# Determination and characterization of genes involved in toxic mechanisms of the prymnesiophyte *Prymnesium parvum*

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# I. Acknowledgements

Albert Einstein once remarked: '*If we knew what we were doing, it wouldn't be research, would it?*' As an awkward Master student beginning my thesis in 2007, this was much the feeling at that point in time. Fortunately, since then I have grown intellectually, despite the nature of scientific research not having changed! I would like to thank first and foremost my supervisors Professor Dr. Allan Cembella and Dr. Uwe John for their support and helpful discussions, not limited to science but sometimes related to general hurdles in life in general. Thanks as well to the entire AG Cembella research group: Annegret, Wolfgang, Bernd, Urban, Sylke, Jan, Philip, Nina, Ines, Haiyan, Karina, Karsten and Aboli for always keeping me in line.

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# II. Summary

This thesis represents a study of the ecophysiology and toxicity of the prymnesiophyte *Prymnesium parvum.* The first aim was to investigate changes in the relative toxicity of *P. parvum* following a series of physiological 'shock' treatments, meant to simulate environmental conditions under which harmful blooms of this species have been observed. As blooms of this haptophyte often occur in dynamic coastal brackish water systems, *Prymnesium parvum* is noted for its physiological flexibility, which may contribute to providing a competitive advantage over other coexisting species. Due to the unconfirmed nature of the compounds involved in toxigenic processes, two bioassays were employed to characterize changes in lytic capacity (extracellular vs. intracellular). These bioassays are considered physiologically relevant, as observed icthyotoxicity occurs through lysis of the gill cell membranes, rendering the fish unable to perform gas-exchange processes and obtain oxygen. Additionally, the gene expression of three polyketide synthase genes (PKS) were analyzed via quantitative PCR (qPCR), based on current chemical characterizations of toxic compounds produced by *P. parvum.*

Low salinity and high irradiance were observed to alter the lytic effects of *P. parvum* on the sensitive cryptophyte *Rhodomonas salina* and erythrocytes. Furthermore, these two shock treatments were found to increase the transcript copy number in selected PKS genes, suggesting a possible correlation between toxicity and the PKS biosynthetic pathway.

Allelochemical mediation has been suggested to affect competition and predatory relationships associated with formation of *P. parvum* blooms. As interactions between species are an integral part of understanding plankton ecology, interspecific interactions between *P. parvum* and three coexisting species were accordingly investigated. Combining bioassays with a functional genomic approach allowed differential characterization of cell-cell contact vs. waterborne cues depending on the organism with which incubated. A unique response on both the levels of toxicity, gene expression profile as well as PKS transcript copy number to the potential predator *Oxhyrris marina* suggest a fundamentally different type of interaction between the two species. Additionally, a dose-response time series experiment showed that changes in gene expression and toxicity did not occur

immediately in *P. parvum,* rather after 60-90 minutes. Such a response by *P. parvum* may in fact signify a co-evolutionarily adaptive defense.

Finally, examination of the effects of phosphorous limitation and low salinity stress on the gene expression profile and lytic capacity showed that the combination of these two stressors induces secretion or extracellular transport of toxic substances to a much higher degree than either stressor individually. Whether this observation is due to changes in membrane integrity due to homeostatic processes needs further research. The pattern of gene expression, however, revealed regulation of among others genes associated with active cellular transport processes, suggesting that maintenance of intracellular-extracellular homeostasis may play a role in the observed toxicity.

In summary, these studies integrate the concepts of ecophysiology and functional genomics, providing a useful platform for further research regarding environmental factors associated with the toxicity of *P. parvum.* As functional genomic methods become more accessible, such approaches illustrate their potential application within the field of harmful algal research.

# III. Zusammenfassung

Die vorliegende Arbeit befasst sich mit der Ökophysiologie sowie der Toxizität des Prymnesiophyten *Prymnesium parvum*. Das Hauptanliegen dieser Arbeit bestand in der Untersuchung der veränderbaren relativen Toxizität von *P. parvum* infolge physiologischer Schockbehandlungen, welche Umweltbedigungen simulieren sollten, unter denen das Auftreten schädlicher Algenblüten dieser Art beobachtet wurde. Da Algenblüten dieses Haptophyten oft in dynamischen Brackwasserküstenökosystemen vorkommen, zeichnet sich *Prymensium parvum* durch seine eurypotenten physiologischen Eigenschaften aus, welche Konkurrenzvorteile gegenüber co-existierenden Arten bieten. Aufgrund der unvollständigen Charakterisierung der in die toxigenen Prozesse involvierten Substanzen wurden zwei Biotests zur Bestimmung des lytischen Wirkungsgrades (extrazellulär versus intrazellulär) dieser Substanzen durchgeführt. Die physiologische Relevanz beider Biotests ergibt sich aufgrund der ichthytoxischen Wirkungsweise welche eine Lyse der Kiemenzellmembranen bewirkt und dadurch Gasaustausch sowie Sauerstoffaufnahme für den Fisch unmöglich macht. Zusätzlich wurde die Genexpression dreier Polyketidsynthase-Gene mittels quantitativer PCR (qPCR) analysiert; die Auswahl dieser Gene basiert auf der momentanen chemischen Charakterisierung der von *Prymnesium parvum* produzierten Substanzen.

Niedrige Salinität sowie hohe Strahlungsintensitäten veränderten den lytischen Wirkungsgrad *Prymensium parvums* gegenüber dem Kryptophyten *Rhodomonas salina*, gleiches zeigte sich gegenüber den Erythrozyten. Zusätzlich zeigten beide Schockbehandlungen eine erhöhte Anzahl an PKS-Gen Transkripten und somit folglich eine mögliche Korrelation von Toxizität und PKS-Biosyntheseweg.

Die Synthese und Verbreitung von Allelochemikalien scheint die mit der Blütenbildung in Verbindung stehenden Prozesse wie Konkurrenz und Prädation in *P. parvum* zu beeinflussen. Da Interaktionen zwischen Arten zu dem zentralen Verständnis der Planktonökologie gehören, wurden interspezifische Interaktionen zwischen *P. parvum* und drei Co-existierenden Arten entsprechend untersucht. Dabei erlaubte die Kombination von Biotests mit funktionellen genomischen Methoden eine differenzielle Charakterisierung

von einerseits direkten Zell-Zell Kontakten gegenüber im Wasser gelösten Signalstoffen. Die dadurch ermittelte Reaktion betreffend der Toxizität sowie auch auf Genexpressions- und PKS-Transkriptebene gegenüber dem potentiellen Prädator *Oxhyrris marina* deutet auf eine grundlegend andere Interaktionsart dieser beiden Arten hin. Darüberhinaus zeigt eine in Form eines Zeitreihenexperimentes durchgeführte Dosis-Wirkungsbeziehung, dass Veränderungen der Genexpression sowie der Toxizität in *P. parvum* nicht sofort erfolgen, sondern erst nach 60 – 90 Minuten eintreten. Diese Reaktionsweise von *P. parvum* deutet auf eine co-evolutiv entstandene, adaptive Verteidigungsstrategie hin.

Die Untersuchung der Effekte von Phosphor-Limitation und erniedrigter Salinität auf die Genexpressionsprofile sowie auf den lytischen Wirkungsgrad zeigten, dass eine Kombination beider Stressoren die Sekretion oder einen extrazellulär gerichteten Transport der toxischen Substanzen zu einem viel höheren Ausmaß bewirkt als jeder Stressor einzeln. Ob dies auf Änderungen der Zellmembranzusammensetzung oder auf homöostatischer Prozesse zurückzuführen ist, benötigt weitere Untersuchungen. Anhand der Genexpressionsmuster zeigt sich jedoch, neben der Regulation anderer Gene, ein Muster welches mit aktiven zellulären Transportprozessen assoziiert werden kann und somit könnte der Aufrechterhaltung der intrazellulären-extrazellulären Homöostase eine tragende Rolle für die beobachtete Toxizitätsänderungen zukommen.

Zusammenfassend kann gesagt werden, dass die vorliegende Arbeit Konzepte der Ökophysiologie und der funktionellen Genomik vereinigt und dadurch eine nützliche Grundlage ist für weitere Forschungen bezüglich der Umweltfaktoren die mit der Toxizität von *P. parvum* in Verbindung stehen. Da funktionelle genomische Methoden immer mehr zugänglich werden, illustrieren Ansätze wie diese welches Potenzial dadurch dem Gebiet der schädlichen Algenforschung zur Verfügung steht.

# IV. Abbreviations

| | |
|---|---|
| ACP | acyl carrier protein |
| AT | acyl transferase |
| cDNA | complementary deoxyribonucleic acid |
| coA | coenzyme a |
| COG | clusters of orthologous groups |
| Ct | threshhold |
| Cy-3 | cyanine-3 |
| Cy-5 | cyanine-5 |
| DEPC | diethylpyrocarbonate |
| DH | dehydratase |
| DNase | deoxyribonuclease |
| dNTP | deoxynucleotide triphosphate |
| DTT | dithiothreitol |
| $EC_{50}$ | half maximal effective concentration |
| ELA | erythrocyte lysis assay |
| ER | enoyl reductase |
| ESD | estimated spherical diameter |
| EST | expressed sequence tag |
| FASs | Fatty acid synthases |
| FBS | fetal bovine serum |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| HABs | Harmful algal blooms |
| IU | international units |
| KS | ketoacyl synthase |
| KR | ketoacyl reductase |
| LPS | lipopolysaccharide |
| MA | major allergen |
| mRNA | messenger ribonucleic acid |
| NSP | nitrile specifier protein |
| Oligo | oligonucleotide |
| PCR | polymerase chain reaction |
| PKS | polyketide synthase |
| Prym1 | Prymnesin 1 |
| Prym2 | Prymnesin 2 |
| psu | practical salinity units |
| qPCR | quantitative polymerase chain reaction |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| RDD | DNase digestion buffer (Qiagen) |
| RLT | RNeasy lysis buffer (Qiagen) |
| RNase | ribonuclease |
| RPE | RNeasy membrane wash buffer (Qiagen) |
| RT | reverse transcription |

| | |
|---|---|
| RW1 | RNeasy high salt membrane binding buffer (Qiagen) |
| tRNA | transfer ribonucleic acid |
| TE | thioesterase |

# 1 General Introduction

## 1.1 Harmful algal blooms

The spectrum of planktonic organisms that can form blooms is broad. By definition, when cell concentrations become significantly higher than the typical background values, this is then termed a bloom (Smayda TJ, 1997). Whether monospecific (primarily one species) or heterospecific (mixed species), blooms that are ecologically detrimental, either posing a threat to human health (Van Dolah F, 2000) and/or monetary losses through detriment to i.e. aquaculture or recreational regions (Tang & Gobler, 2009) are termed harmful algal blooms (HABs). HABs have been noted by civilizations throughout history. The first probable written reference of this occurs in the Bible from approximately 1,000 years B.C.:

*"…all the waters that were in the river were turned to blood. And the fish that were in the river died; and the river stank, and the Egyptians could not drink of the water of the river." (Exodus 7:20-21)*

This historically documented occurrence is probably based on the occurrence of an algal bloom with fish-killing effects. Formation of the bloom may have been caused by an imbalance in the Redfield N:P ratio, leading to oxygen depletion from high respiration rates that occur either at night, during self-shading of the bloom or during bacterial degradation. In any case, this first written record of an algal bloom vividly describes merely the beginning of the negative social and economic impact that today have become all too familiar in coastal areas

There are three general types of HABs, classified by their detrimental effects (Anderson et al., 1998). These are as follows:

(1) Non-toxic blooms that cause discoloration of the water in enclosed as well as water-shed areas. These blooms occasionally can reach such high cell concentrations that oxygen depletion occurs.

(2) Blooms that produce potent toxins that are either sequestered in fish or shellfish, and enter the food chain, eventually reaching and causing various gastrointestinal and neurological detriment to humans.

(3) Blooms that are directly toxic to fish and invertebrates i.e. via mechanical or chemical disruption of oxygen exchange mechanisms at respiratory membranes.

Production of toxic substances by algal species is a worldwide phenomenon. These are termed phycotoxins, and refer to a structurally diverse group of toxic compounds produced by algal species. Phycotoxins can represent a human health hazard, as is the case for several dinoflagellate toxins, however the compounds produced by *Prymnesium parvum* have yet to have been documented with any negative effects on humans. Relatively little information is known about the biological role of the substances in question, which has led to several speculative suggestions. Their role has been suggested to be as a defensive mechanism, perhaps in response to changes in environmental stress and/or predatory threats (Tillmann, 1998). These compounds may also play a role in mixotrophy, a nutritional mode whereby a species is capable of both photosynthesis and phagocytosis to meet cellular energy requirements. Immobilization of prey prior to ingestion is one potential role for toxic compounds. Whatever the function of these compounds, there is evidence that toxicity can vary due to changing environmental conditions. Historically, on

the basis of sharply contrasting laboratory observations, it is difficult to precisely define why these compounds are being produced. Speculation, however, is greatly increasing as genomic investigations begin to provide deeper insights into this area of scientific knowledge.

## 1.2 Prymnesium parvum

The first record of any species now referable to *Prymnesium* is by J. Büttner in 1911, in his paper 'Die farbigen Flagellaten des Kieler Hafens'. He described this organism as *Wysotzkia gladiociliata,* and referred to it as 'another flagellate with three flagella'. While this was not entirely true, as *Prymnesium* has two flagella and one short haptonema, it was a milestone observation at the time (Larsen, 1998). Since then, this alga has been extensively recorded as being associated with seasonal toxic blooms and mass mortality events in aquaculture ponds and in native populations of gill breathing animals (La Claire, 2010). The genus *Prymnesium* currently comprises ten species, four of which are considered to be toxic. *Prymnesium parvum* is one of these four toxic species.

The prymnesiophyte flagellate *Prymnesium parvum* is a mixotrophic species. Phagocytosis of other organisms such as bacteria (Nygaard & Tobiesen, 1993) and other protists (Tillmann, 1998) has been observed. Most of the associated bloom events tend to occur in cooler waters, located in the subtropical and temperate zones between the Tropic of Cancer and the Arctic Circle and between the Tropic of Capricorn and the Antarctic Circle (La Claire, 2010). HABs of *P. parvum* often form in estuarine brackish waters, exhibiting its extremely high tolerance for variations in salinity; however, a large number of blooms are now known to occur in mainland freshwater reservoirs (La Claire, 2010). How *P. parvum*

crossed over from marine to freshwater habitats is currently unknown, however, proposed vectors include contaminated bilge water, bird guano and encystment (La Claire, 2010). Regions affected by *Prymnesium* blooms can be seen in Figure 1.2.1.

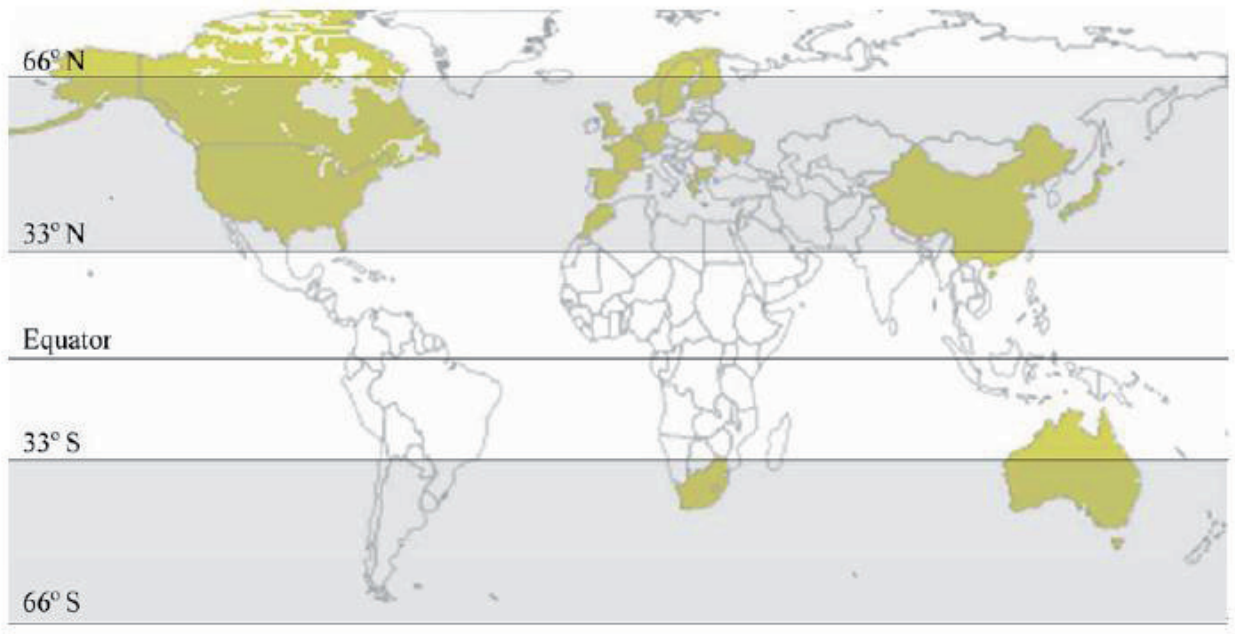


**Figure 1.2.1: Worldwide occurrences of *P. parvum* populations based on countries where reported. (adapted from LaClaire, 2010).**

Blooms of *P. parvum* are often associated with massive fish-kills (Moestrup, 2004; Edvardsen & Larsen, 1998). Besides being toxic to fish, *P. parvum* also produces hemolytic substances that lyse both prokaryotic and eukaryotic cells (Yariv & Hestrin, 1961; Tillmann, 1998). The wide range of toxic effects caused by *P.* parvum suggests that there may be multiple compounds secreted (Shilo, 1967). Igarashi et al. (1999) succeeded in describing the general structure of two polyether compounds as *Prymnesium* toxins, prymnesin-1 and prymnesin-2. These workers did not, however, determine a

straightforward way to quantify these toxic compounds, a difficult task as no commercially available standard exists.

*Prymnesium parvum* both produces and secrete compounds that have toxic effects on other protists and fish. Whether or not and to what extent prymnesins play a role in these observed detrimental effects is, however, not yet clear. How the gene expression profile of this algal species changes depending on the culture conditions has also been recently described (La Claire, 2006). The relative toxicity of *Prymnesium parvum* to other algal species has additionally been shown to be variable, depending on the culture conditions.

### 1.2.1 Phylogeny

After numerous attempts to revise the nomenclature, the family *Prymnesiaceae* was defined, representing one of up to eight recognized members within the order *Prymnesiales*. Figure 1.2.2 shows a phylogenetic tree based on 18s ribosomal RNA sequences (Edvardsen et al. 2000). It is important to note the position of the toxic species, shown exclusively in clade B1.

The two prymnesiophyte genera *Chrysochromulina* and *Prymnesium* are closely related, based on 18s ribosomal RNA (rRNA), as shown in Figure 1.2.2. The genera differ by the length of the haptonema, the structure of their organic surface scales, flagellar insertion and movements (Green et. al., 1982). Despite their morphological differences, several species of these two genera are, according to nucleotide sequence data, more closely related than to any other species within their respective genus (i.e. *P. parvum and C. polylepis)*.

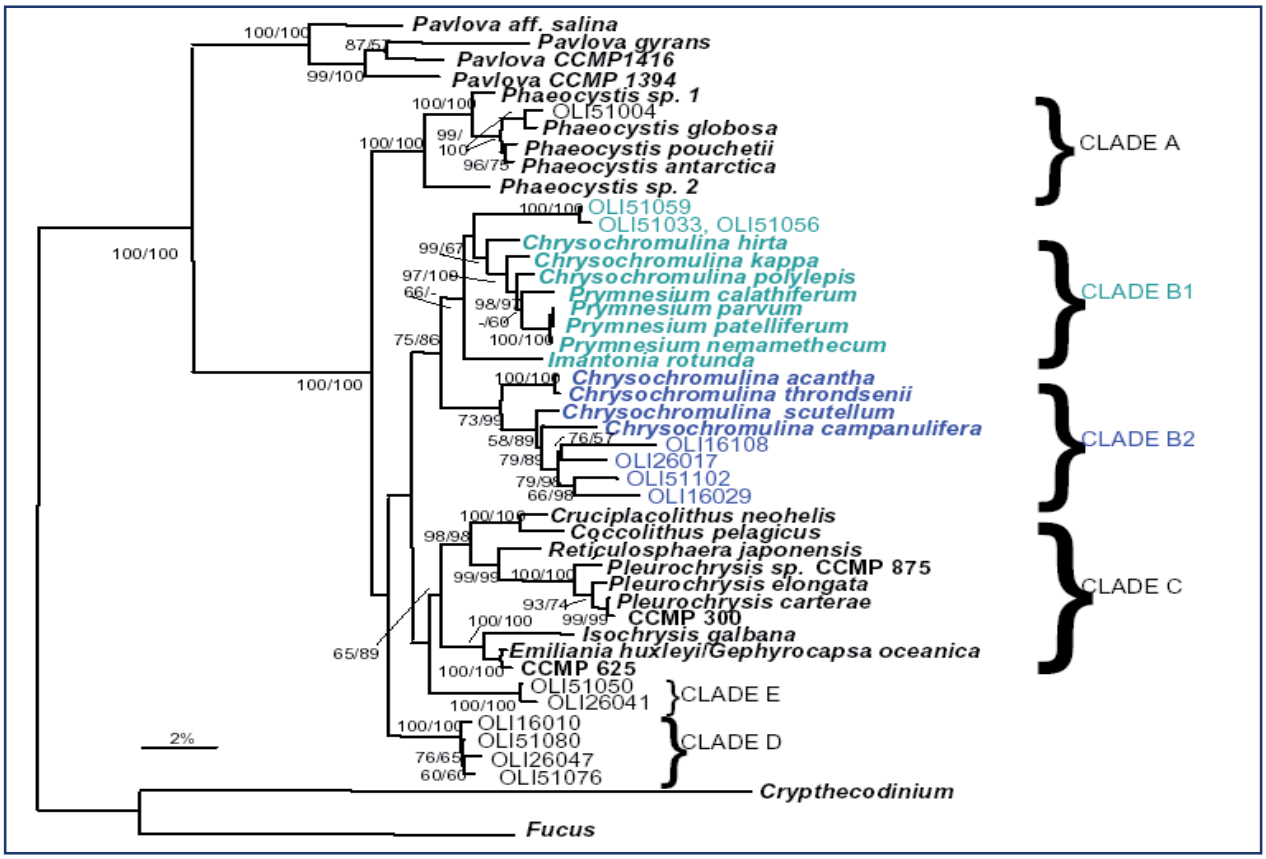


**Figure 1.2.2: Phylogenetic tree based upon maximum likelihood analysis indicating the relationships of the prymnesiophytes. Bootstrap values are indicated at internal nodes (500 replications) for values more than 50% for neighbour-joining and maximum parsimony analyses. Tree is based on 18s ribosomal RNA sequence data (Edvardsen et al., 2000).**

### 1.2.2 Morphology

*Prymnesium parvum* is a unicellular flagellate, with an ellipsoid shape (Lee, 1980; Prescott, 1968). Cells range from 8-11 µm in length, according to Green et al. (1982). Each cell has two flagella of equal length and a haptonema. The flagellae are for motility, whereas the haptonema may be involved in attachment and/or feeding via phagocytosis (figure 1.2.3) (McLaughlin, 1958; Prescott, 1968; Tillmann, 1998). Green et al. (1982) found that the flagella can range from 12-15 µm in length, and the flexible, non-coiling haptonema ranges from 3-5 µm long. Each cell has scales of two types in two layers, with the outer layer having distinctively narrow inflexed rims, whereas those of the inner layer have wide, even more inflexed rims. The scale arrangement and composition is an important phylogenetic diagnostic tool for this species. The flagellum to haptonema ratio is another feature that can be used for phylogenetic identification (Chang & Ryan, 1985).

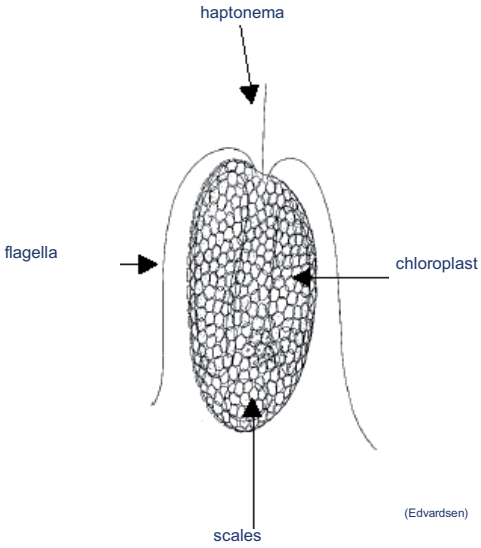


**Figure 1.2.3: Morphological characteristics of the genus *Prymnesium* (Rahat, 1965).**

The nucleus is located centrally between two chloroplasts, one lateral in spatial arrangement whereas the other is parietal (Figure 1.2.3). The chloroplasts are typically yellow-green to olive in color. A double-membrane endoplasmic reticulum (ER) is also present, with the outer membrane being continuous with the nuclear envelope outer membrane (Green, 1982). A large Golgi apparatus is always found between the base of the two flagella and the nucleus (Bold & Wynne, 1985). Finally, a contractile vacuole is sometimes found at the anterior end of *P. parvum* cells (Figure 1.2.3).

### 1.2.2 Life cycle (currently proposed)

It has been suggested that the reproductive life cycle of *P. parvum* alternates in nature (Larsen, 1999). This refers to the "ploidy" or number of copies of chromosomes present in the organism's genome at any given time. In Figure 1.2.4 (Larsen, 1999) it is suggested that the life cycle contains two morphologically different haploid cell types (*P. parvum and P. patelliferum)* and one diploid cell type (*P. parvum).* This is very similar to the proposed life cycle for *C. polylepis*, which is already shown to be related to *P. parvum* through an 18s ribosomal DNA phylogenetic tree (Figure 1.2.2). The two morphologically different diploid cell types are so different that they have been originally described as two different species (Larsen, 1998). One reason for the haploid stage could be as a source of energy conservation, because of the lower nutrient requirements due to the smaller quantity of DNA in haploid cells. It is also thought that sexual reproduction is a part of the *P. Parvum life* cycle under favourable environmental conditions. Sexual reproduction is not known to occur in laboratory culturing of *P. parvum*.

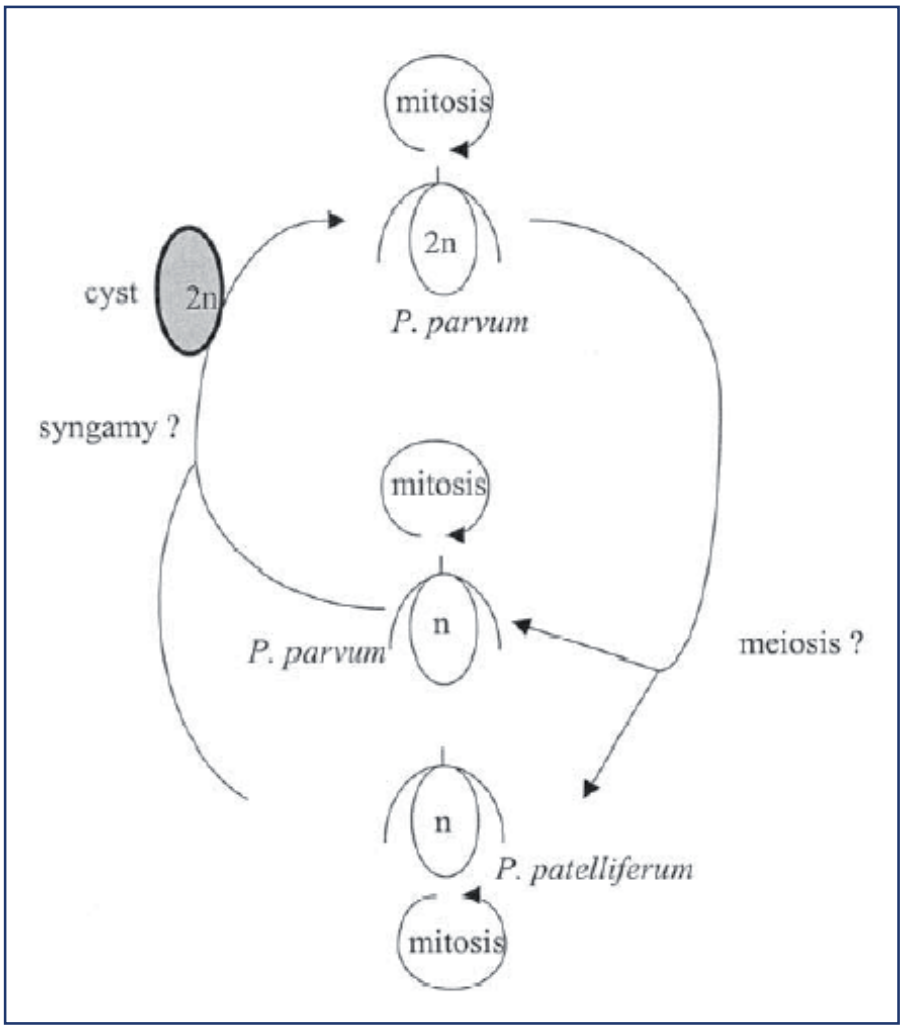


**Figure 1.2.4: The proposed haploid/diploid life cycle of *P. parvum.* Adapted from Larsen, 1999.**

### 1.2.4 Toxicity

The toxins produced by *P. parvum* have been previously shown to be a collection of substances, rather than a single component (Shilo & Sarig, 1989). This collective identity has led to several different chemical and/or structural characterizations. Currently there

are four chemically classified potential components of the *P. parvum* toxin(s): proteolipid (Ulitzur & Shilo, 1970, Dafni et al., 1972), lipopolysaccharide (LPS) (Paster, 1973), galactoglycerolipid (Kozakai et al., 1982), and polyene polyethers (Igarashi et al., 1999).

Prescott (1968) showed a portion of the extracted compound to be proteinaceous, acid labile, non-dialyzable and thermostabile. This characterization was further supported by Ulitzur & Shilo (1970) who suggested that a portion of the toxin is a proteophospholipid. This hypothesis was agreed upon by Dafni et al. (1972). These three analyses were performed using cellular extracts, not whole cell cultures.

Spiegelstein et al. (1969) used two methods to observe the effects of the toxin mixture on *Gambusia,* a large genus of fish in the family *Poeciliidae*. They found that with the immersion method (fish in a toxin solution), the toxicity effect occurs as follows: first the toxin enters the gills (via capillaries), and enters the dorsal aortas, and then travels to the brain. These authors noted that in the intraperitoneal injection method, the toxin first enters the circulatory system whereby it travels to the liver, then enters the hepatic vein, the heart, the aorta and finally the brain. Since a portion of the toxic components was shown to be acid labile, Spiegelstein et al. (1969) further noted that the toxin may be inactivated in the gastrointestinal tract and liver. This supports why the toxin is non-toxic to non-gill breathers, but toxic to gill breathers.

**Figure 1.2.5: Fish kill associated with a bloom of the golden alga, *Prymnesium parvum*, on Lake Whitney in Texas. (photographer: J.Glass/TPWD)**

Paster (1973) noted that the attachment of extracted toxin to gill cell membranes most likely occurs where molecules such as lecithin and cholesterol are found, and that attachment induces a rearrangement on the membrane making it more permeable. He then proposed a portion of the toxin to be lipopolysaccharide, similar to toxins from bacterial cell walls. The fact that these compounds interact with cholesterol in attacking erythrocyte membranes supports this idea (Padilla & Martin, 1973).

After witnessing glycerol enhancement of hemolysin production, Padilla (1970) suggested that overall toxin biosynthesis was dependent on carbohydrate and lipid

metabolism. This author also implied that hemolysin may be a structural part of the cell membrane. The same research found a direct correlation between hemolysin formation, and the presence of membrane vesicles. He further noted that the *P. parvum* toxin only appears under physiological conditions where growth is disturbed and/or growth factors are limited, an important underlying observation for the investigations performed in this Doctoral thesis project. Dafni et al. (1972) finally suggested that the hemolysin portion could be a product of an imbalance in cell membrane metabolism.

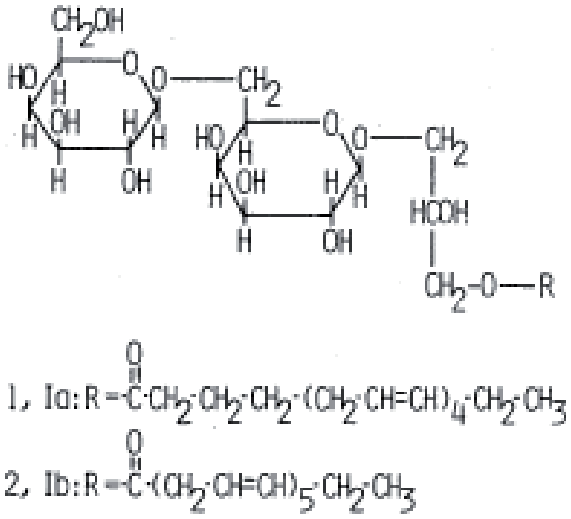


**Figure 1.2.6: Structure of hemolytic component (hemolysin), as described by to Kozakai et al. 1982.**

In a more recent study the hemolytic portion was separated into six components, with the major component, hemolysin I (Figure 1.2.6), being a mixture of 1'-*O*-octadecatetraenoyl-3'-*O*-(6-*O*-*B*-D-galactopyranosyl-*B*-D-galactopyranosyl)-glycerol and 1'-*O*-octadecapentaenoyl-3'-*O*-(6-*O*-*B*-D-galactopyranosyl-*B*-D-galactopyranosyl)-glycerol

(Kozakai et al., 1982). The evidence suggesting a portion of toxic compounds are membrane phospholipid precursors was further supported by a 10-20 fold increase in toxicity per cell (collectively ichthyotoxin, hemolysin, and cytoxin) when phosphate was limited (Shilo & Sarig 1989), potentially due to utilization of available phosphate to biosynthesize toxic compounds.

In 1999 the first structural elucidation of two toxic polyether compounds produced by *P. parvum* was completed by Japanese researchers (Igarashi et al., 1999). These were the first toxic metabolites to be chemically characterized from any isolate of *P. parvum* using modern analytical methods (Igarashi et al., 1996; Igarashi et al., 1999). Prymnesin-1 (prym1) and Prymnesin-2 (prym2) were shown to be polyketides possessing ichthyotoxic and hemolytic activities at nanomolar concentrations (Igarashi et al., 1996; Igarashi et al., 1999). Prymnesins appear to be structurally ladder-like polycyclic ether compounds with several key features (Figure 1.2.7). They have double and triple carbon-carbon bonds in the unsaturated head and tail regions, an amino group, several chlorines, four 1,6-dioxadecalin units, and a variety of sugar moieties (Igarashi et al., 1996; Igarashi et al., 1999). Structurally similar, prymnesins 1 & 2 differ in the number and type of sugar moieties in the tail region (Figure 1.2.7) with prym2 containing a rare L-xylose, an infrequent, yet naturally occurring enantiomer of the sugar xylose. Prym1 was shown to be slightly more polar (due to the addition sugar residues) and therefore elutes ahead of prym2 in reverse phase C-18 chromatography. The characterization by Prescott (1968) can indicate these mentioned properties for only a portion of the compounds since prymnesin 1 and prymnesin 2 are dialyzable based upon molecular size.

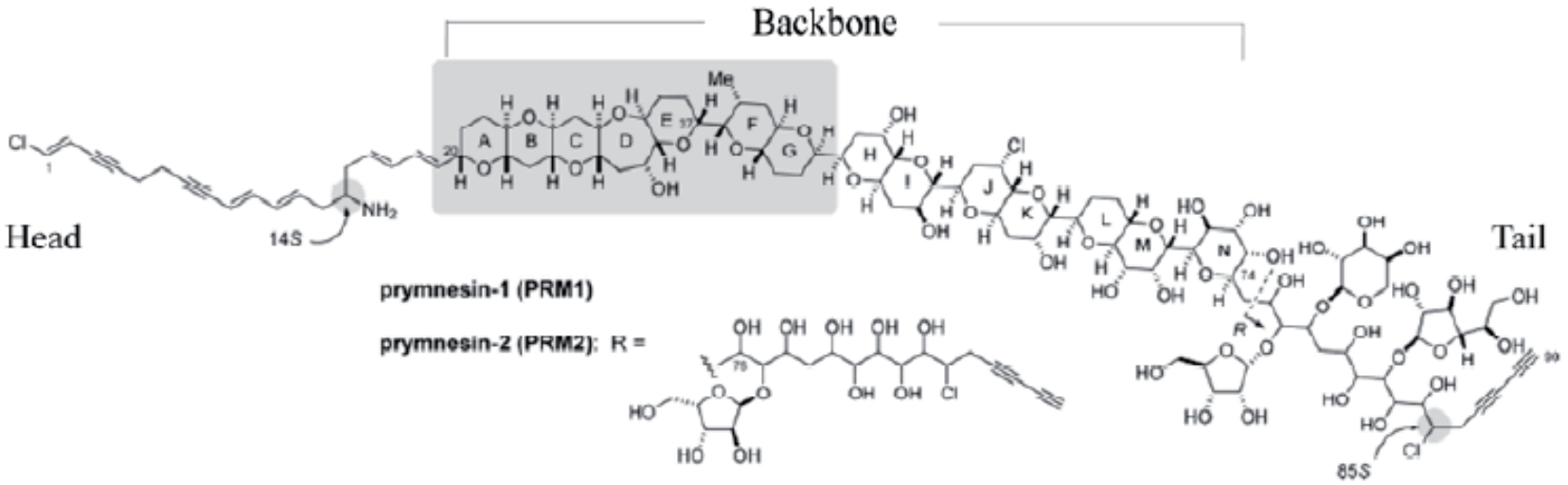


**Fig 1.2.7: Structure of prym1 and prym2, reproduced from LaClaire et al., 2010, with permission from the American Chemical Society (ACS).**

Despite the lack of knowledge concerning the *in vivo* biosynthesis of prym1 and prym2, it is likely that they are derived via the polyketide synthase biosynthetic pathway. Polyketides are a multi-functional family of secondary metabolites produced by fungi, bacteria, higher plants and a few animal lineages. The enzymes associated with their biosynthesis are termed polyketide synthases (PKSs). PKSs are large multi-domain enzymes or enzyme complexes that are related to fatty acid synthases (FASs). The three described types are PKS I, II and II; all of which share an identical set of functional modules: ketoacyl synthase (KS), acyl transferase (AT), ketoacyl reductase (KR), dehyrdratase (DH), enoyl reductase (ER), acyl carrier protein (ACP) and thioesterase (TE) domains. Type I PKSs are further divided into iterative and modular, depending on the mode of biosynthesis they employ. Short chain (branched) fatty acids, amino acids alicyclic and aromatic acids can act as started units. Biosynthesis proceeds through Claisen condensation reactions in a conserved organized manner. Post PKS modifications are also possible, i.e. glycosylation, acylation, alkylation and oxidation. These modifications contribute greatly to the structural diversity of the polyketide family (John et al., 2010).

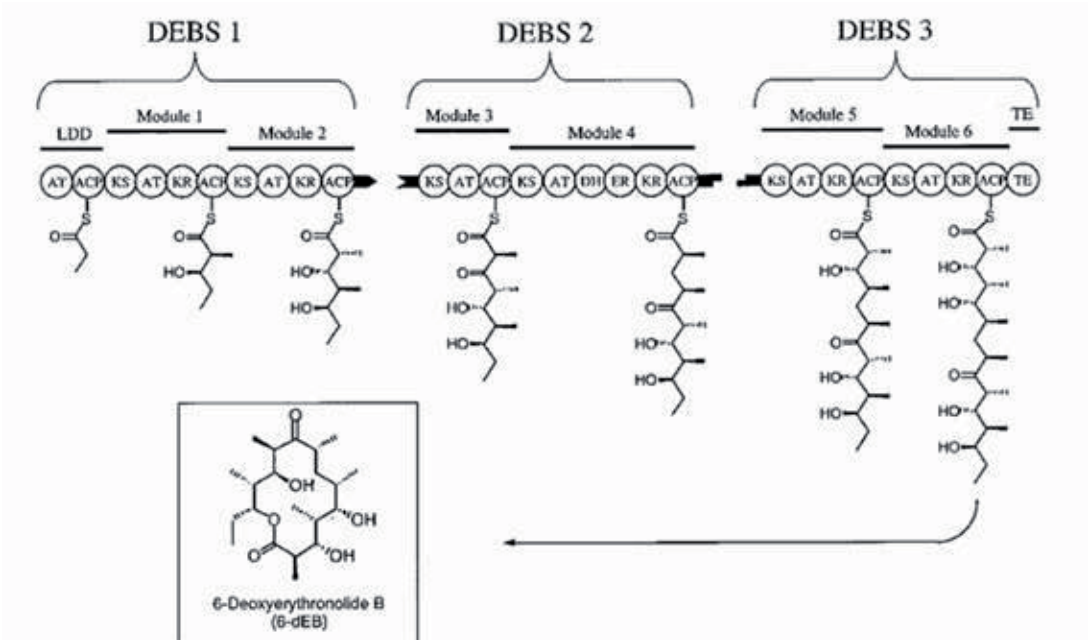


**Figure 1.2.8: An example of polyketide synthesis by a type I modular PKS enzyme (Adapted from Wu et al., 2002)**

### 1.3 Allelopathic role of compounds produced

Members of the genus *Prymnesium* produce and excrete several allelopathic compounds whose function and biosynthesis is not entirely understood. Several possibilities exist concerning the specific function of these compounds. They may reduce grazing from zooplankton (John et al., 2002), or may function allelopathically to reduce or interfere with growth of other phytoplankton (Legrand et al., 2003). When toxicity is low, populations of *Prymnesium* are thought to be controlled by zooplanktonic grazing, however, when enough toxic compounds are secreted into the water, they may act as a chemical defense to repel or kill predators (Tillmann, 2003). Tillmann also suggested the potential of these compounds to immobilize prey prior to phagotrophy.

From an ecological perspective, studies of phytoplankton succession and bloom formation have primarily focused on comparative abiotic effects rather than on individual plankton components (Domingues et al., 2005; Levasseur et al., 1984; Lindenschmidt & Chorus, 1998; Sommer, 1988). In this context, the apparent success of *P. parvum* leading to dominance and bloom formation might be attributed to its physiological flexibility reflected by its ability to grow in a wide range of environmental conditions (Larsen & Bryant, 1998). There is increasing evidence, however, that inter-specific interactions in the plankton play a major role in succession, food web structure and bloom development (Smetacek et al., 2004; Tillmann, 2004). Among these interactions, the capacity to produce toxic or noxious allelochemicals that may deter grazing or affect competition for limiting resources has been increasing recognized as an important regulatory mechanism affecting bloom dynamics of plankton (reviewed by Cembella, 2003; Legrand, 2003). Allelochemicals produced and secreted by *P. parvum* have been shown to kill both competing algal species and their grazers (Tillmann, 2003; Granéli, 2006). Closely related to this "killing capacity" (Tillmann, 2003) is the mixotrophic tendencies of *Prymnesium*, i.e. the ability to ingest immobilized competitors and grazers (Tillmann, 2003; Skovgaard & Hansen, 2003). This strategy to kill (and then eat) your enemies by means of toxic compounds is thought to significantly contribute to the ability of *P. parvum* to form dense and long-lasting blooms.

## 1.4 Prymnesium parvum: nutrient physiology

*Prymnesium parvum* can thrive in a wide range of physiological conditions (La Claire, 2010); however nutrient availability has been shown to play a crucial role in HABs and toxin formation. Agricultural run-off and eutrophication are often associated with an increase in growth for *P. parvum* (Hallegraeff, 1999; Collins, 1978; Holdway et al., 1978). High nitrogen as well as phosphorous loading ultimately leads to an imbalance in nutrient sources, slowing the growth of *Prymnesium,* which is often accompanied by an increase in toxicity (Larsen et al., 1993; Shilo, 1971; Sabour et al., 2000). Several mesocosm experiments have been performed that suggest a decrease in extracellular toxicity, under favorable conditions (Roelke et al., 2007). This has led to discussion that *Prymnesium toxicity* can be therefore be controlled by nutrient manipulation (Legrand et al., 2001).

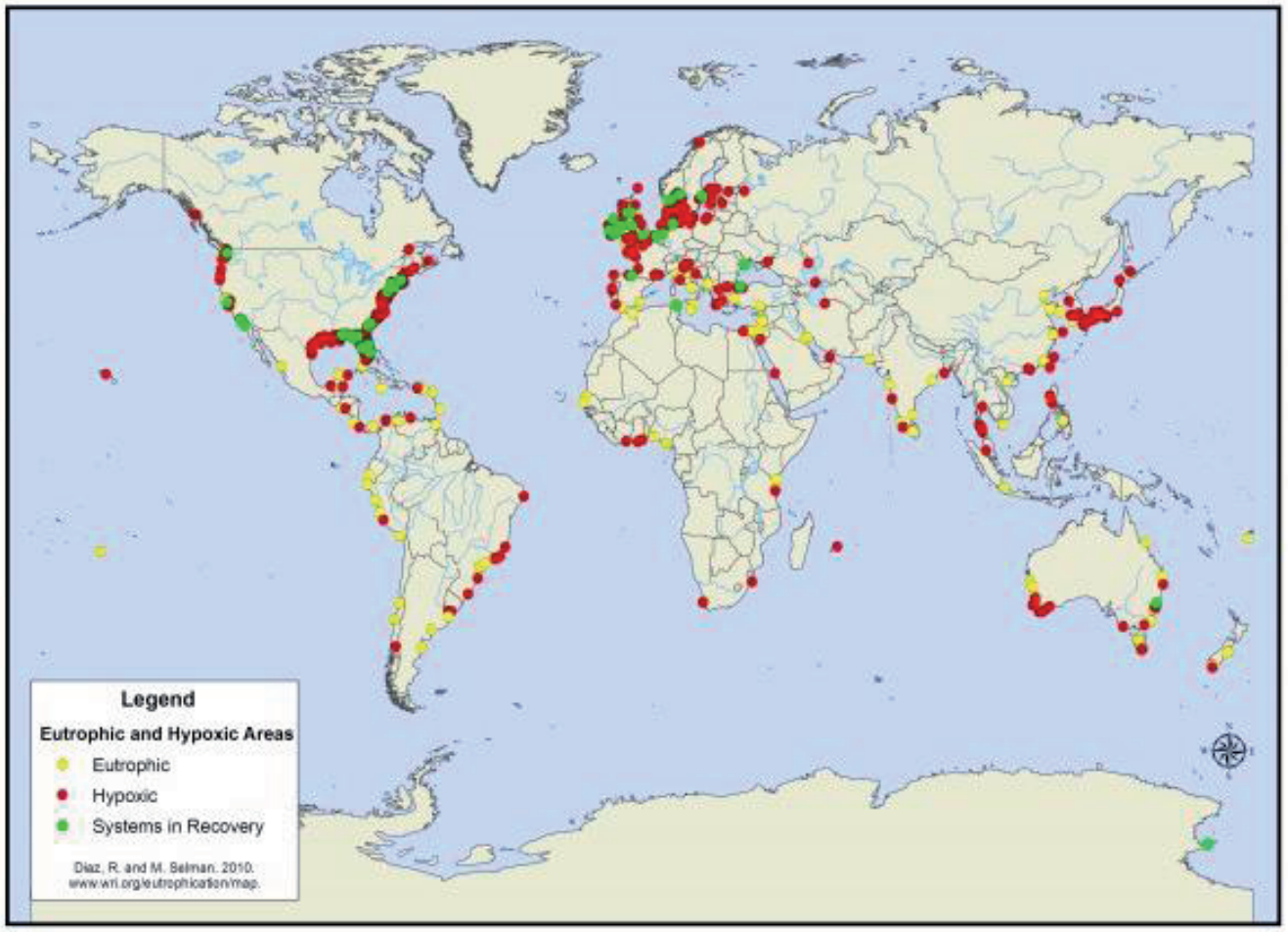


**Figure 1.2.9: World hypoxic and eutrophic coastal areas. (Source http://www.wri.org).**

## 1.5 Functional genomics: upcoming field in harmful algal research

Increasing the knowledge of genes and gene products involved in toxic processes in microalgal species is a rapidly expanding research concept. In the field of harmful algal research, a more comprehensive understanding of the link between an organism's genotype and phenotype (toxicity) is urgently needed, in particular when aspects such as human health are at stake. In the case of *P. parvum,* such approaches are hopeful in elucidating the biosynthetic pathways associated with observed toxicity and lytic effects that we see in lab experiments. We have utilized a microarray platform, derived from a cDNA stress library of *P. parvum,* in an attempt to better understand what is happening at the gene level, in response to factors such as nutrient depletion and allelopathic interactions with coexisting organisms.

Despite the seasonal economic damage *P. parvum* causes through association with fish-kill events, so little is known concerning the exact biological role and mode of action of the toxic substances being produced and excreted. Therefore, elucidating the metabolic story behind this prymnesiophyte during bloom formation is of particular importance, as its toxins, perhaps including prym1 and prym2, may be directly associated with massive fish kills (Edvardsen & Paasche, 1998). Besides the economic impact of these fish kills on aquaculture, other aspects such as tourism are also affected. The current need for a field-probe based system to detect and monitor the presence of this prymnesiophyte in coastal waters, is a driving force behind the functional genomic race to understand the metabolism involved in the toxin production and secretion processes.

Analysis of whole genomes is rapidly becoming a trend that allows new and crucial insights into different aspects of biology (La Claire, 2006). cDNA libraries and expressed

sequence tag [EST] databases developed from them provide an inexpensive overview into the genome of an organism. This overview includes gene expression levels, which may or may not have significance to metabolic processes, such as toxin production. To date, eukaryotic algal complete genome projects comprise only that of the diatom *Thalassiosira pseudonana (*Armbrust et al., 2004), the filamentous seaweed *Ectocarpus Siliculosus* (Cock et al., 2010) and that of the red alga *Cyanidioschyzon merolae* (Metsuzaki et. al., 2004). Despite this low number of completed projects, sequencing and analysis of many algal genomes are very close to completion.

### 1.6 Aim of the thesis

The aim of this thesis was to obtain a more developed characterization of cellular processes potentially involved in toxicity (PKS gene expression), allelopathy, nutrient/resource competition and factors affecting bloom formation in the prymnesiophyte *P. parvum*, using a bioassay-linked functional genomic approach.

### 1.7 Outline of the thesis

This thesis is organized into three core chapters, corresponding to three separate publications where the candidate is first author.

The toxigenic prymnesiophyte *Prymnesium parvum* commonly forms harmful algal blooms in coastal areas, where eutrophication and fluctuation of both abiotic and biotic factors play a role in its ecological success. In **Publication 1**, a series of ecologically relevant physiological shock treatments were applied in an attempt to elucidate effects on the toxicity of *P. parvum.* In order to determine treatment related differences in toxicity,

two separate bioassays were used: a *Rhodomonas salina* assay and an erythrocyte lysis assay (ELA). The first is a measure of secreted lytic capacity, while the latter measures lytic capacity of intracellular compounds. Additionally, gene expression via quantitative real-time PCR (qPCR) was employed to investigate changes in transcript copy number for three polyketide synthase (PKS) genes, due to current chemical characterizations of the compounds Prymnesin 1 (prym1) and Prymnesin 2 (prym2). Through the combination of toxicity bioassays and gene expression analysis, it was possible to associate PKS gene regulation patterns, with changes in toxicity, and associate these to high irradiance stress and low salinity stress. The candidate designed the experimental setup and performed the according RNA isolations, toxicity bioassays as well as qPCR analysis. The candidate analyzed the data and prepared the manuscript.

The lytic compounds produced by *P. parvum* are furthermore thought to play a role in allelopathic interactions, and therefore be important bloom initiation factors. In **Publication 2** an analysis of gene expression and toxicity arising from interspecific interactions between *P. parvum* and three coexisting phytoplankton species was investigated. Incorporating a microarray platform into this study, it was possible to differentiate between gene expression associated with cell-cell contact and gene expression associated with recognition and response to chemical cues. The candidate designed the experimental setup in collaboration with the coauthors and performed RNA isolations, toxicity bioassays, qPCR analysis and microarray hybridizations. Analysis of the data as well as preparation of the manuscript was performed by the candidate.

Taking anthropogenic influences into ecological consideration, Phosphorus limitation is known to increase the toxicity of this prymnesiophyte. Low salinity stress is

also known to be a stressor inducing toxicity in *P. parvum.* **In publication 3** an analysis of toxicity and gene expression related to a combination of Phosphorus limitation stress and low salinity stress was performed. The aim was to use a functional genomic approach to characterize the underlying gene expression associated with changes in toxicity due to these two stressors. With this goal in mind, the candidate designed the experimental setup in collaboration with the coauthors, performed RNA extractions, toxicity assays, nutrient measurements, qPCR as well as microarray hybridizations. Data analysis and writing of the manuscript was additionally performed by the candidate.

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# 2. Publications

**Publication 1**
**Freitag MF, Beszteri SA, Vogel H & John U, (2011). Induced toxicity and polyketide synthase gene expression following physiological shock in the toxigenic *Prymnesium parvum* (Prymnesiophyceae). *Eur J Phycol*, in press**

**Publication 2**
**Freitag MF, Tillmann U, Cembella AD & John U, (2011). Differential responses of the prymnesiophyte *Prymnesium parvum* following interactions with planktonic species. ISME Journal, submitted**

**Publication 3**
**Freitag MF, Tillmann U, Beszteri SA, Cembella AD & John U, (2011). Investigating phosphate limitation and low salinity stressors in the prymnesiophyte *Prymnesium parvum.* In preparation.**

# 2.1 Publication I

## Induced toxicity and polyketide synthase gene expression following physiological shock in the toxigenic *Prymnesium parvum* (Prymnesiophyceae)

### 2.1.1 Abstract

The toxigenic species *Prymnesium parvum* (prymnesiophyceae) is responsible for economically detrimental fishkill events worldwide every year. Although numerous studies concerning the physiology and toxicity of *Prymnesium parvum* exist, the attempt to incorporate gene expression into such data sets is novel. In this study we investigated relative toxicity (intracellular vs. extracellular) and differential gene expression via real-time PCR (qPCR) of three polyketide synthase (PKS) transcripts, based on current hypothesized structural characterizations of toxic compounds produced by prymnesiophyte *P. parvum*. We found that low salinity shock and high irradiation shock increase different aspects of toxicity (intra- vs. extra-cellular) in *Prymnesium*. Furthermore, we found that these two physiological shock treatments induced higher copy numbers in selected polyketide synthase (PKS) genes, suggesting a connection between toxicity and the PKS biosynthetic pathway. Our results demonstrate how PKS is likely to play an important role in toxic processes of *P. parvum.* We anticipate our study to be a starting point for further investigations into the role of PKS in *P. parvum* in response to changing environmental conditions.

### 2.1.2 Introduction

The toxigenic prymnesiophyte *P. parvum* is a worldwide distributed mixotrophic species (Moestrup, 1994). Blooms of *P. parvum* are associated with massive fish-kills (Edvardsen & Paasche, 1998). *P. parvum* produces substances that are directly associated with ichtyotoxicity (lysis of gill cell membranes) and also show lytic activity towards both prokaryotic and eukaryotic single-celled organisms (Yariv & Hestrin, 1961; Tillmann, 2003). Effects of both abiotic and biotic factors have been extensively studied in *P. parvum.* For example, in the presence of a potential grazer such as the dinoflagellate *Oxyhrris marina,* lytic activity of *P. parvum* has been shown to increase towards the small cryptophyte *Rhodomonas salina,* also used in this study as a relative measure of lytic capacity (Tillmann, 2003). Growth phase, cell culture density, temperature, nutrient availability, light intensity as well as salinity have all been shown to cause variations in observed toxicity in addition to a wide range of toxic effects, which suggests there may be multiple compounds responsible for the observed effects (Graneli et al., 2008; Larsen & Bryant, 1998; Graneli et al., 1998, Baker et al., 2007; Shilo, 1967).

Blooms of *P. parvum* are often found in coastal or brackish water areas, where salinity and nutrient availability tend to fluctuate and play a potential role in the variations in toxicity observed in laboratory experiments (Baker et al., 2007). *Prymnesium parvum* is extremely physiologically robust and flexible, and it is this flexibility that may provide a competitive advantage over other coexisting microalgal species that leads to the infamous *P. parvum* associated fish-kill events worldwide. As rapid acclimation of microalgae to environmental changes has previously been shown (Costas et al., 2001; Lopez-Rodas et al.,

2001), our intention was to simulate these rapidly changing environmental conditions through a series of 'shock' experiments.

Physiological 'shock' responses have been demonstrated in many species of bacteria in response to a wide variety of extreme or changing environmental conditions (Grzadkowska & Griffiths, 2001). In marine microalgae, hypoosmotic stress has been shown to induce responses primarily related to impaired photosynthetic capacity (Kirst, 1989). Using photosynthetic machinery as a measurement of response to stress is not representative of how other cellular processes are responding to the disruption in cellular equilibrium. Understanding the relationship between gene expression changes and the corresponding adaptive physiological responses of an organism to environmental cues is crucial in explaining how cells cope with stress (Vilaprinyo et al., 2006).

The structural elucidation of at least a portion of the toxic substances produced by *P. parvum* (Igarashi et al., 1999) revealed two similar compounds: prym1 and prym2. These two structurally polyether compounds were described to possess similar biological activities. Their description raised interest in PKS enzymatic pathways and their potential role(s) in toxic processes described for *P. parvum* (John et al. 2008, LaClaire 2008), as well as for other protists (John et al. 2008, 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), relating their biosynthesis to that of fatty acid compounds (Staunton & Weissmann, 2001; Crawford et al., 2006). Of the known protist PKS enzymes, many have been shown to belong to the same molecular class of biosynthetic pathways, and most marine microalgal species studied so far exhibit two or more functionally different PKS genes (LaClaire, 2006; John et al. 2008; Worden et al. 2009;

Monroe, 2010). As PKS biosynthetic pathways are shown to be involved in brevetoxin (Monroe et al. 2010) and spirolide production (McKinnon et al. 2006), it seems likely that these enzymatic pathways also play a role in the biosynthesis of toxic compounds for *P. parvum*.

Our objectives for this study were to investigate: 1) the effect of short term 'shock' treatments on exhibited toxicity as well as on differential gene expression of three PKS transcripts (obtained from a non-normalized cDNA library constructed by Laclaire et al., 2006) and 2) the extent to which PKS pathways are involved in the biosynthesis and/or secretion of toxic compounds produced by *P. parvum.* Through a combination of bioassays and functional genomic approaches, we are able to correlate changes in toxicity, to changes in expression of select PKS transcripts. We additionally demonstrate that housekeeping genes for a study as is described in this study are not ideal, and that fluctuations in their expression values can lead to misinterpretation of data obtained. The correlation of PKS gene transcripts to changes in toxicity is a novel finding for *P. parvum,* and will serve to fuel future studies further characterizing the role of PKS enzymes in toxic processes in this species.

### 2.1.3 Materials and methods

*Culture conditions and experimental setup*

A toxic clonal strain RL10 of *P. parvum,* isolated in 1993 by Aud Larsen in the Sandsfjord system in Norway (Edvardsen & Larsen, 1998) was used for this study. Strain RL10 was grown in 5 l stock culture in IMR medium. The components of IMR medium (Eppley, 1967) can be viewed in Table 2.1.1-3. Cultures were grown at a salinity of 26 psu under gentle aeration with sterilely filtered air to a concentration of 4.61 x $10^3$ cells $ml^{-1}$, at a constant temperature of 20°C and a light: dark photocycle of 14:10 h. Photon flux density measured inside the flask by a QSL-100 Quantum Scalar Irradiance Meter (Biospherical Instruments, San Diego, USA) was kept at 90 µmol photons $m^{-2}$ $s^{-1}$. Cell concentrations were determined daily using a CASY cell counter (Innovatis AG, Reutlingen, Germany).

Table 2.1.1: Components of IMR medium

| component | final concentration $l^{-1}$ |
|---|---|
| *trace elements** | (see Table 2) |
| vitamins** | (see Table 3) |
| $KNO_3$ | 500 µmol |
| $KH_2PO_4$ | 50 µmol |
| $Na_2SeO_3$ | 500 µmol |
| $Na_2O_3Si \cdot 9H_2O$ | 500 µmol |
| North Sea water | 80% (volume) |
| bi-distilled water | 20% (volume) |

*Table 2.1.2: Trace element stock solutions

| substance | final concentration $l^{-1}$ |
|---|---|
| $Na_2$-EDTA | 6 g |
| $FeCl_3 \cdot 6H_2O$ | 1 g |
| $MnSO_4 \cdot H_2O$ | 620 mg |
| $ZnSO_4 \cdot 7H_2O$ | 250 mg |
| $Na_2MoO_4 \cdot 2H_2O$ | 130 mg |
| $CoCl_2 \cdot 6H_2O$ | 4 mg |
| $CuSO_4 \cdot 5H_2O$ | 4 mg |

**Table 2.1.3: Vitamin stock concentrations

| quantity | vitamin | final concentration per liter |
|---|---|---|
| 1.0 ml | Vit. $B_{12}$ (cyanocobalamin) | 10 µg |
| 1.0 ml | Biotin | 1 µg |
| 100.0 mg | Thiamine HCl | 200 µg |

From the initial stock culture, 400 ml cultures were inoculated at starting concentrations of 1.5 x $10^3$ ± 123 cells $ml^{-1}$ and grown under identical conditions as the stock culture (with exception of no aeration for smaller batch cultures) to a concentration of 3.75 x $10^3$ ± 1,325 cells $ml^{-1}$. This cell concentration was crucial, because (1) the cells were still exponentially growing and (2) it would provide sufficient material for downstream analysis. At this point, 400 ml batch cultures were separated and 'shocked' for 2 h. A summary of physiological 'shock' and control conditions can be seen in Table 2.1.4. All experiments were carried out parallel, in triplicate, with a single control for all samples. Culturing shock parameters were chosen based on known literature tolerance ranges of *P. parvum* (Larsen & Edvardsen, 1998; Graneli et al. 1998; Graneli et al., 2008; Edvardsen & Paasche, 1998; LaClaire, 2006).

Table 2.1.4: Control and physiological 'shock' conditions for replicate 400 ml batch cultures.

| 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 |

*±Temperature adjusted using pre-set water baths. Internal temperature within culture flask was continually monitored through ‚shock' experiment. 25°C internal temperature was achieved in t‹15 min, 5°C was achieved in t‹20 min.*

* *Medium diluted using IMR prepared without North sea water (for identical nutrient/vitamin composition. Magnetic stir bar applied to ensure minimal differences in local salinity within the culture flask.*

*+Separated, and placed under identical conditions in a growth chamber, with altered light source.*

♦*Darkness achieved with alumnimum foil enclosure of the culture flask.*

*Erythrocyte lysis assay*

An erythrocyte lysis assay was performed as described by Eschbach et al. (2001), and was used to the test lytic activity of *P. parvum* whole cell extracts towards erythrocytes.

*Fish husbandry*

Carp (*Cyprinus carpio*) 4-5 years old and weighing 2-3 kg were used for blood collection. Tank and feeding conditions were previously described by Eschbach et al. (2001).

*Blood collection, storage and preparation*

For blood collection and storage, RPMI 1640 culture medium (Sigma) supplemented with fetal bovine serum (FBS) was diluted 10% (v/v) deionized water (Milli-Q filtration system), to adjust its osmotic pressure according to carp serum osmolarity (Mommensen et al., 1994). Syringes were pre-filled with 5 ml diluted RPMI medium, in addition to 50 IU $ml^{-1}$ heparin sodium (Sigma) to avoid clot aggregation formation. Caudal vein puncture was performed on the ventral side of each fish to obtain 5 ml of blood (Stoskopf et al., 1993). Repeated bleeding of the same fish was done with a minimum interval of 4 weeks. Whole fish blood was diluted 1:10 with diluted RPMI medium containing 22.5 IU $ml^{-1}$ heparin sodium (Sigma). Cultures were stored in 25 ml angle necked culture flasks in an upright position at 4° C.

Erythrocyte concentration was determined using a haemocytometer (Superior Marienfeld Laboratory Glassware). Concentration was diluted with assay buffer to $5 \times 10^7$ cells $ml^{-1}$ for use in the assay. Cell solution with appropriate concentration was stored

overnight in RPMI medium, and then centrifuged in an Eppendorf centrifuge at 2000 x g for 5 minutes at 4° C and resuspended in assay buffer the next day immediately prior to assay. After calculation of the desired number of erythrocytes for each sample well, cells were washed twice with assay buffer, and re-centrifuged at the previously mentioned speed, time and temperature. A volume corresponding to 1.0 x $10^7$ *P. parvum* cells from each treatment were harvested via centrifugation, and the cell pellet resuspended in lysis/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, Eschbach et al. 2001). The resuspended pellets (each containing 1.0 x $10^7$ *P. parvum* cells) were then completely lysed via sanitation at the following settings: 50% pulse cycle, 70% amplitude, for 1 min. Cell lysates were pipetted in biotriplicate, as well as technical triplicate, into a 96 conical bottomed optical microtiter plate (Nunc. Wiesbaden, Germany). Pre-washed blood (100 µl) (5.0 x $10^6$ cells) and cell lysate (100 µl) was pipetted into each well. The saponin standard dilutions were pipetted in technical triplicate. The plate was sealed with foil, and was incubated at 15°C for 24 hours. After incubation, each plate was centrifuged for 5 min at 2000 x g and room temperature in an Eppendorf centrifuge, and the supernatant subsequently transferred to a flat bottom optical 96 well microtiter plate (Nunc. Wiesbaden, Germany). The absorption of the released haemoglobin was scanned from 350 to 700 nm with an Ultrospec III UV/Visible photometer using Wavescan Application Software (Pharmacia LKB Biotechnology, Uppsala, Sweden). Lytic activity was calculated in ng saponin equivalents per cell (ng SnE $cell^{-1}$), utilizing the standard saponin from higher plants as an indicator of relative lytic capacity.

*Extracellular and/or secreted toxicity: Rhodomonas salina bioassay*

*Rhodomonas salina* is a sensitive cryptophyte that is commonly used as a measure of lytic capacity for structurally unconfirmed compounds, as is the case for the compounds from *P. parvum.* A dose-response curve is established, and an $EC_{50}$ 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 as described by Tillmann et al. 2008. *Rhodomonas* stock cultures were maintained in F/2 medium as described by Guillard & Ryther, 1962, at 15 ° C and ambient light conditions. 4 ml of a mixture of *P. parvum* (final cell concentrations in decreasing order: $3.75 \times 10^4$ ml$^{-1}$, $2.34 \times 10^4$ ml$^{-1}$, $9.38 \times 10^3$ ml$^{-1}$ and $4.69 \times 10^3$ ml$^{-1}$) and *R. salina* (final cell concentration $1.0 \times 10^5$ ml$^{-1}$) were incubated in glass scintillation vials at 15° C for 24 h in darkness. Vials were then gently mixed by rotating, and 1 ml of mixture was pipetted into an Utermöhl cell sedimentation chamber and fixed with glutaraldehyde (2.5% final concentration). After settling, cells were viewed via epifluorescence microscopy (Zeiss Axiovert 2 Plus, Carl Zeiss AG, Göttingen, Germany) with Zeiss filter-set 14 at 64X magnification. Lysed versus non-lysed cells were easily distinguishable due to pigment auto-fluorescence characteristics (*Prymnesium* - red or *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.

*Statistical significance and standard deviation*

For single data points originating from both bioassays as well as between treatments in the gene expression portion, a *t*-test was used with a significance cut off of

$p<0.05$ to identify significant differences between physiological treatments, observed toxicity and gene fold regulation. Additionally, Figures 1-5 contain error bars, which represent the standard deviation between biological, not technical, replicates.

*Total RNA isolation*

Physiologically shocked triplicate cultures were centrifuged at 3000 x g for 15 min at 20 °C. The supernatant was removed, and the remaining cell pellet was resuspended in 350 µl of buffer RLT (lysis buffer) containing β-mercaptoethanol (Qiagen, Hilden, Germany), and subsequently flash-frozen in liquid nitrogen at -80° C. Samples were then stored at -70° C to minimize activity of potential RNase enzymes and prevent degradation. Prior to starting the protocol 100% ethanol was added to the wash buffer RPE, and β-mercaptoethanol was added as an RNAse inhibitor to the lysis buffer RLT. The amount of starting material was also taken into consideration, following recommendations in the manufacturer's handbook (see Qiagen Plant RNeasy protocol book). Marine protists are known to produce a variety of different secondary metabolites and those such as polysaccharides and phenolic compounds can cause a variety of problems during nucleic acid extraction. In order to obtain high quality RNA only low amounts of cells can be used for extraction, even when the theoretical capacity of the column is not approached.

Flash frozen samples were thawed 'on ice', and approximately two small spatulas full of 0.1 mm diameter glass beads were added to the sample. The cells were disrupted 2 x 30 s using a Qiagen Bead Beater (Hilden, Germany). The homogenate was separated from the glass beads and placed in a QIAshredder column/collection tube and centrifuged for 10 min at maximum speed. Centrifugation through the shredder column functions to remove

cell debris, as well as homogenize the lysate. A small pellet formed at the bottom of the collection tube. The supernatant was very carefully removed and placed in a new centrifuge tube, without disturbing the pellet at the bottom of the tube. Ethanol (250µl-100%) was added to the lysate (0.5 x volume) and mixed by pipetting. The entire sample was loaded onto a new RNeasy column/collection tube, and was spun at 8,000 x g for 30 s. The ethanol added previously functions to bind the RNA to the silica membrane in the column. The flow-through was discarded. 700 µl RW1 buffer was added to the column, and column was centrifuged again at 8,000 x g for 30 s. RWI buffer contains a high guanidine salt concentration that functions to wash the membrane-bound RNA. The flow-through was again discarded. The column was transferred into a new collection tube. Wash buffer RPE containing ethanol (500 µl) was added to the column, and the column was centrifuged as before. The flow-through was discarded. This wash step was repeated once more, including the centrifugation and flow-through discarding step. The column was centrifuged further for 1 min at maximum speed to remove all traces of ethanol present. Any remaining ethanol could interfere with downstream applications of the RNA, i.e. cDNA synthesis. The column was placed next in a new centrifuge tube, 2 x 50 µl of DEPC treated water was pipetted directly to the center of the membrane in order to elute the RNA. The final volume at this point was 100 µl.

*DNase in-tube treatment*

To each sample of 100 µl volume, 10 µl buffer DNase buffer RDD and 5 µl DNAse resuspended in provided nuclease free water (Qiagen) were added. This mixture was incubated for 1 h at room temperature (approximately 23° C).

*RNA Clean-up*

Buffer RLT (350 µl) was added to the DNAse and RNA mixture. The solution was then thoroughly vortex mixed. Ethanol (250 µl-100%) was added to the solution, and the mixture was repeatedly pipetted. The sample (700µl) was applied to a new RNeasy column/collection tube and centrifuged at 8,000 x g for 30 s. Both the flow-through and the collection tube were discarded. The column was washed with 350 µl buffer RW1 (high salt), followed by a DNAse on column digestion. DNAse stock solution (10µl) was added to 70 µl buffer RDD, and was gently flicked, not vortexed, due to the fragile nature of the DNAse enzyme. The entire 80 µl DNAse/buffer RDD solution was applied to the center of the membrane, and was incubated at room temperature for 15 min. 2 x 500 µl buffer RPE washes were performed as previously described, and then the final RNA was eluted either in 50 µl or 2 x 50 µl of DEPC treated water. RNA concentration and quality/integrity was checked using the Nanodrop spectrophotometer and Agilent bioanalyzer (Agilent Technologies, Santa Clara USA).

*Sample purity*

A Nanodrop spectrophotometer was used to determine the purity of the RNA samples obtained. The Nanodrop system is a full spectrum spectrophotometer (220-750 nm). 1 µl of each extracted RNA sample was pipetted onto the spectrophotometer measurement stage for analysis. Polysaccharides absorb at 230 nm, while proteins absorbs at a wavelength of 280. Nucleic acid absorbs at 260 nm, and therefore the ratio of 260/280 indicates protein contamination, and the 260/230 ratio indicates polysaccharide

contamination. It is important that both the 260/280 and 260/230 nm ratios are close to 2.0.

*Sample Integrity*

RNA integrity was measured using gel-chip technology (Agilent). Each chip contains an interconnected set of gel-filled channels that allow for molecular sieving or sorting of nucleic acid samples. Electrodes, which come into contact with the samples when the lid of the bioanalyzer is closed, control the movement of the samples within the gel channels. Each electrode is attached separately to a power source, allowing for very flexible control of the sample movement. RNA of an appropriate concentration and integrity was obtained for all samples, with the exception of the dark treatment.

*In vitro transcription & cDNA synthesis*

Complementary to the gene expression analysis, three typical housekeeping genes (Ubiquitin, GAPDH and Actin) were compared with two genes stemming from the 'small cabbage white' butterfly *Pieris rapae*: major allergen-MA (EU265818) and nitrile specifier protein-nsp (EU265817). These two genes show no sequence similarity to any accession outside of the *Lepidoptera* genus (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 & 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. All primers used in

this study were designed using Primer Express © v 2.0 software using the default settings and synthesized by MWG biotechnologies, Germany.

To obtain mRNA for these two spike genes, *in vitro* transcription was performed with amplified MA & NSP PCR fragments. The reaction components can be viewed in table 2.1.5.

Table 2.1.5: Reaction components for *in vitro* transcription of MA and NSP spike genes

| Component | Volume | [Final] |
|---|---|---|
| 5x T7 RNA Pol. buffer | 10 µl | 1x |
| NTP stock (10 mM each) | 10 µl | 2 µM |
| 10 mM DTT | 5 µl | 5 mM |
| PCR template | 1 µg | 20 µg $ml^{-1}$ |
| Final volume (with water) | | 50 ul |

This reaction mixture was incubated at 37° C for 1 h, after which 5 µl (250 units) T7 RNA polymerase was added to each reaction, followed by 1 h incubation at 37° C. $Na_2EDTA$ (50 µl) was immediately added. The mRNA produced was recovered via the Qiagen RNeasy clean up protocol, which was previously described in the RNA extraction section of the materials and methods.

cDNA was synthesized from 500 ng total RNA of all samples with the Omniscript RT kit (Qiagen, Hilden, Germany) using anchored oligoVN(dT)20 primer (Invitrogen, Paisley, UK) at a final concentration of 25 ng $µl^{-1}$. MA was added at a final concentration of 116 pg $µl^{-1}$ and NSP at 10 fg $µl^{-1}$. RNA samples (500 ng) were diluted to 9.25 µl with RNAse free water. Reaction components are listed in Table 2.1.6. For dark treatment samples, only RNA with very high polysaccharide content in solution was consistently obtained. This can be attributed to degradation of starch within the algal cells, in the absence of light, as has been previously described for the rhodophyte *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.

Table 2.1.6: Components of cDNA synthesis reaction

| Component | Volume |
|---|---|
| 5 mM dNTPs | 1 µl |
| Oligo dT primer | 1 µl |
| 10 x buffer | 2 µl |
| RNAse Out | 0.25 µl |
| Omniscript | 1 µl |
| MA mRNA | 2.64 µl (1.0 ng) |
| NSP mRNA | 2.862 µl (1.0 pg) |
| Final volume | 20 µl |

*Target gene selection and qPCR*

One aim of this study was to characterize three PKS transcripts, originating from *P. parvum,* in response to short-term physiological acclimation. For normalization of these three target genes, we chose two 'foreign' internal reference genes, as well as three commonly accepted housekeeping genes from qPCR related literature. Sequences and names of target genes are given in table 2.1.7. The ratio of the amount of target gene mRNA to the amount of housekeeping gene mRNA was analyzed with a SYBRgreen qPCR reaction, designed according to manufacturer's protocol (Applied Biosystems, Darmstadt, Germany) using 2 µl of a 10-fold diluted cDNA. qPCR reaction details are given in Table 2.1.8. Cycle parameters included an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 59 °C for 1 minute. 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 6 cDNA (originating from the control culture) serial dilution points ($1.0x10^{-3}$-$1.0x10^{-8}$). Percent efficiency was calculated from the slope of the threshold cycle ($C_t$) vs. concentration [cDNA] with equation (I)

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

All PCR efficiencies were 98.88% ≥ x ≥ 92.31% 1.91, all $R^2$ were > 0.94. All samples were run in both biological (independent cultures) as well as technical triplicates. Variation was calculated as averages among technical replicates as well as standard deviation. An R expression ratio was calculated using the $\Delta\Delta C_t$ as described by Pfaffl et al. 2001, incorporating individual reaction efficiencies as correction factors. Calculation of an R expression ratio was performed using the following equation (II)

$$II \qquad Ratio = E_{target}\text{^}[\Delta C_{t\ target}\ (control\text{-}\ sample)]\ /\ E_{MA}\text{^}[\Delta C_{t\ housekeeping}\ (control\text{-}\ sample)]$$

The authors chose this method of quantification, in order to minimize intra and interassay variability, and to aid in a robust comparison between normalization (housekeeping) genes. All calculations were performed using the REST-2009 software platform (Qiagen, Hilden, Germany).

Table 2.1.7: Target genes investigated in this study, and primers used to amplify sequences via qPCR

| *Gene* | *Sequence F(5´-3´)* | *Sequence R(5´-3´)* | *Gene name* | *Amplicon length* |
|---|---|---|---|---|
| MA | AAGAGTGGCCAGCACAGTAGACA | AGCTGCCTCCTTGGAAGCATA | major allergen | 100 bp |
| NSP | TTGACCACTACCCACGGATGA | ACGATCAATCCAGTATGCAACAA | nitrile specific protein | 100 bp |
| GAPDH | GTTGAGGCCGCAGCAATTAC | ATTCGCGTCTTTTCTCCCATAC | Glyceraldehyde-3-phosphate dehydrogenase | 150 bp |
| Ubiquitin | CTCAATGTTCGGGTCAGCAA | CTGGACGGCAAAGTCTGCAT | Ubiquitin conjugating enzyme | 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 2.1.8: Components of qPCR reaction for all samples

| Component | Volume |
|---|---|
| Primer F (10 pM concentration) | 0.25 µl |
| Primer R (10 pM 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 |

*obtained from Applied Biosystems, Darmstadt, Germany

*Evaluation of reference gene stability via geNorm and NormFinder*

To determine differences in stability (variation) between internal reference (MA and 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 based correlative approach. NormFinder is an algorithm that attempts to find the optimum reference genes out of a group of candidate genes. This algorithm can also, in contrast to geNorm, take information of groupings of samples into account, such as untreated vs. treatment . The result is an optimal (pair of) reference gene(s). The resulting pair might have compensating expression, so that one gene, e.g., is slightly over-expressed 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 because reference/housekeeping genes should display non-differential expression across different treatments.

### 2.1.4-5 Results & Discussion

*Toxicity*

In the erythrocyte lysis assay (ELA) as a measure of intracellular toxicity, we found that high light induced the largest significant (t-test, $p<0.05$) increase in lytic capacity relative to a control culture (Figure 2.1.1).

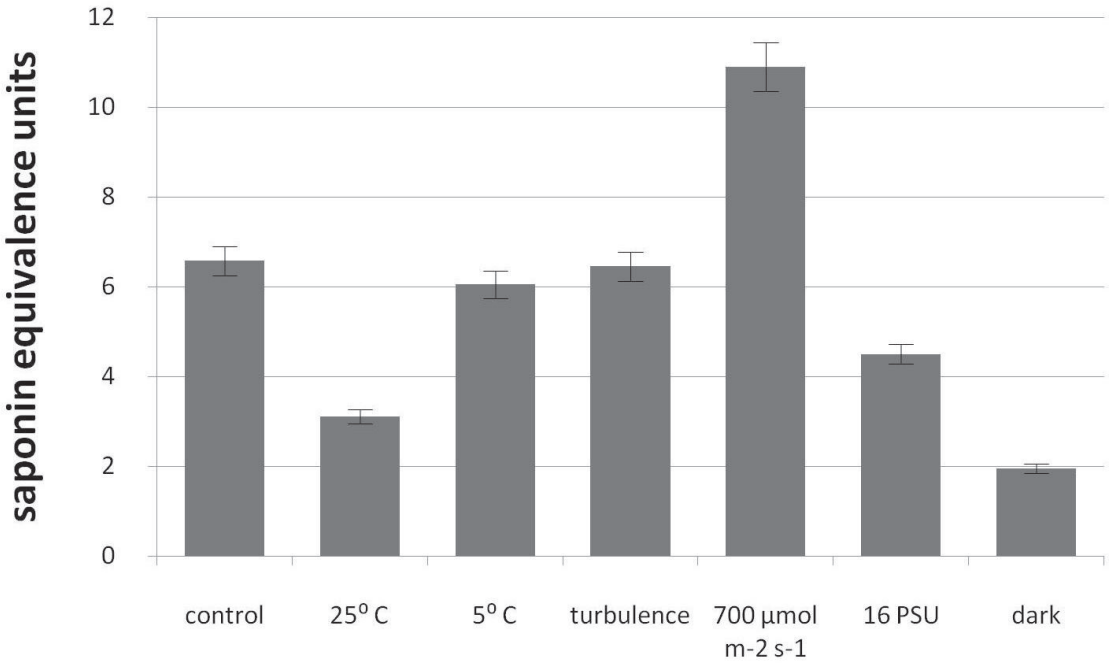



**Figure 2.1.1: Results of erythrocyte lysis assay. Light shock treatment (700 µmol photons $m^{-2}$ $s^{-1}$) shows the highest lytic effect on erythrocytes. Turbulence shows the same effect as the control culture, while the remaining treatments show a decrease in lytic capacity against erythrocytes. All shock treatments were performed for 2 h.**

Exposure to light has been linked to an increase in observed toxicity in *P. parvum* (Shilo & Aschner, 1953). Parnas et al. (1962) found the lytic activity of extracted substances from *P. parvum* to decrease over time with exposure to light. In their

conclusions, however, they made no concrete statements about either the intracellular production or extracellular secretion of toxins. Photosynthetic processes play a major nutritional role for *P. parvum,* and therefore provide energy for cellular processes such as biosynthesis of toxic metabolites. Due to the currently known structural characterizations of compounds derived from *P. parvum* (prym1 & prym2, Igarashi et al., 1996), it is likely that these compounds are biochemically costly to synthesize. For many toxigenic algal species, the effect of light has been directly linked to changes in toxin content per cell, i.e. A*lexandrium catenella* (Proctor et al., 1975), toxin production in *Pseudo-nitschia multiseries* (Bates et al., 1991) as well as observed toxic effects in *P. parvum* (Shilo et al., 1971).

Extracellular or secreted toxicity was investigated using a *Rhodomonas salina* assay, which may or may not be related to the internal toxicity. Prymnesins may play a role in extracellular toxicity, due to several of their described physiochemical properties, however this has not yet been confirmed. These compounds have been described to interact directly with exposed cell membranes, compromising integrity and permitting ion leakage through selective permeation (Manning and LaClaire, 2010). Prymnesin toxicity is furthermore known to be dose-dependent, and to respond in a linear manner when analyzing change in membrane conductance after exposure to these compounds (Manning and LaClaire, 2010). The mechanism by which these compounds are secreted is, however, yet to be described. Observed differences in intracellular versus extracellular toxicity may be due to chemical signalling and recognition, which is a topic of current interest among P*rymnesium* researchers. The effects observed in the *Rhodomonas salina* assay are furthermore those that have an impact on allelochemical interactions, since potential grazers and/or competitors can be affected (Tillmann, 2003).

In the *Rhodomonas* test, the light-shock treatment showed approximately 50% less toxicity (t-test, $p<0.05$) when compared to the control cultures (Table 2.1.9). 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 to the control. Parnas et al. (1962) claimed that the icthyotoxicity of *P. parvum* was inversely proportional to salt concentrations. Even further support of this inverse relationship was later presented (Ulitzer & Shilo, 1966) indicating that the uptake of trypan blue (i.e. cell permeability/toxicity) in the gills of fish decreased after exposure to increased saline conditions -strengthening both previous studies. We were able to show that low salinity shock increases active extracellular process of toxin-secretion of *P. parvum* towards the cryptophyte *R. salina,* although the salinity shock seemed to have no significant increase on the intracellular lytic capacity towards red blood cells of *P. parvum* vs. the control culture (figure 2.1.1).

Table 2.1.9: $EC_{50}$ results for various physiological shock treatments of *P. parvum* strain RL10. Cell concentrations represent concentration of *P. parvum* necessary for 50% mortality of *R. salina.*

| Treatment | $EC_{50}$ *Rhodomonas salina* |
|---|---|
| 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 |

In general the cryptophyte *Rhodomonas salina* responded variably to *P. parvum* cells from different shock treatments, indicating changes in extracellular toxicity and. The results of these two bioassays suggest a difference in the biosynthesis and secretion of the

toxin following different physiological shock treatments. 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. The other shock treatments showed changes in toxicity as well, were however not able to be correlated with the changes observed in gene expression, rendering these results less conclusive in discussion of PKS genes putatively associated with toxic processes in *P. parvum.* A decrease in extracellular salinity may lead to a compromised cellular membrane, subsequently leading to a leakage of intracellular toxin. The difference between active secretion and leakage through a compromised membrane has yet to be distinguished in *Prymnesium parvum*.

*Polyketide synthase gene expression analysis*

In differential gene expression studies, the use of housekeeping genes as endogenous controls can be problematic as they may be implicated in basal metabolic processes depending on the cell type (Thellin O. et al., 1999). We therefore incorporated mRNA from foreign spike genes into our samples, providing stable transcript copy numbers for downstream 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), we determined that both MA and NSP are in general more stable than all other housekeeping genes analyzed (Fig. 2.1.2). Of the two spike genes, NSP was shown to be more stable, with a Normfinder M-value of 0.004, compared to MA with a Normfinder M-value of 0.016 (Fig. 2.1.2). Both algorithms provided similar M-value rankings for the genes investigated.

The evaluation of potential gene expression differences in our samples using a real time PCR approach (qPCR) required data normalization, which is a crucial step for gene transcript quantification analysis (Bustin 2002, Pfaffl 2001). 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 & Larrick, 1992; Bustin, 2000). The qPCR-specific errors in the quantification of mRNA transcripts are easily compounded with 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 and cDNA sample loading variation (Stahlberg 2003, 2004a & 2004b).

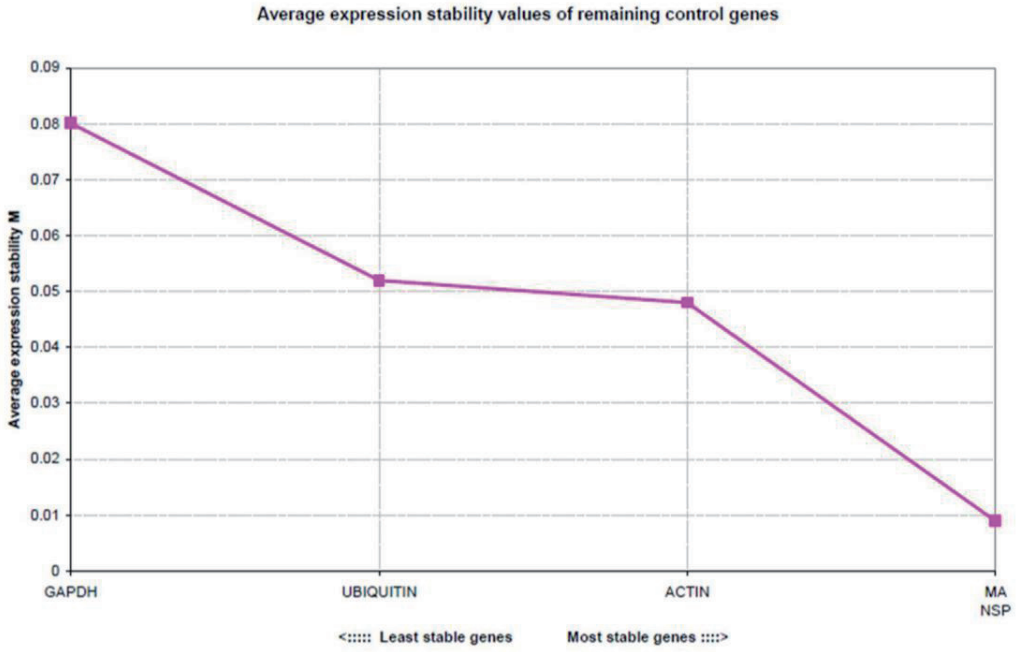



**Figure 2.1.2: Stability value 'M' for housekeeping genes and endogen controls tested, as computed by the Normfinder software. Most stable genes have the lowest 'M' value: NSP & MA.**

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 endogen/housekeeping gene used for normalization. In contrast, the spike gene MA was detected in all samples at a Ct value of 15.75 ± 0.28 (n=18), the second spike gene NSP at a Ct of 26.4 ± 0.29 (n=18) (data not shown); indicating a consistent reverse transcription reaction efficiency for high copy number (MA) and low copy number (NSP) genes across all samples.

Low salinity shock (16 psu) treatment yielded not only an increase in extracellular toxicity towards the cryptophyte *R. salina,* but also an increase in copy number of the PKS 1 gene 6t3 (Figure 2.1.3). This is in contrast to the high light shock treatment caused an increase in lytic capacity towards erythrocytes, possibly representing an increased intracellular concentration of lytic compound, (Figure 2.1.1), and caused an increase in copy number of the PKS 2 gene 7t3 (Figure 2.1.4). The association of particular PKS transcripts with changes in toxic processes indicates not only the uniqueness of at least the two transcripts PKS 1 6t3 and PKS 2 7t3, but also the potential differential roles that these PKS transcripts may play in toxic processes in *P. parvum.* With further characterization of PKS genes in *P. parvum,* one could likely find specific sequential and thus structural based traits that associate a transcript with a particular process, i.e. biosynthesis or transport and/or secretion.

This increase in copy number was apparent, regardless of whether or not normalized against a traditional housekeeping gene, i.e. GAPDH, or utilizing our spike-in endogen control (Figures 2.1.3, 2.1.4 & 2.1.5). Although the trend remains the same, the

amplitude of the data is 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 decreases the confidence interval of a data set relying on these genes for normalization, and thus renders the data open to skepticism.

In contrast to the low salinity shock treatment, the 25 °C treatment yielded smaller changes in PKS relative gene expression, due to minimal variation among housekeeping genes and the spike-in gene NSP. 25° C shocked cultures also showed a lower increase in toxicity in either bioassay tested relative to the control (Table 2.1.9, Figure 2.1.1) compared to other shock treatments. Under the hypothesis that the PKS genes studied here are involved in the biosynthesis of lytic/toxic substances produced by *P. parvum*, a dramatic increase in the PKS copy number after a 25 °C shock treatment was not expected. The biosynthesis of toxic compounds toxins due to increased temperature, however, might additionally be due either to post-transcriptional or translational regulation, or perhaps to the presence of non-active precursors, potentially the activation of "toxin-precursors" that can also occur later under temperature stress.

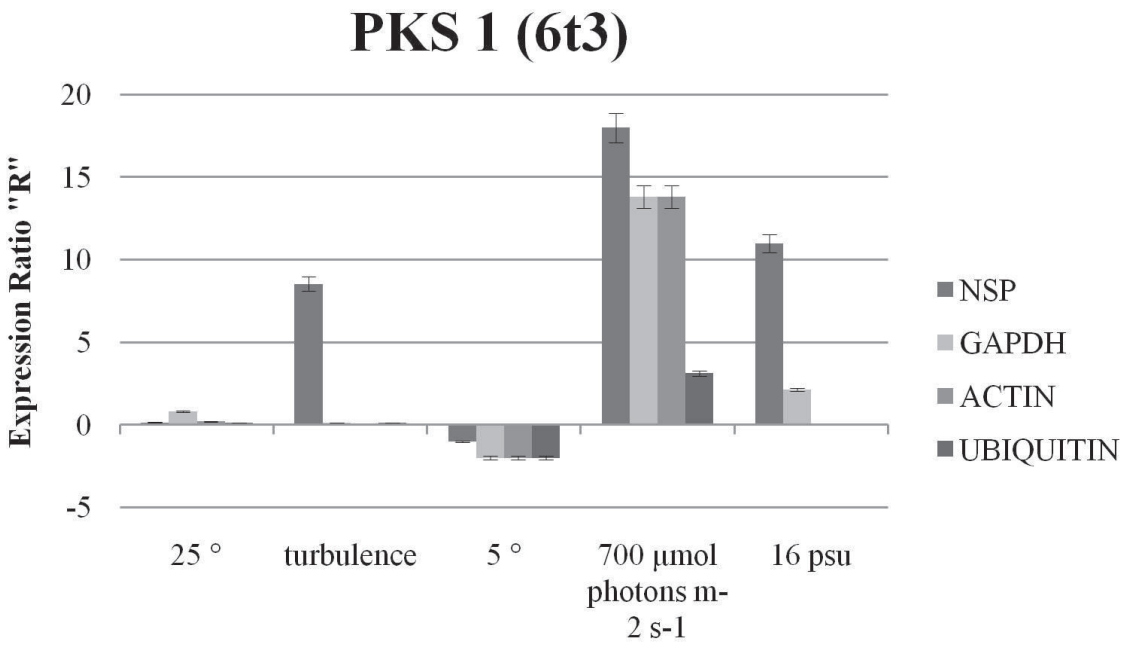



**Fig. 2.1.3: qPCR results for the PKS 1 6t3 gene indicating normalization against three housekeeping genes and one of our internal spike genes (NSP). Data shown is normalized against a control culture [Control (20 °C, 26 psu, 90 µmol photons $m^{-2}$ $s^{-1}$].**

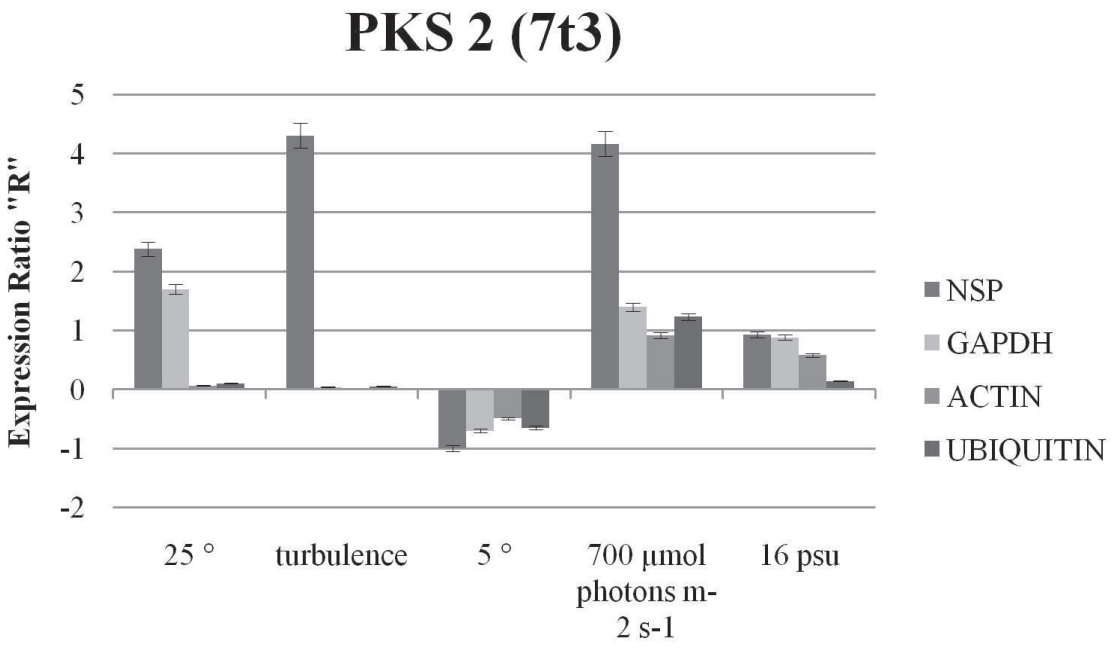



**Figure 2.1.4: qPCR results for the PKS 2 7t3 gene indicating normalization against three housekeeping genes and one of our internal spike genes (NSP). Data shown is normalized against a control culture.**

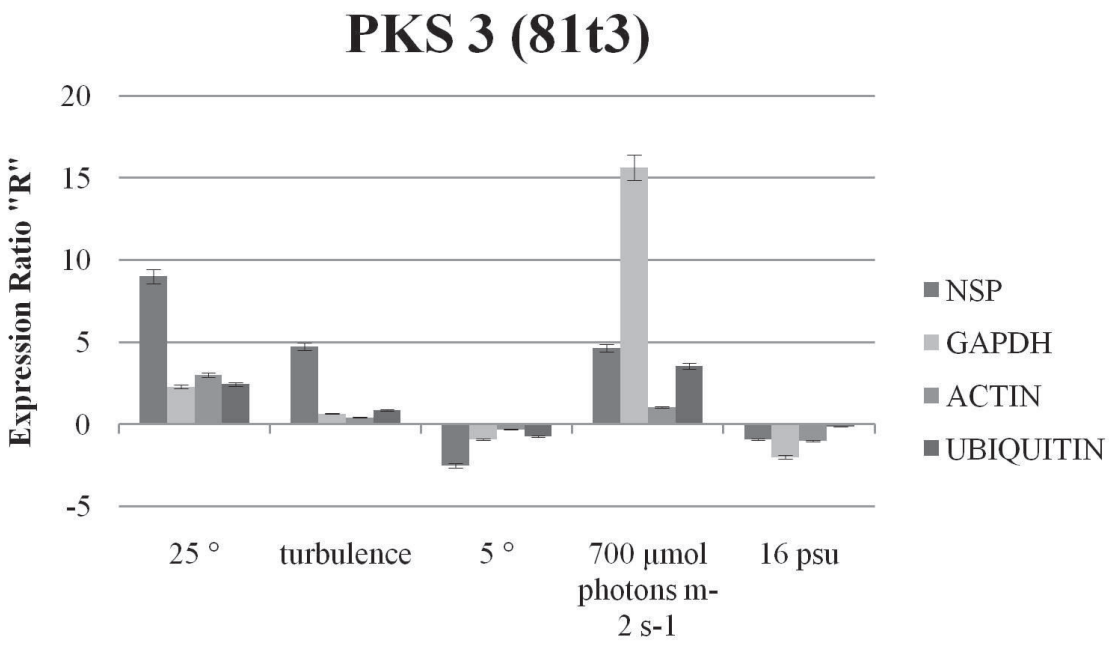



**Figure 2.1.5: qPCR results for PKS 3 81t3 gene investigated indicating normalization against three housekeeping genes and one of our internal spike genes (NSP). Data shown are normalized against a control culture.**

*2.1.6 Conclusions*

We studied the short term impact of environmental changes (shock treatments) on the toxicity and PKS gene expression of *P. parvum.* This topic is of importance because *P. parvum* is known to be competitive in several niches where parameters such as salinity, light and water turbulence undergo rapid change (Edvardsen & Paasche, 1998). Furthermore *P. parvum* is often able to form monospecific algal blooms under these conditions, suggesting the presence of a competitive advantage over coexisting species. Blooms of *P. parvum* often have a strong negative impact on the ecosystem (Larsen & Bryant, 1998). We found high light stress and low salinity stress to be the most relatively influential stresses in toxicity induction (based upon bioassay results) as well as differential gene expression of PKS. The majority of shock treatments induced some level of increase in expression in PKS, suggesting these gene pathways to be of general stress-response importance in *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.

# 2.2 Publication 2

## Differential responses of the prymnesiophyte *Prymnesium parvum* following interactions with planktonic species

### 2.2.1 Abstract

The prymnesiophyte *Prymnesium parvum* is notorious worldwide for formation of toxic blooms associated with fish kills, but the ecological role the toxins play in pelagic food webs remains unresolved. Allelochemical mediation has been suspected to affect competition and/or predation-related interactions involving *P. parvum* blooms. Accordingly, we investigated heterospecific interactions between this prymnesiophyte and three naturally co-occurring planktonic species, the heterotrophic predatory dinoflagellate *Oxyrrhis marina*, and two potential prey species, the photoautotrophic dinoflagellate *Heterocapsa triquetra* and the unicellular cyanobacterium *Chroococcus submarinus*. Combining bioassay-guided toxicity and functional genomic approaches with a specific microarray for *P. parvum* allowed differential characterization of cell-contact and waterborne cue-mediated specific responses to grazing and competition. We identified differential responses in *P. parvum*, depending on the interacting species, in terms of lytic capacity, gene expression profile, as well as transcriptional regulation of polyketide synthase genes (PKS). Microarray analysis identified a unique gene expression pattern in response to both whole-cell culture and filtrate from the potential predator *Oxyhrris marina*, suggesting a qualitatively different interaction compared to that with the potential prey species *H. triquetra* and *C. submarinus*. A further time-series incubation with *O. marina* cells showed that the effects did not occur immediately, but rather after 60-90 min

exposure. Stress derived from competition or grazing pressure is a known factor in co-evolution of species. The differential gene expression of *P. parvum* in response to predators such as *O. marina* versus potential prey species may therefore signify the existence of a co-evolutionarily adaptive defense.

### 2.2.2 Introduction

The prymnesiophytes constitute a predominantly marine group of microalgae with a few genera that play important roles in oceanic carbon recycling. In coastal and brackish waters prymnesiophytes occasionally become dominant members of plankton communities and can even form dense virtually monospecific blooms. Two marine genera *Prymnesium* and *Chrysochromulina* are especially notorious for the production of noxious and/or toxic blooms responsible for massive fish mortalities and ecosystem devastation in coastal and inshore waters, including ponds and lagoons.

From an ecological perspective, studies of phytoplankton succession and bloom formation have primarily focused on comparative abiotic effects rather than on individual plankton components (Domingues et al., 2005; Levasseur et al., 1984; Lindenschmidt & Chorus, 1998; Sommer, 1988). In this context, the apparent success of *Prymnesium parvum* leading to dominance and bloom formation might be attributed to its physiological flexibility reflected by its ability to grow in a wide range of environmental conditions (Larsen & Bryant, 1998). There is increasing evidence, however, that inter-specific interactions in the plankton play a major role in succession, food web structure and bloom development (Smetacek et al., 2004; Tillmann, 2004). Among these interactions, the capacity to produce toxic or noxious allelochemicals that may deter grazing or affect competition for limiting resources has been increasing recognized as an important regulatory mechanism affecting bloom dynamics of plankton (reviewed by Cembella, 2003; Legrand 2003). Allelochemicals produced and secreted by *P. parvum* have been shown to kill both competing algal species and their grazers (Tillmann, 2003, Granéli 2006). Closely related to this "killing capacity" (Tillmann, 2003) is the mixotrophic tendencies of

*Prymnesium*, i.e. the ability to ingest immobilized competitors and grazers (Tillmann 2003; Skovgaard & Hansen 2003). This strategy to kill (and then eat) your enemies by means of toxic compounds is thought to significantly contribute to the ability of *P. parvum* to form dense and long-lasting blooms.

Although multiple toxins may be produced by *P. parvum*, only two definitively toxic metabolites have been isolated and structurally elucidated from this species (Igarashi et al., 1999). The two toxic compounds prym1 and prym2 share a linear polyether structure with similar ichthyotoxic and hemolytic properties. The polyether configuration of these analogues strongly suggests that they are derived via polyketide biosynthetic pathways, thereby raising interest in the putative polyketide synthase (PKS) enzymes involved in their biosynthesis and their biochemical role in toxigenic processes in prymnesiophytes (LaClaire, 2006, John et al., 2008, John et al., 2010).

Polyketides are a family of secondary metabolites whose carbon skeleton is formed through sequential condensation reactions of acyl-coenzyme A (coA), via PKS enzymes evolutionarily related to fatty acid synthases (Staunton & Weissmann, 2001; Crawford et al., 2006). Among the known protist PKS enzymes, many have been shown to be modular PKS types belonging to the same molecular class of biosynthetic pathways; most marine protist species studied so far exhibit two or more functionally different PKS genes (LaClaire, 2006; John et al., 2008, John et al., 2010; Monroe et al., 2010).

Effects of environmental conditions on toxicity as well as the ecological consequences of toxin-related species interactions of *Prymnesium* have been rather well studied (Larsen & Bryant 1998 Tillmann 2003; Uronen et al., 2007; Saponen et al., 2006). Nevertheless, related questions have barely been addressed: Does this responsiveness

come with well definable physiological costs? Is *Prymnesium* able to sense other protists and thus to potentially adjust pathways and processes, e.g. related to toxicity? What are the gene expression mechanisms involved in toxigenesis and how are they regulated?

As for social insect populations, and also for the well defined mechanisms of quorum sensing defined for bacterial interactions (Waters & Basler, 2005, Seeley & Visscher, 2005), one may also expect similar mutually developed strategies of inter-specific chemically mediated sensing among planktonic species in marine ecosystems. For example, in the dinoflagellate *Alexandrium minutum,* selective sensing of waterborne cues has been shown to elicit a differential response in the toxicity of *Alexandrium* cells depending upon the grazer to which they are exposed (Bergkvist et al., 2008). Competitor sensing based on waterborne cues seems therefore to be a very powerful defense strategy to ensure survival of the population (Wolfe et al., 2002).

With specific focus on the importance and/or necessity of physical contact vs. recognition of waterborne cues, we utilized a functional genomic-bioassay linked approach to characterize interactions between *P. parvum* and three potentially coexisting plankton species: the photosynthetic dinoflagellate *Heterocapsa triquetra*, the cyanobacterium *Chroococcus submarinus*, both considered to be possible resource competitors and/or potential prey for *P. parvum*, and the heterotrophic dinoflagellate *Oxyhrris marina*, capable of serving as either predator or potential prey depending on the toxicity status of *P. parvum* (Tillmann, 2003). Changes in toxicity, paired with differential gene expression data provided insights into such processes as induced defense and recognition of and response to coexisting organisms.

### 2.2.3 Materials & Methods

*Microalgal origin and culture conditions*

A toxic clonal strain RL10 of *Prymnesium parvum* was isolated in 1993 from the Norwegian Sandsfjord (Edvardsen & Larsen, 1998). *Prymnesium parvum* cultures were maintained in IMR medium, prepared as described in publication 1 (Eppley, 1972) (see table 2.1.1-2.1.3). IMR medium was prepared using a combination of North Sea water and milliq deionized water (4:1 v:v), to a salinity of 26 PSU, under gentle aeration to a concentration of 3.75 x $10^3$ cells $ml^{-1}$. The heterotrophic dinoflagellate *Oxyhrris marina* (Göttingen culture collection strain B21.89) and the peridinian dinoflagellate *Heterocapsa triquetra* (SCCAP strain K-0481) were cultured in preparation for the experiments in IMR medium (Eppley, 1972) also at a salinity of 26 psu in 100 ml flasks at 15 °C. Stock cultures of *Oxyhrris* in 100 ml flasks were fed upon the chlorophyte *Dunaliella* sp. cultured at 26 psu upon f/10 medium (Guillard & Ryther, 1962). *Oxyhrris* cultures for the experiment were grown at 15 °C to high cell concentrations until they became deprived of food. *Heterocapsa* cultures for the experiment were grown to a concentration of 2.7 x $10^3$ cells $ml^{-1}$. All cultures were kept at a constant temperature of 15°C under a light: dark photocycle of 16:8 h. Photon flux density measured inside the flask by a QSL-100 Quantum Scalar Irradiance Meter (Biospherical Instruments, San Diego, USA) was kept at 90 µmol photons $m^{-2}$ $s^{-1}$. Cell concentrations were determined daily using a CASY cell counter (Innovatis AG, Reutlingen, Germany).

The cyanobacterium *Chroococcus submarinus* (NIVA culture collection strain 331) was maintained in MLA medium (Castro et al., 2004) at salinity 20 psu (achieved using

North Sea water), at 20°C, and grown to a concentration of 1.76 x $10^5$ cells $ml^{-1}$. Cyanobacterium cell concentrations were determined by Neubauer hemocytometer every second day.

*Batch culture Experiment 1*

An initial experiment was conducted to investigate the differential response in *P. parvum* to all three co-existing species, either through cell-cell contact, or via incubation with filtrate with putative chemical cues from the corresponding species. Triplicate 400 ml batch cultures of the *P. parvum* RL10 strain were established from a 5 l stock culture in the exponential growth phase. Batch cultures were maintained with identical growth (IMR medium, 26 PSU) conditions to the stock cultures, without aeration. Filtrate was prepared from all three test species (*O. marina, H. triquetra* and *C. submarinus)* via vacuum filtration via a 0.1µm vacucap at a maximal pressure of 200 mbar to minimize leakage of intracellular compounds. Equal parts by volume (1:1 total volume = 800 ml) of *Prymnesium* culture (final cell concentration: 1.88 x $10^4$ $ml^{-1}$) and coexisting species, either whole cell culture (final concentrations: *O. marina* 500 $ml^{-1}$, *H. triquetra* 1.35 x $10^3$ $ml^{-1}$, *C. submarinus* 8.8 x $10^4$ $ml^{-1}$), or corresponding filtrate from the same volume were incubated together for 2 h. A control culture was included by substituting 400 ml IMR medium for either whole-cell coexisting-species culture or filtrate. After incubation all cultures were harvested by centrifugation at 4,000 x *g* for 15 min at 20 °C**.**

*Dose-exposure series Experiment 2*

The second experiment exclusively focused on the interactions between *O. marina* and *P. parvum* over a time course of exposure. With an identical set-up as in the first experiment (also in triplicate), samples were taken over the course of the total 2 h incubation (at t = 0, 15, 30, 45, 60, 90 and 120 min). Harvesting of the cultures was performed as described for the first experiment. A control identical to that for the first experiment was included. For both Experiments 1 and 2, control and treatment cultures were harvested in parallel.

*Rhodomonas salina lysis assay*

A bioassay was performed with *Rhodomonas salina* strain KAC 30 as a measure of extracellular toxicity as described in publication 1 of this dissertation. *Rhodomonas* stock cultures were maintained in F/2 medium (Guillard & Ryther, 1962) at 15 ° C and ambient light conditions. In brief, 4 ml of a mixture of *P. parvum* (final cell concentrations in decreasing order: $3.75 \times 10^4$ ml$^{-1}$, $2.34 \times 10^4$ ml$^{-1}$, $9.38 \times 10^3$ ml$^{-1}$ and $4.69 \times 10^3$ ml$^{-1}$) and *R. salina* (final cell concentration $1.0 \times 10^5$ ml$^{-1}$) were incubated in glass scintillation vials at 15° C for 24 h in darkness. Vials were then gently mixed by rotating, and 1 ml of mixture was pipetted into an Utermöhl cell sedimentation chamber and fixed with glutaraldehyde (1% final concentration). After settling, cells were viewed via epifluorescence microscopy (Zeiss Axiovert 2 Plus, Carl Zeiss AG, Göttingen, Germany) with Zeiss filter-set 14 at 64 x magnification.. Lysed versus non-lysed cells were easily distinguishable due to pigment auto-fluorescence characteristics (*Prymnesium* - red or *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.

*Erythrocyte lysis assay*

A hemolytic activity bioassay was performed as described in Publication 1 of this dissertation. In brief, an aliquot volume corresponding to 1.0 x $10^7$ cells from each culture was centrifuged at 4,000 x *g* for 10 min at 15 °C and subsequently added to 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). Hemolytic activity was quantified on samples incubated only with filtrate to rule out effects from other intracellular compounds originating from coexisting species. Cell pellets were then completely lysed via sonication. After 24 h incubation, hemolytic activity was measured as 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 based on concentrations of saponin (Sigma Adrich, Hamburg, Germany) in the assay buffer. Results are displayed as $EC_{50}$ value: concentration of corresponding *P. Parvum* cell concentration to cause lysis of 50% erythrocytes in the sample well.

*RNA isolation and processing*

Experimental cultures were centrifuged at 4,000 x *g* for 15 min at 20 °C. The supernatant was removed, and the remaining cell pellet was resuspended in 350 µl of buffer RLT lysis buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol, and subsequently flash-frozen in liquid nitrogen at -80 °C. Samples were then stored at -70 °C

for later extraction. Total RNA was isolated from all samples according to the manufacturer's protocol (see Qiagen Plant RNeasy extraction kit, Qiagen, Hilden, Germany). An additional in-tube DNase treatment was included to facilitate downstream microarray and qPCR processing of samples. RNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany), and the purity estimated by the 260/280 and 260/230 nm absorption ratio (all ratios >2.0). Integrity of the RNA was verified with the lab-on-a-chip Bioanalyzer 2100 system (Agilent Technologies, Boeblingen, Germany).

*Microarray Analysis*

Agilent RNA Spike-In Mix (p/n 5188-5279) was added to the tRNA samples prior to the labelling reactions following the RNA Spike-In Kit protocol (Agilent Technologies, Boeblingen, Germany). Total RNA (500 ng) was amplified, reverse-transcribed and labelled using the two colour low RNA Input fluorescent linear amplification kit (Agilent Technologies, p/n 5184-3523). The Cy-3 and Cy-5 dye incorporation was verified by NanoDrop ND-1000 spectrophotometer. Hybridization was performed onto 4 x 44k microarray slides containing oligonucleotide 60mers designed by the Agilent eArray online platform, using the gene expression hybridization kit two colour (Agilent Technologies, p/n 5188-5242), contained in SureHyb Hybridization Chambers (Agilent p/n G2534A) in a hybridization oven (Agilent p/n G2545A) at 65° C for 17 h. Microarrays were scanned by an Agilent Scanner (p/n G2565BA).

Raw data were extracted with the Agilent Feature Extraction Software version 9.5, incorporating the GE2_105_Dec08 protocol. Feature extraction software served to remove spots that had been flagged 'outliers', 'not known' or 'bad', based on background median

analysis (Storey, 2003). Further analysis of gene expression was performed using GeneSpring GX version 10 software (p/n depending on license).

*SYBR green qPCR analysis*

Plasmid vectors (pDNR-Lib) containing full-length cDNAs of both the nitrile-specifier protein (NSP) and the major allergen (MA) genes of approximately 1.9 kb each from the commonly known 'small cabbage white' butterfly *Pieris rapae* were generated to serve as spike-in controls. Both of these genes are of particular importance in regulation processes regarding plant-insect interactions (Fischer et al., 2008). These plasmid constructs were used as template in PCR reactions to obtain the corresponding DNA fragments. MA and NSP primers were designed using Primer Express © v 2.0 software with the default settings.

*In vitro* transcription was performed according to the manufacturer's protocol with a T7 RNA polymerase (Invitrogen, Paisley, UK) to obtain mRNA for two internal spike reference genes, as described in publication 1 of this dissertation (Freitag et al., 2011 In Press). Spike genes MA (major allergen) and NSP (nitrile- specific protein) were utilized for quantification of results, as well as controlling the cDNA efficiency reaction prior to qPCR analysis. MA was added at a final concentration of 116 pg $\mu l^{-1}$ and NSP at 10 fg $\mu l^{-1}$. cDNA was synthesized from all tRNA samples with the Omniscript RT kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) using anchored oligoVN(dT)20 primer (Invitrogen, Paisley, UK) at a final concentration of 25 ng $\mu l^{-1}$. All primers for qPCR were designed with the Primer Express 2.0 software on default settings (Applied Biosystems, Darmstadt, Germany) and synthesised from MWG Biotechnologies Germany.

Standard PCR primers were designed based on the *Primer 3* platform using default settings (http://frodo.wi.mit.edu/) and synthesised from MWG Biotechnologies (Germany). Primer sequences are available as supplementary material. The SYBR green qPCR reaction was designed according to manufacturer's protocol (Applied Biosystems, Darmstadt, Germany) using 2 µl of a 10-fold diluted cDNA. 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).

Samples were run in biological triplicate to obtain mean values and standard deviation. For each primer pair, a standard curve was established by 10 fold dilutions of the qPCR template, spanning concentration differences of at least four orders of magnitude.

Amplification efficiency of all qPCR reactions was analyzed through linear regression of standard curves, with 6 cDNA (originating from the control culture) serial dilution points ($1.0x10^{-3}$-$1.0x10^{-8}$). Percent efficiency was calculated from the slope of the threshold cycle ($C_t$) vs. concentration [cDNA] with equation (I)

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

All PCR efficiencies were 98.88% ≥ x ≥ 92.31% 1.91, all $R^2$ were > 0.94. All samples were run in both biological (independent cultures) as well as technical triplicates. Variation was calculated as averages among technical replicates as well as standard deviation. An R expression ratio was calculated using the $\Delta\Delta C_t$ as described by Pfaffl et al. 2001, incorporating individual reaction efficiencies as correction factors. Calculation of an R expression ratio was performed using the following equation (II)

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

The authors chose this method of quantification, in order to minimize intra and interassay variability, and to aid in a robust comparison between normalization (housekeeping) genes. All calculations were performed using the REST-2009 software platform (Qiagen, Hilden, Germany).

*Statistical analysis*

Physiological data described are the mean of biological triplicates with the corresponding standard deviation. Significance of physiological data was confirmed using a Student's t-test ($p<0.05$). Microarray expression measurements are given as the geometric mean of three measurements, corresponding to biological triplicates.

### 2.2.4 Results

*Batch culture experiment 1*

*Encounter rates*

An encounter model (Gerritsen & Strickler 1977) was employed to simulate predators, prey and their encounters within the experimental setup. In this model, plankton are assumed to move at a defined speed in a random direction; when they approach to within a critical distance they are considered to 'encounter' each other. Several assumptions for a plausible model were made regarding the cells of *P. parvum* and those of coexisting species. The cells were assumed to be: 1) moving in a homogeneous three-dimensional environment; 2) swimming randomly at constant speeds; 3) randomly distributed.

The encounter rate (Z) of *Prymnesium* to coexisting species was determined according to the following equation (Gerritsen & Strickler, 1977):

$$Z = \frac{\Pi d^2 N}{3} \left[\frac{u^2+3v^2}{v}\right] \quad for\ v > u$$

where d = encounter distance (estimated spherical diameter: esd), N = *P. parvum* cell concentration, v = *P. parvum* swimming speed and u = coexisting species swimming speed. An encounter distance was defined by a fixed estimated spherical diameter (esd) measurement for each species. Encounter rate between *P. parvum* and *C. submarinus* was roughly 800% less frequent than that between *P. parvum* and *O. marina.* Encounter rates are detailed in Figure 2.2.1.

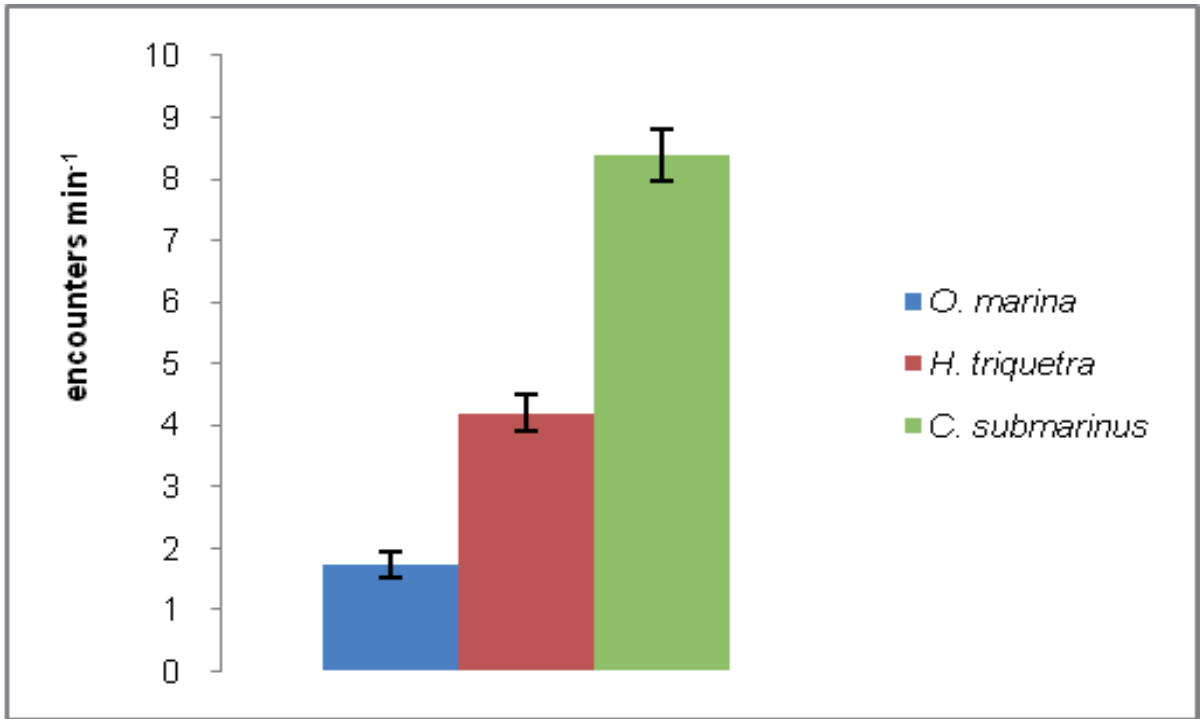



**Figure 2.2.1: Encounter rate (min$^{-1}$) for *Prymnesium parvum* and coexisting species. Estimated spherical diameter (ESD) and average swimming speed values were obtained from the literature: Evans, 1989; Calliaria & Tiselius, 2005; Skovgaard & Hansen, 2003, Henriksen, 2005).**

*Lytic capacity*

Variation in the lytic capacity of *Prymnesium parvum* depended on the coexisting organism and/or chemical cues together with which the prymnesiophyte cells were incubated. Table 2.2.1 shows results from experiment 1 intracellular lytic capacity of erythrocytes, whereas Table 2.2.2 shows results from experiment 1 extracellular or secreted lytic capacity towards *Rhodomonas salina.* Incubation of *P. parvum* cells with *O. marina* and *H. triquetra* filtrates failed to show a significant increase in intracellular lytic

capacity ($p>0.05$, $n = 3$, ANOVA) (11.9 ng SnE cell$^{-1}$ versus 12.5 ng SnE cell$^{-1}$, respectively) relative to the control, and showed a high standard deviation among replicates (Table 2.2.1). Significant changes in lytic capacity were, however, observed after incubation with *C. submarinus* filtrate compared to the control as well as to the other treatments, with a substantial decrease in lytic capacity relative to the control (5.4 ng SnE cell$^{-1}$ respectively, ($p<0.05$, $n =3$, ANOVA) (Table 2.2.1).

Table 2.2.1: Lytic capacity towards erythrocytes of *Prymnesium parvum* following treatment with filtrate of coexisting organisms. Values shown as saponin equivalent units = SnE per cell ( ng SnE cell$^{-1}$) ± std. deviation (n=3).

| Filtrate treatment | Lytic activity |
|---|---|
| *Oxyhrris marina* | 11.9 ± 0.9 |
| *Heterocapsa triquetra* | 12.5 ± 1.0 |
| *Chroococcus submarinus* | 5.4 ± 1.8 |
| Control | 10.8 ± 1.3 |

Incubation with *O. marina* filtrate significantly increased ($p<0.05$, $n = 3$, ANOVA) extracellular or secreted toxicity response towards *R. salina* cells ($EC_{50}$ = 1.3x10$^4$ cells ml$^{-1}$) relative to the control ($EC_{50}$ = 1.8 x 10$^4$ cells ml$^{-1}$ (Table 2.2.2) Incubation with *H. triquetra* filtrate, however, apparently induced only a slight (but not significant) increase in lytic capacity ($EC_{50}$ = 1.7 x 10$^4$ cells ml$^{-1}$) relative to the control, whereas incubation with *C. submarinus* (NIVA 331) decreased the lytic capacity significantly ($EC_{50}$ 2.8 x 10$^4$ cells ml$^{-1}$, $p<0.05$ ANOVA) relative to the same control, $p>0.05$ ANOVA l (table 2.2.2).

Table 2.2.2: lytic activity of *P. parvum* cells towards *R. salina* target cells following incubation with coexisting species filtrates. Values are given as the mean ± standard deviation (*n*=3) of the effective concentration of *P. parvum* cells yielding 50% mortality of *R. salina* cells($EC_{50}$).

| Filtrate | $EC_{50}$ *Rhodomonas salina* |
|---|---|
| *Oxyhrris marina* | $1.3 \times 10^4 \pm 153$ cells $ml^{-1}$ |
| *Heterocapsa triquetra* | $1.7 \times 10^4 \pm 111$ cells $ml^{-1}$ |
| *Chroococcus submarinus* | $2.8 \times 10^4 \pm 226$ cells $ml^{-1}$ |
| Control (IMR medium) | $1.8 \times 10^4 \pm 179$ cells $ml^{-1}$ |

*Microarray analysis and qPCR*

*Prymnesium parvum* exhibited differential gene expression when incubated with both chemical cues contained in filtrate and whole cell culture from the three coexisting species. Observed gene regulation patterns (Figures 2.2.2A & 2.2.2B) in *P. parvum* are qualitatively different between all three coexisting species. A global transcriptomic response was observed for all treatments for both whole cell culture and filtrate incubations: referring to up and down regulatory patterns observed for all treatments. The common response genes among all three organisms comprised 70 whole culture up-regulated, 23 filtrate-up-regulated (Figure 2.2.2A), 423 whole culture down-regulated and 81 filtrate down-regulated (Figure 2.2.2B).

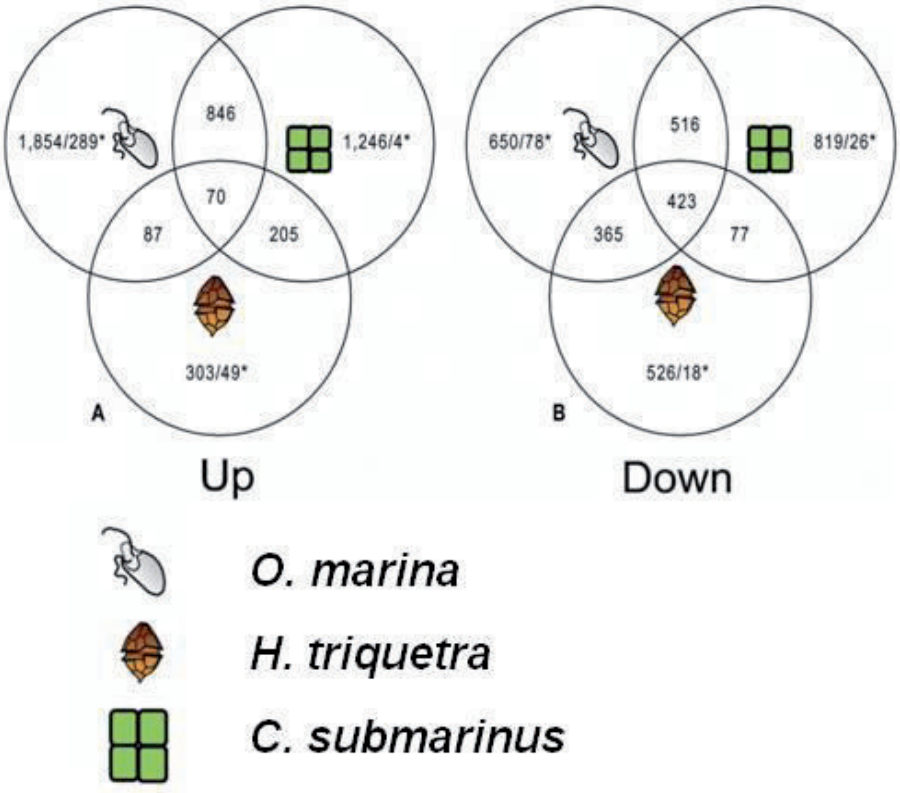



**Figure 2.2.2A & 2.2.2B:**

**A: Numbers of genes differentially up-regulated following incubation of *P. parvum* with three coexisting organisms. '*' indicates those genes differentially regulated following incubation of *P. parvum* with the corresponding filtrate (waterborne signals).**

**B: Number of genes differentially down-regulated following incubation with three coexisting organisms. '*' indicates those genes differentially regulated following incubation of *P. parvum* with the corresponding filtrate (waterborne signals).**

The induced gene expression programme in *P. parvum* following incubation with *O. marina* was the most complex on both quantitative and qualitative levels. *Oxyhrris marina* filtrate induced the highest number of genes regulated among the filtrate-treatments, with

289 up-regulated (Figure 2.2.2A) and 78 down-regulated (Figure 2.2.2B). Similarly, incubation with whole cell culture of *O. marina* also induced the highest number of genes regulated of all three species, with 1,854 up-regulated (Figure 2.2.2A) and 650 down-regulated (Figure 2.2.2B).

Incubation with *Heterocapsa triquetra* filtrate induced 49 uniquely up-regulated genes and 18 down-regulated genes in *P. parvum* (Figure 2.2.2A). This is in contrast to the corresponding whole cell culture which induced up-regulation of 303 genes (Figure 2.2.2A) and down-regulation of 526 genes (Figure 2.2.2B).

Incubation with *Chroococcus* submarinus filtrate induced a slight up-regulation of 4 genes and down-regulation of 26 genes (Figure 2.2.2B). This is again in contrast to the much higher corresponding whole cell culture induced gene up-regulation of 1,246 genes (Figure 2.2.2A) and down-regulation of 819 (Figure 2.2.2B).

Following a qualitative identification of general gene expression pattern trends, the regulated genes were grouped according to organism/treatment with respect to the assigned COG categories. Most genes induced by all three organisms (both culture and filtrate) were readily assignable to one of three COG categories: 1) translation, ribosomal structure and biogenesis; 2) RNA processing and modification; and 3) transcription (Figure 2.2.3A). Notable exceptions included *H. triquetra* culture induction of several cytoskeletal related proteins, *O. marina* filtrate induction of fatty acid metabolism-related genes, as well as *O. marina* culture induction of down-regulation in several posttranslational modification-associated genes (Figure 2.2.3A). COG categorization for the second experiment will be detailed later in the corresponding materials and methods section.

A group of genes associated with fatty acid metabolism, general cellular transport and a calmodulin associated gene were selected from the microarray and verified using qPCR (Figure 2.2.4) Furthermore, three known *P. parvum* derived PKS genes (PKS 6t3, PKS 7t3, and PKS 81t3) (Figure 2.2.4) warranted investigation of their transcriptional regulation due to the putative polyketide structure of toxic prymnesins previously identified in prymnesiophyte (Igarashi et al., 1999). These genes identified from the microarray exhibited comparable results in terms of gene expression fold-change as observed in qPCR analysis (see Table 2.2.3 & Figure 2.2.4).

The PKS 7t3 gene displayed the most drastic increase in expression fold-change relative to the control (approximately 37-fold) following incubation with *O. marina* whole-cell culture, compared with a virtually identical fold-change following incubation with filtrate from this species (Figure 2.2.4). The two remaining PKS transcripts showed regulation of ±5.0 fold change (Figure 2.2.4).

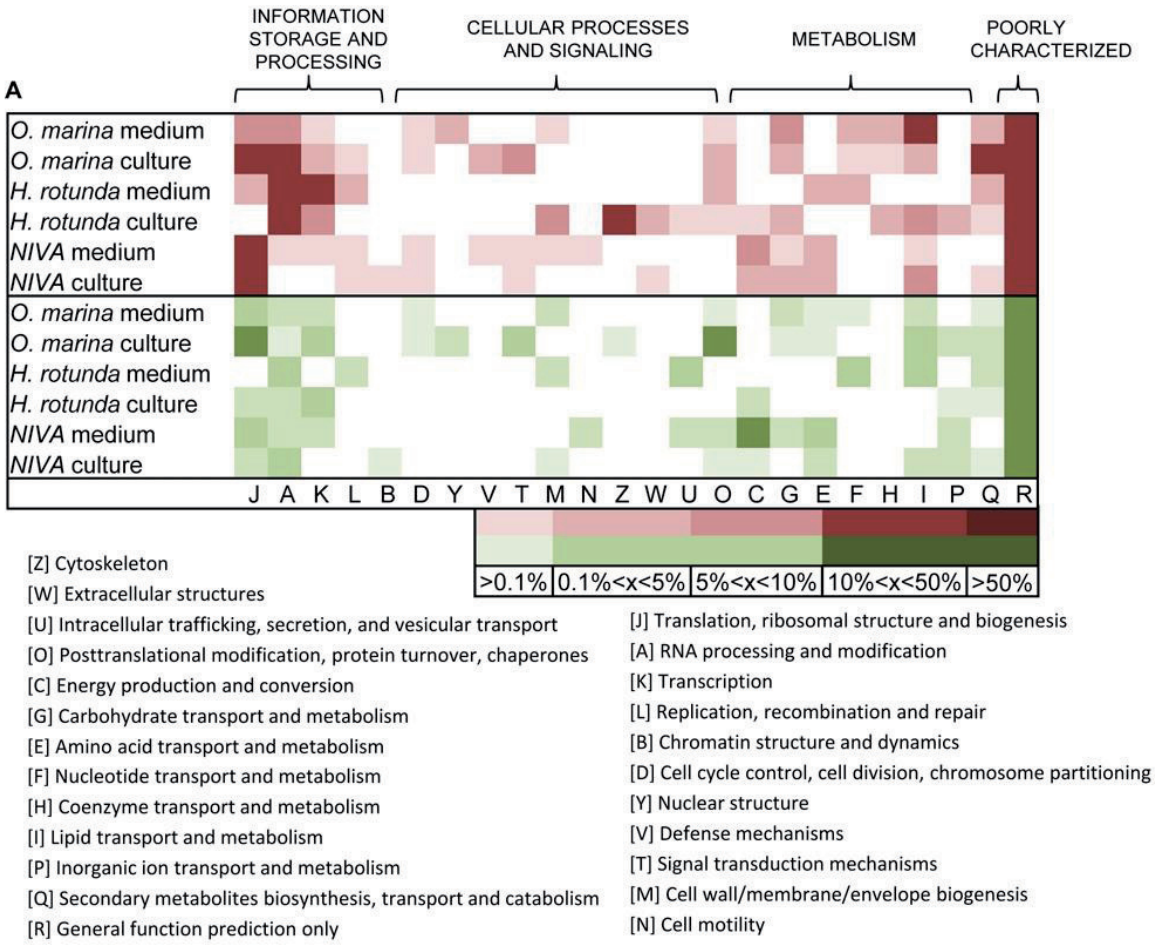
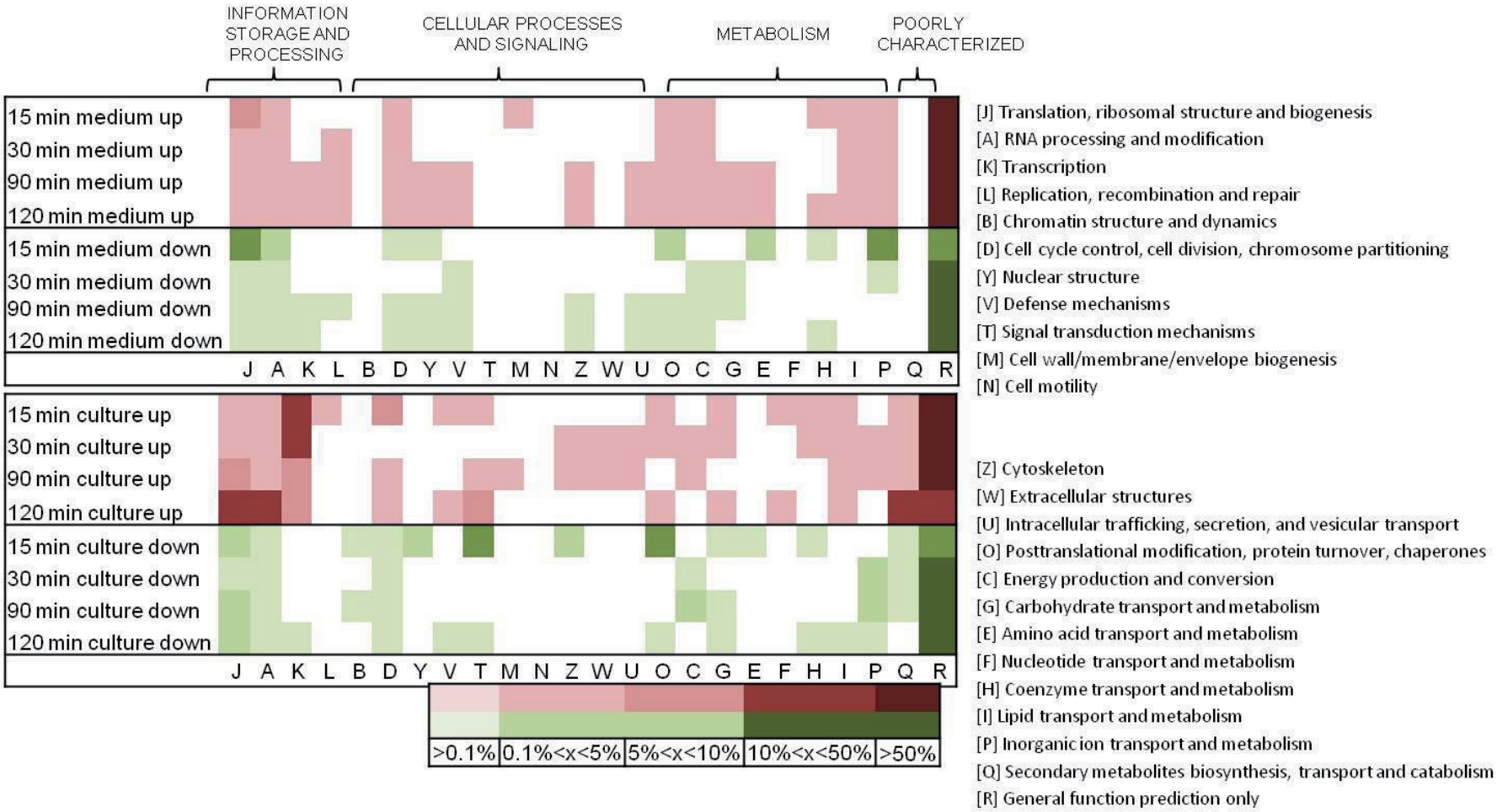



**Figure 2.2.3A: COG category distributions of differentially expressed ESTs as identified by microarray hybridization. Indicated here are the COG category distributions of up- (↑) and down- (↓) regulated ESTs relative to the control treatment for Experiment 1 (A) and Experiment 2 (B). Hue intensity corresponds to the number of ESTs per group, calculated as percentage of the total ESTs grouped into COGs with a known or general function.**


INFORMATION STORAGE AND PROCESSING
CELLULAR PROCESSES AND SIGNALING
METABOLISM
POORLY CHARACTERIZED
15 min medium up
30 min medium up
90 min medium up
120 min medium up
15 min medium down
30 min medium down
90 min medium down
120 min medium down
J A K L B D Y V T M N Z W U O C G E F H I P Q R
15 min culture up
30 min culture up
90 min culture up
120 min culture up
15 min culture down
30 min culture down
90 min culture down
120 min culture down
J A K L B D Y V T M N Z W U O C G E F H I P Q R
>0.1%
0.1%<x<5%
5%<x<10%
10%<x<50%
>50%
[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 mechanisms
[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[Z] Cytoskeleton
[W] Extracellular structures
[U] Intracellular trafficking, secretion, and 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

**B**

Table 2.2.3: qPCR verification of selected genes from microarray hybridization (fold-change values) following exposure of *P. parvum* cells to whole cells or filtrate of coexisting species. *qPCR values are italicized.*

| *Probe identifier* | *Putative gene product* | *O. marina* cells | *O. marina* filtrate | *H. triquetra* cells | *H. triquetra* filtrate | *C. submarinus* cells | *C. submarinus* filtrate |
|---|---|---|---|---|---|---|---|
| *Pparv_reverse_22096* | *Palmitoyl coA Oxidase* | *5.0* | *3.9* | *0.5* | *0.2* | *0.9* | *0.6* |
| *Pparv_reverse_34693* | *Actin* | *2.0* | *1.5* | *0.5* | *0.01* | *2.7* | *1.7* |
| *Pparv_reverse_18989* | *Actin depolymerization factor* | *4.7* | *3.0* | *0.2* | *0.8* | *1.1* | *-2.4* |
| *Pparv_reverse_33186* | *Calmodulin* | *3.4* | *2.5* | *0.7* | *0.6* | *5.2* | *0.6* |
| *Pparv_reverse_27730* | *Membrane protein translocase* | *0.8* | *1.3* | *-0.5* | *0.9* | *0.5* | *-1.8* |
| Pparv_reverse_22096 | Palmitoyl coA Oxidase | 6.0 | 4.3 | 0.3 | 0.5 | 1.2 | 0.8 |
| Pparv_reverse_34693 | Actin | 1.6 | 1.7 | 0.1 | 0.02 | 3.2 | 1.9 |
| Pparv_reverse_18989 | Actin depolymerization factor | 5.2 | 3.1 | 0.2 | 1.0 | 0.9 | -2.7 |
| Pparv_reverse_33186 | Calmodulin | 3.2 | 2.7 | 0.9 | 0.8 | 6.1 | 0.7 |
| Pparv_reverse_27730 | Membrane protein translocase | 1.0 | 1.34 | -1.0 | 1.2 | 0.8 | -2.0 |

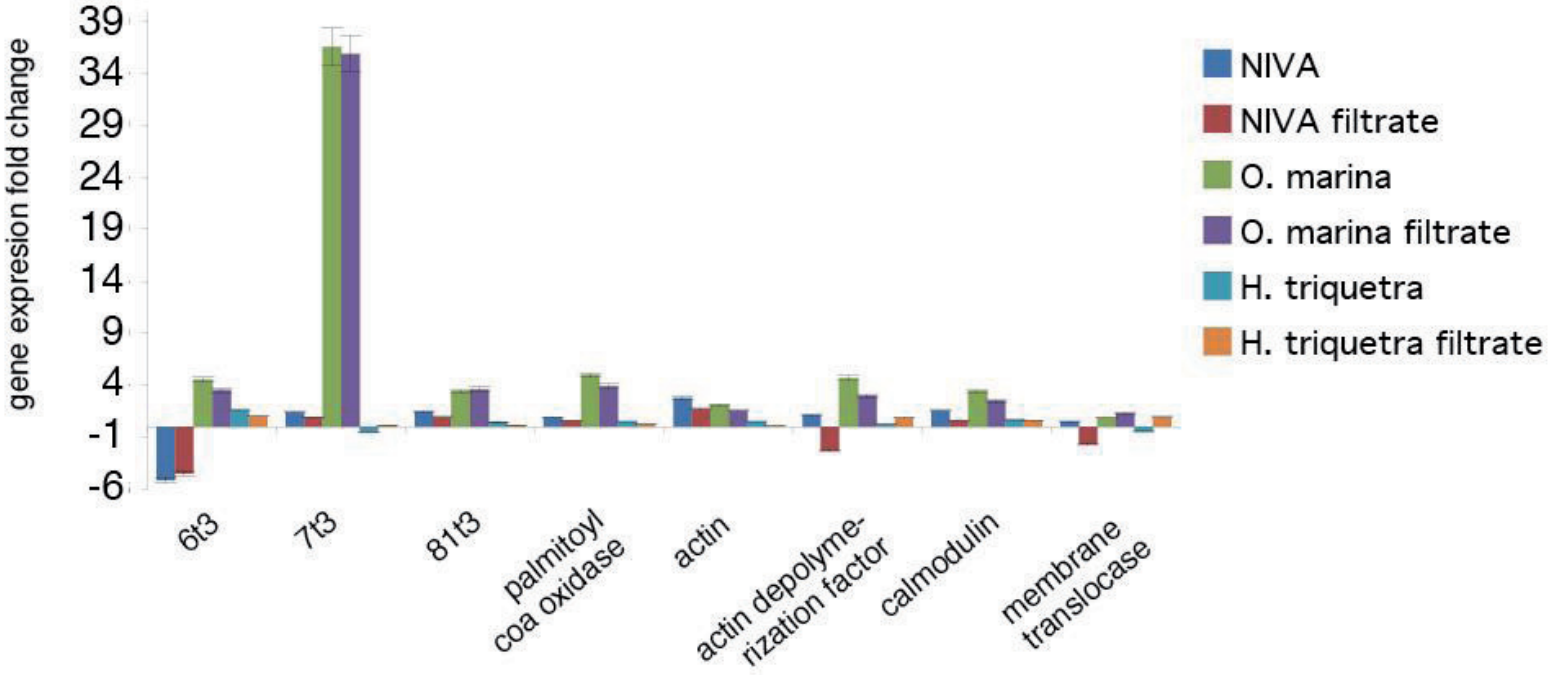
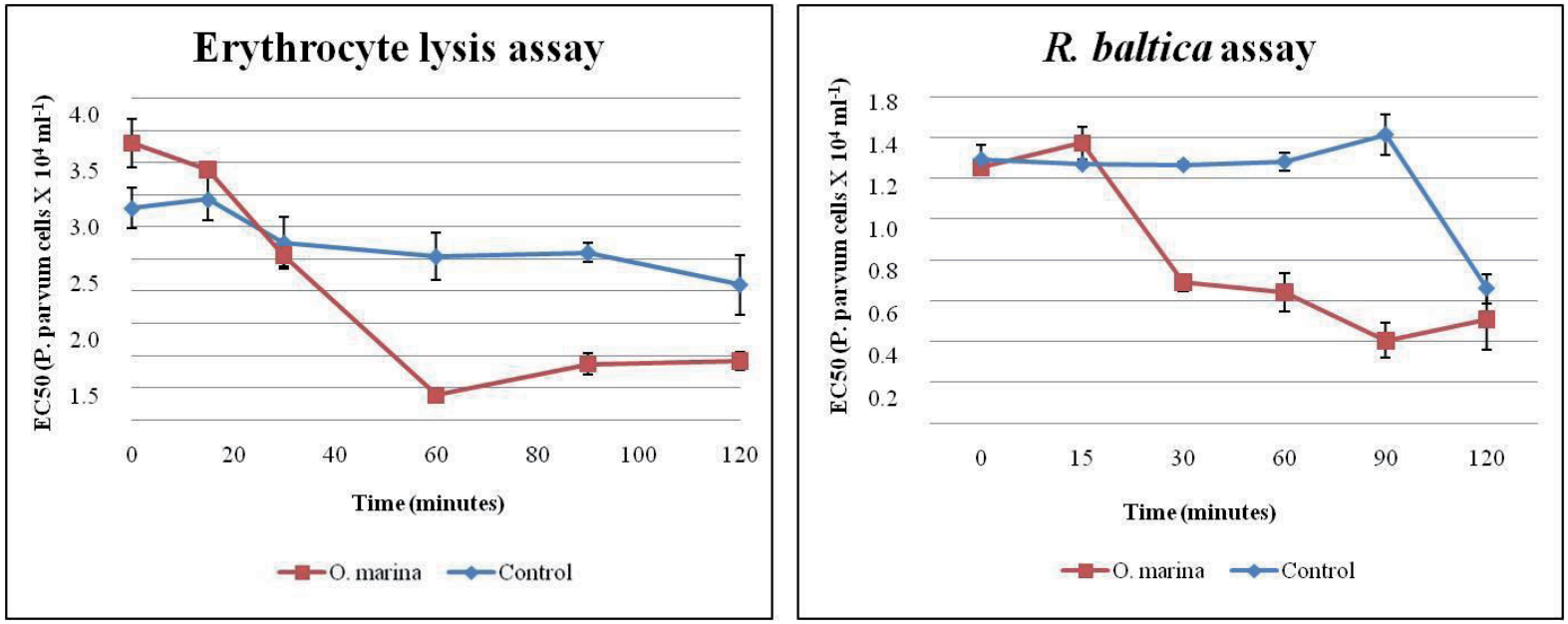



**Figure 2.2.4: qPCR analysis of genes chosen from microarray screening of Experiment 1, based upon *P. parvum* incubation with both culture and filtrate (waterborne cues) of three coexisting organisms.** Error bars represent standard deviation across $n = 3$ biological replicates.




A B

**Figure 2.2.5: A: Results from the *Rhodomonas salina* bioassay for Experiment 2 time-series with *O. marina*, shown as change in $EC_{50}$ over time. Error bars represent standard deviation across *n* = 3 biological replicates. B: Results from the erythrocyte lysis assay for Experiment 2 time-series with *O. marina*, shown as change in $EC_{50}$ over time. Error bars represent standard deviation across *n* = 3 biological replicates. Saponin was used as a lytic standard in this assay.**

*Dose-exposure experiment*

*Lytic capacity*

Extracellular or secreted toxicity/lytic activity towards the cryptophyte *R. salina* was significantly highest relative to the control after 90 minutes incubation with *O. marina* filtrate, yielding an $EC_{50}$ for *Prymnesium parvum* of 5.3 x $10^3$ cells $ml^{-1}$ ( Figure 2.2.5B, $p<0.05$, $n = 3$). After 2 h, the lytic activity of the control decreased but was still significantly lower than that of the treatment ($p<0.05$, $n = 3$).

An initial significant difference in lytic activity towards erythrocytes at t = 0 was observed, despite equal starting *Prymnesium* cell concentrations. The presence of intracellularly stored lytic compounds increased slightly increased over time for both the control and the treatment incubated with *O. marina* filtrate (Figure 2.2.5B). However, after 60 min incubation, the treatment showed a significant increase relative to the control (treatment $EC_{50}$ of 14 x $10^4$ cells $ml^{-1}$ vs. control $EC_{50}$ 2.2 x $10^4$ cells $ml^{-1}$, $p<0.05$, $n = 3$).

*Microarray analysis and qPCR*

Two general response up-regulated genes were identifiable from all time points, despite having unknown functions. Between 30 to 90 minutes, the number of genes up-/down-regulated increased from 398/75 to 1,097/564 when incubated with *O. marina* whole-cell culture, and shifted from 69/16 to 51/45 with *O. marina* filtrate). After 120 minutes, the number of genes regulated reached values similar to those from Experiment 1, providing confirmation of the reproducibility of the initial incubation period (120 minutes). *O. marina* culture induced up-regulation of *P. parvum* genes associated primarily with

translation, transcription and lipid transport and metabolism (Figure 2.2.3B) representing between10-50% of genes with predicted function. This corresponds to the microarray results from the initial experiment. Interestingly, regulation of these aforementioned gene categories occurred throughout the entire series experiment.

As shown in Figure 2.2.6, PKS transcript copy number increased over time, beginning with PKS 6t3 (+4.7-fold) and PKS 7t3 (+7.6-fold) following 30 minutes incubation with *O. marina* whole-cell culture (Figure 2.2.6). After 45 minutes, there was a slight increase in expression of PKS 6t3 in the filtrate-incubated sample. In comparison, the first noticeable induction in PKS 81t3 (whole cell culture +6.3 fold) appeared after 60 minutes, whereas there was a stark induction of PKS 6t3 (whole-cell culture +4.7-fold; filtrate +2.1-fold) and an even more pronounced induction in PKS 7t3 (whole-cell culture +26.2-fold).

Furthermore, filtrate treatment led to an increase (+8-fold) of transcripts of PKS 7t3. After 90 minutes incubation, transcriptional regulation seems to reach a maximum for PKS 6t3 (whole-cell culture +9.2-fold; filtrate +5.1-fold), PKS 7t3 (whole-cell culture +54-fold; filtrate +19 fold) and PKS 81t3 (whole-cell culture +13- fold; filtrate +1.4-fold). Finally, filtrate treatment also led to an increase (+3-fold) of transcript number for PKS 8t3 at 120 minutes, whereas whole-culture treatment yielded a decrease (+3-fold) from the previous time point.

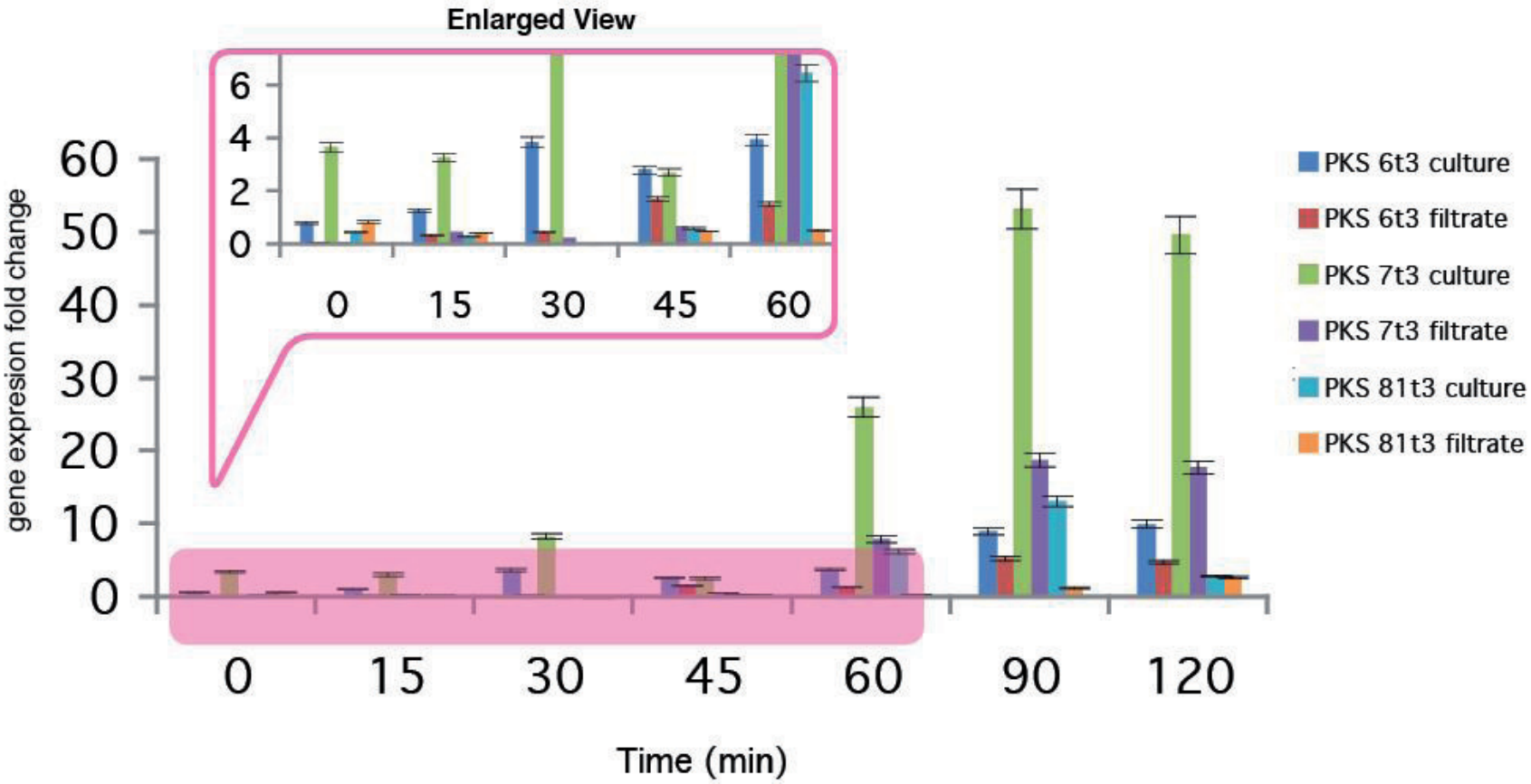



**Figure 2.2.6: qPCR analysis of PKS-associated genes for exposure-dose Experiment 2.** Error bars represent standard deviation across *n* = 3 biological replicates.

### 2.2.5 Discussion

Information transfer via chemicals signals in aquatic sytems has been a research interest for many years. In several cases infochemicals (Dicke & Sabelis, 1988), have demonstrated a defining role in predator-prey interactions competitive processes. For example, infochemicals exuded by carnivorous zooplankton (DeBeauchamp, 1952; Gilbert, 1966, 1967) have been reported to induce defenses in other zooplankton. In freshwater systems, production of toxins or repellent chemicals by cyanobacteria even promotes grazing resistance (Lampert, 1981, 1982; DeMott & Moxter, 1991). Exposure to the freshwater cladoceran *Daphnia* has been shown to induce phenotypic plasticity in the green alga *Scenedesmus* (Hessen & VanDonk, 1993), indicating the potential flexibility of aquatic organisms in response to chemical cues. The evolution of allelochemical substances due to competitive mechanisms among planktonic species has been considered for decades but many issues remain unresolved (Lewis 1986; Jonsson et al. 2009).

*Species-specific differential response*

The significance of encounter rate in predator-prey and competitive interactions in the plankton should not be underestimated. This concept is of ecological importance in our study because entering the chemical sphere vs. recognition of secreted chemical signals may induce different responses in *P. parvum* with respect to co-existing species and their metabolites. In the current experiments, the response of *P. parvum* cells to filtrates of various species are interpreted as a reflection of elicited activity derived from dissolved chemical signatures released by the respective species into the surrounding medium. On the other hand, *P. parvum* responses to direct exposure to intact cells are presumably mediated by cell-contact or close encounters with bioactive compounds retained at the

elicitor cell surface or bound within the phycosphere along a steep concentration gradient. Treatment with filtrate from *O. marina, H. triquetra and C. submarinus* caused differential responses in terms of lytic activity in *P. parvum.* Both *O. marina* and *H. triquetra* filtrates induced an increase in lytic activity of *P. parvum* towards erythrocytes (intracellular lytic capacity) when compared to the control (Table 2.2.1). Different responses suggest a recognition system, in this case: chemical in nature.

However, these same treatments caused significant increases in extracellular lytic activity of *P. parvum* towards the sensitive cryptophyte *R. salina*. An increase of extracellular lytic compounds directly affecting coexisting protists is suggested to be of more ecological relevance compared to an increase of intracellular lytic compounds as reflected by the erythrocyte lysis assay.

Treatment with *C. submarinus* filtrate significantly decreased ($p<0.01$) the lytic activity of *P. parvum* in both the erythrocyte and *Rhodomonas* bioassays (Tables 2.2.1 & 2.2.2). These results, however, are difficult to ascribe to either active regulation or passive decrease in lytic activity. In principle, a decrease of intracellular lytic activity could be explained by a reduced production perhaps accompanied by rapid turnover of the lytic compounds - this would be a “shoot down” attack/defense response based upon a perceived lack of external threat. Alternatively, lytic activity may be subject to intracellular modulation and regulation, e.g. via conformational shifts, that is not directly related to the concentration of the potentially lytic compounds. Finally, the decrease in intracellular activity may reflect a rapid reallocation of compounds by exudation into the surrounding medium, e.g. as a rapid response to potential prey or competitor signals. The latter

mechanism, however, seems unlikely because extracellular lytic activity, as quantified by the *Rhodomonas* bioassay also decreased. Nevertheless, a decrease of extracellular activity might also be due to non-specific binding of potentially lytic components to dissolved organic compounds or even particles.

In this context, lytic activity of *P. parvum* has been shown to decrease by adding increasing amount of target cells (Tillmann, 2003). Many cyanobacteria as well as eukaryotic microalgae are known to exude large amounts of organic material (Hesen, 1993) potentially acting as binding (and thus inactivating) sites for lytic compounds. In addition, decreasing extracellular lytic activity might be due to a fast decomposition of compounds together with reduced production and/or exudation rate (again as a "shoot down response"). As the toxicity of *Prymnesium* is known to be quite unstable on the scale of hours to days (Igarashi 1999, Larsen & Bryant 1998 & Larsen et al. 1993) it is impossible to decide which of the depicted possibilities is the main explanation for the observed decreasing intra- and extracellular lytic activity.

From an ecological perspective, a possible reason for this decrease in lytic activity in *P. parvum* exposed to the cyanobacterium and/or its extracellular metabolites is the lack of predatory or competitor threat posed by *C. submarinus.* Coexistence of *P. parvum* and *C. submarinus* may have rendered a mutual tolerance towards respective chemical signatures. In fact, cyanobacteria have been found to be among the most tolerant groups of coexisting organisms in response to *P. parvum* allelochemicals (filtrate) in a natural community experiment (Fistarol et al., 2003). Nevertheless, the large number of genes up- and down-regulated as found by microarray hybridization (Figures 2.2.2A & 2.2.2B) following treatment with both whole-cell culture and filtrate of *C. submarinus* does suggest the

recognition of cyanobacterial chemical signals by *P. parvum.* Although a similar number of regulated genes were noted following treatment with *O. marina* and a lower number for *H. triquetra* than for the cyanobacterium (Figures 2.2.2 A & 2.2.2B, *P. parvum* reacted by increasing lytic activity. This response can be interpreted as recognition of two coexisting species that pose either a potential predatory threat (*O. marina)* or competition, i.e. for nutrients and/or other limiting resources *(H. triquetra).*

The ability to differentiate among coexisting species and their potential threats may be dependent on variation in chemical signal strength over time, allowing planktonic species to allocate their metabolic energy/costs based on whether the signals come from competitors, prey or predators or from innocuous sources (Carlsson & Taffs, 2010; Strauss et al., 2002). Prey-predator interactions represent a very strong selective pressure and can therefore co-evolve in a more sharply defined relationship than between mere competitors. Nevertheless, such interactions are complex and are not always unidirectional in the plankton. For example, Tillmann et al. (2003) showed that the heterotroph *O. marina* can voraciously feed on *Prymnesium* (thus the dinoflagellate is a predatory danger), but in an intriguing reversal of fortune depending on the toxicity of *Prymnesium*, the dinoflagellate can be lysed and phagocytized by the prymnesiophyte. Survival therefore entails a complex interplay between physical constraints and selective pressures, such as those posed by predation.

In marine ecosystems, both microalgae (Paul & Van Alstyne, 1992) and macroalgae (Rothaeusler et al., 2005) have been shown to display induced defense mechanisms related to differential gene expression, however, with some degree of variability. Waterborne cues of copepods induce toxicity and changes in gene expression profiles in the dinoflagellate

*Alexandrium* spp. (Yang et al, 2010, Wohlrab et al. 2010 accepted). Even selective grazing and bio-recognition of prey in *O. marina* has been thought to be attributable to noxious chemicals produced by prey species, such as *P. parvum* (Martel, 2008). A bio-recognition system that allows for recognition and processing of *O. marina* chemical cues before actual physical encounter occurs is therefore plausible.

The functional genomic data obtained in this *P. parvum* study indicate that there is a qualitative difference between gene regulation in this prymnesiophyte in the presence of intact cells of coexisting species versus exposure to the corresponding filtrates. For all three coexisting species, the raw number of genes expressed differentially was much higher than for the filtrate (Figures 2.2.2A & 2.2.2B). After COG classification, the differentially expressed gene classes show striking similarity between culture and filtrate treatments, the primary difference being a qualitative decrease in gene number regulated following filtrate incubation (Figures 2.2.2A & 2.2.2B). Genes classified as transcription- and translation-associated are of particular interest, assuming that these genes were differentially expressed in response to an exogenous stimulus, e.g., with the coexisting species as source.

In this study the differential gene expression data on *P. parvum* indicate that there is a difference in gene expression induced by chemical waterborne cues vs. intact coexisting cells. Despite the fact that there are fewer genes regulated in the filtrate treatments (Figures 2.2.2A & 2.2.2B) than for whole cell exposure, these results are consistent with the presence of chemical cues, and their recognition by *P. parvum.*

*Exposure time*

The second experimental setup allowed for consideration of the effects of time and exposure to *O. marina* on *P. parvum.* The relevance of an exposure- and time-dependent response describing of the interaction between *P. parvum* and *O. marina* must be considered in the content of both parameters of time and change in toxicity from the second experiment, as well as the changes in gene expression regulation (Figures 2.2.3B & 2.2.6). The effects we observed as increased or decreased lytic activity could be related to differing levels of exposure (dose-dependence) as well as to differences in exposure time. We argue therefore that it makes metabolic sense that *Prymnesium* does not immediately respond to the presence of co-existing cells. In our interpretation, the required dose of chemical cues from competitor/predator cells must reach a threshold level before *Prymnesium* merits activating its defense. Allocation of energy either to growth or a switch to defense-related physiology represents a balance with associated bioenergetic costs (Carlsson and Taffs, 2010; Strauss et al., 2002). Defense mechanisms and induction of toxic processes are no doubt costly to the organism, and thus warrant finely tuned control. Hence it is important not only to differentiate between different species and between cell contact and chemical cues, as demonstrated in our first experiment, but also that the signals reach a certain time- or concentration-dependent threshold to be sensed before the "defense machinery" is activated.

A similar system has been described for the marine bacterium *Vibrio fischeri,* which produces bioluminescence only at high cell densities, yet can be induced at low cell densities by being placed in 'spent' high cell density filtrate (Bassler et al., 1997). The signaling molecule responsible for this 'auto induction' was later found to be an acylated homoserine lactone (Bodman et al., 2008). This type of recognition has been termed

'quorum sensing', and specifically refers cell density-linked, coordinated gene expression in populations that experience threshold signal concentrations to induce a synchronized population response (Fuqua et al, 1994). For an individual cell a direct grazer attack (mechanical contact) a direct defense system (such as trichocysts and/or escape) would be required. In contrast, threshold induction systems support the survival of the population and hence the gene pool and although this does not directly benefit the individual cell, altruism in natural systems cannot be ruled out.

Regarding PKS gene expression and corresponding changes in toxicity showed that following incubation with *O. marina,* an increase in both extracellular and intracellular toxicity is apparent in *P. parvum*. Since the experimental time was relatively short, and the cell densities identical, we can rule out the effect of pH on relative toxicity (Schmidt and Hansen, 2001). Hence, the induction of toxicity observed was significant, and can be related to the treatment itself. The induced toxicity exhibits a similar trend to that of the qPCR gene fold-change expression data obtained for PKS, and in particular for PKS 7t3. Such circumstantial evidence supports the importance of PKS biosynthetic pathways in toxic processes of *P. parvum*, although this does not directly demonstrate that the allelochemicals are polyketides.

**Conclusions**

This study sheds light on heterospecific interactions between the toxic prymnesiophyte *Prymnesium parvum*, and three coexisting species: *Oxyhrris marina, Heterocapsa triquetra* and *Chroococcus submarinus*. We found the interactions to be species-specific and to differ in complexity, based upon a combined functional genomic-bioassay linked experimental approach. The results of the treatment with the potential predator *O. marina*, in contrast to the response to whole cells and filtrate of the other coexisting species, may be attributable to a co-evolutionary mechanism developed in *P. parvum* in response to grazing pressure and stress from *O. marina*. Such pressure has previously been described for yeast as a driving force behind genomic diversity and regulation (Chu et al., 1998). The experimental design implemented in this study has also allowed for determination of the importance of cell-cell physical contact vs. recognition of waterborne cues and the time dependence of chemical signalling effects on *P. parvum.* Finally, the fact that PKS genes show transcriptional regulation supports the role of polyketide pathways in toxic processes in *P. parvum.* This integrated study furthered understanding of recognition and responses to signalling molecules in *P. parvum*, with broader implications of the ecological role and evolution of chemical signalling pathways in plankton assemblages.

# 2.1 Publication 3

## Investigating phosphorus limitation and low salinity stressors in the prymnesiophyte *Prymnesium parvum*

### 2.3.1 Abstract

It has previously been shown that low saline culturing conditions increase the relative toxicity of *Prymnesium*. Whether or not this involves an increase in the production of these toxic compounds is still unknown. More recently, nutrient deficiency (N&P) has been shown to enhance the toxicity of *Prymnesium* as well. In this study, a combination of low saline aqueous medium and phosphorous limitation is used to investigate if the combination of these two physiological factors can even further enhance *Prymnesium's* toxicity. The *Prymnesium parvum* strain K252 was cultured at both 26 and 5 psu, with or without addition of an organic phosphate source to the culture medium. Intracellular production of lytic compounds of *Prymnesium* cultures was measured using an Erythrocyte Lysis Assay (ELA). In contrast, extracellular compound secretion was investigated through mortality rates of *Rhodomonas salina treated* with the differentially cultured *Prymnesium.* The combination of low salinity and phosphorous deficiency proved to enhance the toxicity of this *Prymnesium* strain the most. These results support the idea the production and/or secretion of lytic compounds in *Prymnesium parvum* may provide a competitive advantage under phosphorous limited conditions as well as under fluctuating salinity.

### 2.3.2 Introduction

The earliest description of a *Prymnesium parvum* related fish-kill event dates back to the 1920s as described by Liebert & Deerns (1920) in Holland. 9 years later a similar event was observed in Denmark, where the culprit was identified as *Prymnesium parvum* Carter. Otterstroem and Nielsen (1940) further confirmed that the toxicity observed was due to an extracellular, thermolabile toxin. Blooms of prymnesiophytes have since then been frequently associated with massive both ecologically and economically detrimental fish kills (Otterstrøm & Nielsen, 1940; Shilo, 1971; Shilo, 1967; Edvaardsen & Paasche, 1998; Moestrup, 1994)).

Although the species in traditionally described as being euryhaline (Shilo, 1971), these dense, detrimental blooms have been described primarily in coastal or brackish water systems (Parnas & Abbott, 1965; Skulberg et al., 1993). Studies investigating the roles of environmental and physiological factors' effects on the toxicity of this prymnesiophyte have become numerous. Parnas et al. (1962) claimed that the activity of extracted ichthyotoxin of *P. parvum* is inversely proportional to salt concentrations. Ulitzer & Shilo (1964) found with whole cell culture experiments that a decrease in salinity induces an increase in ichthyotoxicity, and that ichthyotoxicity decreases as salinity increases. More recently Larsen & Bryant (1998) investigated several *Prymnesium* strains and concluded that salinity has a strong effect on relative toxicity using a brine shrimp *Artemia* bioassay. However, for all strains, no general pattern concerning the relation of salinity and relative toxicity could be determined.

Phosphate sources in the growth medium have also been found to display an inverse relationship to toxicity. Dafni et al. (1972) found that a decrease in phosphate caused an

increase in toxicity. These authors hypothesized that a phosphate-limited environment may cause a flaw in biosynthesis of membrane phospholipids, thus leading to a higher membrane permeability, and leakage of compounds that possess a lytic capacity. In particular, they noted that the cell volume of *Prymnesium parvum* decreased under such conditions, further indicating a membrane disturbance. Furthermore, Paster (1973) found *P. parvum* to be more toxic when grown in phosphate-poor medium. More recently, a massive fish kill in the Sandsfjord system in Norway was attributed to phosphate-limited growth of *P. parvum* (Kaartvedt et al., 1991). Johansson & Graneli (1999) described increases in toxicity related to both nitrogen and phosphate limitation. They further hypothesized that an unbalanced N:P ratio, caused by nutrient input or eutrophic conditions, could be one factor governing toxicity in this prymnesiophyte. Although the authors admit that the reason for toxin production is unknown, they suggest it may have something to do with competition for resources during nutrient limitation.

The documentation of monospecific blooms of *P. parvum* highly suggests the presence of a competitive advantage over other co-existing phytoplankton species. *Prymnesium parvum* blooms often occur in eutrophic areas, such as coastal waters, where run-off can alter the N:P ratio (Collins, 1978). This observation, in conjunction with observed increases in toxicity under nutrient stress (Paster, 1973; Johansson & Graneli, 1999; Kaartvedt et al., 1991) suggests that *P. parvum* is able to outcompete other phytoplankton species for limited resources. This advantage is most likely not based solely on growth rate, as *P. parvum* has been previously shown to display moderate growth rates under a variety of physiological conditions (Holdway et al., 1978; brand, 1984; Larsen & Bryant, 1998), perhaps rather on production or secretion of allelochemical compounds that

have an effect on coexisting species. Despite the rigor and number of studies, our understanding of environmental factors and their effects on toxin production and toxicity in *P. parvum* is still quite poor.

A major hurdle in furthering the understanding of regulation of toxicity in *P. parvum* is that the observed toxicity varies both in nature and in culture (Ulitzur & Shilo, 1966; Dafni et al., 1972; Larsen et al., 1993). Indeed toxin production has been shown not to be a basal part of metabolism in phytoplankton (Plumley, 1997), but rather dependent on environmental conditions. *Prymnesium parvum* blooms often occur in eutrophic areas, such as coastal waters where run-off can alter the N:P ratio (Collins, 1978). The accepted ecological reference for C:N:P ratios is termed the Redfield Ratio, as first described by Alfred C. Redfield in 1934. This ratio refers to the global elemental composition of marine organic matter, of C:N:P 106:16:1 (Redfield, 1934). Since nutrient availability as well as ratios can have a significant impact on phytoplankton growth, and thereby phytoplankton interactions, changes in nutrient levels may in fact alter toxin biosynthesis.

In the current study we examined the combined versus individual effects of phosphorous limitation and low salinity stress on the toxicity of *P. parvum* (strain K0252). This particular strain was of ecological relevance due to the tidal nature of its geographical origin (Norman Bay) demonstrating eutrophic conditions, as well as fluctuations in salinity.

We investigated the effects of low salinity and phosphorous limitation on the physiological processes of growth and observed toxicity. Salinity as well as phosphorous limitation was shown to influence the growth rate of *P. parvum* strain K0252 cultures. Utilizing a functional genomic bioassay-linked approach, we also observed the combination of phosphorous limitation with low salinity stress to increase the lytic capacity/toxicity of

*P. parvum* in a non-linear manner. This is, to our knowledge, the first example of an experimental system involving *P. parvum* where toxicity is inducible to such a high degree. Lastly, this study lays the groundwork for future functional genomic studies involving *P. parvum*, in an attempt to better understand the ecology of this harmful algal species.

### 2.3.3 Materials & methods

*Algal culture conditions*

A non-axenic toxic clonal strain of *Prymnesium parvum f. patelliferum* (K0252), isolated by Ø. Moestrup from Wilsons Promontory, Norman Bay, Victoria, Australia on 07.12.1987, was grown in IMR medium as described in Publication 1 of this dissertation (4:1, v:v, North Sea seawater: MilliQ deionized water) in 5 l stock culture. This strain was chosen based on results from preliminary experiments on lytic capacity towards erythrocytes and *Rhodomonas salina.* The components of IMR medium (Eppley, 1967) are given in Table 2.1.3-5 (Publication 1). Salinity of the IMR medium was adjusted either with North Sea seawater volume, or with NaCl, to minimize phosphorous from increased volume of North Sea water. Phosphorous limitation was achieved by withholding $KH_2PO_4$ from the culture medium.

Stock cultures were grown in conditions corresponding to those of the experimental treatments (Table 2.3.1). Four experimental treatments were carried out, one of which served as a control (26 psu, P-replete) (Table 2.3.1.). Experimental cultures were grown in 5 l Duran bottles (Schott AG, Mainz, Germany) under gentle aeration with sterile-filtered air, at a constant temperature of 20°C and a light: dark photocycle of 14:10 h. Sampling was performed using a combination of sterile tube-vacuum system (as described in Eschbach et al., 2005) to minimize bacterial growth, and centrifugation of exponential growth phase cultures. Experimental cultures were inoculated with starting concentrations of $1.5x10^3 \pm 535$ cells $ml^{-1}$, and were sampled four times throughout the experiment. Nutrient sampling points included early and late exponential, and early and

late stationary growth (days 1, 4, 6 and 12, Figure 2.3.3). Samples for gene expression analysis (qPCR and microarray) and toxicity measurements via bioassay were taken on day 4 (exponential growth phase, Figure 2.3.3). Sampled cultures were centrifuged at 3000 x

Table 2.3.1: Experimental treatments.

| **Treatment** | **Description** |
|---|---|
| 26 psu, P-replete | 20 °C, 90 µmol photons $m^{-2}$ $s^{-1}$, 26 psu |
| 26 psu, P-deplete* | 20 °C, 90 µmol photons $m^{-2}$ $s^{-1}$, 26 psu, no $KH_2PO_4$ added to culture medium |
| 5 psu, P-replete | 20 °C, 90 µmol photons $m^{-2}$ $s^{-1}$, 5 psu |
| 5 psu P-deplete | 20 °C, 90 µmol photons $m^{-2}$ $s^{-1}$, 5 psu, no $KH_2PO_4$ added to culture medium |

*** Prepared by adding NaCl to 5 psu P-limited medium, to avoid addition of trace amounts of phosphate present in North Sea seawater.**

g for 15 minutes at 20 °C. The supernatant was removed, and the remaining cell pellet was resuspended in 350 µl of buffer RLT lysis buffer containing β-mercaptoethanol (Qiagen, Hilden, Germany), and subsequently flash-frozen in liquid nitrogen at -80° C. Samples were then stored at -70° C to minimize activity of potential RNase enzymes and to prevent degradation. Irradiance was kept at 90 µmol photons $m^{-2}$ $s^{-1}$ and was measured as described in Publication 1 using a Quantum Scalar Irradiance Meter (Biospherical Instruments, San Diego, USA). Cell concentrations were determined daily using a CASY cell counter (Innovatis AG, Reutlingen, Germany).

*Erythrocyte lysis assay*

An erythrocyte lysis assay was performed as described in Publication 1, and was used to the test lytic activity of *P. parvum* whole cell extracts towards erythrocytes. A volume corresponding to 1.0 x $10^7$ *P. parvum* cells from each treatment were harvested via centrifugation and the cell pellet resuspended in lysis/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, Eschbach et al. 2001). The resuspended pellets each containing 1.0 x $10^7$ *P. parvum* cells were then completely lysed via ultrasonication at the following settings: 50% pulse cycle, 70% amplitude, for 1 min. Lytic activity was calculated in ng saponin equivalents per cell (ng SnE $cell^{-1}$), utilizing the standard saponin from higher plants as an indicator of relative lytic capacity.

*Extracellular and/or secreted toxicity:* Rhodomonas salina *bioassay*

A *Rhodomonas salina* assay was performed as described in Publication 1 to characterize differential extracellular/secreted toxicity of *P. parvum.* 4 ml of a mixture of *P. parvum* (final cell concentrations in decreasing order: 3.75 x $10^4$ $ml^{-1}$, 2.34 x $10^4$ $ml^{-1}$, 9.38 x $10^3$ $ml^{-1}$ and 4.69 x $10^3$ $ml^{-1}$) and *R. salina* (final cell concentration 1.0 x $10^5$ $ml^{-1}$) were incubated in glass scintillation vials at 15° C for 24 h in darkness. Vials were then gently mixed by rotating, and 1 ml of mixture was pipetted into an Utermöhl cell sedimentation chamber and fixed with glutaraldehyde (2.5% final concentration). After settling, cells were viewed via epifluorescence microscopy (Zeiss Axiovert 2 Plus, Carl Zeiss AG, Göttingen, Germany) with Zeiss filter-set 14 at 64X magnification. Lysed versus non-lysed cells were easily distinguishable due to pigment auto-fluorescence characteristics (*Prymnesium* - red

or *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.

*Nutrient analysis*

Filtered medium samples 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 analyzed by continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany). For total dissolved phosphorus and nitrogen, the analysis was preceded by digestion with peroxodisulphate in an autoclave. Samples for particulate nutrient analysis were filtered on pre-combusted glass fiber GF/F filters (Whatmann, Omnilab, Bremen, Germany) and stored at -20°C. Filters for particulate 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 phosphorus was measured photometrically by continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany) after digestion with peroxide and sulphuric acid (Eberlein et al., , 1980). Mean C/N values were calculated from the C/N measurements for individual filters; C/P and N/P values were determined from the average of all possible pairs of measurements for each culture at a given sampling point.

*RNA isolation*

RNA isolation was performed as described in Publication 1, according to the protocol in the RNeasy Plant total RNA extraction kit (Qiagen, Hilden, Germany). Prior to starting the protocol 100% ethanol was added to the wash buffer RPE, and β-mercaptoethanol was added as an RNAse inhibitor to the lysis buffer RLT. The amount of starting material was also taken into consideration, following recommendations in the manufacturer's handbook (see Qiagen Plant RNeasy protocol book).

Flash frozen samples were thawed 'on ice', and approximately two small spatulas full of 0.1 mm diameter glass beads were added to the sample. The cells were disrupted 2 x 30 s using a Qiagen Bead Beater (Hilden, Germany). The homogenate was separated from the glass beads and placed in a QIAshredder column/collection tube and centrifuged for 10 min at maximum speed. Centrifugation through the shredder column functions to remove cell debris, as well as homogenize the lysate. A small pellet formed at the bottom of the collection tube. The supernatant was very carefully removed and placed in a new centrifuge tube, without disturbing the pellet at the bottom of the tube. Ethanol (250µl-100%) was added to the lysate (0.5 x volume) and mixed by pipetting. The entire sample was loaded onto a new RNeasy column/collection tube, and was spun at 8,000 x g for 30 s. The ethanol added previously functions to bind the RNA to the silica membrane in the column. The flow-through was discarded. 700 µl RW1 buffer was added to the column to wash the membrane-bound RNA, and the column was centrifuged again at 8,000 x g for 30 s. The flow-through was again discarded. The column was transferred into a new collection tube. Wash buffer RPE containing ethanol (500 µl) was added to the column, and the column was centrifuged as before. The flow-through was discarded. This wash step

was repeated once more, including the centrifugation and flow-through discarding step. The column was centrifuged further for 1 min at maximum speed to remove all traces of ethanol that could interfere with downstream applications of the RNA, i.e. cDNA synthesis. The column was placed in a new centrifuge tube, and 2 x 50 µl of DEPC- treated water was pipetted directly onto the center of the membrane to elute the RNA. The final volume was 100 µl.

*DNase in-tube treatment*

To each sample of 100 µl volume, 10 µl buffer DNase buffer RDD and 5 µl DNAse resuspended in provided nuclease free water (Qiagen) were added. This mixture was incubated for 1 h at room temperature (approximately 23 °C).

*RNA Clean-up*

Buffer RLT (350 µl) was added to the DNAse and RNA mixture. The solution was then thoroughly vortex mixed. Ethanol (250 µl-100%) was added to the solution, and the mixture was repeatedly pipetted. The sample (700µl) was applied to a new RNeasy column/collection tube and centrifuged at 8,000 x g for 30 s. Both the flow-through and the collection tube were discarded. The column was washed with 350 µl buffer RW1 (high salt), followed by a DNAse on column digestion. DNAse stock solution (10µl) was added to 70 µl buffer RDD, and was gently flicked, not vortexed, due to the fragility of the DNAse enzyme. The entire 80 µl DNAse/buffer RDD solution was applied to the center of the membrane, and was incubated at room temperature for 15 min. 2 x 500 µl buffer RPE washes were performed as previously described, and then the final RNA was eluted in

either 50 µl or 2 x 50 µl of DEPC-treated water. RNA concentration and quality/integrity was checked using the Nanodrop spectrophotometer and Agilent bioanalyzer (Agilent Technologies, Santa Clara, USA).

*Sample concentration and purity*

Sample concentration and purity were determined as described in Publication 1, using a Nanodrop spectrophotometer.

*Sample Integrity*

RNA integrity was measured as described in Publication 1, using gel-chip technology (Agilent). RNA of an appropriate concentration and integrity was obtained for all samples, with the exception of the dark treatment.

*SYBR green qPCR analysis*

qPCR analysis was performed as described in Publication 1. Plasmid vectors (pDNR-Lib) containing full-length cDNAs of both the nitrile-specifier protein (NSP) and the major allergen (MA) genes of approximately 1.9 kb each from the commonly known 'small cabbage white' butterfly *Pieris rapae* were generated to serve as spike-in controls. MA and NSP primers were designed using Primer Express © v 2.0 software with the default settings.

*In vitro* transcription was performed according to the manufacturer's protocol with a T7 RNA polymerase (Invitrogen, Paisley, UK) to obtain mRNA for two internal spike reference genes, as described in Publication 1. Spike genes MA (major allergen) and NSP

(nitrile-specific protein) were utilized for quantification of results, as well as controlling the cDNA efficiency reaction prior to qPCR analysis. MA was added at a final concentration of 116 pg $\mu l^{-1}$ and NSP at 10 fg $\mu l^{-1}$. cDNA was synthesized from all tRNA samples with the Omniscript RT kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) using anchored oligoVN(dT)20 primer (Invitrogen, Paisley, UK) at a final concentration of 25 ng $\mu l^{-1}$. All primers for qPCR were designed with the Primer Express 2.0 software on default settings (Applied Biosystems, Darmstadt, Germany) and synthesised from MWG Biotechnologies Germany. Standard PCR primers were designed based on the *Primer 3* platform using default settings (http://frodo.wi.mit.edu/) and synthesised from MWG Biotechnologies (Germany). Primer sequences are available as supplementary material. The SYBR green qPCR reaction was designed according to manufacturer's protocol (Applied Biosystems, Darmstadt, Germany) using 2 µl of a 10-fold diluted cDNA. 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 6 cDNA (originating from the control culture) serial dilution points ($1.0 \times 10^{-3}$ to $1.0 \times 10^{-8}$). Percent efficiency was calculated from the slope of the threshold cycle ($C_t$) vs. concentration [cDNA] with equation (I)

$$E = 10^{-1/slope} \qquad (I)$$

All PCR efficiencies were 98.88% ≥ x ≥ 92.31% 1.91, all $R^2$ values were >0.94. Samples were run in both biological (independent cultures) as well as technical triplicates. Variation was calculated as averages among technical replicates as well as standard deviation. An R expression ratio was calculated using the $\Delta\Delta C_t$ as described by Pfaffl et al. 2001, incorporating individual reaction efficiencies as correction factors. Calculation of an R expression ratio was performed using the following equation (II)

*II* $Ratio = E_{target}\text{^}[\Delta C_{t\ target}\ (control\text{-}\ sample)]\ /\ E_{MA}\text{^}[\Delta C_{t\ housekeeping}\ (control\text{-}\ sample)]$

This quantitative method was chosen to minimize intra- and interassay variability. All calculations were performed using the REST-2009 software platform (Qiagen, Hilden, Germany).

*Microarray analysis*

Microarray analysis was performed as described in Publication 1. Agilent RNA Spike-In Mix (p/n 5188-5279) was added to the tRNA samples prior to the labelling reactions following the RNA Spike-In Kit protocol (Agilent Technologies, Boeblingen, Germany). Total RNA (500 ng) was amplified, reverse-transcribed and labelled using the two colour low RNA Input fluorescent linear amplification kit (Agilent Technologies, p/n 5184-3523). The Cy-3 and Cy-5 dye incorporation was verified by NanoDrop ND-1000 spectrophotometer. Hybridization was performed onto 4 x 44k microarray slides containing oligonucleotide 60mers designed by the Agilent eArray online platform, using the gene expression hybridization kit two colour (Agilent Technologies, p/n 5188-5242), contained in SureHyb Hybridization Chambers (Agilent p/n G2534A) in a hybridization

oven (Agilent p/n G2545A) at 65° C for 17 h. Microarrays were scanned by an Agilent Scanner (p/n G2565BA).

Raw data were extracted with the Agilent Feature Extraction Software version 9.5, incorporating the GE2_105_Dec08 protocol. Feature extraction software served to remove spots that had been flagged 'outliers', 'not known' or 'bad', based on background median analysis (Storey, 2003). Further analysis of gene expression was performed using GeneSpring GX version 10 software (p/n depending on license).

*Statistical analysis*

Physiological data described are the mean of biological triplicates with the corresponding standard deviation. Significance of physiological data was confirmed using a Student's t-test ($p<0.05$). Normal distribution of data was analyzed by the Shapiro-Wilk test as implemented in R. Microarray expression measurements are given as the geometric mean of three measurements, corresponding to biological triplicates.

### 2.3.4 Results

*Growth and physiological assessment*

All cultures displayed a short initial lag phase from inoculation to approximately 2 days following initiation of the experimental (Figure 2.3.3). All cultures showed similar initial growth patterns until 4 days (Figure 2.3.3). P limitation occurred after 4 days, where the two P replete cultures continue to grow exponentially, whereas the two P deplete cultures reach a stationary growth phase. Mean growth rate was calculated for all four treatments between days 4 and 11 (last culture to reach stationary growth phase), using the following equation:

$$\text{Growth rate: } K' = \text{Ln } (N2 / N1) / (t2 - t1)$$

where N1 and N2 = biomass at time (t1) and time (t2) respectively (Levasseur et al., 1993). Mean growth rates can be seen in Table 2.3.2.

Table 2.3.2: Exponential mean growth rates.

| Treatment | Mean growth rate (days 4-11) ± st. dev. |
|---|---|
| 26 psu, P-replete | 11.80 ± 0.34 |
| 26 psu, P-deplete* | 9.04 ± 0.28 |
| 5 psu, P-replete | 8.65 ± 0.17 |
| 5 psu P-deplete | 11.44 ± 0.22 |

As expected, P-deplete cultures (after day 4) demonstrate a lower growth rate during the exponential growth phase. Interestingly, 5 psu P-replete cultures demonstrated a lower exponential growth rate than 26 psu P-replete cultures: suggesting salinity may play a role in hindering cell division in *P. parvum.* Also as expected, pH measurements showed a trend

towards higher values with increasing cell concentration, and were not dependant on salinity (Figure 2.3.4).

P-limited cultures contained reduced concentrations of dissolved phosphate (Figure 2.3.5). P-limited cultures depleted the available phosphorous by Day 4, as indicated by a reduction in cell division (Figure 2.3.6), a significant increase in the particulate organic C:P ratio (Student's t-test, $p<0.05$)(Figure 2.3.6) and a significant increase in the particulate N:P ratio (Student's t-test, $p<0.05$)(Figure 2.3.6). Intracellular particulate N levels and C:N ratios, however, were not significantly different between P-limited and non-limited cultures (Student's t-test, $p>0.05$) (Figure 2.3.6).

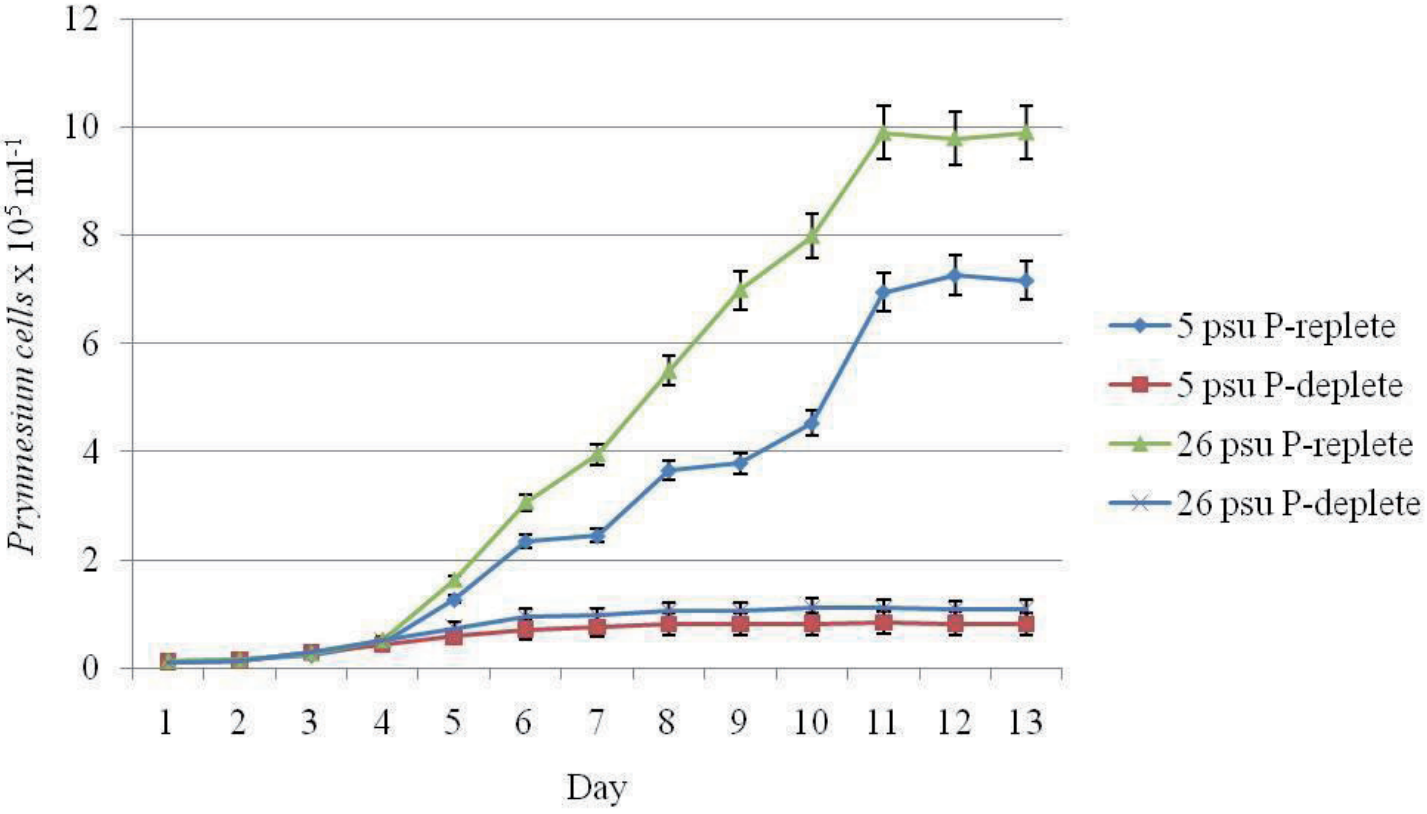



**Figure 2.3.3: Growth of experimental *P. parvum* cultures. After day 4, available phosphorous is depleted by cultures. Mean exponential growth rates of all cultures can be viewed in Table 2.3.2. Phosphorous limitation limited cell growth (two lower curves). 5 psu P-replete displayed a slightly lower mean exponential growth rate than 26 psu P-replete (see Table 2.3.2) indicating the potential role of salinity in hindering cell division in *P. parvum*.**

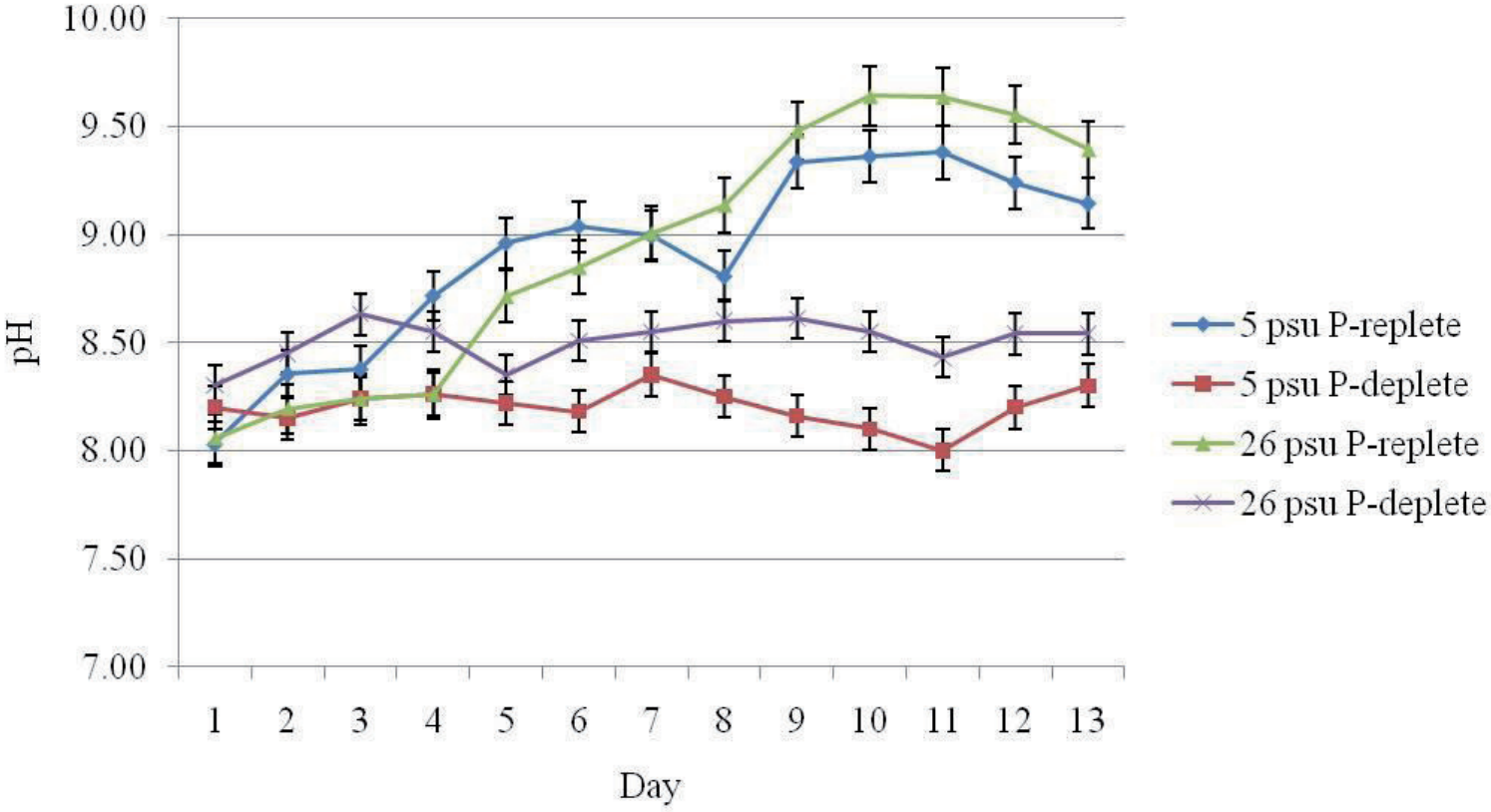



**Figure 2.3.4: mean pH measurements throughout experiment for all four treatments. Error bars represent standard deviation between three biological replicates. Trend indicates higher pH values, with higher cell concentration, as expected. pH maximum reached in 26 psu P-replete culture between days 10 and 11. pH minimum reached in 5 psu P-deplete culture at day 11.**

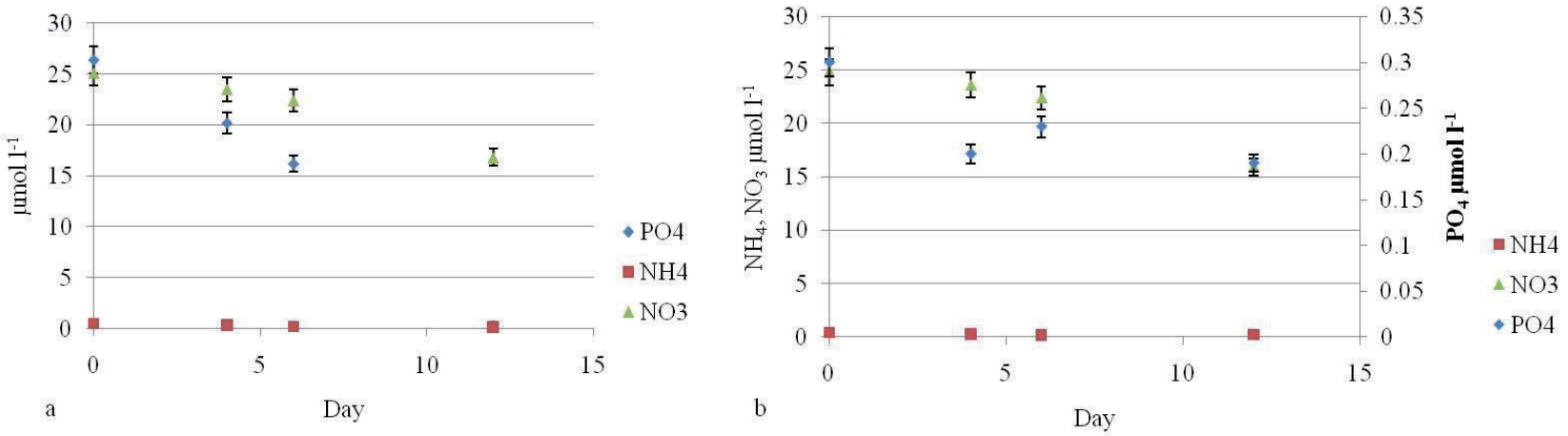



**Figure 2.3.5:** ***Prymnesium parvum*** **dissolved nutrient ratios (Dissolved $PO_4$, $NO_3$and $NH_4$).**

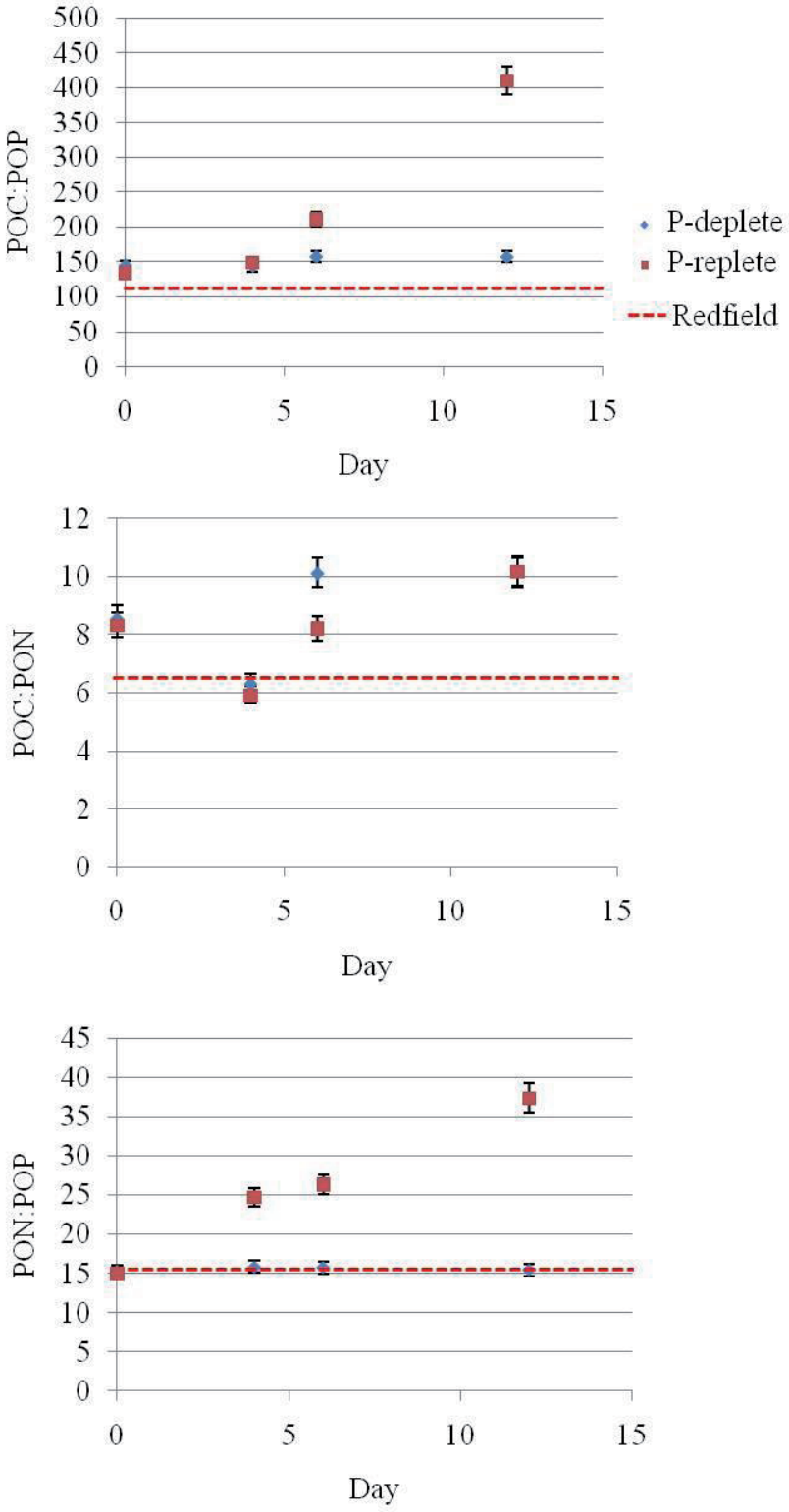



**Figure 2.3.6: *Prymnesium parvum* organic nutrient ratios (atomic N:P, C:N and C:P). Redfield ratio is indicated by dashed red line (C:N 106:1, C:N 6:1 and N:P 16:1).**

*Lytic activity*

Extracellular or secreted toxicity/lytic activity towards the cryptophyte *R. salina* was significantly highest relative to the control in the low salinity P-deplete (5 psu, -P) cultures, indicating an $EC_{50}$ for *Prymnesium parvum* of 116 $^{\pm}$39 cells $ml^{-1}$. This is a significant increase (Student's t-test, $p<0.05$) of approximately +1940 fold in lytic activity compared to the control culture (26 psu), which yielded an $EC_{50}$ of 2.25 x $10^5$ ±4732 cells $ml^{-1}$ (Table 2.3.3). Low salinity cultures (5 psu) gave an $EC_{50}$ of 1.32 x $10^3$ ± 256 cells $ml^{-1}$, whereas for P-limited cultures (26 psu, –P) showed an $EC_{50}$ of 3.56 x $10^4$ ±1264 cells $ml^{-1}$. These differences from the control were both significant (Student's t-test, $p<0.05$).

Observed differences among treatments in lytic activity towards erythrocytes were not as large as the differences in extracellular or secreted lytic activity between treatments. Low salinity P-limited cultures showed a significant increase in lytic activity of 22.56 ng SnE $cell^{-1}$ (Student's t-test, $p<0.05$) (Figure 2.3.7) compared to lytic activity for the control culture of 15.4 ng SnE $cell^{-1}$. Low salinity cultures (5 psu) and P-limited cultures (26 PSU, –P) did not show significant changes in lytic activity towards erythrocytes compared to the control (14.39 ng SnE $cell^{-1}$ and 15.76 ng SnE $cell^{-1}$, respectively).

Table 2.3.3: $EC_{50}$ results Phosphate limitation and low salinity treatments of *P. parvum* strain K0252. $EC_{50}$ is defined as the P*. parvum* cell concentration causing 50% mortality of *R. salina* cells**.**

| **Treatment** | **$EC_{50}$ *Rhodomonas salina* (mean ± standard deviation cells $ml^{-1}$)** |
|---|---|
| 5 psu P-replete | 1.32 x $10^3$ ± 256 |
| 26 psu P-replete | 2.25 x $10^5$ ± 4732 |
| 5 psu P-deplete | 1.16 x $10^2$ ± 39 |
| 26 psu P-deplete | 3.56 x $10^4$± 1264 |

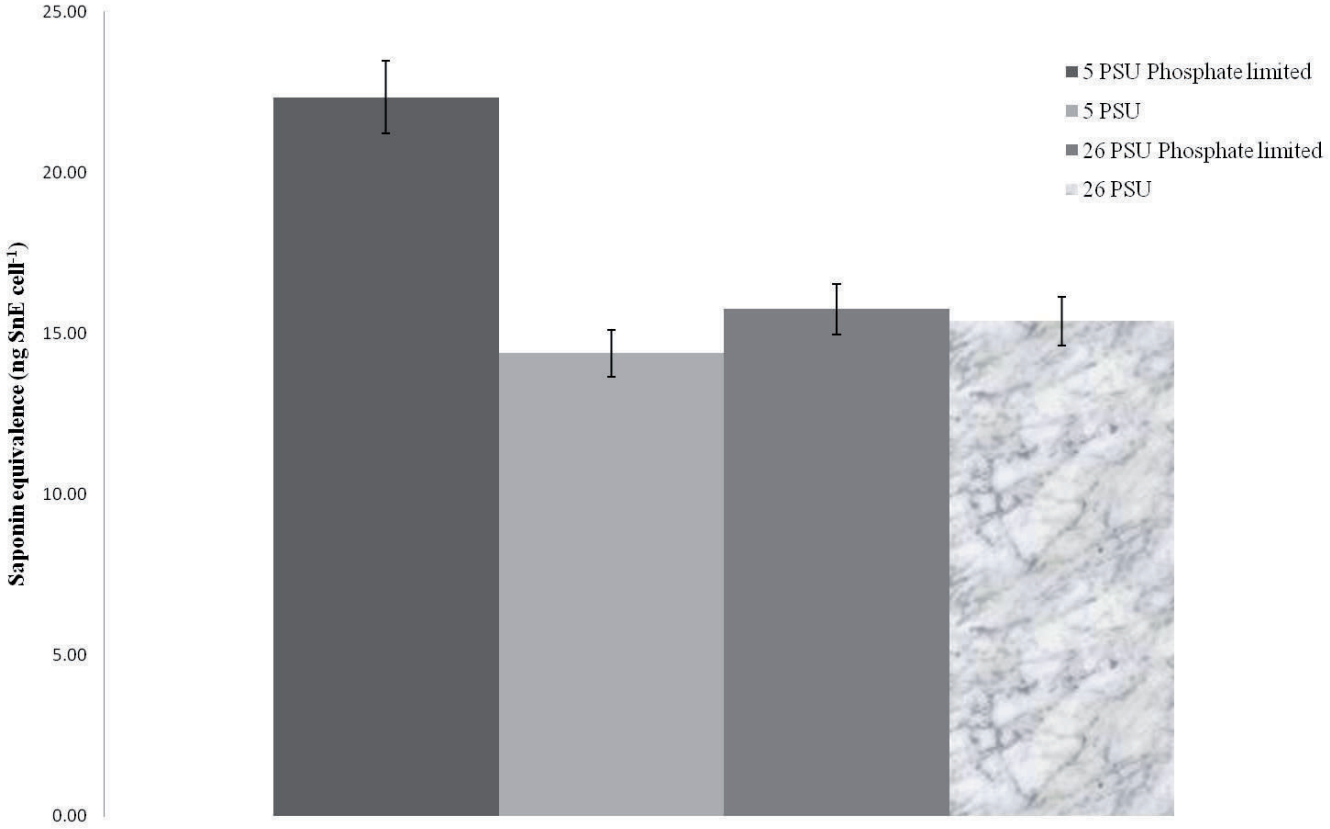



**Figure 2.3.7: Lytic activity of experimental cultures towards erythrocytes. Error bars represent standard deviation among biological triplicates.**

*Gene expression*

A total of 2,788 genes were identified as differentially expressed among the three treatments (5 psu P-replete, 5 psu P-deplete –P & 26 psu P-deplete –P), and the control (26 psu P-replete) as a reference probe, harvested in exponential growth phase. The highest number of genes regulated was observed in 5 psu P-replete with 1409 upregulated (Figure 2.3.7). Indications of a more refined, less global response in gene regulation were observed for the 26 psu P-deplete treatment, with 18 upregulated and 30 downregulated (Figure 2.3.7). These identified sets of genes were used to select genes relevant to nutrient and salinity stress, general growth processes and cellular transport. A comparison of gene expression ratios for these genes is shown in Table 2.3.4. The microarray hybridization scheme applied in this study allowed for selection of differentially regulated genes that could be associated with single factors, i.e. due to a decrease in salinity or P-limitation, as well as due to a combination of these factors (Figure 2.3.7). Low salinity induced differential upregulation in genes related to general cellular transport and cellular skeletal function (actin, caltractin) and a protein phosphatase (Table 2.3.4). Low salinity induced downregulation in a phosphate acyltransferase, a triosephosphate isomerase, a very strong downregulation of a sodium symporter membrane transport protein, and an even stronger downregulation of a Ras-related protein (Table 2.3.4). Phosphorus limitation induced an upregulation in a tetraphosphate hydrolase, a pyrophosphate powered membrane bound proton pump, actophorin, caltractin and a mitochondrial inner membrane transport protein (Table 2.3.4). Nutrient stress also induced a strong downregulation in a phosphate acytransferase and particularly a nearly 416 fold downregulation of N-acetylneuraminate phosphate synthase. The combination of low salinity and P-limitation induced upregulation

in several of the same genes as the individual treatments, including a pyrophosphate powered membrane-bound proton pump, caltractin, a mitochondrial inner membrane transport protein, actophorin, in addition to a protein phosphatase (Table 2.3.4). Downregulation of a lesser degree compared to 26 psu P-deplete was observed for a membrane potassium channel, a phosphate acytransferase, a triosephosphate isomerase, a sodium symporter as well as N-acetylneuraminate phosphate synthase (Table 2.3.4).

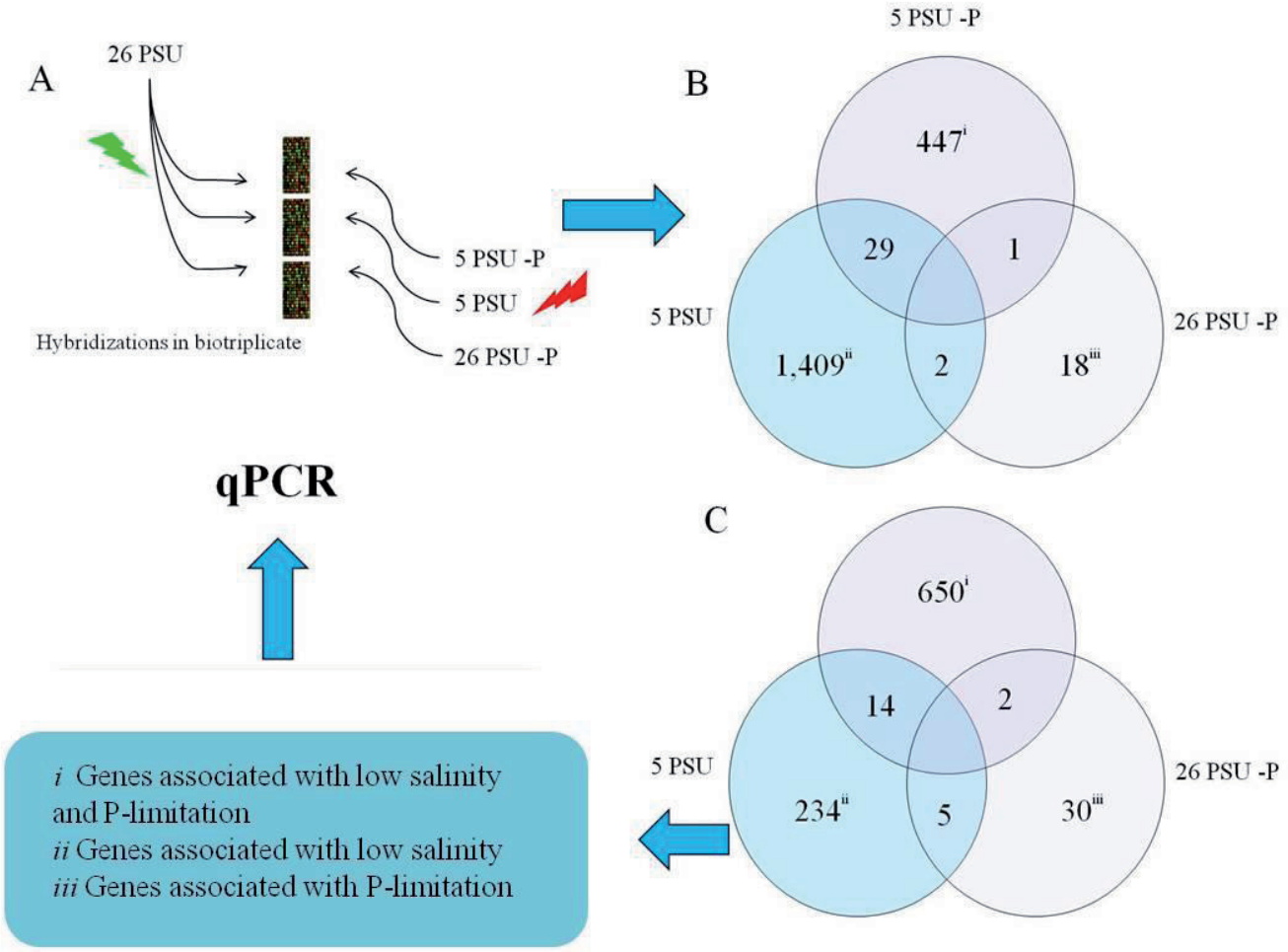



**Figure 2.3.7: Workflow of gene expression portion of P-limitation and low salinity stressor study on *P. parvum* strain K0252. A. Microarray hybridization scheme B. Upregulated genes found for three treatments (normalized against 26 PSU-control). C. Downregulated genes found for three treatments (normalized against 26 PSU-control). Genes chosen in three categories for verification via qPCR.**

Table 2.3.4: Comparison of qPCR and microarray expression ratios (fold change normalized against control 26 psu P-replete) for a selected set of physiologically relevant genes. *qPCR expression ratios are italicized.* ± standard deviation noted between biological triplicates. Values in red represent those above a SAMS-based 2.5 fold threshold of differential regulation from the control sample. Values in red are above an arbitrarily chosen cut off value of 2.5 fold change.

| | *Probe identifier* | *Putative gene product* | *5 psu P deplete* | *26 psu P-deplete* | *5 psu* | 5 psu P-deplete | 26 psu P-deplete | 5 psu |
|---|---|---|---|---|---|---|---|---|
| **Upregulated** | Prymnesium-C-a-69k09.m13f | Tetraphosphate hydrolase | *1.65±0.01* | *3.31±0.11* | *1.15±0.02* | 2.45±0.32 | 3.64±0.14 | 0.98±0.023 |
| | Prymnesium-C-a-29o24.m13f | Pyrophosphate proton pump-membrane bound | *6.85±0.16* | *4.91±0.08* | *0.62±0.01* | 7.23±0.24 | 5.21±0.15 | 8.12±0.22 |
| | Prymnesium-C-a-102n10.m13r | Protein phosphatase | *3.27±0.02* | *1.00±0.01* | *1.47±0.03* | 3.66±0.42 | 0.81±0.23 | 1.73±0.29 |
| | Prymnesium-C-a-99e17.m13f | Actin | *1.82±0.04* | *1.19±0.012* | *6.29±0.17* | 2.11±0.02 | 2.11±0.02 | 7.88±0.013 |
| | Prymnesium-C-a-100n19.m13r | Actophorin | *3.05±0.09* | *3.97±0.16* | *2.22±0.14* | 2.78±0.03 | 4.23±0.021 | 2.99±0.01 |
| | Prymnesium-C-a-90h09.m13f | Caltractin | *45.33±1.41* | *5.57±0.10* | *9.84±2.24* | 51.1±2.70 | 6.8±1.03 | 11.2±1.02 |
| | Prymnesium-C-a-59e12.m13r | Mitochondrial inner membrane transport subunit | *3.62±0.17* | *11.6±0.22* | *3.14±0.10* | 4.22±0.68 | 13.3±2.1 | 2.88±0.01 |
| | Prymnesium-C-a-102n10.m13r | Protein phosphatase | *2.56±0.08* | *1.38±0.02* | *15.9±1.2* | 2.40±0.01 | 0.98±0.03 | 18.2±0.02 |
| | Prymnesium-C-a-72j22.m13f | Hypothetical protein R144.6 in chromosome III. | *1.82±0.02* | *0.82±0.03* | *1.84±0.06* | 1.22±0.05 | 0.23±0.01 | 2.82±0.04 |
| | Prymnesium-C-a-96b01.m13r | hypothetical protein protein\|o_sativa | *0.78±0.06* | *1.15±0.08* | *0.86±0.06* | 0.92±0.16 | 1.56±0.18 | 0.45±0.04 |
| **Downregulated** | Prymnesium-C-a-90h02.m13f | *Membrane potassium channel* | *-2.80±0.06* | *-0.76±0.01-* | *-0.52±0.01* | -4.12±0.15 | -1.23±0.12 | -0.21±0.01 |
| | Prymnesium-C-a-89l24.m13f | *Inositol 1,4,5-trisphosphate receptor type 2* | *-0.11±0.01* | *-0.28±0.02* | *-0.38±0.05* | -1.29±0.13 | -0.78±0.02 | -2.12±0.03 |
| | Prymnesium-C-a-88c06.m13f | *Phosphate acyltransferase* | *-81.8±2.45* | *-64.7±6.9* | *-7.05±0.29* | -71.1±1.96 | -52.1±3.44 | -9.09±1.3 |
| | Prymnesium-C-a-05g02.m13f | Triosephosphate isomerase | *-4.55±0.11* | *-18.1±0.30* | *-8.69±0.14* | -5.34±2.33 | -21.6±3.94 | -3.45±1.24 |
| | Prymnesium-C-a-86k17.m13f | Sodium symporter | *-8.28±0.64* | *-39.5±1.60* | *-52.2±2.80* | -9.21±1.71 | -51.3±1.9 | -8.87±2.93 |
| | Prymnesium-C-a-46e07.m13r | Ras related protein | *-126.7±8.80* | *-67.5±2.5* | *-69.5±7.14* | -144.2±10.2 | -56.3±5.85 | -88.2±9.4 |
| | Prymnesium-C-a-62b03.m13f | N-acetylneuraminate phosphate synthase | *-65.9±7.11* | *-415.7±20.7* | *-3.11±0.18* | -49.3±5.12 | -388.2±0.21 | -4.02±0.34 |

### 2.3.5 Discussion

Knowledge about the ecological role of phycotoxins is still scarce, despite decades of research. Whether or not the mode of action of known phycotoxins in mammalian systems reflects (in whole or in part) ecological function as allelochemicals remains under debate. The argument that allelochemicals may regulate growth and survival of coexisting species, particularly under growth limiting conditions, such as nutrient depletion, is nevertheless compelling. In certain cases, phycotoxins have been shown by several studies to have a negative effect on zooplankton (Ives, 1985; Huntley et al., 1986) as well as on other microalgae (Windust et al., 1996; Keating, 1977). Specifically, the compounds produced by *P. parvum* have been shown to effect gill breathing organisms (Shilo, 1967), while also displaying effects on copepods (Nejsgaard & Solberg, 1996) and other microalgae (Arlstad, 1991).

*Growth and physiology*

In this study we have used cellular particulate nutrient content (C, N & P) as well as dissolved nutrient levels ($NO_3$, $PO_4$ & $NH_4$) as indicators of P-limitation. The nutrient status of the environment within which phytoplankton grow influences their respective cellular elemental composition and ratios (Harrison et al., 1988). One effect of nutrient limitation is the reduction of intracellular levels of the limiting nutrient thereby reflected in the elemental ratios (Cembella et al., 1984; Sakshaug and Olsen, 1986; Darley, 1988). If the C-supply is replete, under P- or N-limitation the cellular levels of C increase due to residual C following cell division (Cembella et al., 1984). In our study, cellular particulate nutrient content (C, N, P) as well as dissolved nutrient ($NO_3^-$, $PO_4^{-3}$ and $NH_4^{+)}$ concentrations in the

growth medium served as indicators of P-limitation. The cellular carbon content of P-limited *P. parvum* cells did not differ significantly from that of the P-replete control culture, indicating that P-limitation has only a slight effect on the intracellular carbon content.

For both nutrient limited and replete control cultures, nutrient quotas and molar ratios (C:N, C:P and N:P) were within the range of literature values for *P. parvum* (Uronen et al., 2005, Graneli et Johansson, 2003). Phosphorus-limited cultures showed expected increased in molar ratios (C:P and N:P) whereas P-replete control cultures showed only slight deviations from the canonical Redfield ratio C:N:P 106:16:1 (Figs. 2.3.7-2.3.9), widely considered to represent balanced growth and developmental conditions in natural phytoplankton populations. A clear separation in growth curves was visible between P-limited and non-limited control cultures (Fig. 2.3.3), indicating that growth limitation was indeed attributable to the restriction in P-supply.

*Lytic activity*

Mixotrophic flagellates such as *Prymnesium parvum* are both photosynthetic and able to take up particulate food. It has previously been speculated that *Prymnesium* species utilize phagotrophy as a mechanism to obtain essential growth factors, i.e. nutrients for use in photosynthetic growth (Caron et al., 1993; Arenovski et al., 1995; Legrand et al., 1998; Stoecker et al., 1998). Feeding may therefore supply the organism with nitrogen and phosphorous when concentrations of dissolved inorganic nutrients in the surrounding water are limiting (Skovgaard et al., 2006). It is plausible that *Prymnesium parvum* may incorporate mixotrophic tendencies into its feeding regime, in an attempt i.e. to obtain phosphorous, when faced with growth limiting phosphorous concentrations (Nygaard &

Tobiesen, 1993, Tillmann, 2007). This hypothesis (Tillmann, 2007) may explain changes in extracellular or secreted lytic activity in cultures stressed by P-limitation. An increase in lytic capacity in *P. parvum* cultures would therefore be expected in response to P-limitation, if in fact this method is effective to immobilize and ingest prey to obtain organic-P. The observed increase in lytic capacity associated with the combination of low salinity and P-limitation is, however, a novel observation. This may be explained as an attempt by *P. parvum* cells to maintain membrane homeostasis in the presence of low extracellular ion (e.g. $Na^{+}$) concentrations. Increasing the permeability or "leakiness" of the external cell membrane may increase secretion of intracellular compounds that possess lytic capacity and may also interfere with the function of $PO_4^{-3}$ ion transporters. Moreover, the phenomenon of increasing lytic activity may be due to increased release of lytic compounds, but this mechanism is not necessarily adaptive. The response could be an artifact of increased membrane permeability and loss of membrane integrity leading to enhanced diffusion of lytic compounds into the extracellular environment. In the erythrocyte lysis assay, the intracellular lytic activity does increase under the combination of low salinity and P-limitation, but not nearly to the same extent as observed in the *Rhodomonas salina* bioassay, which is diagnostic for extracellular activity. This indicates that in fact there is an increase in lytic activity of intracellular compounds (however not proportional to the increase observed in the *R. salina* bioassay) and supports the idea that the observed increases in extracellular lytic capacity may be due to a compromised less selective cellular membrane. Whether or not this increase in activity is linked to an increase in biosynthesis of the same compounds must be further elucidated.

*Gene expression*

Our microarray hybridization scheme allowed for qualitative identification of groups of genes potentially associated with physiological stress factors, namely low salinity and P-limitation. Overlapping genes found between treatments indicate however that the processes of coping with low salinity and P-limitation are not regulated by strictly distinct pathways. The identification of 43 genes differentially expressed between 5 psu P-replete and 5 psu P–deplete treatments provides circumstantial evidence that genes may be specifically regulated by P-nutrient status. Nevertheless, the identification of 7 differentially expressed genes (up and downregulated) been the between 26 psu P-replete and 5 psu P-replete indicates that the stressor of low salinity also alters gene regulation on the transcriptional level (Figure 2.3.11). 26 psu P-deplete and 5 psu P-deplete had 3 commonly differentially regulated genes (up and downregulated) suggesting these genes may play a role in *Prymnesium's* response to low salinity stress. From this qualitative analysis, we can discern two principles: 1) specific regulatory pathways associated with effects of P- limitation versus low salinity are not easily decipherable, and 2) the combination of these two stressors likely involves regulation on another level, such as post translational modification. However, our conclusive interpretation is limited by the relatively low number of available annotated sequences for this toxigenic prymnesiophyte, and is subject to change considerably with a significantly higher functional annotation. In any case, confirmation of selected genes via qPCR reveals a similar finding, in that there seems to be little specificity on the level of transcriptional regulation concerning the individual stress factors of P-limitation and low salinity.

Early previous studies have shown that the relationship between growth and toxicity in *P. parvum* is not simple, and our current work underscores this complexity. High toxicity has been observed with very low cell numbers, contrasting with other cases where massive growth of *P. parvum* did not produce any observable toxic effects in nature (Shilo, 1967). It is therefore evident that growth and toxicity are regulated by different factors in this prymnesiophyte. Comparing the capacity of *P. parvum* to produce toxins under various environmental conditions has illustrated that growth and toxicity have different optimal requirements (Shilo, 1971). More specifically, it has long been known that toxicity of this species is increased when growth conditions are limiting (Dafni et al., 1972). These earlier observations are supported by our findings, as non-P-limited cultures exhibited smaller increases in lytic capacity than P-depleted cells over time in batch growth mode. The effect on extracellular toxicity observed for the combination of low salinity and P-limitation is however not easily decipherable from our transcriptomic analysis, in terms of its regulatory basis.

Our results confirm that *P. parvum* does alter its physiology and metabolism when P-resources are limiting for growth. These metabolic shifts are reflected through an increase in lytic capacity towards *Rhodomonas salina*, an increase in hemolytic activity, and differential gene regulation between treatments and the P-replete control. From an ecological perspective, it is likely although not definitive that these metabolic responses and increased lytic activity represent a selective competitive advantage under nutrient-limited growth conditions. A general transcriptomic approach, supplemented with more detailed comparative expression analysis of key regulatory genes provide a platform for

further understanding of growth and toxicity of *Prymnesium parvum* in natural populations.

## Synthesis

The toxic prymnesiophyte *Prymnesium parvum* is a harmful algal bloom species with a complex life cycle, physiology as well as ecology (Barkoh & Fries, 2010). It has a haploid-diploid life cycle, two flagellated stages as well as a non-motile form (Larsen, 1998; Johnsen et al., 2010). These organisms synthesize their own food when inorganic nitrogen and phosphorous are abundant (Nicholls, 2003); however when one of both of these nutrients are limited, they release a cocktail of chemical compounds (collectively termed prymnesins) that may serve various purposes. Prymnesins lyse or break up cells of other organisms to release available nutrients (Estep & McIntyre, 1989) or even immobilize prey for *P. parvum* to ingest whole (Nygaard & Tobiesen, 1993; Johansson & Granéli, 1999; Tillman, 2003). Prymnesins also play a potential role in deterring potential grazers as well as killing or inhibiting the growth of coexisting species (Tillmann, 2003; Uronen et al., 2005, Granéli et al., 2008). Observed changes in toxicity and gene expression patterns from the aforementioned studies provide evidence that *P. parvum* does possess a competitive advantage in certain systems.

Research focusing on *P. parvum* has been conducted since the late 1930's, yet no clear understanding currently exists concerning the ecology and factors effecting toxicity. The current dissertation exploits recent advances in genomics in combination with toxicity assays, and expands current knowledge, particularly concerning the transcriptional regulation of PKS in response to specific abiotic stressors and the association with changes in toxicity is valuable ecological information concerning this toxic prymnesiophyte. The work performed in this thesis represents the foundation for understanding genotypic and phenotypic relationships in the toxigenic *P. parvum.* The ecology of this haptophyte is currently poorly understood, despite the existence of studies investigating factors such as allelopathy and nutrient limitation. The most

obvious casualty of *P. parvum* toxicity being fishkill events worldwide (Ulitzur and Shilo, 1966; Paster, 1973; Linam et al., 1991), other invertebrates such as planktonic algal species and bacteria are also negatively affected (Sarig, 1971; Nygaard and Tobiesen, 1993; Fisterol et al., 2003). The latter may be involved in processes such as planktonic community structure, of which an improved understanding is crucial to predicting and responding to economically detrimental bloom events.

### 3.1 Molecular advances in harmful algal research

*Prymnesium parvum* is as crucial as all other microalgal species to global productivity and biogeochemical cycling however the genomic understanding of these organisms is still currently at an immature stage relative to comparable projects involving human and plant genetics. Despite limitations in HAB genomic analyses, it is nonetheless crucial to discuss which microalgal species are be examined using genomic techniques, the information obtained and what this information can tell us about relevant structural, functional, developmental and even evolutionary aspects of these organisms (Grossman, 2005). Collaborative studies incorporating traditional phycological approaches and functional genomic experimental piplines are providing the further insight needed to better understand the underlying ecology of HABs.

One of the primary goals of functional genomic studies, as applied to harmful algal bloom research, is to describe the gene(s) or gene products associated with toxin production that could subsequently be used as markers of toxigenic blooms (Plumley, 1997). A second important goal is to identify genetic expression signatures associated with ecophysiological responses to known conditions in the natural environment (Kudela et al., 2010). Using a stress derived cDNA library, I have addressed both these primary goals in the three aforementioned

publications. Through these studies, novel genomic characterizations for the toxic prymnesiophyte have been made.

Application of molecular and functional genomic tools allowed for further characterization of factors involved in bloom initiation and development. Response to abiotic shock treatments induced toxicity, particularly high irradiation and low salinity, which was able to be correlated with transcriptional regulation of PKS genes. This is a novel characterization for *P. parvum.* Microarray gene expression profiling aided in unraveling alleopathic interactions by indicating qualitative transcriptional regulatory patterns, distinguishing cell-cell contact vs. recognition of chemical cues. These patterns helped to explain the ecological niche in which *P. parvum* lives, the ways in which gene content have been arranged and potentially modified by evolution in response to predator or prey encounters. Additionally, PKS transcriptional regulation analysis via qPCR was able to be associated with changes in *P. parvum's* allelopathic behavior and lytic capacity.

### 3.2 Evolutionary significance of interspecific interactions between *P. parvum* and coexisting planktonic species and

Interaction of two species rarely indicates a shared interest, either in a particular resource or in niche selection. More often we see the growth of one of these species affected by the other, likely in an attempt to outcompete. In particular, interspecific interactions between members of different species i.e. competing for the same resource or space warrant a competitive advantage of one over the other. Production of allelochemicals in this sense can sometimes be considered a defense mechanism, and could potentially play a role in structuring the phytoplankton community. In contrast, an increase in growth rate or nutrient uptake independent from

production of allelochemical compounds could also provide a competitive advantage. Increased toxicity in *P. parvum* under phosphorus limitation potentially serves both these purposes. Production of alleopathic compounds could be envisioned as a method by which to retard the uptake of valuable nutrient by other coexisting species.

As shown by Granéli and Hansen (2006), production and/or release of chemical compounds may in fact be an evolutionarily developed response to competition, in the presence of co-existing species. Evolutionary biology suggests that these developed responses are associated with metabolic cost constraints which we have attempted to observe in our studies, either through the gene expression profile, or relative toxicity in *P. parvum.* Chemicals associated with defense in phytoplankton are very often complex secondary metabolites, whose biosynthesis require a plethora of cellular machinery and energy sources. However, organisms such as *Alexandrium tamarense* that have been studied do not seem to show a reduction in i.e. growth rate (Tillmann et al., 2009). We can therefore presume that using growth rate as a sole indicator of the costs involved with the production of chemical defense compounds is an insufficient method of characterization. Even if there is a cost in terms of growth rate, this may be compensated for via i.e. production of allelopathic compounds.

There are many aspects which speak for evolutionary development playing a role in responses such as described in Publication 2 (i.e. increased lytic activity, higher qualititative gene expression regulation). The term “co-evolution” is highly debated by researchers in the sense that coexisting species may have parallel developed mechanisms by which they attempt to maintain the competitive advantage in limiting systems. This term refers specifically to selection that occurs as a result of interactions between species (e.g. predation or parasitism) where we see evolutionary transmission of physiological traits in both species involved (Freeman and Herron,

2007). This principle would theoretically involve recognition on either the infochemical or physical encounter (e.g. via cellular surface receptors), leading to selection of individuals within a population that are able to respond appropriately to pressure such as grazing or even nutrient limitation.

In Publication 2, we demonstrated the difference between cell-cell physical encounter and recognition of chemical cues for *P. parvum*, both on the level of transcriptional regulation (gene expression) and toxicity. We furthermore demonstrated that *P. parvum's* response is differential, depending on the organism which it encounters. *O. marina* is a potential predator. It is plausible that recognition by *P. parvum* of molecules produced by *O. marina* contributes to the metabolic response we observed (increased toxicity and PKS gene expression). Such a recognition system could have evolutionary implications and indicate a coevolved response by both organisms involved. Furthermore, there is a principal difference in *P. parvum's* physiological response when confronted with cells vs. chemical cues from the same competitor. This response also leads us to believe that recognition of predator cells has a stronger effect on toxicity and related gene regulation in *P. parvum.*

### 3.3 Possible role of polyketide synthase enzymes (PKS) in toxic processes originating from *P. parvum*

Due to the putative polyketide structure of Prymnesin-1 and Prymnesin-2 proposed by Igarashi et al. (1996), we focused the qPCR portion of our gene expression analyses on three PKS transcripts, identified from a cDNA library (LaClaire 2006). The role of PKS enzymes in the biosynthesis of toxic compounds for *P. parvum* is not confirmed, however further bioassay-guided chemical analyses are currently underway (Schug et al., 2010). Despite the lack of *in*

*vivo* knowledge concerning biosynthesis of toxic compounds in *P. parvum,* it is likely that the polyketide synthase pathway plays a role based on current characterizations of toxic compounds produced by *P. parvum.* The data obtained in Publications 1 and 2 of this dissertation support the importance of this biosynthetic pathway in toxic processes.

In Publication 1, we demonstrated that high light and low salinity stress induce both the highest transcriptional regulation in select PKS transcripts, as well as the largest increases in toxicity, both extracellular and intracellular. Here we observed two general trends in differential regulation. A global regulation pattern was observed for all shock treatments applied, suggesting that polyketide synthase enzymes may be involved in general stress responses in *P. parvum.* In contrast, higher regulatory patterns were observed for the shock treatments of high light and low salinity. These two shock treatments also induced toxicity, as observed in both the *Rhodomonas salina* bioassay as well as the erythrocyte lysis assay. The causality relationship between toxicity and PKS gene expression is however questionable, as transcriptional regulation in other shock treatments was observed as well.

As presented in Publication 2, we found evidence that the same three PKS transcripts may serve allelopathic or chemical defense purposes in *P. parvum.* In particular in the presence of *O. marina* cells and filtrate, the increase in both PKS transcript copy number over time (Dose exposure experiment) and of relative toxicity (both extracellular and intracellular) strongly suggests a relationship between PKS gene regulation and a change in the phenotype, namely an increase in toxicity. This relationship however needs further study in order to be confirmed.

### 3.4 Phosphorus limitation and low salinity as a toxigenic trigger

As global eutrophic zones increase, the role of nutrient limitation has become an important topic when discussing factors involved in bloom formation and toxicity of the haptophyte *P. parvum.* Imbalances in nutrients such as phosphorous and nitrogen have been shown to decrease the growth rate of *P. parvum,* ultimately leading to an increase in toxicity of this microalgal organism (Hallegraeff, 1999; Collins, 1978; Holdway et al., 1978). Control of toxicity via nutrient limitation is therefore a very relevant issue, and must be addressed further to gain a more complete understanding of *Prymnesium parvum's* ecology (Legrand et al., 2001).

Considering *P. parvum's* notorious physiological flexibility, it is relevant to consider the process of mixotrophy when discussing nutrient limitation and observed changes in toxicity. For example, the observed increase in lytic capacity presented in Publication 3 seems logical, if one considers the release of organic phosphorus achieved through this process. This may also be viewed as a competitive advantage, in nutrient limited situations. This is also supported by observed increases in both extracellular and intracellular toxicity observed in *P. parvum* under P-nutrient limited conditions. Investigating the combinatory effects of low salinity and phosphorus limitation is however a novel experimental design. The data obtained for Publication 3 strongly suggest that these two physiological factors play collaborative roles in toxigenic processes in *P. parvum.*

Of particular interest is the phenomenon that under the combined stressors of low salinity and phosphorous limitation, extracellular toxicity (*R. salina* bioassay) increases over 1000 fold, when compared to intracellular toxicity (erythrocyte lysis assay). This suggests either compromised membrane conditions under phosphorus limitation, or an increase in active extracellular transport of bioactive compounds.

Functional genomic analysis revealed that in fact there are pathways associated with both individual stressors, however, deciphering the gene regulation individually is a daunting task. Due to the limited nature of the data set (stress-derived cDNA library) it is possible that an increase in annotatable genes may change this analysis dramatically. This would not however change the significant changes in extracellular toxicity observed under P-depleted and low salinity conditions.

### 3.5 Future perspectives

Functional genomic approaches are limited in the sense that identification of genes and gene products is a database-limited process. Non-model organisms are therefore at a disadvantage, due to time and financial constraints regarding the elucidation of the genome. The three aforementioned studies take advantage of current available information concerning the genome of *P. parvum,* however their limitations must be acknowledged. As more information becomes available, such studies must be further developed to reduce the gap between speculation and fact. To compensate for such shortcomings, it would be necessary to have better gene annotations available for functional genomic analysis. This would greatly improve the search for relevant genes and gene products involved in toxic processes.

Regarding the coevolutionary development of infochemical sensing and response thereto, there are no experiments to date specifically testing this principle in phytoplankton and protists. To test such a principle, it would be necessary to have an experimental model involving the organism of interest i.e. *P. parvum*, and two other organisms, one with which *Prymnesium* has shared an ecological niche and one from a completely isolated niche where no *Prymnesium* has been observed. This type of experimental setup would provide the evolutionary basis on which

to further analyze coevolutionary development of such traits as predation and defense. Observed variations in toxicity such as those seen in Publication 2 provide a useful platform for further investigations. When an allelochemical producer can have negative, neutral as well as positive effects this would suggest that target organisms can in fact possess a developed tolerance, similar to that observed in hosts and parasites. In order to test this principle, it would be necessary to compare the allelopathic effects of i.e. *P. parvum* on an organism it has coevolved with, vs. an organism it has not coevolved with.

Furthermore, until the toxic compounds produced by *P. parvum* are fully characterized, researchers working with *Prymnesium* parvum must rely on relative bioassays as indicators of differential toxicity, such as the two used in this work (*R. salina* bioassay and erythrocyte lysis assay). The inability to measure distinct chemical compounds in correlation with observed toxicity is a handicap in terms of concrete characterization of toxic processes in this haptophyte, and is currently a limiting factor in all studies involving this organism. After the spectrum of compounds produced by *P. parvum* is identified and chemically characterized, studies involving this haptophyte can become less speculative and more toxicological in nature.

Understanding the complex ecology of *P. parvum* is a task that will require understanding not only of toxicity, but also the metabolic basis behind this. Techniques such as microarrays as a screening tool for relevant genes are useful in identifying which pathways are regulated. Once relevant pathways are identified, molecular methods such as fluorescent microscopy may help to identify i.e. localization of enzyme or protein activity. Knowing where active cellular processes are localized would help to understand the physiological phenomena. The work in this thesis represents an initial incorporation of this interdisciniplary approach, and will provide the framework for researchers working with *P. parvum* to further investigate the relationship

between toxicity, genes and gene products, as well as to develop a better understanding of the ecology of this haptophyte. Mitigation of harmful algal blooms requires both precise molecular genomic as well as ecological knowledge of triggers and environmental factors that catalyze these events. Interdisciplinary approaches are the most effective way to gain this knowledge, and will no doubt greatly contribute to future understanding of the complex ecology of *P. parvum.*

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