed with the RNeasy Plant mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, by performing the optional DNAse digestion on the spin column. RNA purity was assessed by UV-spectrophotometry at 260/230 and 260/280

nm wavelength in a Nano-drop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany) and RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Boeblingen, Germany).

2.6 cDNA library construction, EST sequencing and microarray analysis

2.6.1 cDNA library construction and EST sequencing and annotation

Total RNA (4,550 ng) of each treatment as listed in Table 1 was pooled and sent to Vertis Biotechnologie AG (Freising-Weihenstephan, Germany) for cDNA library construction. The cDNA was synthesized according to their standard protocol for full-length enriched cDNA synthesis from poly A+ RNA purified from the total RNA and finally electroporated into competent *Escherichia coli* cells, resulting in an estimated total of 1.4 x 10^6 clones in the primary library.

treatment	salinity psu	light µmol photons m^{2s-1}	temperature (℃)	medium IMR
replete control	26	200	20	IMR
low temperature	26	200	5	IMR
high temperature	26	200	25	IMR
low salinity	5	200	20	IMR
high salinity	50	200	20	IMR
low light	26	7	20	IMR
P depleted	26	200	20	IMR-P
N depleted	26	200	20	IMR-N

Table 1. Conditions used for the cDNA library construction

Approximately 11 x 10^3 colonies were picked, and DNA was extracted by magnetic beads on a robotic platform (Qiagen, Hildesheim, Germany). Plasmid inserts were sequenced from both ends using Big Dye Chemistry (Applied Biosystems, Darmstadt, Germany) and separated on an ABI Prism 3700xl sequencer (Applied Biosystems, Darmstadt, Germany). In

total 18,428 sequencing reactions were performed of which 15,591 passed the quality check and were considered as ESTs for the annotation pipeline. These were clustered with a sequential assembly using decreasing identity thresholds (gap4 assembler, Staden Package) to avoid misassemblies due to polyA tails. Clustering of all ESTs yielded 6,381 contigs and singletons of good quality.

Contig and singleton sequences were loaded into the SAMS (Sequence Analysis and Management System, Center for Biotechnology, University of Bielefeld) for automated annotation based on BLAST comparisons against KEGG, KOG, SwissProt, InterPro and the Genbank nt and nr databases. Metabolic enzyme coding transcripts were identified using the metaSHARK tool (Pinney et al., 2005) with settings adapted to detect gene fragments (genewise run with the –init wing option).

2.6.2 Comparative analysis with other prymnesiophytes

For comparison, we performed the same analysis with the transcript sequences available for *Isochrysis galbana* (Patron et al., 2006) *Emiliania huxleyi* (http://genome.jgi-psf.org) and for the previously published EST library from *P. parvum* (La Claire, 2006). Contig sequence data from cDNA libraries and transcript model data from the genome sequencing project for *E. huxleyi* at JGI were analyzed in functional categories, applying trpsblastn (Altschul et al., 1997) against KOG/euNOG databases of eukaryote-specific sequence orthologues from eggNOG (Muller et al., 2010). Significant best hits (e-value<10^{-10}) were tabulated contig-wise and counts of common functions were computed and displayed as Venn-diagrams in R (Team, 2008) using the gplots R-package from CRAN (http://cran.r-project.org/web/packages/gplots/index. html).

KOG and euNOG databases of reference alignments from the Eggnog-2 website (version 2, http://eggnog.embl.de//) were downloaded and converted into rpsblast-formatted databases. For this purpose HMMer (Eddy, 1998) was applied to emit consensus sequences from the

HMM-models of the alignments obtained from hmmbuild. Alignments were converted to position-specific scoring matrices (PSSMs) by blastpgp, using the consensus sequence as query. The resulting collections of PSSMs were assembled into rpsblast-compatible databases for the KOG and euNOG subsets of orthologies via formatrpsdb. HMMer version 2.3.2 was used in combination with BLAST programs of version 2.2.16.

2.6.3 Oligonucleotide design

Based on the assembled EST dataset, 60-nucleotide-hybridization probes were designed for all annotated and non-annotated contigs, at intervals of about 250 bp along the DNA sequence, oriented both in 5'-3' and in 3'-5' direction. The resulting 21,570 oligonucleotide sequences were synthesized on a 44K microarray. Based on the hybridization signal 2-3 probes per contig were selected. The selection criteria were reproducibility of the signal within the triplicate of arrays, as monitored by self-hybridization. Final microarrays for gene expression profiling were generated by applying the selected subset of probes by Agilent (Agilent Technologies, Palo Alto, CA) using the SurePrint technology in the 44K format.

2.7 Microarray experiment

A "reference sample" approach was used for the microarray hybridization experiments to enable gene expression level comparisons across all treatments. The reference sample consisted of a mix of *P. parvum* RNA samples, originating from different treatments also applied for the cDNA library synthesis. All arrays were hybridized and analyzed in biological triplicates, i.e., originating from three independent batch cultures.

The two-color microarray-based gene expression analysis protocol and equipment employed according to recommendations of the manufacturer (Agilent Technologies, Waldbronn, Germany). 250 ng of total RNA was amplified, reverse-transcribed and labeled with the two-color low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies, Waldbronn, Germany). The Cy-3 and Cy-5 dye incorporation was verified by NanoDrop ND-

1000 spectrophotometer. Hybridization was performed onto 4 x 44k microarray slides, with the two color gene expression hybridization kit in SureHyb Hybridization Chambers in a hybridization oven at 65 °C for 17 h. Microarrays were scanned by an Agilent Scanner (Waldbronn, Germany). Raw data were extracted with the Agilent Feature Extraction Software version 9.5. Feature extraction software served to remove spots that had been flagged 'outliers', 'not known' or 'bad', based on background median analysis (Storey, 2003).

The program MeV (Saeed et al., 2006) was used for statistical analyzes of the microarray data. To ensure statistical and biological significance, gene expression differences that proved statistically significant according to a SAM (Statistical Analysis of Microarray) analysis and showing at least two-fold expression differences between treatment and control were identified.

3. Results

3.1 EST library and functional annotation

3.1.1 **Prymnesium parvum** *non redundant genes*

Pooling of total RNA samples isolated from different treatments (Table 1) yielded a high diversity of transcripts and allowed construction of a normalized cDNA library for *P. parvum*. After sequencing, quality clipping and assembly 6,381 tentative unique genes (TUG) were identified.

Further profile-based functional annotation of the generated TUGs for metabolic enzymes, using the metashark tool with an e-value cut-off of 10^{-5}, identified 392 TUGs as members of KEGG metabolic pathways (annotated with EC numbers). Analysis of the EST library published by La Claire (2006); using the same protocol and stringency, yielded annotation for 372 genes. 187 EC numbers were present in both libraries, while 185 EC numbers were unique to the previously published data set, and 205 were unique to the library

described here. Altogether, the number of KEGG metabolic enzymes identified from *P. parvum* has been expanded from 372 (EST library constructed by (La Claire, 2006) to 597 (Appendix, Table 2). KEGG metabolic pathways represented by ESTs from either *Prymnesium* library are depicted in Appendix, Table 2.

Twelve and six pathways (represented by 21 and 29 enzymes) were found in our and in the previous EST library respectively, which were not present in the other *P. parvum* library (Table 3).

ESTTAL	**EC numbers**
Benzoate degradation via hydroxylation	2
Biosynthesis of vancomycin group antibiotics	1
Caffeine metabolism	8
Carotenoid biosynthesis	1
D-Alanine metabolism	1
D-Arginine and D-ornithine metabolism	1
Glycosaminoglycan degradation	1
Indole and ipecac alkaloid biosynthesis	1
Keratan sulfate biosynthesis	1
Penicillin and cephalosporin biosynthesis	1
Puromycin biosynthesis	1
Terpenoid biosynthesis	2
library La Claire 2006	
Biosynthesis of ansamycins	1
Caprolactam degradation	2
Diterpenoid biosynthesis	1
Folate biosynthesis	1
Geraniol degradation	1
Phosphatidylinositol signaling system	23

Table 3. Enzymes identified in the two *P. parvum* libraries, which are not present in the other library

3.1.2 Comparative analysis of prymnesiophytes

BLAST-based sequence comparisons performed with five sets of prymnesiophyte EST libraries (*Isochrysis galbana* (Patron et al., 2006), *Pavlova lutheri* (Patron et al., 2006),

Chrysochromulina polylepis (John et al., 2010), *Prymnesium parvum* I (La Claire, 2006), and *Prymnesium parvum* II (this study) and the transcript sequences of *Emiliania huxleyi* (http://bioinfo.csusm.edu/Coccolithophorids/Emiliana-huxleyi/http://genome.jgi-psf.org) revealed many congruencies and apparent homologies (Table 4). In comparison to existing prymnesiophyte sequences, 2,882 found hits with an e-value better than $1e^{-5}$ in the *E. huxleyi* genome, compared with 1,820 and 1,179 in the EST libraries of *P. parvum* (La Claire, 2006) and *I. galbana* (Patron et al, 2006).

species	contigs	significant hits	median contig length
Prymnesium parvum	6381	2953	818
Prymnesium parvum	3380	1563	790
Isochrysis galbana	12276	3686	489
Chrysochromulina polylepis	2207	707	745
Pavlova lutheri	13068	5264	490
Emiliania huxleyi	39126	18699	903

Table 4. Statistics of EST sequences from cDNA libraries of species used for the analysis

When comparing the five EST libraries, 1,526 genes were found in common among these four prymnesiophytes. Furthermore, the two *P. parvum* libraries had 88 functions in common, which appear to have no counterpart in the other species investigated in this study (Figure 1).

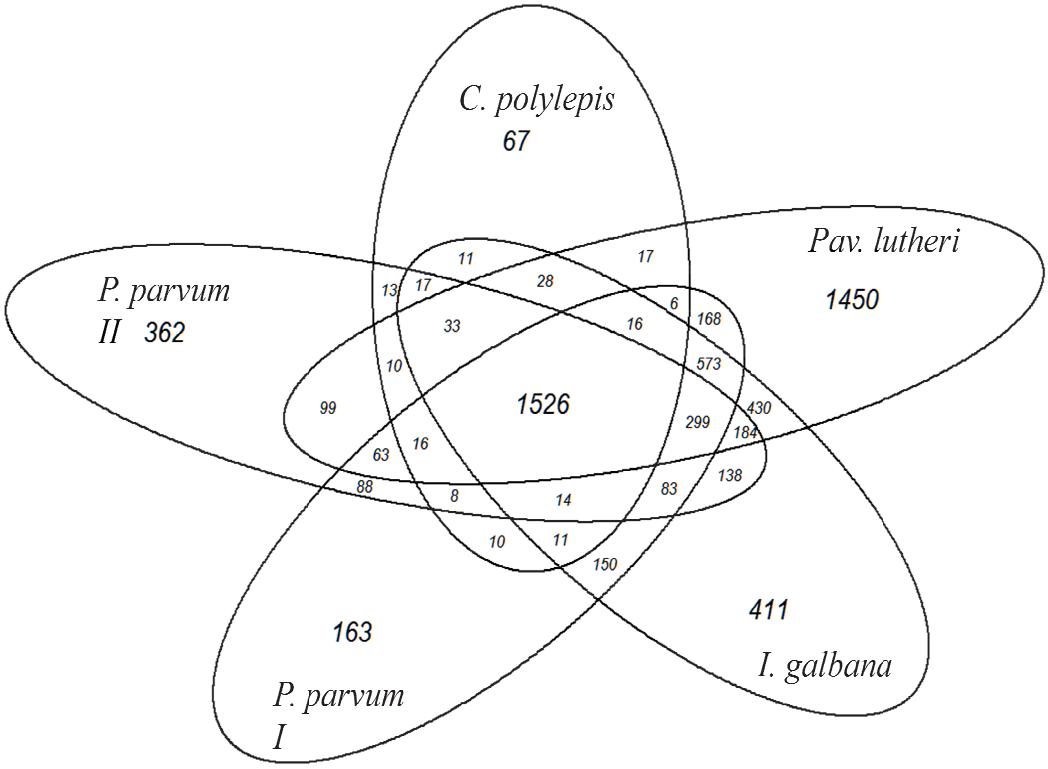

Figure 1. Functional coverage comparisons of the haptophytes *I. galbana*, *C. polylepis*, *Pavlova lutheri*, *P. parvum I* (La Claire), *P. parvum II* (this work). Common and library-specific hit-distributions of transcripts to KOG/euNOG orthologies with an e-value of 10^{-10} or less are shown.

Comparison between the transcript sequences of *E. huxleyi* and the four other prymnesiophyte species showed that 478 functions are present in all species. A further 1,695 functions were found in *P. parvum* and *E. huxleyi* only, and an additional seven orthologies were shared between the EST libraries of the two toxic species *P. parvum* and *C. polylepis* (Figure 2).

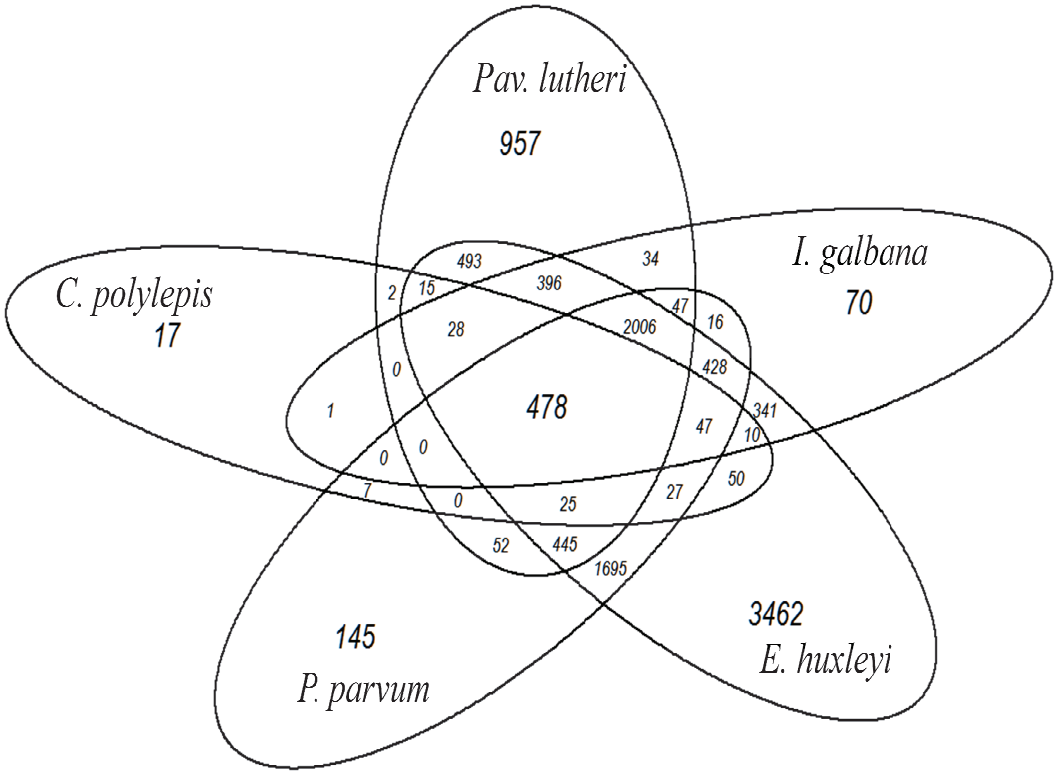

Figure2. Comparative analysis of KOG/eu NOG based functional annotated genes (e-value of 10^{-10} or less) in the haptophytes *I. galbana*, *C. polylepis*, *Pavlova lutheri*, *P. parvum* (EST libraries merged) in relation to the best transcript models from *E. huxleyi.*

The contigs found in the EST libraries and the transcript sequences from *E. huxleyi* were annotated and sorted into KOG functional categories (Figure 3). Interestingly, in the category J (translation, ribosomal structure and biogenesis), we obtained less contigs than are present in the other libraries, whereas our combined *P. parvum* set of ESTs represents more functions involved in defense mechanisms (category V) than either *P. lutheri* or *I. galbana* (Figure 3).

3.1.3 Genes involved in nitrogen or phosphorus metabolism, vesicle transport and growth

We searched the tentative unigene TUG set for genes that are hypothesized to be related to the metabolism, uptake and storage of the nutrients N and P (Table 5), cell division and growth

(Table 6), as well as vesicle transport /phagotrophy (Table 7) as well as cell division, growth (Table 6). In the case of genes putatively involved in nutrient metabolism, eighteen fragments showed significant hits to annotated genes based on similarity with an e-value below 10^{-10}. The best hits of more than half of the N- and P-relevant genes from the NCBI non-redundant database were to those of microalgal origin. Fourteen and six genes were found to be involved in the uptake, transport and storage of N and P, respectively. Table 6 and 7 comprise a subset of nineteen genes involved in cell division and transcription, translation and vesicle transport, respectively.

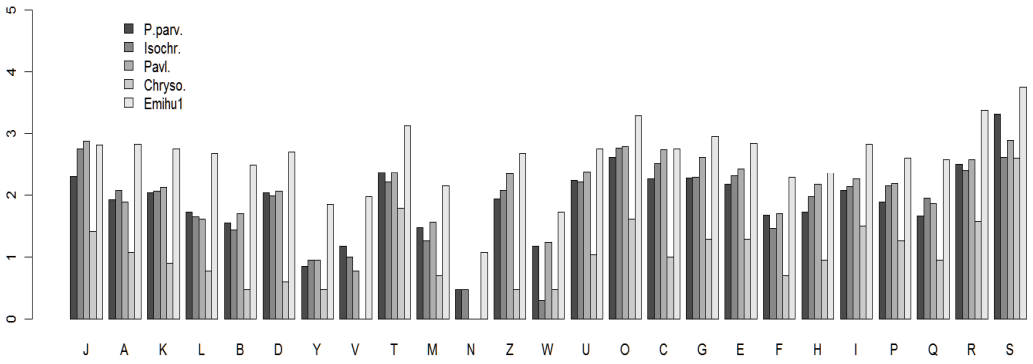

Figure 3. Abundances of significant hits to KOG-categorized orthologies euNOG and KOG on logarithmic scale. Functional (KOG) categories (given as capital letters) are used to group the hit-counts of the respective library. (J Translation, ribosomal structure and biogenesis, A RNA processing and modification, K Transcription, L Replication, recombination and repair, B Chromatin structure and dynamics, D Cell cycle control, cell division, chromosome partitioning, Y Nuclear structure, V Defense mechanisms, T Signal transduction, M Cell wall/membrane/envelope biogenesis, N Cell motility, Z Cytoskeleton, W Extracellular structures, U Intracellular trafficking, secretion, vesicular transport, O Posttranslational modification, protein turnover, chaperones, C Energy production and conversion, G Carbohydrate transport and metabolism, E Amino acid transport and metabolism, F Nucleotide transport and metabolism, H Coenzyme transport and metabolism, I Lipid transport and metabolism, P Inorganic ion transport and metabolism, Q Secondary metabolites biosynthesis, transport and catabolism, R General function prediction only, S Function unknown

organism hit in nr	e value nr	annotation	e value	expression
domonas reinhardtii	4.E-08	KEGG: Ammonium transporter	5.00E-05	**P ↓**
domonas reinhardtii	5E-12	KEGG: probable high affinity ammonium transporter	2.00E-09	*
s (Silurana) tropicalis	6E-37	KOG: Ammonium transporter	6.00E-37	**P ↓**
iosira pseudonana	1E-80	KEGG: formate/nitrite transporter	1.00E-46	**nd**
a yezoensis	5E-15	KEGG: high-affinity nitrate transporter, putative	2.00E-14	**P ↓**
ntrum minimum	8E-32	KEGG: formate transporter	7.00E-23	**R ↑**
iosira pseudonana	1E-66	KEGG: Formate/nitrite family of transporters	7.00E-39	**P ↑**
myces maris	6E-19	KEGG: glutamate synthase small subunit	2.00E-16	**nd**
ceros compressus	1E-60	KEGG: glnA; Glutamine synthase	1.00E-34	**nd**
phaera watsonii	9E-23	KEGG: glutamine amidotransferas	1.00E-22	**nd**
iosira pseudonana	2E-42	KEGG: Glutamine synthase	5.00E-25	**nd**
pora owczarzaki	2.E-29	KOG: Copper amine oxidase	4.00E-30	**P ↓**
iosira pseudonana	5E-31	KOG: Aspartate aminotransferase	3.00E-23	**nd**
agus cuniculus	1E-67	KOG: Aspartate aminotransferase	6.00E-69	**nd**
iosira pseudonana	4E-33	KEGG: asparaginase [EC:3.5.1.1]	3.00E-22	**nd**
a huxleyi	8E-58	KEGG: phosphate-repressible phosphate permease	1.00E-24	**P ↑, R ↓**
accharomyces pombe	3E-13	KEGG: probable inorganic phosphate transporter	4.00E-14	*
a huxleyi virus 86	1E-38	KEGG: putative phosphate/sulphate permease	6.00E-22	*
ga brevicollis	1.E-20	KOG: Purple acid phosphatase	8.00E-11	**P ↑**
pus siliculosus	6.E-10	KEGG: phosphatidic acid phosphatase	4.00E-10	**N, R ↑**
occus anophagefferens	5.E-49	KEGG: arsC; arsenate reductase	6.00E-21	**R, N ↓ P ↑**

orage and transport of nitrogen and phosphorus in the EST library of *P. parvum*. Best hits from nr (non-redundant sequences database, s up and downregulation, nd no difference in gene expression in the exponential vs. stationary phase within treatment. Genes marked ad signal on the microarray.

	E-value nr	best hit	E-value	expression
	1.E-104	KEGG: proliferating cell nuclear antigen	4.00E-98	**all ↓**
	7.E-110	KEGG: replication factor	6.00E-108	**all ↓**
	2.E-31	KEGG: replication factor	6.00E-32	**nd**
	2.E-28	KOG: meiosis protein Mei2	6.00E-28	**nd**
	2.E-11	KOG: meiosis protein Mei2	1.00E-12	**nd**
	1.E-11	KOG: DNA-dependent RNA polymerase	2.00E-13	**nd**
	8.E-44	KOG: DNA polymerase	1.00E-40	**N, P ↓**
	2.E-105	KOG: Nucleosome remodeling protein	3.00E-107	**P ↓**
	8.E-34	SP: DNA replication complex G	4.00E-30	**all ↓**
	6.E-88	KEGG: similar to cell division protein	3.00E-88	**all ↓**
	3.E-30	KEGG: Cyclin-dependent kinases	1.00E-27	**all ↓**
	1.E-88	KEGG: RNA helicase	4.00E-51	**all ↓**
	7.E-110	KEGG: Replication factor C subunit 3	6.00E-108	**all ↓**
	1.E-104	KEGG: Proliferating cell nuclear antigen 2	4.00E-98	**all ↓**
i	4.E-77	KOG: DNA polymerase delta	2.00E-72	**all ↓**
s	2.E-29	KEGG: POLA2; polymerase	2.00E-25	**all ↓**

e EST library, involved in cell proliferation, mitosis, meiosis, DNA/RNA transcription. Best hits from the nr (non-redundant sequences,

↑ ↓ depicts up and downregulation, nd : no difference in gene expression in the exponential vs. stationary phase within treatment.

	E-value (nr)	best hit	E-value	expression
	8.00E-17	KOG: Vacuolar protein sorting associated protein	3.00E-15	**N** ↑
	3.00E-30	KOG: Multidrug resistance-associated protein	9.00E-31	**N , C** ↑
	4.00E-76	KOG: Protein required for fusion of vesicles	7.00E-70	**P** ↑
rinus	1.00E-27	KOG: GTP-binding ADP-ribosylation factor	8.00E-29	**P** ↑
	2.E-101	KEGG: dynein, axonema	2.00E-100	**N** ↑
erta	2.00E-33	KOG: Microtubule-associated anchor protein	7.00E-34	**P** ↑
nana	6.00E-38	KEGG: hemolysin III	1.00E-25	**nd**
orffii	3.00E-13	KOG: SNARE protein SED5/S	1.00E-12	**nd**
	5.00E-33	KOG: importin	2.00E-32	**nd**
uca	8.00E-46	KEGG: karyopherin (importin)	1.00E-46	**P** ↓
	4.00E-45	KOG: Karyopherin (importin)	2.00E-43	**nd**
	2.00E-44	KEGG: exportin	2.00E-43	**nd**
	1.00E-68	KEGG: karyopherin alpha	6.00E-64	**nd**
gefferens	2.00E-60	KOG: Clathrin adaptor complex	1.00E-57	**nd**
	3.00E-22	KEGG: clathrin, heavy polypeptide	4.00E-20	**nd**
aki	9.00E-25	KOG: Vesicle coat complex	9.00E-24	**nd**
ae	5.00E-36	KOG: Clathrin coat dissociation kinase	4.00E-36	**nd**
	2.00E-33	KEGG: autophagy related protein	5.00E-33	**P** ↑
	1.00E-44	SP: Autophagy-related protein 18	3.00E-44	**nd**

ST library involved in vesicle transport, secretion and mixotrophy. Best hits from nr (non-redundant sequences, GenBank) database are egulation, nd : no difference in gene expression

3.2 Nutrient status of Prymnesium parvum cell cultures

The concentration of dissolved inorganic N and P (measured in the form of NO_2^-, NO_3^- and PO_4^{2-}) in the medium decreased with increasing cell concentration of the cultures (Figure 4). At the first sampling point on Day 2, as the cells reached a concentration of about 5 x $10^4 ml^{-1}$, the nitrogen deprived cultures had used up about one third, whereas the N and P replete and P deprived cells had consumed about ten percent of their original N resource. When the N-depleted cells reached stationary phase, the medium contained <0.5 µM NO_3^- and NO_2^- , whereas the consumption of PO_4^{3-} was not measurable. On the other hand, in the medium of the P-deprived cultures, an excess of NO_2^- and NO_3^- remained, but the final PO_4^{3-} concentration was <0.5 µM. The nutrient-replete control cultures used up almost all the N present in the form of NO_2^- and NO_3^-, but had an excess of available P measured as PO_4^{3-}. Surprisingly, the concentration of NH_4 in the medium increased in both the nutrient-replete and P-depletion experiments, whereas it remained stable during developing N starvation. The measurement of the particulate nutrients also confirmed nutrient limitation in the stationary phases of the respective depleted cultures (Table 8).

	PON	st. dev.	POC	st. dev.	POP	st. dev.
replete I	3.82	0.60	46.97	3.40	0.95	0.08
replete II	2.68	0.15	33.94	0.98	0.40	0.04
replete III	2.15	0.12	42.45	0.76	0.29	0.01
P depleted I	3.10	0.36	43.40	1.92	0.53	0.02
P depleted II	2.84	0.13	33.10	1.65	0.31	0.03
P depleted III	2.59	0.23	66.71	4.80	0.07	0.01
N depleted I	4.42	0.22	62.90	2.09	1.01	0.09
N depleted II	1.81	0.29	29.68	2.43	0.36	0.03
N depleted III	1.69	0.29	32.51	3.07	0.44	0.04

Table 5. Particulate N, P and C measurements of N or P deprived and replete *P. parvum* cells. I, II and III represents early exponential, mid-exponential-early stationary and stationary growth stage, respectively.

3.3 Growth and toxicity of Prymnesium parvum

During early growth stages, the nutrient deprived cultures displayed similar growth and physiological characteristics to the nutrient replete controls. Cell concentrations increased exponentially until Day 4 after inoculation (Figure 4). The N-deprived cultures stopped growing on Day 5, whereas the P starved cells reached stationary phase on Day 10. The nutrient replete control cultures grew for eleven days and entered stationary phase at a cell concentration of about 1.3 x 10^6 cells ml^{-1}. The final concentrations of the starved cultures were lower (1.4 x 10^5 and 4.5 x 10^5 cells ml^{-1}, respectively, for the N and P starved cells). When the limiting nutrient was added, the cells resumed exponential growth in both N- and P-depleted cultures (Figure 4), confirming that limitation by the corresponding nutrient was the cause of entering the stationary growth phase in the depleted cultures in a reversible manner.

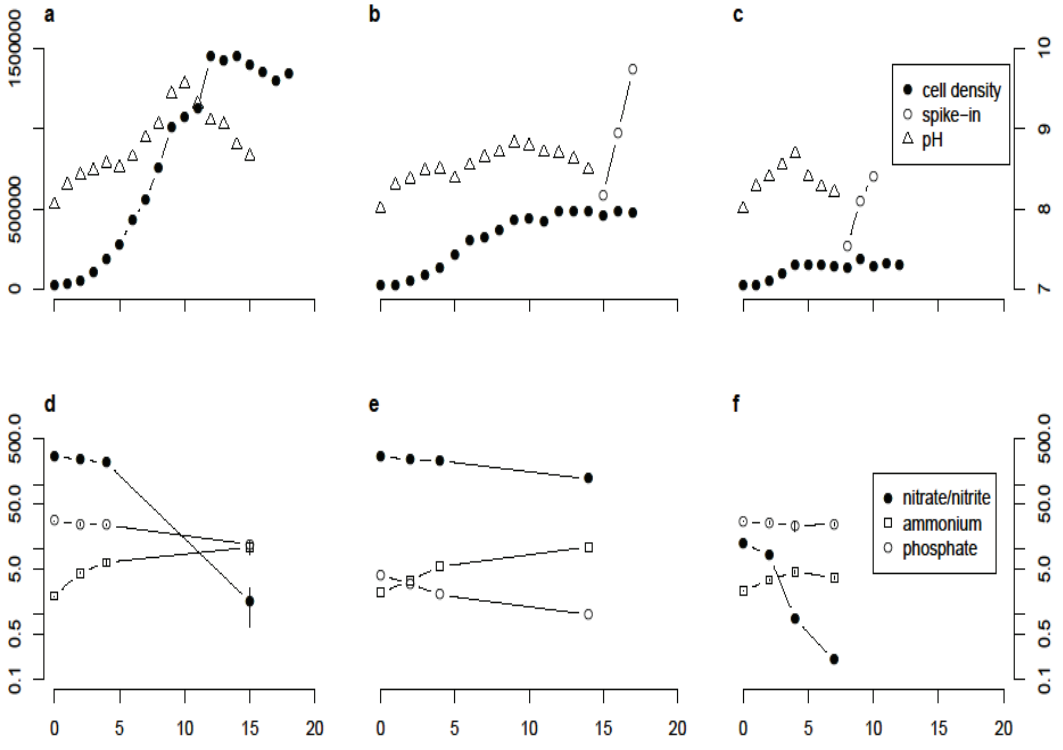

Figure 4. Growth, pH and dissolved nutrient measurements of *P. parvum* a-c: daily cell concentration measurements and the pH of the cultures. d-f: dissolved nutrient concentrations in the early exponential, mid exponential and stationary growth phases on a logarithmic scale (in µM). Treatments: a, d: nutrient replete control; b, e: phosphate depletion; c, f: nitrate depletion. Subplots in the same rows and columns are scaled identically on their Y and X axes, respectively. X axes: time in days; Y axes: cell densities for solid and empty circles in cells / ml (axis labels for all subplots on the right hand side), pH for triangles (labels for all subplots on the right hand side).

In the early growth stages, the pH of all cultures increased similarly to about 8.5 by Day 4. On Day 5, the pH of the N-deprived cells decreased slightly, and in the following two days, it decreased to 8.2. In the P-deprived cultures, the pH increased further to 8.8 until Day 9 and then slowly decreased until Day 14 (to 8.5). The pH of the nutrient replete cultures, on the other hand, increased until Day 10 (reaching 9.55) and then decreased to 8.6 by Day 14 (Figure 4).

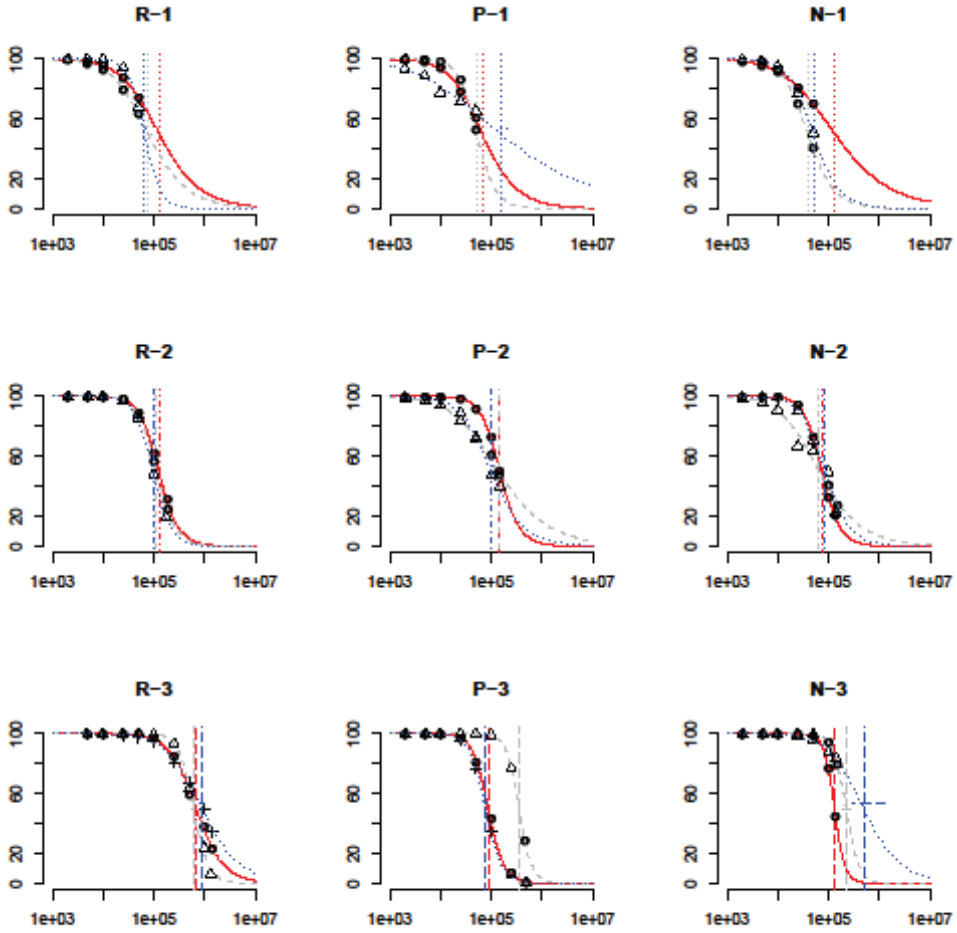

Figure 5. Extracellular toxicity of *Prymnesium parvum* against the cryptomonad *Rhodomonas salina* under different treatments and growth phases. Observed survival proportions (symbols) and posterior median dose-response curves fitted to *Rhodomonas salina* survival data as function of *Prymnesium parvum* cell concentration using a Bayesian approach. Panel a-c depicts the early exponential, panel d-f the mid exponential and panel g-i stationary growth phase. Treatments: first column: nutrient replete control (R); second column: phosphate depletion (P); third column: nitrate depletion (N). X axes: *P. parvum* cell density (cells ml^{-1}); Y axes: *R. salina* viability (percent). Vertical lines represent the posterior median of EC50, and horizontal lines at 50 % height represent the 95 % highest posterior density interval of EC50. The red, blue and gray colors correspond to the three replicates on each plot.

The toxicity of *P. parvum* cells was determined with whole cell bioassay methods to assess both allelopathic capacity and intracellular toxicity. Generally, no difference in allelopathic capacity, -measured with *Rhodomonas* bioassay, was observed in the early- and mid-exponential growth phase among the treatments. At the first sampling point (early exponential growth), extracellular toxicity was not very pronounced. The undiluted samples caused about 40% lysis of *Rhodomonas* cells, irrespective of treatment; the EC_{50} values were estimated at around 10^5. Similarly, at the second sampling point, there was no significant difference in the extracellular toxicity of deprived versus replete cells. Again, the EC_{50} value was close to 10^5 for all treatments (Figure 5).

However, toxicity in the stationary phase differed markedly among the three treatments. The nutrient replete cultures grew to cell concentrations beyond 10^6 ml^{-1} and the allelopathic capacity decreased (EC_{50} values rose to between 6 and $8x10^5$). Whereas the N depleted cells showed a similar decrease in toxicity, the extracellular toxicity of the P starved cells remained unaltered in the stationary phase.

The results of the fish erythrocyte lysis assay to quantify the intracellular toxicity (Figure 6) indicated that, in general, intracellular toxicity remained unchanged throughout the growth curve in the case of N deprived cells, whereas it decreased in nutrient replete cultures in stationary phase. On the other hand, P-starvation greatly enhanced the intracellular toxicity in *P. parvum*. Throughout the experiment, higher toxicity was observed in P-deprived than nutrient replete cells, with toxicity of the P-depleted cultures highest in the stationary phase (Figure 6).

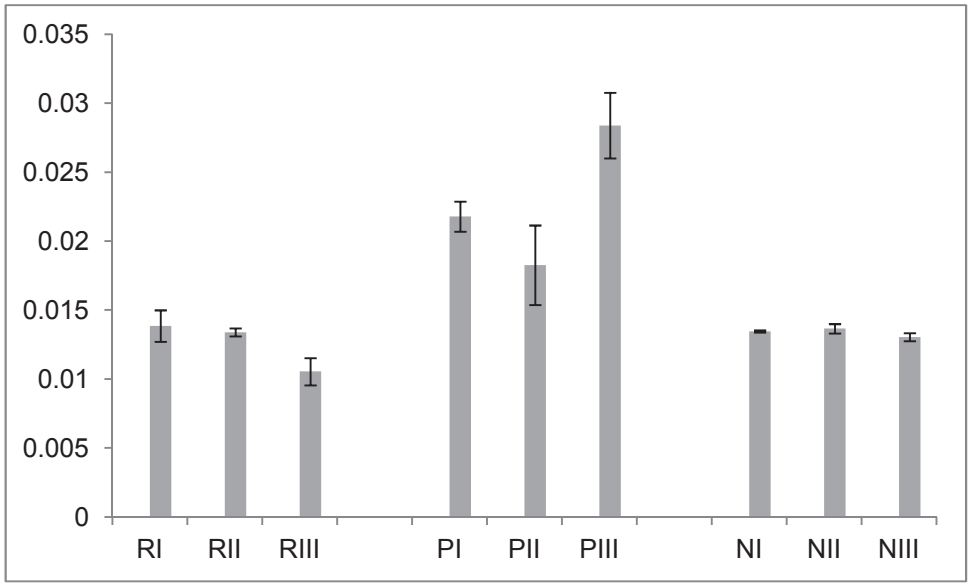

Figure 6. Intracellular toxicity of *P. parvum* given in ng saponin equivalent cell^{-1}, mean of biological triplicates; with standard deviation shown. Treatments: R: nutrient replete control; P: phosphate depletion; N: nitrate depletion. Subplots in the same rows and columns are scaled identically on their Y and X axes, respectively. X axes: treatments; Y axes: saponin equivalent (ng cell^{-1})

3.4 Gene expression pattern analysis with microarrays

Of the approximately 6,300 sequences in our data set, 1742 were identified as differentially expressed between at least one combination of the three physiological growth phases of the nutrient replete or deprived cultures. When searching for growth dependent gene expression patterns, we found the highest number of differentially expressed genes when comparing cells in early exponential versus stationary growth phases. There was a much smaller difference observed in expression patterns when comparing the two exponential phases or the mid-exponential with stationary phase (Figure 7).

We observed the up- and downregulation of 64 and 139 genes, respectively, in all stationary phase cells as compared to the early exponential phase within treatments (Figure 8). Unfortunately, but as expected no annotation could be assigned to the vast majority of the upregulated genes. Interestingly, two bicarbonate transporter genes were identified among the ESTs and both were found to be upregulated. One fragment showed elevated expression in all treatments, approximately six-fold in the deprived versus 14-fold in the replete stationary

phase cells, whereas expression of the other fragment was elevated only in the N-depleted and nutrient replete cultures, at six and 14-fold upregulation, respectively.

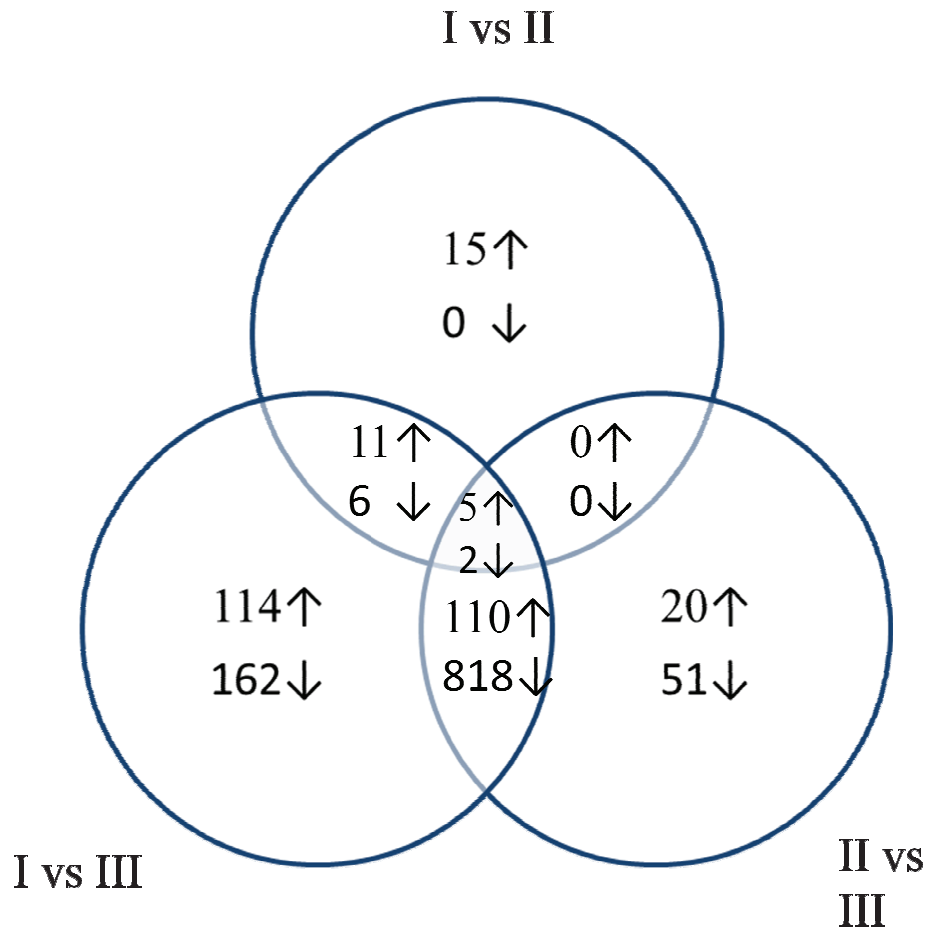

Figure 7. Growth phase dependent gene expression of *P. parvum*. ↑↓ depicts up- and downregulation of genes between different growth phases irrespective of treatment.

Among the downregulated genes, we found those involved in cell division, mitosis, DNA/RNA transcription, translation, photosynthesis (Table 6). The expression of the genes putatively involved in vesicle transport and secretion showed also a growth-phase dependent pattern (Table 7). Almost all genes that exhibited differential expression were upregulated in the stationary phase of nutrient depleted cells.

Many genes (total 327) were differentially expressed only in the P-deprivation treatment, with 173 genes uniquely up and 154 downregulated in the P-limited stationary phase cells. Among the upregulated genes, we identified specific responses, such as the elevated mRNA

concentration of a phosphate repressible phosphate permease (40 fold increase), and acid phosphatase (13 fold increase), an arsenate reductase (4.6 fold increase) and a triose phosphate/phosphate translocator (4 fold increase) (Table 5). Among downregulated genes, we observed reduced expression levels of translation initiation factors and ribosomal proteins, and of many unknown genes, only under P starvation.

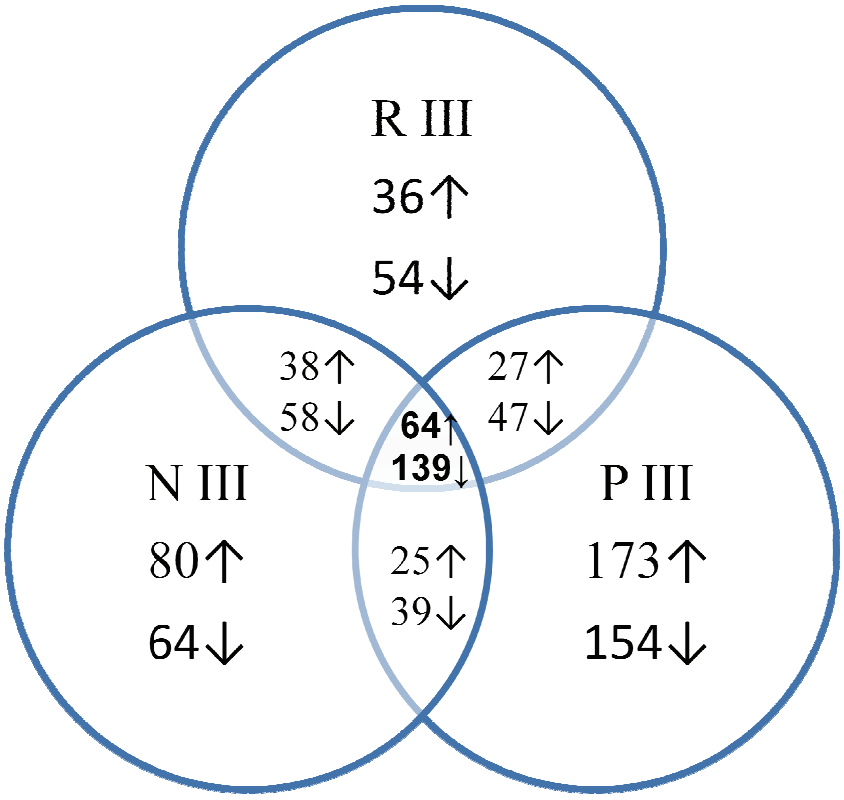

Figure 8. Treatment specific gene expression differences in *P. parvum* Genes differentially regulated in the stationary phase of nitrogen (NIII), phosphorus (PIII) depleted and replete (RIII) cultures ↑↓ depicts up and downregulation of genes between starved and replete stationary cells.

In the stationary growth phase of the N-depletion experiment, 80 genes were uniquely upregulated, whereas 64 were downregulated as compared to the exponential phase. No function could be assigned to any of the upregulated genes. No upregulation of any genes putatively involved in acclimation to N-starvation (Table 5) was observed. Nevertheless, some genes such as a Photosystem II stability/assembly factor, nuclear transportation factor, and an actin regulating protein were downregulated in the N-starved stationary phase cells.

4. Discussion

4.1 Comparison of EST libraries for prymnesiophytes

The normalized EST library of the toxic prymnesiophyte *P. parvum* includes genes expressed in a variety of physiological states induced under a variety of environmental regimes. Alteration of nutrient concentrations and ratios along the growth curve provided significant insights into the transcriptome of this toxic species. Comparison with several EST datasets available for prymnesiophytes including the non-toxic *Isochrysis galbana* (Patron et al., 2006), *Pavlova lutheri* (Patron et al., 2006), and *Emiliania huxleyi*, and the toxic species *Chrysochromulina polylepis* (John et al., 2010) serves to distinguish gene functions common to prymnesiophytes from toxin-specific elements. Our comparison of the two *P. parvum* libraries, based on their functional annotation, increased the number of KEGG metabolic enzymes identified from this species from 372, as identified in the EST library constructed by (La Claire, 2006), to 597 (Table 2). This highlights the importance of sampling RNA species over a wide range of environmental regimes. In any case, when comparing *P. parvum* genes to other prymnesiophyte sequences, we observed a similar distribution of functional categories similar to that in non-toxic *Pavlova lutheri* and *I. galbana* (Figure 3). On the one hand, the categories `defense mechanisms´ (V) and `function unknown´ (S) appears to be overrepresented in the *P. parvum* libraries. On the other hand, in the category `translation, ribosomal structures and biogenesis´ (J) less hits than in the other species were found in the libraries of *P. parvum*. This phenomenon is probably due to the fact that our EST library was normalized, whereas that of *I. galbana* and *P. lutheri* was not, thus enabling us to catch more of the “gene space”.

4.2 Nutrient effects on toxicity and gene expression

4.2.1 Effect of phosphorus starvation

The allelopathic capacity or extracellular toxicity of *P. parvum* against different organisms, including the brine shrimp *Artemia salina* (Granéli and Johansson, 2003a; Larsen and Bryant, 1998; Larsen et al., 1993; Meldahl et al., 1995), the cryptomonad *Rhodomonas baltica/salina*: (Barreiro et al., 2005; Skovgaard and Hansen, 2003), and fish (*Pimephales promelas*): (Baker et al., 2007; Henrikson et al., 2010; Schug et al., 2010; Valenti Jr et al., 2010) has been monitored in many studies. In a consistent pattern, the extra- and intracellular toxicity of P-starved cells has been elevated in comparison to P-replete cells (Granéli and Johansson, 2003b; Johansson and Graneli, 1999b; Uronen et al., 2005). In accord, we also found enhanced overall toxicity in the stationary phase of the P-starved cells, whereas no elevated toxic effect was observed in case of the N-depleted and nutrient-replete treatments, even in stationary phase.

In addition to the shift up in toxicity, we also found a marked transcriptional response to P-starvation. The identified phosphate-repressible phosphate permease (Prymnesium-C-a-45e11.m13r) (Table 5) was upregulated 40 fold in the stationary phosphorus starved cells. This fragment shows high similarity to a phosphate repressible phosphate permease from *Emiliania huxleyi* (Table 5), the expression of which also increases under P-starvation (Dyhrman et al., 2006). Furthermore, our finding is in accordance with the observations of Moseley and co-workers who found three different phosphate transporter genes upregulated in phosphate depleted *Chlamydomonas reinhardtii* cells (Moseley et al., 2006). Also, a phosphate transporter gene (TcPHO) from the prasinophyte alga *Tetraselmis chui* was upregulated in phosphorus starved cells (Chung et al., 2003). Two acid phosphatases were found in our EST database (Prymnesium-C-a-102j12.m13f, Prymnesium-C-a-48g10.m13f).

The expression of one fragment (Prymnesium-C-a-102j12.m13f) was highly elevated in phosphorus starved cells (13 fold), whereas the other fragment (Prymnesium-C-a-48g10.m13f) was upregulated in the stationary nutrient replete and nitrogen starved cells, and showed no regulation under phosphorus depletion. In *Chlorella*, *Emiliania* and *Chlamydomonas*, the expression of acid phosphatases and phosphohydrolases was elevated in low nutrient concentrations, more so under nitrogen depletion than under phosphate limitation (Abel et al., 2002; Kruskopf and Du Plessis, 2004; Riegman et al., 2000; Ticconi and Abel, 2004). These enzymes either hydrolyze phosphate esters to access extracellular, organically bound phosphate nutrients under acidic conditions, or they are involved in intracellular relocation of phosphate.

Also, high levels of phosphorus are required for various chloroplast functions. In *Chlamydomonas* phosphate is transported from the cytosol into the chloroplast, where it is incorporated into starch bodies. Translocators of phosphate/triose or hexose phosphate are activated when phosphorus is limiting (Sharkey et al., 2004). In case of *P. parvum* the expression of a putative glucose-6-phosphate/phosphate or phosphoenolpyruvate/phosphate antiporter was even in the phosphorus starved cells during all physiological phases. However, the expression of the same gene was reduced in the stationary replete and nitrogen depleted cells. These findings suggest that *Prymnesium parvum* reacts to P limitation specifically on the gene expression level, a strategy known to be applied by other microalgae too (Dyhrman et al., 2006; Wurch et al., 2011).

We identified two genes which presumably are encoded by an arsenic detoxification operon from *Prymnesium parvum*. The arsH gene (Prymnesium-C-a-62c01.m13f) was upregulated during phosphorus starvation (5 fold ↑), but the expression of arsenate reductase gene arsC remained unaltered in the phosphate starvation experiment, whereas it was downregulated in the stationary phase of the nitrogen starved and the nutrient supplied

stationary cells. The arsC gene was clearly shown to be an arsenate reductase required for arsenate detoxification in *Sinorhizobium meliloti* (Yang et al., 2005). Moreover, Sanders & Windom observed the arsenic uptake rates of the diatom *Skeletonema costatum* (Sanders and Windom, 1980). The uptake of arsenic in this species depends on the concentration of As(V) and phosphate in the medium. Increasing concentration of arsenic causes elevated uptake, whereas increased phosphate concentrations cause decreased arsenic uptake rates. The total intracellular arsenic concentrations were influenced by phosphate addition as well. Arsenic concentrations in cells grown under relatively high phosphorus concentration were an order of magnitude lower than in cells grown under phosphate limited conditions (Sanders and Windom, 1980). These authors concluded that since arsenate is a chemical analogue of phosphate, arsenate and phosphate compete for uptake by algal cells. Although we do not know the amount of arsenic in the seawater used in the experiment, we assume that under phosphorus limiting conditions elevated arsenic uptake took place, which in turn caused elevated expression of the arsenate reductase gene.

3.2.2 Nitrogen starvation

Some researchers found enhanced intracellular toxicity under nitrogen starved *Prymnesium parvum* cells (Johansson and Graneli, 1999b; Uronen et al., 2005). According to others, the toxicity of N depleted cells does not differ significantly from that of the nutrient replete cells (Lindehoff et al., 2009; Sopanen et al., 2008). Our findings concerning the intracellular toxicity confirm the latter observation (Figure 6). Also, the allelopathic capacity of nitrogen starved *P. parvum* cells remained unchanged in all physiological phases. It did not differ from that of the nutrient supplied cultures in the first two sampling points either; whereas a decrease could be observed in case of the stationary replete cells (Figure 5).

We investigated the effect of nitrogen starvation on the gene expression pattern of *P. parvum* with the microarray approach. Similar gene expression studies were performed with *Aureococcus anophagefferens* (Wurch et al., 2011), *Alexandrium minutum* (Yang et al., 2011) and the haptophyte *E. huxleyi* (Dyhrman et al., 2006). These latter authors applied the long serial analysis of gene expression approach (SAGE), to study the effect of nitrogen or phosphorus depletion on *E. huxleyi*. They identified 38 genes upregulated under nitrogen starvation, but to most of them no function could be assigned. In our study 504 genes were differentially regulated in the nitrogen depleted stationary cells as compared to the depleted exponential cells (Figure 8). Out of the 207 up and 300 downregulated genes, 80 are uniquely up and 64 downregulated in the nitrogen starved stationary cells. To none of the upregulated genes were we able to assign function based on sequence similarity. Fourteen genes (Table 5) were identified in our database, putatively involved in nitrogen transportation and storage, the expression of which was monitored. Surprisingly, no clear nitrogen starvation response could be seen on the gene expression level of these candidate genes. The expression of nitrate and ammonium transporter genes was found unaltered in nitrogen depleted and replete stationary cells, but went down under phosphorus depletion. Kang and co-workers (Kang et al., 2007; Kang et al., 2009) observed an increase of mRNA level of both nitrate and ammonium transporter genes in nitrogen depleted, to nitrate conditioned *Isochrysis galbana* cells, but when ammonium and nitrate was provided, the expression of both transporter genes decreased. The expression of a glutamine synthase gene in *I. galbana* was upregulated twofold in nitrogen depleted cells, whereas no differential regulation was shown in the presence of ammonium (Kang et al., 2007). In *P. parvum* neither glutamine nor glutamate synthase genes were differently regulated under nitrogen starvation. Bruhn and co-workers on the other hand, found no increase in the expression of cytosolic glutamate synthases under N

deficient conditions in *E. huxleyi* cells (Bruhn et al., 2010). This contradiction may be due to the investigation of different type of glutamate synthase genes e.g. cytosolic or plastidic.

An increasing amount of ammonium was measured in the medium as the cultures grew denser and the pH got higher. The highest amount was measured in the stationary replete cells and stationary phosphorus depleted cells. Interestingly, even the nitrogen depleted cells liberated some ammonium. *Prymnesium parvum* was shown to possess cell surface L-amino acid oxidase(s), which oxidize amino acids and amines from organic matter, liberating ammonium, which is subsequently taken up (Palenik and Morel, 1991). No amine oxidase enzyme activity was observed by these authors in the presence of ammonium whereas the presence of nitrate and nitrogen starvation triggered higher enzyme activity (Palenik and Morel, 1991). In agreement with this a copper dependent amine oxidase (Prymnesium-C-a-20e07.m13f, Table 5) was identified from *P. parvum.* It was downregulated in the phosphorus depleted stationary cells, whereas no difference in the gene expression was observed in the nitrogen depleted and replete stationary cells. Also, the two putative ammonium transporter genes showed even expression levels during the whole experiment in the nitrogen starved and in the replete cells, but they were downregulated in the stationary phosphorus depleted cells. The increased amount of ammonium in the medium might be due to increased amine oxidase activity, under low nitrate levels in the medium (nitrogen depleted and replete cultures). Alternatively, it could have leaked out of disintegrating cells under all treatments.

Shilo and co-worker investigated the effect of ammonium or acetic acid on *P. parvum* cells (Shilo, 1953; Shilo and Shilo, 1962). They found a temperature, salinity and pH dependent lytic effect on *P. parvum*. The same amount of ammonium has a pH dependent effect, the higher the pH is, the higher more pronounced is the lytic effect, the cells swell and finally burst (Shilo and Shilo, 1962). Temperature and salinity has a similar effect as pH, the higher the temperature/salinity is, the higher rate of swelling is observed in the presence of

ammonium. The pH dependence of the lysis can be explained by the fact that the membrane is permeable to undissociated electrolyte, but not to the ion. Shilo claims, that an osmotic mechanism plays a role in this lytic process, the ammonia accumulates in the cells until the internal osmotic pressure causes the burst of the cell envelope (Shilo and Shilo, 1962). In our experiment the pH value of the replete stationary cells was 9.55. Under this pH 70% of the overall ammonium is in form of ammonia, 30% as ammonium. At this pH, ~ 100 μM ammonium sulphate would be needed to cause total lysis of *Prymnesium* cells. We detected one tenth of this amount (Figure 4) in the culture flasks, which very likely caused partial swelling/lysis of *P. parvum* cells.

A marked decrease was observed in the expression of cytochrome and light harvesting related genes under all treatments in the stationary phase. In nitrogen starved cells it may be a treatment specific response, knowing the nitrogen dependency of photosynthesis. Indeed, it was observed in many algal species, that shortage on nitrogen results in lower amount of chlorophyll per cell (de Groot et al., 2003). Gene expression analyses of other microalgae support this finding (Yang et al, 2011 in press, (Dyhrman et al., 2006). Due to low photosynthetic capacity of the cells, increased amount of reactive oxygen species are often observed. In accordance with these findings, the expression of an alternative oxidase (Prymnesium-C-a-19e01.m13f) was found unaltered in N starved cells, whereas its expression decreased in replete or P starved stationary cells.

4.2.3 Phagotrophy

P. parvum although primarily photosynthetic, was shown to be able to feed on bacteria (Nygaard and Tobiesen, 1993), and other organisms (Martin-Cereceda et al., 2003; Tillmann, 2003). This mode of nutrition is called mixotrophy. Tillmann observed the formation of elongated pseudopodia, when *Prymnesium parvum* cells were incubated with prey organisms

(Tillmann, 1998). He postulated that the induction of these structures is mediated by chemical signals, since he observed pseudopodia in cells not in direct contact with potential food particles. When encountered a food particle, the pseudopodium enclosed it and it was taken up into a vacuole at the posterior of the *P. parvum* cells (Tillmann, 1998). Carvalho and co-worker observed elevated feeding frequency (phagotrophy) in nitrogen or phosphorus starved cells as compared to replete cells (Carvalho and Granéli, 2010). These authors describe phagotrophy even in unialgal cultures of *P. parvum*, where no prey organism was added. In this case, *P. parvum* presumably fed on bacteria, organic particles (dead cells) or weak *Prymnesium* cells (see also (Nygaard and Tobiesen, 1993; Skovgaard and Hansen, 2003). Sillo and co-workers identified more than 400 genes which are differentially regulated in *Dictyostelium* by phagocytosis (Sillo et al., 2008). However, few genes could be identified coding for proteins directly involved in phagocytosis. Among them are fragments related to cell adhesion, receptor proteins or genes involved in vesicle transport (Sillo et al., 2008). We identified in our EST library several fragments putatively involved in vesicle transport and secretion such as autophagy related proteins, exportin/importin fragments, clathrin and vesicle coat proteins (Table 6). The upregulation of genes involved in vesicle transport and secretion could be related to enhanced toxicity of stationary cells. However, another explanation would be the higher frequency of phagotrophy, feeding cannibalistically on disintegrated cells.

4.2.4 Genes expressed in the different growth phases

A high number of genes were regulated in a growth phase linked manner. Altogether 64 and 139 genes were up and down regulated in all stationary cells as compared to the exponentially growing cells. The gene expression differed more in a growth phase dependent

manner, than between the different treatments at the same physiological state. We could not assign any function to most of the genes up regulated in the stationary cells under all treatments. This is not surprising, considering that the functional identification of these is achieved through similarity comparisons. Still, the general pattern of overall decrease in the expression of genes involved in photosynthesis, cell proliferation and signal transduction could be observed, demonstrating the reduced cellular anabolic processes in stationary cells. Interestingly, a bicarbonate- transporter was upregulated to different extent in all treatments. Higher upregulation (14 fold) was observed in the stationary replete cultures, where the pH of the medium got well over 9 (9.5), whereas in the starved cultures the upregulation was six fold, and the pH of the cultures were 8.8 and 8.6 in case for phosphorus and nitrogen starvation respectively. This high pH is a common phenomenon in nature, since during a dense *P. parvum* bloom the pH can get as high, or even higher than 9, (Lindholm and Virtanen, 1992; Michaloudi et al., 2009). As the cells grow and divide the pH of the cultures increases, because the algae use up the dissolved carbon in the medium (Figure 4). At pH 8 and 9 bicarbonate is the predominant carbon species in the medium, and as the concentration of dissolved carbon in the medium decreases, the expression of the bicarbonate transporter increases in order to compensate and support higher rates of uptake.

5. CONCLUSIONS

A normalized EST library was constructed and served as a genetic basis for gene expression studies of the toxic Haptophyta *Prymnesium parvum*. We monitored the physiological and gene expression responses of *Prymnesium parvum* to low N or P levels. We observed an overall higher toxicity under phosphorus starvation, but not under nitrogen

limitation as compared to nutrient replete cells. Also, genes involved in the transport, mobilization and intracellular relocation of P were identified and found upregulated in phosphorus starved cells. Several genes were identified which are involved in the acquisition and storage of nitrogen, but surprisingly these were not differentially regulated under N starvation. Still, many unknown genes showed elevated mRNA levels in N limited cells. We propose that the genes, which are upregulated in N- or P-deprived cells, can be used as sensitive indicators for potential bloom events.

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8. APPENDIX

		KEGG pathway	EC numbers ESTTAL	EC numbers library La Claire
0	map00950	Alkaloid biosynthesis I	2	1
0	map00960	Alkaloid biosynthesis II	1	1
0	map00520	Amino sugar and nucleotide sugar metabolism	3	3
0	map00970	Aminoacyl-tRNA biosynthesis	5	7
0	map00330	Arginine and proline metabolism	12	14
3	map00053	Ascorbate and aldarate metabolism	4	5
0	map00410	beta-Alanine metabolism	3	4
0	map00100	Biosynthesis of steroids	12	6
0	map01040	Biosynthesis of unsaturated fatty acids	3	6
2	map00534	Chondroitin sulfate biosynthesis	1	1
0	map00020	Citrate cycle (TCA cycle)	9	11
0	map00460	Cyanoamino acid metabolism	4	2
2	map00270	Cysteine metabolism	3	1
2	map00982	Drug metabolism - cytochrome P450	3	3
3	map00983	Drug metabolism - other enzymes	4	4
1	map00061	Fatty acid biosynthesis	4	4
2	map00062	Fatty acid elongation in mitochondria	2	4
1	map00071	Fatty acid metabolism	6	9
1	map00051	Fructose and mannose metabolism	10	9
2	map00052	Galactose metabolism	6	6
1	map00361	gamma-Hexachlorocyclohexane degradation	3	2
0	map00480	Glutathione metabolism	9	7
0	map00010	Glycolysis / Gluconeogenesis	12	15
4	map00534	Heparan sulfate biosynthesis	4	2
0	map00340	Histidine metabolism	3	1
3	map00900	Limonene and pinene degradation	1	1
0	map00540	Lipopolysaccharide biosynthesis	2	1
0	map00300	Lysine biosynthesis	3	4
0	map00310	Lysine degradation	7	8
0	map01100	Metabolic pathways	95	221
	map00980	Metabolism of xenobiotics by cytochrome P450	2	3

map00270	Methionine metabolism	3	4
map00761	Nicotinate and nicotinamide metabolism	1	1
map00401	Novobiocin biosynthesis	1	1
map00770	Pantothenate and CoA biosynthesis	1	4
map00040	Pentose and glucuronate interconversions	5	4
map00030	Pentose phosphate pathway	8	10
map00360	Phenylalanine metabolism	5	2
map00400	Phenylalanine, tyrosine and tryptophan biosynthesis	4	6
map00523	Polyketide sugar unit biosynthesis	1	1
map00860	Porphyrin and chlorophyll metabolism	6	8
map00230	Purine metabolism	47	26
map00240	Pyrimidine metabolism	38	2
map00830	Retinol metabolism	1	1
map00450	Selenoamino acid metabolism	5	4
map00500	Starch and sucrose metabolism	3	1
map00521	Streptomycin biosynthesis	1	3
map00072	Synthesis and degradation of ketone bodies	2	3
map00430	Taurine and hypotaurine metabolism	1	3
map00380	Tryptophan metabolism	6	5
map00350	Tyrosine metabolism	7	6
map00290	Valine, leucine and isoleucine biosynthesis	17	18

Table 2. Comparison of KEGG pathway genes found in the two *P.parvum* libraries

VIII. SYNTHESIS

Functional and comparative genomic approaches have proven appropriate tools in the identification of toxicity and growth related genes in *C. polylepis* and *P. parvum*. Linking ecophysiological data with gene expression studies yielded novel insights into the cellular backgrounds of bloom formation. We tested the hypotheses concerning the involvement of PKS genes in the production of *C. polylepis* and *P. parvum* toxins, and followed up the effects of macronutrient starvation in *P. parvum*.

8.1 Transcriptomic databases of P. parvum and C. polylepis

Two normalized EST libraries were assembled, resulting in ca. 6300 and ~2200 tentative unigenes for *P. parvum* and *C. polylepis* respectively, similar to other haptophyte EST sequencing projects, e.g. *Pavlova lutheri* (7600) and *Isochrysis galbana* (12200) ESTs (Patron et al., 2006).

The generated EST databases were automatically annotated through similarity searches. Approximately75 % and more than 80 % of the *C. polylepis* and *P. parvum* ESTs respectively fall into the category `poorly characterized´. This is not unusual for microalgal EST libraries, as no annotation could be assigned based on similarity to 70% or more of dinoflagellate ESTs (Hackett et al., 2005; Lidie et al., 2005; Yang et al., 2010).

The comparison of *P. parvum* and *C. polylepis* EST to available Haptophyta data sets (the non-toxic *Isochrysis galbana* and *Pavlova lutheri* (Patron et al., 2006), and *Emiliania huxleyi* (http://genome.jgi.doe.gov/Emihu1/Emihu1.home.html) and the toxic *P. parvum* (La Claire, 2006)), allowed the establishment of similarity matrices and the identification of fragments which are present only in toxic prymnesiophytes. 1,526 genes with an assigned function were found in common among these four Haptophyta species, which are genes involved in core cellular functions and metabolic pathways, such as chaperones, proteasome proteins and

unknowns. Thirteen fragments were found only in the toxic species *C. polylepis* and *P. parvum*, most of them poorly characterized. The comparative analyses also identified 362 genes in the *P. parvum* libraries which appear to have no counterpart in the other species. Interestingly many of the fragments present in *P. parvum* only are involved in transcription or translation related processes, or are participants of cell cycle events. These genes are probably not unique to *P. parvum*; it is more likely that they are highly enriched in this library as compared to the others due to different approaches applied for library construction. Among the many uncharacterized proteins found only in *P. parvum* there may be candidates involved in species-specific adaptations such as phagotrophy.

We estimated the genome size of *C. polylepis* at around 230 Mb. which is similar to that of *E. huxleyi* (170Mb), and *P. parvum* was also estimated to fall into this range (G. Glöckner pers. comm.). These estimates are intermediate between small diatom genomes (40-80 Mb for currently sequenced species) and macroalgae *Fucus* (530 Mb), which is much larger. This relatively large genome size in Prymnesiophyceae may be due to the presence of haplo-diplontic life cycle, characteristic for this algal class (Edvardsen and Medlin, 1998; Larsen and Edvardsen, 1998; Rousseau et al., 1994), whereas no such could be observed in the sister class Pavlovophyceae, represented by smaller genome size (Nosenko et al., 2007). The observed moderate genome size combined with sinking prices of second generation sequencing would allow for an affordable genome sequencing project of both *C. polylepis* and *P. parvum*.

We screened both EST databases for PKS genes that have been suspected to be involved in toxin biosynthesis. Eight putative PKS gene fragments were detected among the *C. polylepis* sequence tags. Interestingly, although we could identify four putative PKS fragments in the previously published *P. parvum* database (La Claire, 2006), the EST library generated during this work for *P. parvum* contained none. This discrepancy is probably due to

methodological differences, namely the use of oligo-dT primers for reverse transcription in our case. The application of these primers in case of long, modular transcripts such as PKS genes leads to poor results, whereas it is adequate in case of non modular genes, because it delivers long, nearly full length sequences. Phylogenetic analysis of *C. polylepis* PKS genes sorted two PKS candidates (PKS 4 and 7) of eight into the Prymnesiophyceae clade, supported by good bootstrap values. This result suggests the presence of the conventional, modular PKS I structure, which was found typical for protists (John et al., 2008). As to the origin of the other putative PKS genes: since the cultures were not grown axenically the presence of bacteria could account for the finding of bacterial type PKS genes.

This thesis work successfully established the following:

- Normalized transcriptomic databases were constructed for *P. parvum* and *C. polylepis*
- Putative PKS gene fragments were identified from *C. polylepis* and *P. parvum*
- A `core genome set´ of Haptophyta species was identified with the comparative genomic approach
- Thirteen functional orthologs were present only in the toxic prymnesiophytes (*P. parvum* and *C. polylepis*) investigated in this study

8.2 Toxicity and PKS gene expression of P. parvum and C. polylepis

We hypothesized that the exhibited toxicity of *C. polylepis* shows a positive correlation with the expression of PKS gene(s). In accord, we found low PKS gene expression in the middle of the dark phase, and much higher towards the end of the dark phase. This result is consistent with the data of Eschbach and co-workers (2005), who found elevated

toxicity at the transition from dark to light phase and observed a marked decrease in toxicity in the middle of the dark phase. The drop in toxicity corresponded to the cell division event in the G2+M phase, as demonstrated with flow cytometric analysis. An increase in toxicity was observed as the cells started to produce the toxin(s) in the beginning of the G1 and light phase. Still, it remains to be resolved wether the enhanced toxicity is cell cycle related or is a light mediated response. Similar trend was observed in dinoflagellates, where toxin production happened exclusively in the G1 phase in *Alexandrium fundyense* (Taroncher-Oldenburg et al., 1997), whereas the shade adapted benthic species *Prorocentrum lima* (Pan et al., 1999) was found to synthesize different toxin compounds in the G1 and S phases. We observed a positive correlation between elevated toxicity and PKS gene expression, delivering indirect support for the involvement of the latter in toxin synthesis. Of course, further studies will be necessary to causally link these two phenomena.

In Chapter II we focused on *P. parvum*. In order to link observed phenotype (toxicity) with cellular processes putatively involved in toxin synthesis, we monitored toxicity and measured the expression of three PKS genes as a function of different abiotic treatments.

A high induction of intracellular toxicity was observed under high light whereas low salinity was found to induce elevated extracellular toxicity in the two hours treatment respectively. High irradiation increases the toxicity of *P. parvum* (Shilo & Aschner, 1953) and an inverse relationship between salinity and extracellular toxicity in *P. parvum* has also been reported previously by Parnas and co-workers (1962). On the other hand, and again in accord with previous studies, e.g. Larsen and Byant (1998) and Ulitzur & Shilo (1964) our bioassay results showed no temperature impact on toxicity. These findings are also in accordance with field observations, according to which freshwater inflow (Kaartvedt et al., 1991) promotes toxicity in *P. parvum*.

All three PKS genes were differentially expressed under at least one treatment, and different PKS fragments showed in part different expression patterns across the treatments. It is likely that the three fragments investigated originate from three different gene copies; or alternatively, the modules of one single gene might operate independently, as was demonstrated for bacterial PKS genes (Tsantrizos and Yang, 2000). This could explain the different gene expression patterns obtained.

Notably, PKS expression level was not strictly associated with toxicity. Interestingly, elevated expression level of genes PKS 2 (7t3) and PKS 3 (81t3) was found under high temperature and bubbling, but these treatments did not trigger higher toxicity. Since PKS genes are known to produce a large variety of compounds with a wide range of biological activities, it is likely that PKS 2 (7t3) and 3 (81t3) are involved in the synthesis of as yet to date uncharacterized (probably non-lytic) compounds. The seemingly unspecific (not toxicity related) upregulation of PKS 2 and 3 and the production of the unknown substance may be a general stress response in *P. parvum*.

High light intensity caused elevated expression of all PKS fragments investigated; accompanied by increase in intracellular toxicity. Under low salinity, which promoted elevated extracellular toxicity, we measured elevated expression of PKS1 (6t3). The expression of PKS1 (6t3) was found to correlate with the observed increase in toxicity; this indicates the involvement of this gene in the production and/or release of the bioactive substances in *P. parvum*.

A qPCR method based small scale gene expression study (Chapter II) has been performed with *P. parvum* PKS genes, the expression of which was monitored as a function of six short term treatments. To test the strength of an `alien´ reference gene in the normalization process, we incorporated foreign mRNA into the RNA samples before cDNA synthesis and qPCR measurement. Three housekeeping genes exhibited varying numbers of

mRNA levels under different treatments thus proved their unsuitability for normalization processes, whereas the copy numbers of the foreign reference gene remained stable in all samples. The use of external mRNA of insect origin in the measurement of plaktonic species allows for correction of differences in RNA quality and thus cDNA synthesis, their reliability in normalization purposes enhances the potential of qPCR measurements.

With respect to toxicity and PKS gene expression the following conclusions can be drawn:

- PKS genes show treatment dependent gene expression patterns, which in the case of *P. parvum* is not consistent for all fragments investigated.
- Ther is a positive correlation between treatment dependent elevated toxicity and upregulation of PKS genes in both species.
- The data obtained in Chapter I and II provided evidence about a positive correlation between enhanced toxicity and elevated gene expression of PKS genes, thus support the hypothesis about the involvement of PKS genes in the biosynthesis of the toxin.
- The addition of foreign mRNA allows for normalization in gene expression studies of marine microalgae.

8.3 Gene expression differences in P. parvum associated with growth and nutrient limitation

Studies of the ecological role of toxicity for *P. parvum* pointed out that toxin production is apparently part of an alternative nutrient acquisition strategy of this species, when dissolved inorganic nutrient concentrations decrease to sub-optimal levels. At low N and P levels, cultured *P. parvum* starts producing lytic substances, thereby immobilizing and even lysing potential predator cells which would otherwise consume *P. parvum* (Tillmann, 2003). *P. parvum* can then feed on both decaying predators and released organic nutrients. In this ecological context, it is not surprising that toxic blooms of *P. parvum* have usually been reported under imbalanced nutrient conditions. The detailed cellular responses of this species

to nutrient limitation and their regulation were, however, little known previously. Chapter III addressed this gap by a functional genomic approach, namely linking gene expression profiles with physiological observations in *P. parvum* under bloom promoting conditions (N or P limitation).

Interestingly, gene expression differed more markedly among physiological (growth) stages, than between different treatments. This was mostly represented by the downregulation of genes related to cell cycle (transcription, translation cell division etc) or photosynthesis. Similar results have been observed in dinoflagellates (Toulza et al., 2010; Yang et al., 2010).

High N:P ratios have been observed in most *P. parvum* blooms. We hypothesized that P or N deprivation triggers the upregulation of genes involved in the acquisition, storage, etc of these macronutrients. Accordingly, P-deprived cells demonstrated elevated toxicity as compared to non-limited cells, which probably led to elevated phagotrophy. We also observed treatment specific regulation of 327 genes as compared with exponentially growing, nutrient replete cells. As expected and seen in case of the closely related *E. huxleyi* (Dyhrman et al., 2006), several genes involved in the acquisition and transport of P were found upregulated. However, under N depletion no enhanced toxicity was observed and only 144 genes were differentially regulated in *P. parvum*. To most of the fragments no annotation could be assigned, as was also the case for *E. huxleyi* (Dyhrman et al., 2006), and the expression of the genes identified among the ESTs as related to N uptake and storage remained unaltered. This finding is very relevant, since it is in conflict with the results of small-scale expression analysis studies, performed with *I. galbana*. In this closely related non-toxic, and more importantly non-mixotrophic organism the expression of nitrate and ammonium transporter genes was upregulated under N starvation (Kang et al., 2007). I postulated that *P. parvum* developed alternative ways to cope with N starvation, and the upregulated unknown genes may be related to alternative N acquisition strategies such as phagotrophy.

In recent bloom forecast modern molecular biological tools are applied with increasing frequency (Töbe et al., 2006). I identified genes highly indicative of nutrient limitation in *P. parvum*, which stress leads to enhanced toxin production in *P. parvum*. Feeding on released organic matter and decaying sympatric species *Prymnesium* is able to maintain growth, thus developing into a toxic algal bloom. Genes indicative of nutrient limitation can be used as proxies in bloom forecast.

This study (Chapter III) yielded the following insights:

- N- or P-depletion causes unaltered and elevated toxicity in stationary phase cells respectively, whereas stationary, nutrient replete cells exhibit decreased toxicity as compared to exponentially growing replete cells.
- P limitation results in the specific upregulation of P acquisition-related genes.
- N starvation leads to the upregulation of genes with unknown functions, whereas N acquisition genes are not differentially regulated.
- P-acquisition genes may serve as marker proxies in bloom monitoring processes.

In the course of my thesis I linked physiological observations with gene expression studies, and found evidence concerning the involvement of PKS genes in toxin production in *C. polylepis* and *P. parvum*.

IX. OUTLOOK

Since the first bloom reports of *P. parvum* and *C. polylepis*, a considerable amount of (even if somewhat contradictory) knowledge has been developed about bloom dynamics, ecology and physiology of these toxic species. However, some of the key questions about toxicity and bloom formation of these species are still open.

Clear evidence is lacking about the composition and nature of the toxin(s) produced by either species. Until the active compounds are not characterized, their quantification is impossible. In order to elucidate the structure of the active compounds of *C. polylepis* or *P. parvum,* a bioassay-guided analytical and physico-chemical (e.g., chromatographic combined with mass spectrometric) approach should be applied. Once the substances responsible for the lytic effect are identified, quantification of these compounds with analytical chemical methods will be possible. This could replace the bioassays and the quantification of toxin content would be more reliable and reproducible.

The structure of the toxins alone cannot provide direct evidence about biosynthetic pathways, but once the structure of the toxic compounds is resolved, combined efforts of genomic and physiological studies can pinpoint enzymes involved in their biosynthesis. Gene expression studies of putative toxin genes correlated to toxicity could give insights about their involvement in toxicity.

Identification of genes involved in certain cellular processes (for example stress response, growth or toxicity) can also be facilitated by comparisons of strains of the same taxa, which highly express and lack the phenotype in question. This approach has been followed by researchers comparing non-toxic and toxic strains of the same species (Yang et al., 2010), but for algae for which (as in the case of *P. parvum*) only toxic strains are available, treatments causing high and low toxicity should be compared. Large scale gene expression or proteomic comparative studies have the potential to reveal the differences in the transcriptome or

proteome of the compared strains. With bioinformatic methods the identification of genes related to certain phenotype or environmental condition (such as toxicity or elevated pH) will relatively easily be facilitated.

For the isolation and further characterization of putative toxin related genes the construction of a genomic library is mandatory. In case of long, modular sequences (such as PKS genes), cosmid/fosmid or bacterial artificial chromosome (BAC) libraries, which contain long (up to 40 kbp) genomic DNA fragments are good choices for full-length gene isolation. The expression of the putative toxin genes in yeast or bacteria should be monitored, and if the toxic phenotype can be observed by heterologous expression, the role of the investigated gene in toxicity will be proven.

The construction of a mutant database through random mutagenesis induced by for example UV radiation or the establishment of a knock-out system would allow for gene silencing; this would greatly enhance the possibilities of functional gene characterization. The latter approach allows for targeted disruption of previously identified genes/gene families, and has been used with gene/function identification in many organisms previously, such as higher plants, bacteria or the chlorophyte alga *Chlamydomonas*. For the successful transformation of algal cells it is necessary to develop a protocol how to grow the species in question on a solid surface. Cells growing on a solid surface can be subjected to particle bombardment, during which a construct bearing marker genes and a silencing construct can be introduced into the cells. As a result of silencing, the expression of the targeted gene will be highly reduced or absent. The growth and toxicity of e.g. a PKS knock-out mutant with no PKS gene(s) transcripts could then be compared to wild-type strains with normal PKS expression levels. Reduced or no toxicity in case of the mutant cells would confirm the role of PKS genes in toxin synthesis.

The autecological problem – the limited representation of intraspecific variability in studies performed with single clonal strains derived from natural populations and then maintained in laboratory culture, often over long periods of time, is a fundamental, rather challenging issue of this research field. The differences in inducible toxicity among strains compared are considerable; this points out that phenotypic (and underlying genetic) diversity of natural populations is probably substantial. To test the predictability, variability and the role of environmental impacts on the phenotypic expression, instead of using one or few "representative" clones kept in the laboratory after isolation from a certain region, a large numbers of isolates from different populations from all over the world should be investigated. Alternatively, studies performed at intermediate scales between high-diversity, low-control field observations and low-diversity, highly controlled clonal strain experiments, for instance mesocosm experiments or laboratory manipulations of genetically diverse populations, might provide a useful link. Toxicity/toxin expression among different populations and in different members of the populations should be compared, to observe heterogeneity in the population. This diversity may be an important feature in the natural selection of particular strains responsible for bloom formation or stabilization, and population dynamics under bloom forming conditions.

Diploid and haploid *C. polylepis* strains are known to have different environmental optima and thus may reflect niche specific adaptation. Expression studies with 1N and 2N strains should be used to identify underlying processes involved in the adaptation to different ecological niches applying transcriptional/proteomic comparisons.

Functional genomic approaches in non-model organisms are limited to genes which have been identified in transcriptomic databases. The EST approach to identify expressed genes is an appropriate tool, although it is bound to contain only a subset of the investigated transcriptome, and therefore it delivers incomplete genetic information. A first step towards a

more complete functional characterization of *P. parvum* and *C. polylepis* could be a full genome sequencing project. Such an undertaking would deliver the sequence of the whole genome of the species in question, which would allow for further gene expression studies and the construction of gene silenced mutants could be achieved. Based on a genome sequence, further investigations into the regulation of cellular mechanisms could be conducted, for example through the identification of binding sites of enhancer or suppressor proteins. Through the establishment of pyrosequencing, which allows for the production of a large amount of sequence information in single, highly parallel sequencing runs, such an achievement for relatively low costs might soon become feasible.

Comparative functional genomic approaches which link ecophysiology to gene expression data in harmful algal species have an enormous potential to identify genes involved in bloom related processes and toxigenesis. Knowledge about physiological cues triggering toxic blooms, and cellular processes involved in growth and the regulation, induction of toxin production will significantly contribute to better understanding of bloom dynamics of *C. polylepis* and *P. parvum*.

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