Hemolytic Activity in the Euryhaline Fish-Killing Phytoflagellate Prymnesium parvum under Environmental Stresses

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Abstract

Harmful algal blooms (HABs) are vast expanses of noxious or toxic phytoplankton that periodically dominate coastal ocean waters or freshwater systems. An especially damaging type of HAB are the species that kill fish. In this thesis, a potentially invasive marine fish-killing flagellate, *Prymnesium parvum*, was investigated for its tolerance to freshwater conditions. This species has invaded some freshwater systems in the southern United States. The thesis examines if the growth rate and toxicity of *Prymnesium parvum* remain high under low salinities similar to freshwater systems. A hemolytic lysis assay was used as a proxy for toxicity. The findings presented here indicate that the three *Prymnesium parvum* isolates shared a similar growth and toxicity response, regardless of origin. All isolates could grow well at low salinities and all retain their toxicity. Findings from this thesis indicate that should this species “invade” and establish itself in the Great Lakes, it could thrive and exhibit a toxic phenotype.

Keywords

marine phytoplankton, HABs, harmful algal blooms, *Prymnesium parvum*, hemolytic activity, salinity, light intensity, temperature, nutrients, regulation
Co-Authorship Statement

It is anticipated that this thesis in an abbreviated form will be submitted for publication. The authors of the publication will be myself and my supervisor, Dr. Charles Trick.
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>UTEX</td>
<td>The University of Texas at Austin Culture Collection of Algae (Austin, TX)</td>
</tr>
<tr>
<td>ESAW</td>
<td>enriched seawater artificial water</td>
</tr>
<tr>
<td>HSD</td>
<td>Tukey’s Honestly Significant Difference</td>
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<tr>
<td>( \log_{10} )</td>
<td>logarithm base 10</td>
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<tr>
<td>( \mu \text{M} )</td>
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<tr>
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<td>parts per thousand</td>
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<tr>
<td>( \mu \text{mol} )</td>
<td>micromole</td>
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<tr>
<td>( g )</td>
<td>G-force</td>
</tr>
<tr>
<td>( \mu \text{m} )</td>
<td>micrometer</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>( \mu )</td>
<td>maximal growth rate of cells in batch culture</td>
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<tr>
<td>yield</td>
<td>maximal cell yield in batch culture</td>
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Preface

“In one drop of water are found all the secrets of all the oceans.”

---- Kahlil Gibran

“Even the upper end of the river believes in the ocean.”

--- William Stafford
1. INTRODUCTION

1.1. Harmful algal blooms

Algal blooms, caused by the proliferation of microalgae in aquatic ecosystems due to the abundance of nutrients, can be an important food source for many species and can play a key role in maintaining ecosystems. However, the addition of nutrients (and, indeed, other abiotic and biotic factors) will cause the enhanced growth of selected strains that can often impact an ecosystem in a negative fashion. This includes the accumulation of species that are poorly consumed by the existing food chain, species that impart poor water esthetics and deoxygenation of waters or sediments, and species that contain toxins that are released into the drinking water or accumulate in associated biota. The accumulation of algae that have detrimental effects on the environment and human health, and cause economic losses to fisheries, aquaculture and tourism (Smayda 1997; Hallegraeff 1993) are referred to as harmful algal blooms (HABs). The toxicity of harmful algal bloom species varies depending on algal taxa and the different environmental conditions to which the algae are exposed. Even the most potentially harmful algal taxa are not always toxic because certain conditions may not induce algal toxicity (Van de Waal et al. 2014).

Modern day HAB events are a consequence of modified coastal environments (Anderson et al. 2002; Kudela et al. 2008; Davidson et al. 2012). During HAB events, members of the phytoplankton community that exert adverse effects on the surrounding ecology replace the dominant species of the phytoplankton community (Smayda 1997). The presence of HAB species may result in an alteration of the food chain, a change in water quality or the presence of a marine toxin (Anderson et al. 2002; Horner et al. 1997; Van Dolah 2000). These alterations can negatively influence the relationship between humans and coastal resources (Khan et al. 1997; Tiffany et al. 2001; Kempton et al. 2008). While
there have been reports of HAB events for hundreds of years, modern day HABs are thought to be the result of human modification of coastal water quality (Glibert et al. 2005; Quayle 1969; Hallegraeff 1993; Wells et al. 2015).

There is a consensus that the frequency of HAB events has increased over the last few decades (Smayda 1990; Hallegraeff 2010). Factors that are often implicated in this increased frequency include coastal eutrophication (Anderson et al. 2002; Kudela et al. 2008), such as the increased deposition of new nitrogen to coastal waters (e.g., urea-based fertilizers) (Glibert et al. 2005). Other large-scale factors include climate change, the stabilization of coastal surface waters, and the general increase in human population and the subsequent rise in run-off from coastal societies (Wells et al. 2015; Michalak 2016). There is, at present, no single factor that describes the increased frequency of HAB events.

Harmful algal blooms (HABs) can be generalized into one of three categories. One type of bloom is formed by non-toxic algal species that accumulate a high biomass, and discolour the surface of the water but the cells do not produce a specific toxin (Gomes et al. 2014; Hallegraeff 1993; Hallegraeff 2010). However, these blooms can be harmful by creating anoxic conditions as the bloom decomposes, leading to the death of many fish and invertebrates (Hallegraeff 2010). The depletion of oxygen may be caused by a high respiration rate by the algae or by bacteria during the decay of the bloom (Hallegraeff 1993). The most typical species for this type of bloom is *Notiluca scintillans*, a large (up to 2000 µm in diameter) (Horner 2002) kidney- or balloon-shaped Dinoflagellate. As *N. scintillans* is a cosmopolitan cold-water and temperate species, large blooms frequently occur but in unpredictable locations and seasons (Uhig and Sahling 1990).

The second type of bloom is formed by algal species that have the ability to produce neurotoxins that can bioaccumulate or transfer through the food chain to humans via shellfish and fish consumption (Backer et al. 2005; Isbister and Kiernan 2005). The toxins
of these species can cause many gastrointestinal and neurological illnesses (Anderson et al. 2012). Species of the dinoflagellate genus *Alexandrium* can produce toxins which accumulate in shellfish. These toxins from dinoflagellates can cause paralytic shellfish poisoning (PSP) if affected shellfish are ingested (Hallegraeff 1993; Plumley 1997). Neurotoxins are not limited to dinoflagellates. One emerging toxic phytoplankton genus is *Pseudo-nitzschia* spp. A ubiquitous species, massive blooms have been occurring over the last 20 years, primarily in upwelling areas (Trainer et al. 2015). Under certain environmental conditions members of this genus produce the neurotoxin domoic acid that bioaccumulates through the food web and is associated with the death of marine mammals and humans.

In contrast to the first two bloom categories which involve a wide diversity of taxonomically weakly related species, the third type of algal bloom involves the ichthyotoxic algae, which have a limited taxonomic diversity and a limited number of species with HAB attributes. Most are members of the Raphidophyceae (*Chattonella, Heterosigma, Fibrocapsa*) or Prymnesiophyceae (*Prymnesium, Chrysochromulina*, and *Phaeocystis*) (Edvardsen and Imai 2006). Despite the similarity in physical structure (biflagellated, monad-shaped cells), and ecology (cosmopolitan, with a broad salinity tolerance), 18S rRNA sequence analysis does not support a close genetic relationship between the Prymnesiophyceae and the Raphidophyceae (Potter et al. 1997). Although separated taxonomically, members of these two groups appear to kill fish in a similar, and complicated, fashion. The underlying forms of toxicity of these species (Fig. 1.1) can vary from producing extracellular reactive oxygen species (ROS), cytotoxins or hemolysins to causing mechanical irritation from cell-derived mucus or direct cell contact (Hallegraeff 1993; Hallegraeff 2010; Kim et al. 1999; Kim et al. 2002; Neely and Campbell 2006; Twiner and Trick 2000). Some species of the diatom genus *Chaetoceros* possess barbed spines that cause direct physical damage to the gills of marine animals, while species of the raphidophyte genus *Chattonella* release harmful substances associated with the rupturing of gills, edema or death of aquatic organisms (Hallegraeff 1993; Hallegraeff 2010; Taylor et al. 1994).
Physiological activities associated with a generalized fish kill flagellated phytoplankter. Algal cells attach to the gill of the fish. Cells use exoenzymes to embed into the tissue of the gill. Cells produce neurotoxins and reactive oxygen species that lead to asphyxiation of the fish. Cells release hemolysins that lyse erythrocytes, in order to scavenge nitrogen and iron (Trick, unpublished).

1.2. Prymnesiophytes

There are approximately 300 species of Prymnesiophytes (a.k.a. Haptophytes) (Edvardsen and Imai 2006; Granéli et al. 2012). Harmful forms of Prymnesiophytes include *Prymnesium, Chrysochromulina,* and *Phaeocystis* (Edvardsen and Imai 2006). *Chrysochromulina* produces highly toxic blooms of moderate biomass, while *Phaeocystis* is non-toxic but produces blooms of high biomass (Hallegraeff 1993; Smith et al. 2014). *Prymnesium* forms blooms which are dense and almost monospecific. Blooms are also associated with toxic compounds with ichthyotoxic properties (Granéli et al. 2012). There are six confirmed species of *Prymnesium.* Four are potential fish killers: *P. calathiferum, P. faveolatum, P. parvum* and *P. zebrinum* (Edvardsen and Imai 2006).
1.3. *Prymnesium parvum*

1.3.1. Taxonomy and cellular structure

*Prymnesium parvum* is classified in the class *Prymnesiophyceae*. It is unicellular, subspherical to elongate, and is approximately 8-15 \( \mu \text{m} \) long and 4-10 \( \mu \text{m} \) wide (Green et al. 2007). *P. parvum* has two flagella (12-20 \( \mu \text{m} \) long) used for movement and has a structure, unique to the Haptophytes, called a haptonema (3-5 \( \mu \text{m} \) long). The haptonema is often confused with a flagellum, but is different in function and arrangement of microtubules (Green et al. 2007). The haptonema is used for attaching to surfaces and cells, and, putatively, for injecting toxins (Granéli et al. 2012).

The primary pigments within *P. parvum* cells are chlorophyll a and c, fucoxanthin, 19-hexanoyloxyfucoxanthin, 19-butanoyloxyfucoxanthin and 19-hexanoyloxyfucoxanthin. The xanthin pigments give the cells their golden-brown appearance (Beltrami et al. 2007).

1.3.2. Physiological ecology

*Prymnesium parvum* can tolerate a broad range of temperatures, between 2-30°C, and is widely distributed throughout temperate regions (Edvardsen and Paasche 1998; Valenti et al. 2010). Cells grows best when exposed to moderate to low light (< 100 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)). However, there have been some incidences where cells preferred higher light (> 100 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)) (Larsen and Bryant 1998; Parnas et al. 1962). *P. parvum* is also euryhaline, enabling cells to inhabit marine, estuarine and inland systems at salinities of 0.8-100 ppt (Edvardsen and Paasche 1998; Valenti et al. 2010). *P. parvum* blooms occur in low salinity and brackish or coastal waters, particularly in eutrophic, warm waters (Edvardsen and Paasche 1998; Igarashi et al. 1999). *P. parvum* blooms are
associated with golden coloured waters and cause damage to both fish culture and wild biota (Granéli et al. 2012; Yasumoto 2001).

Harmful *Prymnesium* blooms have occurred in Europe, Israel, China, North America, Russia, Morocco and Australia (Guo et al. 1996; Edvardsen and Paasche 1998). The first recorded blooms of *P. parvum* in North America were identified in Texas (Roelke et al. 2010). Since then, recurrent *P. parvum* blooms have occurred in rivers, lakes and reservoirs in Texas at salinities up to 1 ppt (Edvardsen and Imai 2006). Most blooms in Texas have occurred in the cooler fall to spring months, suggesting that temperature is an important factor in the occurrence of *P. parvum* blooms. The amount of light received in Texas reservoirs varies with season, and salinity varies with location, suggesting that these are also important factors contributing to bloom formation (Baker et al. 2007). *P. parvum* blooms have resulted in more than an estimated 30 million fish killed and tens of millions of dollars in lost revenue in Texas reservoirs alone (Brooks et al., 2010). *P. parvum* has also invaded reservoirs and river systems in 15 other states, spanning west to California and north to North Carolina (Roelke et al. 2010).

1.3.3. Toxicology

Reports indicate that blooms can exist in either toxic or non-toxic states and that the toxicity can change over time within a single bloom (Bertin et al. 2012). Bioactive secondary metabolites have been isolated and linked to cell-based toxicity. These metabolites include two polyether compounds called prymnesins (Igarashi et al. 1998; Igarashi et al. 1996; Igarashi et al. 1999; Yasumoto 2001). Intriguingly, there has been no evidence of the presence of these types of compounds in *P. parvum* blooms.

However, when blooms of *P. parvum* form there is evidence of an impressive array of activities: ichthyotoxic, neurotoxic, cytotoxic, hepatotoxic, antibacterial, hemolytic and allelopathic activities toward many organisms (Larsen and Bryant 1998; Granéli and
Johansson 2003; Granéli 2006; Granéli et al. 2012; Yariv and Hestrin 1961). The multitude of biological activities displayed by *Prymnesium* may be caused by one multifaceted toxin or may be caused by a mixture of different toxins (Shilo 1967). In addition, the activities are not constitutively present but each varies under different environmental conditions (Johansson and Granéli 1999).

The ichthyotoxic activity of *Prymnesium* is targeted to the tissues of gills, which leads to a loss of selective permeability and disturbance in ion balance (Johansson and Granéli 1999; Vasas et al. 2012). The putative mechanism of action of the neurotoxin is to block neuronal and muscle action potentials (Festoff 1975). Excessive amounts of extracellular reactive oxygen species (ROS), such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (•OH), alter gill structure and function. Neurotoxin and ROS production then leads to the asphyxiation of the affected organism (Oda et al. 1997; Twiner and Trick 2000). It is also believed that blood vessels are damaged in order to scavenge nitrogen and iron from the blood, leading to the lysis of erythrocytes (Ling and Trick 2010).

1.3.3.1.  *Regulation of Toxicity: Unbalanced Growth*

*Prymnesium* toxic activities vary under different growth conditions. Toxin production occurs when cells are physiologically stressed (Manning and La Claire II 2010). Toxicity has been found to be affected primarily by light, temperature, salinity, and phosphorus (P) and nitrogen (N) limitation. Other factors that affect toxicity include cell density, growth phase, pH, cofactors (divalent cations and streptomycin), cationic polyamines, and strong aeration (Edvardsen and Imai 2006; Granéli 2006; Paster 1973; Manning and La Claire II 2010; Shilo 1967; Shilo 1971; Valenti et al. 2010).

Balanced growth is when cellular components are produced at constant rates that are relative to environmental conditions, thus allowing for maximum rates of cell division (Willey et al. 2013). If environmental conditions are altered, unbalanced growth may
occur. Unbalanced growth may occur when the rate of cellular division is lower than the rate of nutrient acquisition and formation of metabolites in the cell. Nutrient limitation leads to a slowing of cellular division, while carbon accumulation remains high, leading to an accumulation of secondary metabolites, such as toxins (Granéli and Flynn 2006; Johansson and Granéli 1999; Plumley 1997). Limiting environmental conditions and the resulting decrease in growth rate have been associated with an increase in Prymnesium toxicity (Manning and La Claire II 2010). Prymnesins are carbon-rich compounds, so it is possible that they are produced to store excess carbon that is made available during photosynthesis under nutrient limiting conditions (Granéli 2006). This increase in prymnesin production may then lead to an increase in toxicity.

1.3.3.2. Regulation of Toxicity: Light Intensity

Several studies have reported that prymnesin synthesis and activity are influenced by irradiance quality and intensity (Guo et al. 1996; Larsen et al. 2012; Shilo 1967). Reich et al. (1965) determined that cultures exposed to constant light lacked ichthyotoxicity, but demonstrated hemolytic activity. One explanation for this apparent disparity is that the ichthyotoxic compounds were inactivated or degraded quicker than the hemolytic compounds. On the other hand, this may have occurred because there are more factors responsible for hemolytic activity than there are for ichthyotoxicity (Reich et al. 1965). Parnas et al. (1962) observed an inactivation of exotoxins when exposed to UV and visible wavelengths of light. However, intracellular toxins were not affected (Parnas et al. 1962). This suggests that these toxin compounds may be protected or stored in vesicles (Manning and La Claire II 2010). To add to this complication, Larsen et al. (2012) revealed that different isolates of P. parvum responded differently to light with regards to hemolytic activity.

The poor correlation between light exposure and toxicity may be due to mixotrophy in some isolates of P. parvum; thus, some isolates feed in the absence of light and may not
require light for toxin production, once there is enough food to provide carbon skeletons for the synthesis of prynmesin (Manning and La Claire II 2010). Contrary to this theory, Shilo (1967) did not observe toxin synthesis in heterotrophic conditions in the dark. However, cell multiplication did occur. Cultures grown in the dark and then exposed to 24 hours of light displayed hemolytic activity (Shilo 1967).

1.3.3.3. **Regulation of Toxicity: Temperature**

The ideal temperatures for *P. parvum* growth are between 25 and 30°C (Granéli et al. 2012). Toxin production has mostly been observed at temperatures of 5-30°C (Baker et al. 2007; Edvardsen and Imai 2006; Larsen and Bryant 1998). However, the ideal temperatures for growth and toxin production vary among different isolates (Manning and La Claire II 2010). Some studies have shown that *P. parvum* toxicity is not affected by temperature, while other studies have found that toxicity is increased by extreme (low and high) temperatures (Granéli et al. 2012; Larsen et al. 2012). Baker et al. (2007) observed an interaction between temperature and salinity, with the optimum temperature for growth decreasing with a decrease in salinity. This shift may help explain the occurrence of blooms during different seasons in different locations (Baker et al. 2007). Toxicity is believed to increase when the alga is exposed to temperatures that are not ideal for growth (Granéli et al. 2012).

1.3.3.4. **Regulation of Toxicity: Salinity**

The effect of salinity on toxicity has been the focus of many studies due to the increased prevalence of *Prymnesium* blooms in low salinity waters (Manning and La Claire II 2010). Most reported blooms have occurred in highly mineralized waters at salinities between 3-8 ppt (Guo et al. 1996; Manning and La Claire II 2010). The ideal salinity for the growth of *P. parvum* has been observed to be between 3-50 ppt, while salinities between 50-120 ppt inhibit toxin production (Manning and La Claire II 2010; Paster
1973). However, variations in optimal salinities for growth have been observed among isolates (Larsen and Bryant 1998).

Some studies have revealed that toxicity increases when salinity is not ideal for growth, while other studies have found no effect of salinity on toxicity (Manning and La Claire II 2010; Granéli et al. 2012; Larsen et al. 2012). The growth of *P. parvum* is negatively affected by low salinities, with growth being greatly reduced below 1 ppt (Baker et al. 2007; Baker et al. 2009). Baker et al. 2007 observed maximum toxicity to juvenile fish at extreme (low and high) values of salinity, while maximum growth and abundance were observed at moderate salinities. Paster (1973) found ichthyotoxicity to be highest in cultures grown at a salinity of 0.75 ppt and lowest in cultures grown at 4.5 ppt. At salinities higher than 4.5 ppt, no ichthyotoxicity was observed (Paster 1973). However, the absence of secreted toxins is not directly linked to intracellular toxin production, as toxins can still be extracted from the cells themselves (Paster 1973).

Hemolytic activity has been found to be inversely correlated with salinity. The highest hemolytic activity was observed for cultures grown at 10 ppt (Manning and La Claire II 2010; Larsen et al. 2012). In contrast, Larsen et al. (2012) observed a decrease in toxicity with an increase in salinity. However, this may have been caused by the different isolates responding differently to environmental conditions (Larsen et al. 2012).

1.3.3.5. Regulation of Toxicity: Nutrients

Nutrient limitation leads to a decrease in the intracellular levels of the limiting nutrient within phytoplankton (Johansson and Granéli 1999). With regards to *P. parvum*, N and P deficiencies lead to increased toxin production (Granéli et al. 2012). In a study conducted by Johansson and Granéli (1999), N and P limitation both caused increased toxicity in *P. parvum*. Carbon content (associated with nutrient limitation) in P-limited *P. parvum* cells did not differ significantly from that of non-limited cells, suggesting that P only has a
moderate limiting effect (Johansson and Granéli 1999). In contrast, other studies have shown that a P deficiency leads to a greater increase in toxicity than did N deficiency (Granéli et al. 2012).

Anthropogenic inputs, such as agricultural runoff, into aquatic systems increase N and P levels (Manning and La Claire II 2010; Granéli et al. 2012). This results in an increase in the growth rate of *P. parvum* and the formation of blooms (Manning and La Claire II 2010). Ultimately, this leads to a nutrient imbalance which decreases the growth rate of *P. parvum* and increases its toxicity (Manning and La Claire II 2010; Granéli et al. 2012).

Most studies focus on the effects on macro-nutrient (N and P) limitation on growth and toxicity. However, the limitation of micro-nutrients, such as Fe, also affects the growth of *Prymnesium*. Therefore, it can be presumed that micro-nutrient limitation would also affect toxicity. Studies have shown that during iron (Fe) limitation phytoplankton alter their Fe acquisition strategy by reducing Fe (III) chelates and ingesting insoluble Fe (Rahman et al. 2014). Rahman et al. (2014) showed that *P. parvum* exhibited slower growth in Fe-deplete media. They also showed that *P. parvum* differentially synthesizes some proteins under Fe-limitation, which may help with altering their Fe acquisition strategy to adapt to the Fe-limitation (Rahman et al. 2014).

1.3.3.6. Regulation of Toxicity: Cell Density

It has been documented that *Prymnesium* blooms can reach densities of $10^6$ to $10^7$ cells/L (Roelke et al. 2010). Some studies claim that cell density is a significant factor that affects *P. parvum* toxicity (Larsen et al. 1998), while other studies insist that cell density does not have a large effect on toxicity (Valenti et al. 2010). One study demonstrated a positive correlation between the density of *P. parvum* cells and the amount of exotoxins produced (Paster 1973). Granéli (2006) found that only *P. parvum* cells growing under steady-state conditions showed increasing toxicity with increasing cell density. However,
other studies suggest that growth and toxicity are regulated by different factors (Manning and La Claire II 2010). In support of the latter, Guo et al. (1996) observed that cell density and the presence of extracellular toxins had no defined relationship. There have also been cases where *P. parvum* blooms of low density are highly toxic, and dense blooms display no toxicity (Shilo and Aschner 1953; Shilo 1967).

1.3.3.7. Regulation of Toxicity: Growth Phase

The toxicity of *P. parvum* is believed to vary with its growth phases (lag phase, exponential phase, and stationary phase) (Manning and La Claire II 2010). Shilo (1967) observed that cultures grown in suitable media synthesized the greatest amount of toxins during the late exponential growth phase, and this continued throughout the stationary phase. Additionally, Guo et al. (1996) discovered that ichthyotoxicity decreased during the rapid growth phase (exponential phase) and increased when *P. parvum* cells reached limiting conditions (stationary phase). Paster (1973) reported prymnesin synthesis throughout *P. parvum*’s life cycle and release of the compounds only after cell death and disintegration of the cellular components. Secondary metabolism usually increases when balanced growth has stopped, so this may explain the increase in *P. parvum* toxin production during stationary phase (Plumley 1997).

1.4. Measuring Toxicity

Commonly used methods for measuring toxicity include, direct measurements of the compound and indirect measurements of the mode of action or properties of the compound. Direct measurements include techniques such as mass spectroscopy, chromatography and capillary electrophoresis (Holland 2008). These techniques are used for separating crude, impure samples and do not provide any indication of the mode of action or toxicity of the compound. Indirect measurements include, bioassays (toxicity to other species), enzyme inhibition and activation assays, radioimmunoassays, gill cell
viability assays, mammalian cell viability assays and hemolytic assays (Bertin et al. 2012; Holland 2008; Larsen et al. 1998; Johansson and Granéli 1999; Granéli 2006; Valenti et al. 2010).

1.5. Hemolytic Activity

The measurement of hemolytic activity is the most sensitive way to assay prymnesin (Paster 1973). Hemolytic activity is directly proportional to the overall toxicity of the organism. The hemolytic reaction consists of two temperature-dependent phases: pre-lytic and lytic (Paster 1973). The pre-lytic phase is characterized by the binding of prymnesin to the erythrocyte membrane, causing the membrane to transform and become more permeable. The release of hemoglobin does not occur until the lytic phase, where rapid hemolysis occurs until a maximum effect is reached (Paster 1973). The compounds lecithin and cholesterol inhibit the pre-lytic phase at low concentrations, indicating that inhibition is not due to osmotic stabilization (Paster 1973). It appears to be a competitive inhibition reaction between the inhibitor and prymnesin. The compounds compete for the same target site. Lysis inhibition occurs via the formation of a prymnesin-inhibitor complex (Paster 1973). Since cholesterol and lecithin are two components of the erythrocyte membrane, the membrane is assumed to be the attaching site for prymnesin (Paster 1973).

1.6. Expansion of *P. parvum* in North America

Fish kills caused by *P. parvum* blooms were first identified in Europe (Baker et al. 2007). It has been suggested that in the late 1800s, *P. parvum* was responsible for fish kills in the Baltic Sea, however, this was not confirmed (Manning and La Claire II 2010). In 1920, Liebert and Deerns reported fish kills in Holland caused by a phytoflagellate toxin (Manning and La Claire II 2010; Shilo and Aschner 1953). This was followed by a study in 1939 by Otterstroem and Steeman-Nielsen that identified *P. parvum* as the cause of a
similar fish-killing event in Denmark. Reich and Aschner then reported the occurrence of *P. parvum* blooms in Israel in 1947 (Baker et al. 2007; Shilo and Aschner 1953). Blooms were later reported in many other regions worldwide, such as the coastal waters of Norway, China, Holland, Spain, Russia, Morocco and Australia (Baker et al. 2007; Edvardsen and Paasche 1998; Guo et al. 1996; Shilo and Aschner 1953). In 1985, the first blooms of *P. parvum* in North America were identified in the Pecos River in Texas (Baker et al. 2007; Roelke et al. 2010). Since then, reports of fish kills in freshwater systems due to *P. parvum* have been increasing (Manning and La Claire II 2010). Recurrent blooms of *P. parvum* continue to occur in rivers, lakes and reservoirs in Texas (Edvardsen and Imai 2006). Furthermore, *P. parvum* has invaded reservoirs and river systems in 15 other states, spanning from California to North Carolina (Roelke et al. 2010).

1.7. Study statement

1.7.1. Hypothesis

The principal hypothesis of this study is that *P. parvum* maintains ichthyotoxic activities when exposed to sub-optimal growth conditions (conditions that do not support maximal growth). As species expand into new locations, the species must maintain a positive net growth and the physiological characteristics that are associated with toxicity. The environmental conditions that the cells will encounter in freshwater systems include reduced salinity, altered nitrogen supply and modified iron supply. For this reason, the growth and toxicity of *P. parvum* were tested under these conditions.
1.7.2. Objectives

To test the above hypothesis, multiple objectives were set. The first objective was to establish the impact of salinity and light intensity on growth and hemolytic activity of three *P. parvum* isolates. Obtained knowledge will help in the prediction of the expansion of *P. parvum* from coastal waters to freshwaters and will provide evidence on whether the cells remain toxic or if toxicity per cell is enhanced under sub-optimal saline conditions.

The second objective was to detect how changing temperature affects growth and hemolytic activity. Freshwater systems (in the summer periods) tend to be significantly warmer than coastal marine environments and could serve as a barrier to expansion if elevated temperatures depressed growth significantly. Similarly, elevated temperatures may have no impact on growth, but the levels of toxicity may be affected. Thus, the relationship between temperature and toxicity is key in understanding the consequence of the expansion of *P. parvum*.

The third and fourth objectives were to gauge the toxicity and growth of *P. parvum* under altered N-source (ammonium, nitrate, urea) and iron conditions (growth replete and growth deplete). N-sources and iron concentrations are locally or regionally variable, and thus this knowledge will indicate if there are either nutrient barriers or locations of enhancement in the mosaic of freshwater conditions.
2. MATERIALS AND METHODS

2.1. Algal cultures and culturing conditions

Three isolates of *P. parvum* were chosen for this study (Table 2.1). Isolates of *P. parvum* varied in their location of origin and time spent in culture (Table 2.1). All isolates were obtained and maintained as uni-algal, non-axenic isolates. Isolates UTEX 995, UTEX 2797, and UTEX 2827 were obtained from The University of Texas at Austin Culture Collection of Algae (UTEX). Each isolate was reported to have fish-killing attributes. UTEX 995 represents a species that is common in Europe and thrives in estuarine conditions. The other two isolates (UTEX 2797 and UTEX 2827) represent newly observed bloom species that are expanding into freshwater systems of the southern United States.

Stock cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL of culture medium. Cells were maintained in nutrient enriched artificial seawater (ESAW) (Harrison et al. 1980), supplemented with f/20 nutrients and metals and vitamins (Guillard 1975). The medium was modified by omitting the addition of silica. All glassware and media were sterilized by autoclaving (135°C and 30 psi, 60 min duration). Cultures were incubated without shaking in a growth chamber at 18°C under a continuous light flux of 90-100 μmol photons m⁻² s⁻¹. Irradiance was supplied by cool white fluorescent lamps. Irradiance was measured using a Quantum Scalar Laboratory 2100 irradiance sensor (Biospherical Instruments, San Diego, CA).
Table 2.1 Origins and isolation dates of the *Prymnesium parvum* isolates obtained from the UTEX Culture Collection of Algae.

<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Geographic Origin</th>
<th>Year of Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>995</td>
<td>River Blackwater, Essex, England</td>
<td>1952</td>
</tr>
<tr>
<td>2797</td>
<td>Texas Colorado River, Texas, USA</td>
<td>2002</td>
</tr>
<tr>
<td>2827</td>
<td>Oyster Rake Pond, near Charleston, South Carolina, USA</td>
<td>2002</td>
</tr>
</tbody>
</table>

2.2. Experimental design

For all experiments, stock cultures served as inoculum, with 10% (v/v) added to multiple flasks containing experimental culture medium. Experimental cultures were maintained in autoclaved culture maintenance medium (listed above). Cultures were sampled daily for biomass measurements and cells from specific cultures were harvested for use in the erythrocyte lysis assay (ELA). For all experiments, cells were first acclimated in stock cultures under the appropriate growth conditions and the growth of these cultures was measured. Cells from these stock cultures were then used as inoculum for experimental cultures. Three replicate flasks per treatment were used for all experiments. All experiments were performed two to three times.

2.3. Biomass measurements

For growth experiments, direct cell counts were performed daily using a Turner Designs C6 flow cytometer (Becton-Dickinson, NJ USA) to calculate cell density (cells·mL⁻¹).
Cell counts were determined using the FL-3 channel (chlorophyll-a cell\(^{-1}\)) and the FSC (forward scatter for particle density). The growth rate (\(\mu, \text{d}^{-1}\)) of \textit{P. parvum} cultures was determined using the following equation:

\[
\mu = \frac{\ln(N_t) - \ln(N_0)}{\Delta t}
\]

where \(N_t\) is the population size at time (t) point two, \(N_0\) is the population size at time (t) point one and \(\Delta t\) is the difference between time point two and time point one. The yield (\(Y, \text{in units cells} \cdot \text{mL}^{-1}\)) of \textit{P. parvum} cultures was determined by averaging the cell densities from three consecutive days once the cultures entered stationary phase.

### 2.4. Preparation of algal samples

\textit{P. parvum} isolates 995, 2797 and 2827 were chosen because they have been reported by UTEX to have fish-killing attributes, and therefore may be hemolytic. Additionally, they allow for differences in growth and hemolytic activity between isolates from different geographic locations (Table 2.1) to be investigated. Algal samples for the Erythrocyte Lysis Assay (ELA) were prepared following a modified protocol of Eschbach et al. (2001). Samples were prepared from exponentially growing cultures (3 days after inoculation). Cell counts of the algal cultures were performed using a Turner Designs C6 flow cytometer. Cell cultures for all experiments were diluted to 15 x 10\(^3\) cells·mL\(^{-1}\) and 10 x 10\(^3\) cells·mL\(^{-1}\) in ELA buffer (150 mM NaCl, 3.2 mM KCl, 1.25 mM MgSO\(_4\), 12.2 mM TRIS base, and pH adjusted to 7.4). Intact algal cells were used to determine whether the haptonema is necessary for delivering the hemolytic compounds to red blood cells. Lysed algal cells were used to determine if the compounds are stored within the cells. Appropriate densities of intact and lysed algal cells in a volume of 15 mL were used. Algal cells were lysed by sonication (90 s on ice, at an output power of 6 with a Virsonic
100 Ultrasonic Cell Disrupter (VirTis Company, Gardiner, NY). Microscopic observation confirmed the complete lysis of cells.

2.5. Preparation of erythrocytes

Rabbit blood [50% whole blood: 50% Alsever’s (isotonic salt solution), v:v] was obtained from PML Microbiologials, Wilsonville, Oregon and stored in the dark at 4-8°C. The protocol for erythrocyte preparation from Eschbach et al. (2001) was followed. There were two methodological changes: the erythrocytes were centrifuged at a speed of 1000 g instead of 2000 g and rabbit erythrocytes were used instead of carp erythrocytes. Erythrocytes were centrifuged in a 13 mL centrifuge tube at 1000 g for 5 min at 4˚C. A Beckman GH-3.8/GH-3.8A swing-out rotor (Beckman Coulter, Fullerton, CA) was used. Pelleted erythrocytes were washed twice in ELA buffer by vortexing the sample, followed by centrifugation. Erythrocyte suspensions were diluted to $10^7$ cell counts in ELA buffer. Erythrocytes were counted using a hemocytometer.

2.6. Erythrocyte lysis assay

The protocol for the ELA from Eschbach et al. (2001) was followed. However, it was performed using larger volumes in larger vessels instead of 96-well microtitre plates. Equivolume amounts of erythrocytes and intact or lysed algal cells were incubated in 1.5 mL clear, conical bottom polypropylene microfuge tubes (Axygen Scientific, Union City, CA). A total volume of 1 mL was used for the assay and final cell densities of $5 \times 10^6$ cells·mL$^{-1}$ erythrocytes, and either $5 \times 10^3$ cells·mL$^{-1}$ and $7.5 \times 10^3$ cells·mL$^{-1}$ algal cells were obtained. Whole erythrocytes and lysed erythrocytes (sonicated for 90 s on ice at an output power of 6) were incubated in ELA buffer alone and used as negative and positive controls, respectively. Lysed erythrocytes were examined with a microscope to confirm complete lysis. Algal samples incubated in ELA buffer alone were controls for
any background absorbance of algal cells in the samples. Samples were incubated for six hours at 18-20°C under a continuous light flux of 30-50 µmol photons m⁻² s⁻¹.

After incubation, each sample was centrifuged at 2000 g for 5 min. at 18°C (Eppendorf 5415 D, Eppendorf North America Inc., Westbury, NY) and the supernatants were transferred into polymethylmethacrylate disposable cuvettes with a path length of 10 mm. The absorbance of hemoglobin molecules released from the erythrocytes was measured with a CARY 300 spectrophotometer (Agilent Technologies, Santa Clara, CA) at a wavelength of 414 nm. Lysed erythrocytes release hemoglobin into solution proportional to the amount of cell lysis. The hemolytic activity of each experimental sample was expressed as the percentage of hemolysis relative to the positive and negative controls. The following equation was used:

\[
\% \text{ hemolysis relative to control} = (100) \times \frac{E_{414} - A_{414} - N_{414}}{P_{414}}
\]  

(2)

where \(E_{414}\), \(A_{414}\), \(N_{414}\), \(P_{414}\) is the absorption at 414 nm of the experimental sample (erythrocytes incubated with algal sample), algal sample, negative control (whole erythrocytes) and positive control (lysed erythrocytes), respectively. Samples with a percentage of hemolysis above zero were considered hemolytic, and samples at zero were considered non-hemolytic.

2.7. Standardization of ELA design

2.7.1. Erythrocyte lysis by saponin

Saponin (Sigma-Aldrich, St. Louis, MO), a glycoside extract from the soapbark tree \((Quillaja saponaria)\) was used as a positive control to fully lyse the erythrocytes. It is a
chemically defined hemolytic agent used to standardize the ELA. Erythrocytes were incubated with a range of saponin concentrations (0-20 µg·mL⁻¹) under the same conditions described for the ELA, and a standard curve was obtained.

2.7.2. Incubation time

Eschbach et al. (2001) reported total lysis of erythrocytes by *P. parvum* after an incubation time of 4 h. However, to further establish an incubation time for the detection of *P. parvum* hemolytic activity, intact *P. parvum* isolate 2797 cells were exposed to erythrocytes for three different periods (2, 4, and 6 h).

2.7.3. Incubation irradiance

To determine the appropriate light intensity at which to perform the ELA experiments, different samples were incubated at varying intensities (0-90 µmol photons m⁻² s⁻¹). The hemolytic activity of *P. parvum* isolate 2797 was compared to the saponin treatment, *Heterosigma akashiwo* isolate 513 (a known ichthyotoxic flagellate), and *Tetraselmis suecica* isolate 906 (a known non-toxic flagellate) as further controls for the ELA experiment. Intact cells were used for each algal sample.

2.7.4. Incubation temperature

It has been reported that *P. parvum* hemolytic activity is temperature dependent (Paster 1973). For this reason, different samples were incubated at a range of temperatures (12-38°C) to determine the appropriate temperature to perform the ELA experiments. The hemolytic activity of *P. parvum* isolate 2797 was compared to the saponin treatment, *H. akashiwo* isolate 513 and *Tetraselmis suecica* isolate 906 as further controls for the ELA experiment. Intact cells were used for each algal sample.
2.8. Salinity and light intensity experiments

Stock cultures of the *Prymnesium* isolates were usually maintained in f/20 media at a salinity of 30‰ under a continuous light flux of 90-100 µmol photons m\(^{-2}\) s\(^{-1}\). For the salinity and light intensity experiments, stock cultures were maintained in f/20 media of varying salinities (1-30‰), under a range of intensities (10-180 µmol photons m\(^{-2}\) s\(^{-1}\)). These stock cultures were allowed to grow for three days at the appropriate conditions before being inoculated for experimental cultures into the appropriate medium under the appropriate light intensity.

2.9. Temperature experiments

Stock cultures of the *Prymnesium* isolates were usually maintained at 18-20°C. However, for the temperature experiment stock cultures were grown at temperatures of 12°C, 18°C and 23°C. These stock cultures were allowed to grow for three days before being used for inoculum for experimental cultures that were then grown at the appropriate temperature.

2.10. Nitrogen experiments

*Prymnesium* isolates were usually maintained in f/20 medium with 88 µmol nitrate. For the N experiments, cultures were first starved of N to ensure that growth and the production of hemolytic metabolites were due to the N source provided, rather than reserves of the previous N source. To do this, cells in N replete medium were inoculated into f/20 medium minus the nitrate. These cultures were grown for seven days before being inoculated into f/20 media with one of three different N sources – nitrate, ammonium and urea – at varying concentrations, 25-500 µmol N. Stock cultures were allowed to grow for three days before being inoculated into different flasks with the same media for experimental cultures.
2.11. Iron chelator experiments

Cultures of *Prymnesium* isolates were usually maintained in f/20 medium with 11.7 µM Fe. Desferroxamine B (DFB), a chemical chelator that binds to Fe at a 1:1 ratio, was added to the f/20 media at concentrations ranging from 0-100 µM. A larger concentration of DFB resulted in a lower amount of free Fe in the media and a higher amount of chelated Fe. Stock cultures were transferred from the Fe replete medium into the media with the appropriate concentration of DFB. These cultures were allowed to grow for seven days before being used as inoculum for experimental cultures.

2.12. Statistical analyses

Statistical analyses were performed using the statistical protocols and the “ez” statistical package (Lawrence 2015) in R 3.3.0 (R Core Team 2013). One-way or two-way ANOVAs (α ≤ 0.05) were used for growth experiments to compare growth rates and yields where appropriate. All significant ANOVAs were followed by Tukey’s multiple comparison tests (α ≤ 0.05) to examine differences. Two-way ANOVAs that yielded a non-significant interaction were rerun without the interaction to examine main effects. Split-plot ANOVAs (α ≤ 0.05) were used to evaluate the hemolytic experiments. P-values obtained from the Greenhouse-Geisser Correction (α ≤ 0.05) were used for tests that violated sphericity. Significant interactions were further investigated with two-way ANOVAs and Tukey’s multiple comparison tests. Before conducting the ANOVA, the percentage values (0-100%) were changed to the proportion of hemolysis (0-1.0), and negative values were set to zero prior to transformation. The data were then transformed with the arcsine transformation.
3. RESULTS

3.1. Standardization of ELA design

To optimize the detection of the hemolytic activity of *P. parvum*, preliminary experiments were performed to determine incubation conditions (time, temperature and irradiance), and algal sample preparation (growth phase and density). ELA experiments were performed, with slightly modified conditions, in order to determine optimal conditions for the detection of hemolytic activity of *P. parvum*.

3.1.1. Saponin-induced erythrocyte lysis

Saponin, a known hemolytic substance, was used as a positive control for the ELA. Saponin had a significant effect on hemolysis (Fig. 3.1; one-way ANOVA, $F_{7,8} = 36.95$, $p < 0.001$), causing concentration-dependent hemolysis up to ca. 2 µg·mL$^{-1}$. Beyond this concentration, hemolysis plateaued at the maximal level (Fig. 3.1). Complete hemolysis was observed at ca. 3 µg·mL$^{-1}$ of saponin and 50% hemolysis was observed ca. 1 µg·mL$^{-1}$ (Fig. 3.1).
3.1.2. Algal samples and incubation conditions

3.1.2.1. *Algal sample preparation*

A representative growth curve (Fig. 3.2) of *P. parvum* isolates grown in f/20 enriched ESAW shows the differences and similarities in growth between the isolates. It also demonstrates the exponential and stationary growth phases. For all ELA experiments, samples were prepared from cultures in exponential phase (day 3). Stationary phase for isolates 2797 and 2827 occurred after ca. 5 days of growth and for isolate 995, after ca. 7 days. The growth rates for isolates 995, 2797 and 2827 were determined to be ca. 0.317 day\(^{-1}\), 0.527 day\(^{-1}\), and 0.648 day\(^{-1}\), respectively (Fig. 3.3). Isolate 995 was significantly slower growing than isolates 2797 and 2827 (Fig. 3.3; one-way ANOVA, \(F_{2,6} = 10.4, p <\) 0.05).
Yields for isolates 995, 2797 and 2827 were determined to be ca. 16.7 x 10^4 cells·mL\(^{-1}\), 40.1 x 10^4 cells·mL\(^{-1}\), and 38.2 x 10^4 cells·mL\(^{-1}\), respectively (Fig. 3.4). Isolate 995 also had a significantly lower yield than isolates 2797 and 2827 (Fig. 3.4; one-way ANOVA, F\(_{2,6}\) = 4.68, p = 0.05).

Figure 3.2 Growth curves of *P. parvum* isolates grown in f/20 enriched ESAW. Growth is expressed as log\(_{10}\) of cell density. Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Exponential and stationary phases are labelled.
Figure 3.3 Growth rates ($\mu$) of *P. parvum* isolates grown in f/20 enriched ESAW. Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with an asterisk (*) are statistically different from the other bars ($p < 0.05$) according to Tukey’s multiple comparison test.

Figure 3.4 Yields (Y) of *P. parvum* isolates grown in f/20 enriched ESAW. Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with an asterisk (*) are statistically different ($p < 0.05$) from the other bars according to Tukey’s multiple comparison test.
3.1.2.2. **Incubation time**

Incubation time had no significant effect on hemolytic activity (Fig. 3.5; one-way ANOVA, $F_{2,3} = 0.306, p = 0.757$). Hemolytic activity was independent of incubation time, provided incubation time was at least 2 h. However, at 6 h less variability between samples occurred (Fig. 3.5). For this reason, an incubation time of 6 h was employed for all subsequent experiments.

![Figure 3.5](image)

**Figure 3.5** Effect of incubation time (2-6 h) on hemolytic activity of intact *P. parvum* isolate 2797 cells. Values represent the average of two assays (each performed in triplicate) ± standard deviation (n = 2). Incubation time did not have a significant effect on hemolytic activity ($p > 0.05$).

3.1.2.3. **Incubation irradiance**

Incubation irradiance did not significantly affect hemolysis (Fig. 3.6; two-way ANOVA, $F_{4,32} = 1.17, p = 0.344$). However, significant differences were observed in the hemolytic activity of the following samples: *P. parvum* and *H. akashiwo*, *P. parvum* and *T. suecica*, *H. akashiwo* and *T. suecica*, saponin and *H. akashiwo*, and saponin and *T. suecica* (Fig. 3.6; two-way ANOVA, $F_{3,32} = 210.7, p < 0.001$). *P. parvum* and saponin displayed higher
hemolytic activity than *H. akashiwo* and *T. suecica*. Although incubation irradiance did not significantly affect hemolytic activity, samples for all subsequent ELA experiments were still incubated under 30-50 µmol photons m⁻² s⁻¹ light. This is because it has been reported that light is necessary for toxin activation for several algal species (Kuroda et al. 2005).

![Image of a bar chart showing the effect of incubation irradiance on hemolysis of different algal species and saponin. The graph indicates that saponin and *P. parvum* were more hemolytic than *H. akashiwo* and *T. suecica*, and *H. akashiwo* was more hemolytic than *T. suecica*.](image)

**Figure 3.6** Effect of incubation irradiance (0-90 µmol photons m⁻² s⁻¹) on hemolytic activity of intact *P. parvum* (isolate 2797), *H. akashiwo* (isolate 513) and *T. suecica* (isolate 906) cells, and saponin. Values represent the average of two assays (each performed in triplicate) ± standard deviation (n = 2). Incubation irradiance did not have a significant effect on hemolytic activity (p > 0.05). However, as indicated by the asterisks (******), statistical differences (p < 0.001) existed between samples according to Tukey’s multiple comparison test. Saponin and *P. parvum* were more hemolytic than *H. akashiwo* and *T. suecica*, and *H. akashiwo* was more hemolytic than *T. suecica*.

### 3.1.2.4. Incubation temperature

Both incubation temperature and algal sample had significant effects on hemolysis (Fig. 3.7; two-way ANOVA, F₃,₁₆ = 39.47, p < 0.001; F₃,₁₆ = 101.9, p < 0.001). Samples incubated at 38°C displayed significantly lower hemolytic activity than the samples
incubated at the other temperatures (Fig. 3.7; two-way ANOVA, \( F_{3,16} = 39.47, p < 0.001 \)). This may have been due to denaturing of the toxins. Significant differences were observed in the hemolytic activity of the following samples: \( P. \) \( parvum \) and \( H. \) \( akashiwo \), \( P. \) \( parvum \) and \( T. \) \( suecica \), \( H. \) \( akashiwo \) and \( T. \) \( suecica \), saponin and \( H. \) \( akashiwo \), and saponin and \( T. \) \( suecica \) (Fig. 3.7; two-way ANOVA, \( F_{3,16} = 101.9, p < 0.001 \)). \( P. \) \( parvum \) and saponin treatment elicited higher hemolytic activity than \( H. \) \( akashiwo \) and \( T. \) \( suecica \). Samples of \( P. \) \( parvum \) and saponin showed greater hemolytic activity at 12 and 18°C, while samples of \( H. \) \( akashiwo \) showed greater activity at 26°C and \( T. \) \( suecica \) showed consistently low activity at all temperatures (Fig. 3.7). An incubation temperature range of 18-20°C was used for all subsequent ELA experiments. This temperature range displayed maximal hemolytic activity and was also used for growing cultures of \( P. \) \( parvum \) isolates.

Figure 3.7 Effect of incubation temperature (12-38°C) on hemolytic activity of intact \( P. \) \( parvum \) (isolate 2797), \( H. \) \( akashiwo \) (isolate 513) and \( T. \) \( suecica \) (isolate 906) cells, and saponin. Values represent the average of two assays (each performed in triplicate) ± standard deviation (n = 2). Samples incubated at 38°C were statistically less hemolytic than samples incubated at other temperatures. Saponin and \( P. \) \( parvum \) were more hemolytic than \( H. \) \( akashiwo \) and \( T. \) \( suecica \), and \( H. \) \( akashiwo \) was more hemolytic than \( T. \) \( suecica \). Bars labelled with asterisks (***), are statistically different (p < 0.001) according to Tukey’s multiple comparison test.
3.1.2.5. *Algal density and growth phase*

Algal cell density was found to significantly affect the hemolytic activity of all isolates (Fig. 3.8; $F_{3,18} = 56.2$, $p < 0.001$), with hemolytic activity increasing with cell density. This trend was observed for both intact and lysed cells. Significant interactions between isolate, cell type (intact or lysed cells) and density were also observed ($F_{6,18} = 11.75$, $p = 0.001$). Two-Way ANOVA and Tukey’s multiple comparison analyses ($\alpha \leq 0.05$) were performed to examine these interactions. Both intact and lysed cells of isolates from North America (2797 and 2827) behaved similarly (Fig. 3.8; $F_{2,66} = 2.96$, $p > 0.05$). Overall, intact cells of isolates 2797 and 2827 displayed greater hemolytic activity than that of isolate 995 (Fig. 3.8; $F_{2,66} = 2.96$, $p < 0.001$). However, the lysis of cells showed that all isolates possessed the same potential for hemolysis (Fig. 3.8; $F_{2,66} = 2.96$, $p > 0.05$). This was particularly apparent at higher cell densities ($3.5 \times 10^3$ – $7.5 \times 10^3$ cells·mL$^{-1}$). Overall, no significant difference in hemolytic activity between intact and lysed cells for isolates 2797 and 2827 was observed ($F_{2,66} = 2.96$, $p > 0.05$). However, lysed cells of isolate 995 displayed greater hemolytic activity than intact cells ($F_{2,66} = 2.96$, $p = 0.013$).
Figure 3.8 Effect of cell density ($3.5 \times 10^3 – 7.5 \times 10^3$ cells·mL$^{-1}$) on hemolytic activity of intact (A) and lysed (B) *P. parvum* cells. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with asterisks (***) are statistically different (p < 0.001) to the other bars according to Tukey’s multiple comparison test.
The hemolytic activity of the *Prymnesium* isolates during exponential phase (day 3) and stationary phase (day 10) were compared. For all isolates, no significant difference was observed between the hemolytic activity of cultures in exponential and stationary phase, for either intact or lysed cells (Fig. 3.9; $F_{1,6} = 0.169$, $p = 0.695$). Intact cells from cultures of the North American isolates in exponential and stationary phase showed more hemolytic activity than cultures of isolate 995 in exponential phase (Fig. 3.9; $F_{2,6} = 5.50$, $p = 0.04$). However, intact cells from cultures of isolate 995 in stationary phase showed comparable hemolytic activity to that of the North American cultures (Fig. 3.9; $F_{2,84} = 1.10$, $p > 0.05$).
Figure 3.9 Effect of growth phase on hemolytic activity of varying densities (3.5 x 10^3 – 7.5 x 10^3 cells·mL\(^{-1}\)) of intact *P. parvum* cells. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with an asterisk (*) are statistically different (p < 0.05) to the other bars according to Tukey’s multiple comparison test.
3.2. Effect of salinity and light intensity on growth and hemolytic activity

3.2.1. Effect of salinity and light intensity on growth

3.2.1.1. Isolate 995

For isolate 995, cultures grown in a salinity of 1‰ had lower growth rates than cultures grown in 3-30‰ (Fig. 3.10; two-way ANOVA, $F_{6,59} = 19.2, p \leq 0.001$). Cultures grown in a salinity of 3‰ had lower growth rates than those grown in 10-30‰ ($F_{6,59} = 19.2, p < 0.001$), and cultures in 5‰ had lower growth rates than 15-30‰ (Fig. 3.10; $F_{6,59} = 19.2, p < 0.01$). The cultures of isolate 995 grown under 10 μmol photons m$^{-2}$ s$^{-1}$ had lower growth rates than those grown under 30 and 50 μmol photons m$^{-2}$ s$^{-1}$ (Fig. 3.10; two-way ANOVA, $F_{4,59} = 4.92, p \leq 0.05$). At a salinity of 1‰, cultures accumulated yields lower than the yields observed at 5-30‰ (Fig. 3.11; two-way ANOVA, $F_{6,59} = 49.5, p \leq 0.01$). At 3‰, cultures achieved yields lower than those of cultures grown in 10-30‰ (Fig. 3.11; $F_{6,59} = 49.5, p < 0.001$). As well, cultures in 5‰ had lower yields than cultures in 10-30‰ media (Fig. 3.11; $F_{6,59} = 49.5, p < 0.001$). Finally, cultures grown under 10, 90 and 180 μmol photons m$^{-2}$ s$^{-1}$ accumulated lower yields than cultures grown under 30 and 50 μmol photons m$^{-2}$ s$^{-1}$ (Fig. 3.11; two-way ANOVA, $F_{4,59} = 9.93, p \leq 0.05$).

3.2.1.2. Isolate 2797

The lowest growth rates and yields were observed for cultures of isolate 2797 grown at a salinity of 1‰ (Fig. 3.10-3.11; two-way ANOVA, $F_{6,94} = 29.3, p < 0.001$; $F_{6,94} = 40.05, p < 0.001$). Cultures grown at a salinity of 3‰ had lower growth rates (Fig 3.10; $F_{6,94} = 29.3, p < 0.01$) than cultures grown at 10-30‰, and lower yields (Fig. 3.11; $F_{6,94} = 40.05, p < 0.001$) than cultures grown at 5-30‰. As well, cultures grown at 5‰ had lower growth rates than those grown at 20 and 30‰ (Fig. 3.10; $F_{6,94} = 29.3, p < 0.05$). Cultures
grown under 10 μmol photons m\(^{-2}\) s\(^{-1}\) had lower growth rates than those grown under 50-180 μmol photons m\(^{-2}\) s\(^{-1}\) (Fig. 3.10; two-way ANOVA, F\(_{4,94}\) = 5.41, p < 0.01). No significant effect of light intensity on yield was observed for isolate 2797 (Fig. 3.11; two-way ANOVA, F\(_{4,94}\) = 2.71, p > 0.05).

3.2.1.3. Isolate 2827

The growth rates of cultures of isolate 2827 grown in 1‰ medium were lower than that of cultures grown in 3-30‰ (Fig. 3.10; two-way ANOVA, F\(_{6,94}\) = 25.6, p < 0.001). Cultures grown in 3‰ had lower growth rates than that of cultures grown in 10-30‰ (F\(_{6,94}\) = 25.6, p < 0.01), and cultures in 5‰ had lower growth rates than those in 15 and 20‰ (Fig. 3.10; F\(_{6,94}\) = 25.6, p < 0.01). Growth rates of cultures grown under 10 and 30 μmol photons m\(^{-2}\) s\(^{-1}\) were lower than those grown under 50-180 μmol photons m\(^{-2}\) s\(^{-1}\) (Fig. 3.10; two-way ANOVA, F\(_{6,94}\) = 37.1, p < 0.001). The yields attained by cultures grown in 1‰ medium were lower than that of cultures grown in 3-30‰ (Fig. 3.11; two-way ANOVA, F\(_{6,94}\) = 23.6, p < 0.001). Cultures grown under 10 and 30 μmol photons m\(^{-2}\) s\(^{-1}\) had lower yields than those grown under 90 μmol photons m\(^{-2}\) s\(^{-1}\) (Fig. 3.11; two-way ANOVA, F\(_{4,94}\) = 4.14, p < 0.01).
Figure 3.10 Growth rates (µ) of P. parvum isolates (995, A; 2797, B; 2827, C) at a range of salinities (1-30‰) and light intensities (10-180 µmol photons m⁻² s⁻¹). Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Asterisks (* = p < 0.05, ** = p < 0.01, *** = p < 0.001) indicate statistical differences according to Tukey’s multiple comparison test.
Figure 3.11 Yields (Y) of *P. parvum* isolates (995, A; 2797, B; 2827, C) at a range of salinities (1-30‰) and light intensities (10-180 µmol photons m⁻² s⁻¹). Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Asterisks (* = p < 0.05, ** = p < 0.01, *** = p < 0.001) indicate statistical differences according to Tukey’s multiple comparison test.
3.2.2. Effect of salinity and light intensity on hemolytic activity

3.2.2.1. *Isolate 995*

Salinity did not significantly affect the hemolytic activity of isolate 995 (Fig. 3.12; $F_{6,42} = 0.586$, $p = 0.739$). However, hemolysis decreased with increasing light intensity (Fig. 3.12; $F_{4,42} = 9.63$, $p < 0.001$). Hemolysis also increased with cell density, with 7500 cells·mL$^{-1}$ having more hemolytic activity than 5000 cells·mL$^{-1}$ ($F_{1,42} = 60.9$, $p < 0.001$). Lysed cells were also more hemolytic than intact cells ($F_{4,42} = 10.7$, $p < 0.001$).
Figure 3.12 Hemolytic activity of 5 x 10^3 (A, B) and 7.5 x 10^3 cells·mL^-1 (C, D) of P. parvum isolate 995. Hemolytic activity of intact (A, C) and lysed (B, D) cells grown in a range of salinities (1-30‰) under 10-180 μmol photons m^-2 s^-1. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with asterisks (****) are statistically different (p < 0.001) according to Tukey’s multiple comparison test. Salinity did not significantly affect hemolytic activity (p > 0.05).
3.2.2.2. Isolate 2797

For isolate 2797, interactions were observed between salinity, cell density ($F_{6,70} = 7.68, p < 0.001$) and cell type ($F_{6,70} = 4.82, p < 0.001$), as well as light and cell type (Fig. 3.13; $F_{4,70} = 3.79, p = 0.007$). Less hemolytic activity was observed at 5000 cells·mL$^{-1}$ than 7500 cells·mL$^{-1}$ ($F_{1,70} = 138.1, p < 0.001$). Cultures grown in lower salinities (1 and 5‰) were more hemolytic than cultures grown in higher salinities (15-30‰) (Fig. 3.13; $F_{6,70} = 8.46, p < 0.001$). Cultures grown in 20 and 30‰ were less hemolytic than cultures grown in 3 and 10‰ (Fig. 3.13; $F_{6,70} = 8.46, p < 0.001$). As well, those grown in 15‰ were more hemolytic than those grown in 30‰ (Fig. 3.13; $F_{6,70} = 8.46, p < 0.001$). Lysed cells displayed more hemolytic activity than intact cells ($F_{1,70} = 14.3, p < 0.001$). Cultures grown under 10 µmol photons m$^{-2}$ s$^{-1}$ were less hemolytic than cultures grown under 30-180 µmol photons m$^{-2}$ s$^{-1}$ (Fig. 3.13; $F_{4,70} = 15.5, p < 0.001$). Cultures grown under 30 µmol photons m$^{-2}$ s$^{-1}$ were more hemolytic than cultures grown under 50-180 µmol photons m$^{-2}$ s$^{-1}$ (Fig. 3.13; $F_{4,70} = 15.5, p < 0.001$). As well, cultures grown under 50 µmol photons m$^{-2}$ s$^{-1}$ were more hemolytic than cultures grown under 180 µmol photons m$^{-2}$ s$^{-1}$ (Fig. 3.13; $F_{4,70} = 15.5, p < 0.05$).
**Figure A**

- Salinity (‰) range: 1 to 30
- Hemolysis (%) range: 0 to 120
- Photon intensity levels: 10, 30, 50, 90, 180 µmol photons m⁻² s⁻¹

**Figure B**

- Salinity (‰) range: 1 to 30
- Hemolysis (%) range: 0 to 120
- Photon intensity levels: 10, 30, 50, 90, 180 µmol photons m⁻² s⁻¹
Figure 3.13 Hemolytic activity of 5 x 10^3 (A, B) and 7.5 x 10^3 cells·mL^-1 (C, D) of *P. parvum* isolate 2797. Hemolytic activity of intact (A, C) and lysed (B, D) cells grown in a range of salinities (1-30‰) under 10-180 µmol photons m^-2 s^-1. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with asterisks (****) are statistically different (p < 0.001) according to Tukey’s multiple comparison test. Salinity did not significantly affect hemolytic activity (p > 0.05).
3.2.2.3. *Isolate 2827*

Salinity did not have a significant effect on the hemolytic activity of isolate 2827 (Fig. 3.14; $F_{6,70} = 1.09$, $p = 0.380$). However, light intensity was found to significantly affect hemolytic activity ($F_{4,70} = 5.25$, $p < 0.001$). Cultures grown under 30-180 μmol photons m$^{-2}$s$^{-1}$ showed more hemolysis than cultures grown in 10 μmol photons m$^{-2}$s$^{-1}$ (Fig. 3.14; $F_{4,70} = 6.43$, $p < 0.001$). Less hemolytic activity was observed at 5000 cells·mL$^{-1}$ than 7500 cells·mL$^{-1}$ ($F_{1,70} = 146.0$, $p < 0.001$). Lysed cells displayed more hemolytic activity than intact cells ($F_{1,70} = 70.0$, $p < 0.001$).
Figure 3.14 Hemolytic activity of 5 x 10^3 (A, B) and 7.5 x 10^3 cells·mL⁻¹ (C, D) of *P. parvum* isolate 2827. Hemolytic activity of intact (A, C) and lysed (B, D) cells grown in a range of salinities (1-30‰) under 10-180 µmol photons·m⁻²·s⁻¹. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with asterisk (****) are statistically different (p < 0.001) according to Tukey’s multiple comparison test. Salinity did not significantly affect hemolytic activity (p > 0.05).
3.3. Effect of temperature on growth and hemolytic activity

3.3.1. Effect of temperature on growth

Cultures grown at 12°C had lower growth rates and yields than cultures grown at 18 and 23°C. However, temperature did not have a significant effect on the growth rates (Fig. 3.15; one-way ANOVA, $F_{2,3} = 1.053, p = 0.45$; $F_{2,6} = 2.42, p = 0.17$) and yields (Fig. 3.16; one-way ANOVA, $F_{2,3} = 0.993, p = 0.467$; $F_{2,6} = 2.42, p = 0.17$) of cultures of isolate 995 or isolate 2827. A significant effect of temperature was only observed for isolate 2797. The growth rates of cultures grown at 18 and 23°C were significantly different to the rates of those grown at 12°C ($F_{2,6} = 19.5, p = 0.002$; $F_{2,6} = 19.5, p = 0.009$), but no significance was found between 18 and 23°C (Fig. 3.15; one-way ANOVA, $F_{2,6} = 19.5, p = 0.379$). As well, no significant difference was found between the yields of these cultures (Fig. 3.16; one-way ANOVA, $F_{2,6} = 1.52, p = 0.292$).
Figure 3.15  Growth rates ($\mu$) of *P. parvum* isolates at a range of temperatures (12-23°C). Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). An asterisk (*) indicates cultures of isolate 2797 grown at 12°C are statistically different (p < 0.05) to cultures grown in 18-23°C according to Tukey’s multiple comparison test.

Figure 3.16  Yields (Y) of *P. parvum* isolates at a range of temperatures (12-23°C). Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). No significant effect of temperature on yield was observed.
3.3.2. Effect of temperature on hemolytic activity

*Tetraselmis suecica* is a marine flagellate that is known to be non-toxic. This species was used as a control for the temperature assay. *T. suecica* did not display hemolytic activity, whereas *Prymnesium* isolates did (Fig. 3.17; p < 0.001). The hemolytic activity of isolate 995 was significantly affected by temperature (Fig. 3.17; $F_{2,6} = 15.3, p = 0.004$). Lysed cells were more hemolytic than intact cells ($F_{1,6} = 187.2, p < 0.001$). Intact cells showed an increase in hemolytic activity with an increase in density ($F_{1,6} = 34.2, p = 0.001$). The hemolytic activity of isolate 2797 was not significantly affected by temperature (Fig. 3.17; $F_{2,12} = 0.563, p = 0.584$). Lysed cells were more hemolytic than intact cells ($F_{1,12} = 15.9, p = 0.004$). The hemolytic activity of isolate 2827 was significantly affected by temperature, with hemolytic activity increasing with temperature (23 and 18°C) (Fig. 3.17; $F_{2,12} = 12.3, p = 0.001$). Lysed cells were more hemolytic than intact cells ($F_{1,12} = 93.8, p < 0.001$). Intact cells showed an increase in hemolytic activity with an increase in density ($F_{1,12} = 25.8, p < 0.001$).
Figure 3.17  Hemolytic activity of $5 \times 10^3$ cells·mL$^{-1}$ (A) and $7.5 \times 10^3$ cells·mL$^{-1}$ (B) intact and lysed *P. parvum* and *T. suecica* cells grown in a range of temperatures (12-23°C). Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Asterisks (* = p < 0.05, ** = p < 0.01, *** = p< 0.001) indicate statistical differences according to Tukey’s multiple comparison test.
3.4. Effect of nitrogen on growth and hemolytic activity

3.4.1. Effect of nitrogen source on growth

3.4.1.1. *Isolate 995*

The growth rate of isolate 995 was significantly affected by N source and concentration (Fig. 3.18; two-way ANOVA, $F_{2,18} = 10.2$, $p = 0.001$; $F_{4,18} = 6.01$, $p = 0.003$). As well, an interaction between N source and concentration was observed (two-way ANOVA, $F_{8,18} = 2.64$, $p = 0.04$). The growth rates of cultures grown in 25-100 µmol of urea were significantly lower than cultures grown in 150 µmol ammonium, as well those grown in 50 and 100 µmol of urea were lower than those grown in 50 µmol ammonium (Fig. 3.18; $F_{8,18} = 2.64$, $p = 0.046$, $p < 0.05$). Cultures grown in 25-100 µmol urea had lower growth rates than cultures grown in 500 µmol urea (Fig. 3.18; $F_{8,18} = 2.64$, $p = 0.046$, $p < 0.05$). The growth rates of cultures grown in 100 µmol nitrate were significantly different to that of cultures grown with 50 µmol urea (Fig. 3.18; $F_{2,18} = 10.2$, $p = 0.04$). Nitrogen concentration ($F_{4,26} = 4.52$, $p < 0.001$) and source ($F_{2,26} = 4.22$, $p = 0.026$) were found to individually affect yield, with 150 µmol being significantly different to 25-100 µmol ($F_{4,26} = 4.52$, $p \leq 0.05$), and nitrate being significantly different to urea (Fig. 3.19; two-way ANOVA, $F_{2,26} = 4.22$, $p = 0.022$).

3.4.1.2. *Isolate 2797*

For isolate 2797, cultures with urea as their N source had lower growth rates (Fig. 3.18; two-way ANOVA, $F_{2,29} = 9.27$, $p = 0.004$; $p = 0.001$) and yields (Fig. 3.19; two-way ANOVA, $F_{2,21} = 63.1$, $p < 0.001$) than those grown with ammonium and nitrate. Furthermore, cultures grown with nitrate and ammonium were found to have comparable growth rates (Fig. 3.18; $F_{2,29} = 9.27$, $p = 0.915$), but cultures grown with nitrate produced a higher yield (Fig. 3.19; $F_{2,21} = 63.1$, $p < 0.001$). Cultures grown in concentrations of N lower than 100 µmol N had lower growth rates and yields due to N limitation, while
cultures grown at concentrations higher than 150 µmol N had lower growth rates and yields due to P limitation. However, only cultures grown in 25 µmol N were found to have significantly different growth rates to cultures grown in 100-500 µmol N (Fig. 3.18; F4,21 = 35.9, p = 0.013; p = 0.012; p = 0.003). Cultures grown in 25 and 50 µmol N had significantly different yields to cultures grown in 100-500 µmol N (Fig. 3.19; F4,21 = 35.9, p < 0.001). However, this difference was dependent on N source (F4,21 = 5.6, p < 0.001). Cultures grown in 25 and 50 µmol ammonium or nitrate had lower yields than those grown in higher concentrations of ammonium or nitrate (100-500 µmol) (F4,21 = 5.6, p ≤ 0.01). As well, cultures grown in 25-100 µmol urea had lower yields than those grown in 500 µmol urea (F4,21 = 5.6, p < 0.05).

3.4.1.3. Isolate 2827

Cultures of isolate 2827 with urea as their N source had lower growth rates (Fig. 3.18; two-way ANOVA, F2,35 = 10.2, p = 0.001) than those grown with ammonium and nitrate. The yields for cultures grown in each N source were significantly different, with cultures grown in nitrate having the highest yield and urea the lowest (Fig. 3.19; two-way ANOVA, F2,27 = 141.8 p ≤ 0.001). As with isolate 2797, cultures of 2827 grown in concentrations of N lower than 100 µmol N had lower growth rates and yields due to N limitation, while cultures grown at concentrations higher than 150 µmol N had lower growth rates and yields due to P limitation. Cultures grown in 25 µmol N were found to have significantly different growth rates to cultures grown in 50-500 µmol N (Fig. 3.18; F4,35 = 14.6, p ≤ 0.001). Additionally, cultures grown in 50 and 100 µmol urea had lower growth rates than those grown in higher concentrations of ammonium or nitrate (100-500 µmol) (Fig. 19; F4,35 = 14.6, p < 0.05). As well, cultures grown in 25-100 µmol urea had lower growth rates than those grown in 500 µmol urea (Fig. 3.18; F4,35 = 14.6, p ≤ 0.05). Cultures grown in 25 and 50 µmol N had significantly different yields to cultures grown in 100-500 µmol N (Fig. 3.19; F4,27 = 92.85, p < 0.001). Furthermore, cultures grown in 100 µmol N were significantly different to cultures grown in 150 and 500 µmol N (Fig. 3.19; F4,27 = 92.85, p < 0.001).
Figure 3.18 Growth rates (µ) of *P. parvum* isolates (995, A; 2797, B; 2827, C) in different N sources (NO₃, urea, NH₄) at different concentrations (25-500 µmol). Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Asterisks (* = p < 0.05, ** = p < 0.01) indicate statistical differences according to Tukey’s multiple comparison test.
Figure 3.19 Yields (Y) of *P. parvum* isolates (995, A; 2797, B; 2827, C) in different N sources (NO₃, urea, NH₄) at different concentrations (25-500 µmol). Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Asterisks (* = p < 0.05, ** = p < 0.01, *** = p< 0.001) indicate statistical differences according to Tukey’s multiple comparison test.
3.4.2. Effect of nitrogen source on hemolytic activity

3.4.2.1. *Isolate 995*

Hemolytic activity of isolate 995 was significantly affected by both the N source ($F_{2,15} = 29.6, p = 0.001$) and the concentration of N (Fig. 3.20; $F_{4,15} = 18.8, p = 0.001$). Overall, cultures grown in nitrate and urea were more hemolytic than cultures grown in ammonium (Fig. 3.20; $F_{2,15} = 29.6, p = 0.001$). However, at 500 µmol, the hemolytic activity of cultures grown in urea decreased (Fig. 3.20; $F_{8,15} = 11.9, p < 0.001$). All N sources were found to induce similar hemolytic activity in cultures grown in 25 µmol of N (Fig. 3.20; $F_{8,15} = 11.9, p > 0.05$). Hemolytic activity was similar in all concentrations of nitrate. Intact cells of cultures grown in 25 µmol urea were more hemolytic than cultures grown in 50-500 µmol (Fig. 3.20; $F_{4,15} = 6.71, p = 0.002$). Intact cells of cultures grown in 100 µmol urea were more hemolytic than cultures grown in 150 and 500 µmol (Fig. 3.20; $F_{4,15} = 6.71, p = 0.002$). Lysed cells of cultures grown in 500 µmol urea were less hemolytic than cultures grown in other concentrations (25-150 µmol) (Fig. 3.20; $F_{4,15} = 6.71, p = 0.002$). Cultures grown in 25 µmol ammonium were more hemolytic than cultures grown in 50-500 µmol ammonium (Fig. 3.20; $F_{8,15} = 11.9, p < 0.001$). Lysed cells of cultures grown in 25 and 150 µmol ammonium were significantly more hemolytic than cultures grown in other concentrations of ammonium (Fig. 3.20; $F_{4,15} = 6.71, p = 0.002$). Hemolytic activity was found to increase with an increase in algal cell density, and in general, lysed cells were more hemolytic ($F_{1,15} = 96.3, p < 0.001; F_{1,15} = 6.13, p = 0.02$).
Figure 3.20 Hemolytic activity of $5 \times 10^3$ (A, B) and $7.5 \times 10^3$ cells·mL$^{-1}$ (C, D) of *P. parvum* isolate 995. Hemolytic activity of intact (A, C) and lysed (B, D) cells grown in 25-500 µmol nitrate, ammonium and urea. Values represent the average of three assays (each performed in triplicate) ± standard deviation $(n = 3)$. Asterisks (**) = $p < 0.01$, (***) = $p < 0.001$ indicate statistical differences according to Tukey’s multiple comparison test.
3.4.2.2. *Isolate 2797*

Hemolytic activity of isolate 2797 was significantly affected by both the N source (F\(_{2,30} = 22.1, p < 0.001\)) and the concentration of N (Fig. 3.21; F\(_{4,30} = 39.5, p < 0.001\)). Overall, cultures grown in nitrate and urea were more hemolytic than cultures grown in ammonium (Fig. 3.21; F\(_{2,30} = 22.1, p < 0.001\)). However, at 150 and 500 µmol, the hemolytic activity of intact cells from cultures grown in urea decreased (Fig. 3.21; F\(_{8,30} = 10.3, p < 0.001\)). All N sources were found to induce similar hemolytic activity in cultures grown in 25 µmol of N (Fig. 3.21; F\(_{8,30} = 10.3, p > 0.05\)). Hemolytic activity was similar in all concentrations of nitrate. Intact cells of cultures grown in 25-100 µmol urea were more hemolytic than cultures grown in 150 and 500 µmol (Fig. 3.21; F\(_{4,15} = 23.1, p = 0.001\)). Intact cells of cultures grown in 25 and 50 µmol ammonium were more hemolytic than cultures grown in 100-500 µmol ammonium (Fig. 3.21; F\(_{8,15} = 10.3, p < 0.001\)). Lysed cells of cultures grown in 25-100 µmol ammonium were significantly more hemolytic than cultures grown in other concentrations of ammonium (Fig. 3.21; F\(_{8,30} = 10.3, p < 0.001\)). Hemolytic activity was found to increase with an increase in algal cell density (F\(_{1,30} = 34.1, p < 0.001\)).
Figure 3.21 Hemolytic activity of $5 \times 10^3$ (A, B) and $7.5 \times 10^3$ cells·mL$^{-1}$ (C, D) of *P. parvum* isolate 2797. Hemolytic activity of intact (A, C) and lysed (B, D) cells grown in 25-500 µmol nitrate, ammonium and urea. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Asterisks (* = p < 0.05, *** = p < 0.001) indicate statistical differences according to Tukey’s multiple comparison test.
3.4.2.3. *Isolate 2827*

Hemolytic activity of isolate 2827 was significantly affected by the concentration of N (Fig. 3.22; $F_{4,30} = 8.64$, $p < 0.001$). It was also significantly affected by the source of N, but this effect was dependent on the concentration of N (Fig. 3.22; $F_{8,30} = 26.4$, $p < 0.001$). The intact cells of cultures grown in 25 and 50 µmol N, displayed similar hemolytic activity, regardless of N source. However, the hemolytic activity of intact cells from cultures grown in ammonium decreased at 100-500 µmol, and at 500 µmol urea, hemolytic activity of intact cells decreased (Fig. 3.22; $F_{8,30} = 26.4$, $p < 0.001$). Hemolytic activity was similar for all concentrations of nitrate. Intact cells of cultures grown in 25-150 µmol urea were more hemolytic than cultures grown in 500 µmol (Fig. 3.22; $F_{4,15} = 23.1$, $p = 0.001$). The hemolytic activity of lysed cells were similar at all concentrations for all N sources. Hemolytic activity was found to increase with algal cell density ($F_{1,30} = 13.1$, $p = 0.001$).
Figure 3.22  Hemolytic activity of $5 \times 10^3$ (A, B) and $7.5 \times 10^3$ cells·mL$^{-1}$ (C, D) of *P. parvum* isolate 2827. Hemolytic activity of intact (A, C) and lysed (B, D) cells grown in 25-500 µmol nitrate, ammonium and urea. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with asterisks (***$p$ < 0.001) are statistically different according to Tukey’s multiple comparison test. No significant effect was observed for lysed cells.
3.5. Effect of an iron chelator on growth and hemolytic activity

3.5.1. Effect of an iron chelator on growth

The addition of the Fe chelator, DFB, affected the growth rates and yields of isolates 2797 and 2827. For isolate 2797, cultures grown in media with 0-30 µM DFB had higher growth rates than those grown with 50-100 µM DFB (Fig. 3.23; one-way ANOVA, $F_{6,14} = 28.7, p \leq 0.001$) and higher yields than cultures grown with 75-100 µM DFB (Fig. 3.24; one-way ANOVA, $F_{6,14} = 9.49, p < 0.05$). Isolate 2827 had a similar response, growth rates of cultures grown in 0-50 µM DFB were higher than those in 75 µM, and cultures grown in 0-5 µM DFB also had higher rates than those in 100 µM DFB (Fig. 3.23; $F_{6,14} = 6.62, p < 0.05$). Yields of cultures grown in 0-50 µM DFB were higher than those grown in 75-100 µM DFB (Fig. 3.24; $F_{6,14} = 10.5, p < 0.05$). In contrast, cultures of isolate 995 maintained a similar growth rate (Fig. 3.23; $F_{6,7} = 0.08, p = 0.997$) and yield (Fig. 3.24; $F_{6,7} = 1.77, p = 0.236$) at all concentrations of DFB.
Figure 3.23 Growth rates ($\mu$) of *P. parvum* isolates in different concentrations (0-100 $\mu$M) of the Fe chelator, DFB. Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Asterisks (*) = $p < 0.05$, ** = $p < 0.01$) indicate statistical differences according to Tukey’s multiple comparison test. Growth of isolate 2797 cultures grown in 50-100 $\mu$M DFB was lower than that of cultures grown in 0-30 $\mu$M. Growth of isolate 2827 cultures grown in 75-100 $\mu$M DFB was lower than that of cultures grown in 0-50 $\mu$M.

Figure 3.24 Yields (Y) of *P. parvum* isolates in different concentrations (0-100 $\mu$M) of the Fe chelator, DFB. Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n = 3). Bars labelled with an asterisk (*) are statistically different ($p < 0.05$) according to Tukey’s multiple comparison test. Growth of cultures of isolates 2797 and 2827 grown in 75-100 $\mu$M DFB was lower than that of cultures grown in 0-50 $\mu$M.
3.5.2. Effect of an iron chelator on hemolytic activity

3.5.2.1. *Isolate 995*

The Fe chelator, DFB, had a significant effect on the hemolytic activity of isolate 995 (Fig. 3.25; $F_{6,7} = 7.03$, $p = 0.01$). Hemolysis caused by intact and lysed cells was significantly different, with lysed cells being more hemolytic (Fig. 3.25; $F_{1,7} = 30.0$, $p < 0.001$). The hemolytic activity of intact cells decreased with an increase in DFB (Fig. 3.25; $F_{6,7} = 7.03$, $p = 0.01$). Intact cells of cultures grown in 0 µM DFB were more hemolytic than cells grown in 30-100 µM DFB (Fig. 3.25; $F_{1,7} = 30.0$, $p < 0.001$). Intact cells grown in 5, 10 and 30 µM DFB were more hemolytic than cells grown in 50-100 µM DFB (Fig. 3.25; $F_{1,7} = 30.0$, $p < 0.001$). Lysed cells from cultures grown in 0-30 and 100 µM DFB were significantly more hemolytic than lysed cells grown in 50-75 µM (Fig. 3.25; $F_{1,7} = 30.0$, $p < 0.001$).

3.5.2.2. *Isolate 2797*

DFB had a significant effect on the hemolytic activity of isolate 2797 (Fig. 3.25; $F_{6,14} = 11.1$, $p < 0.001$). The hemolytic activity of intact cells decreased with an increase in the concentration of DFB in the media (Fig. 3.25; $F_{6,14} = 8.93$, $p < 0.001$). Intact cells of cultures grown in 0 µM DFB were more hemolytic than cultures grown in 30-100 µM DFB, and those grown in 5 and 10 µM DFB were more hemolytic than cells grown in 50-100 µM DFB (Fig. 3.25; $F_{6,14} = 8.93$, $p < 0.001$). Except for 50 µM DFB, the hemolytic activity of intact and lysed cells from cultures grown in the same concentration of DFB did not display any significant difference (Fig. 3.25; $F_{6,14} = 8.93$, $p < 0.001$).
3.5.2.3. *Isolate 2827*

The hemolytic activity of isolate 2827 was significantly affected by the addition of DFB (Fig. 3.25; $F_{6,14} = 9.11, p < 0.001$). The hemolytic activity of intact cells decreased with an increase in the concentration of DFB in the media (Fig. 3.25; $F_{6,14} = 8.93, p < 0.001$). Intact cells of cultures grown in 0 and 5 µM DFB were more hemolytic than cells grown in 30-100 µM DFB (Fig. 3.25; $F_{6,14} = 10.2, p < 0.001$). Intact and lysed cells grown in 0 and 5 µM DFB displayed similar hemolysis. However, lysed cells from cultures grown in 10-100 µM DFB were significantly more hemolytic than intact cells from these cultures (Fig. 3.25; $F_{6,14} = 10.2, p < 0.001$).
Figure 3.25 Hemolytic activity of $5 \times 10^3$ cells·mL$^{-1}$ (A) and of $7.5 \times 10^3$ cells·mL$^{-1}$ (B) intact and lysed *P. parvum* cells grown in varying concentrations (0-100 µM) of the Fe chelator, DFB. Values represent the average of three assays (each performed in triplicate) ± standard deviation (n = 3). Asterisks (* = p < 0.05, ** = p < 0.01, *** = p< 0.001) indicate statistical differences according to Tukey’s multiple comparison test.
Table 3.1 Summary of conditions for maximal growth and hemolytic activity of *Prymnesium parvum* for each experimental variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Isolate 995</th>
<th>Isolate 2797</th>
<th>Isolate 2827</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salinity</strong></td>
<td><em>Growth</em></td>
<td>10 - 30 ‰</td>
<td>10 - 30 ‰</td>
</tr>
<tr>
<td></td>
<td><em>Hemolytic Activity</em></td>
<td>1 - 3 ‰</td>
<td>1 - 5 ‰</td>
</tr>
<tr>
<td><strong>Light Intensity</strong></td>
<td><em>Growth</em></td>
<td>30 - 50 µmol photons m⁻² s⁻¹</td>
<td>30 - 90 µmol photons m⁻² s⁻¹</td>
</tr>
<tr>
<td></td>
<td><em>Hemolytic Activity</em></td>
<td>10 - 50 µmol photons m⁻² s⁻¹</td>
<td>30 - 90 µmol photons m⁻² s⁻¹</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td><em>Growth</em></td>
<td>12 - 23 °C</td>
<td>18 - 23 °C</td>
</tr>
<tr>
<td></td>
<td><em>Hemolytic Activity</em></td>
<td>23 °C</td>
<td>23 °C</td>
</tr>
<tr>
<td><strong>Nitrogen</strong></td>
<td><em>Growth</em></td>
<td>*Nitrate: 150 µmol</td>
<td>*Nitrate: 150 µmol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urea: 500 µmol</td>
<td>Ammonium: 150 µmol</td>
</tr>
<tr>
<td></td>
<td><em>Hemolytic Activity</em></td>
<td>*Nitrate: 25 &amp; 500 µmol</td>
<td>*Nitrate: 50 - 150 µmol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urea: 25 &amp; 100 µmol</td>
<td>Ammonium: 25 µmol</td>
</tr>
<tr>
<td><strong>Iron</strong></td>
<td><em>Growth</em></td>
<td>0 - 5 µM DFB</td>
<td>0 - 30 µM DFB</td>
</tr>
<tr>
<td></td>
<td><em>Hemolytic Activity</em></td>
<td>0 µM DFB</td>
<td>0 - 5 µM DFB</td>
</tr>
</tbody>
</table>

Asterisk (*) indicates the optimal nitrogen source for growth and hemolytic activity.
4. DISCUSSION

4.1. Standardization of ELA design

The hemolytic compounds produced by *P. parvum* are of a relatively unknown structure, so detection and quantification of these substances are achieved by *in vitro* bioassays, such as the ELA. These bioassays are based on the mode of action of these substances: their ability to lyse erythrocytes (Eschbach et al. 2001). There are three components involved in the ELA: 1) preparation of the algal sample, 2) incubation of the algal sample with erythrocytes and 3) detection and quantification of erythrocyte lysis. For this study, each component was standardized for the detection of the hemolytic activity of *P. parvum* (Table 4.1).
Table 4.1 Summary of erythrocyte lysis assay (ELA) components for detection and quantification of hemolytic activity of *Prymnesium parvum*.

<table>
<thead>
<tr>
<th>ELA Component</th>
<th>Parameter Tested</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algal sample preparation</td>
<td>Algal sample type</td>
<td>Intact and lysed cells</td>
</tr>
<tr>
<td></td>
<td>Growth phase</td>
<td>Exponential</td>
</tr>
<tr>
<td>Incubation conditions</td>
<td>Final rabbit erythrocyte</td>
<td>$5 \times 10^6$ cells·mL$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td>$5 \times 10^3$ cells·mL$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>Final algal concentration</td>
<td>$7.5 \times 10^3$ cells·mL$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>Irradiance</td>
<td>30 - 50 μmol photons m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>18 °C</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>6 h</td>
</tr>
<tr>
<td>Detection of hemolytic activity</td>
<td>Absorption wavelength</td>
<td>414 nm</td>
</tr>
</tbody>
</table>

4.1.1. Detection and quantification of hemolytic activity

4.1.1.1. Photometric detection of hemolysis

In previous studies, the hemolytic activity of ichthyotoxic algae was determined photometrically through the measurement of released hemoglobin at wavelengths of 414, 540 or 560 nm (Eschbach et al. 2001). For this study, 414 nm was used because lysed rabbit erythrocytes have been found to have an absorption maximum at that wavelength that is 10-fold higher than at 540 and 560 nm (Fig. 4.1) (Ling and Trick 2010; Trick
unpublished). This has also been found for fish and human erythrocytes (Eschbach et al. 2001). Absorption at 414 nm has been observed to be linearly correlated to erythrocyte lysis up to $5 \times 10^6 \text{cells}\cdot\text{mL}^{-1}$, so hemolytic activity can be quantified through measurement of absorption at this wavelength (Ling and Trick 2010). The standard erythrocyte concentration for the ELA used was $5 \times 10^6 \text{cells}\cdot\text{mL}^{-1}$.

![Absorption spectra of lysed erythrocytes, intact erythrocytes and lysed algal cells (Heterocapsa pygmaea, for example) (Trick, unpublished). Arrows indicate the maxima absorption peaks.](image)

**Figure 4.1** Absorption spectra of lysed erythrocytes, intact erythrocytes and lysed algal cells (Heterocapsa pygmaea, for example) (Trick, unpublished). Arrows indicate the maxima absorption peaks.

### 4.1.1.2. Potential interference in hemolytic activity detection

Sources of interference include background absorption of algal samples and intact erythrocytes at 414 nm. Algal sample (algal samples alone) and negative (intact erythrocytes alone) controls were used for the ELA to account for these sources. Negative controls also accounted for erythrocyte autolysis. This is the lysis of erythrocytes due to assay conditions such as incubation temperature, irradiance and time, instead of the algal hemolytic compounds. In addition, intact erythrocytes were removed from the supernatant before measurement at 414 nm since they can interfere with hemolytic activity detection.
To enhance the pelleting of the intact erythrocytes, small volume conical microfuge tubes were used to conduct the ELA.

### 4.1.1.3. Algal sample

Intact and lysed cells were used for the ELA. Since it is believed that *P. parvum* actively releases toxic compounds using its haptonema, intact cells were used for the ELA to represent the active release of hemolytic compounds by *P. parvum* cells. Lysed cells were also used because previous studies performed on *H. akashiwo* and *Chattonella marina*, two species that are also ichthyotoxic flagellates, found that lysed algal cells displayed the most hemolytic activity (Kuroda et al. 2005; Ling and Trick 2010). The lysed cell samples represent the release of all hemolytic compounds contained within the algal cells. Intact and lysed cells displayed hemolytic activity in a cell density-dependent manner (Fig. 3.8). Lysed cells displayed higher amounts of hemolytic activity than intact cells, indicating that the haptonema is not necessary for the delivery of hemolytic compounds and that the compounds reside within the cells. In congruence with this, Ling and Trick (2010) found that intact cells of *H. akashiwo* showed low or no hemolytic activity, while lysed cells showed a high amount of hemolytic activity. Several studies have demonstrated the extracellular existence of *P. parvum* toxins (Yariv and Hestrin 1961). However, these toxins, including hemolysins, can be extracted from intact *P. parvum* cells in quantities that are larger than the quantities present in supernatants. The lysis of cells was found to increase the amount of hemolysin released (Shilo and Rosenberg 1960).

### 4.1.1.4. Selection of incubation conditions

To further standardize the ELA for quantifying the hemolytic activity of *P. parvum*, an optimal incubation time, temperature and light intensity had to be determined. An incubation time of 6 h was chosen because hemolytic activity decreased slightly as incubation time increased, but shorter incubation times resulted in more variability (Fig.
Similarly, Eschbach et al. (2001) observed erythrocyte lysis after 4 h of incubation with *P. parvum* extract. An incubation temperature range of 18-20°C was chosen because higher temperatures resulted in a decrease of hemolytic activity, possibly due to the denaturing of the hemolytic compounds (Fig. 3.7). As well, this is the temperature range where maximal hemolytic activity was observed. An incubation irradiance of 30-50 µmol photons m⁻² s⁻¹ was chosen because light has been reported to be necessary for toxin activation in several algal species (Kuroda et al. 2005; Shilo 1967; Yariv and Hestrin 1961) (Fig. 3.6). However, toxin production for *P. parvum* has been found to occur in the dark with the addition of glycerol (Martin and Padilla 1973). The incubation conditions selected were also similar to those used by Kuroda et al. (2005), who selected 5 h, 23°C and 30 µmol photons m⁻² s⁻¹ for testing the hemolytic activity of the ichthyotoxic flagellates *Fibrocapsa japonica*, *C. marina*, *H. akashiwo* and *Olisthodiscus luteus*. Furthermore, the selected temperature and light conditions coincide with culturing conditions for *P. parvum*, and are therefore more relevant to real bloom scenarios.

4.1.1.5. **Effect of isolate variability on hemolytic activity**

This study demonstrates that variability exists in the hemolytic activity between isolates from different geographic locations. All isolates tested were hemolytic. However, intact cells of isolate 995 from Europe were less potent than the cells of isolates 2797 and 2827 (Fig. 3.8), from North America. Isolates 2797 and 2827 displayed similar potency of hemolytic activity (Fig. 3.8), which can be attributed to their close geographic proximity (2797 from Texas and 2827 from South Carolina). In contrast, isolates of *P. parvum* showed similar hemolytic activity for the lysed cell samples (Fig. 3.8), demonstrating that each isolate possessed the same potential to be hemolytic. The growth of isolates 2797 and 2827 were similar under all tested conditions, while some differences were observed between these isolates and isolate 995, further indicating the variability between geographically distinct isolates. In congruence with the findings of this study, Larsen et al. (1998) also found differences in the growth and toxicity of *P. parvum* isolates. Furthermore, Larsen and Bryant (1998) reported not only a difference in toxicity between
isolates, but a change in the level of toxicity for each isolate with each experiment. This was suggested to be due to genetic variations between isolates from different geographic locations arising from responses by each isolate to different environmental conditions (Larsen and Bryant 1998; Larsen et al. 1993).

The hemolytic activity of intact and lysed cells of all isolates increased with an increase in algal cell density (Fig. 3.8). However, most studies have shown that toxicity can occur when algal density is very low and it can be absent when algal density is very high (Shilo 1967). This indicates that the relationship between algal cell density and toxicity is not simple, and growth and toxicity may be regulated by different factors (Dafni et al. 1972; Johansson and Granéli 1999). Growth phase was not found to affect the hemolytic activity of *Prymnesium* isolates (Fig. 3.9). The hemolytic activity of cells in both the stationary and exponential phases were comparable. However, many studies have found that older, denser populations of *P. parvum* were more toxic than young, sparse populations (Dafni et al. 1972; Granéli and Salomon 2010). These contradictory results may be explained by the findings of Shilo and Rosenberg (1960), who showed that the *P. parvum* ichthyotoxin (which has been the focus of most studies) accumulated during stationary phase, while hemolysins accumulated during exponential phase.

### 4.2. Effect of salinity and light intensity on growth and hemolytic activity

*P. parvum* was able to grow over a range of salinities (Fig. 3.10-3.11). This is in agreement with previous studies that have shown that *P. parvum* is euryhaline and, therefore, can thrive over a wide range of salinities (Dickson and Kirst 1987; Larsen and Bryant 1998; Larsen et al. 1993; McLaughlin 1958; Padilla 1970). *P. parvum* isolates were able to grow over a wide range of salinities (1-30‰), with lower growth rates at lower salinities (1 and 3‰) and maximal growth rates at higher salinities (10-30‰) (Fig. 3.10). Larsen and Bryant (1998) also found that *P. parvum* was able to survive at salinities of 3-30‰. As well, they observed variations in optimal salinities between isolates (Larsen and Bryant 1998). All *P. parvum* isolates were capable of growing under
a range of light intensities (10-180 µmol photons m$^{-2}$ s$^{-1}$), with lower growth rates when grown under lower intensities (10 and 30 µmol photons m$^{-2}$ s$^{-1}$) (Fig. 3.10). This is in agreement with previous studies, which also observed growth over a wide range of light intensities. These studies found an increase in growth with an increase in light intensity, until a saturation point of approximately 100-200 µmol photons m$^{-2}$ s$^{-1}$ (Dickson and Kirst 1987; Larsen and Bryant 1998; Larsen et al. 1993; Padilla 1970).

In this study, variability was observed between the yields of each isolate. The lowest yields for all isolates were observed for cultures grown at lower salinities (1 and 3‰). However, a substantial biomass was still maintained at these salinities (Fig. 3.11). For isolate 995, cultures grown under the lowest and highest light intensities (10, 90 and 180 µmol photons m$^{-2}$ s$^{-1}$) had the lowest yield (Fig. 3.11). Yields of cultures of isolate 2797, remained relatively similar at all light intensities (Fig. 3.11). Cultures of isolate 2827 grown under 10 and 30 µmol photons m$^{-2}$ s$^{-1}$ displayed the lowest yields (Fig. 3.11). These results further demonstrate the ability of *P. parvum* to grow over a wide range of salinities and light intensities. It also suggests that there is a saturation point for isolate 995 at approximately 90 µmol photons m$^{-2}$ s$^{-1}$ and 180 µmol photons·m$^{-2}$·s$^{-1}$ for isolate 2827.

Salinity did not have a significant effect on the hemolytic activity of isolates 995 and 2827. However, light intensity was found to have a significant effect. For isolate 995, hemolysis was inversely related to light intensity, with less hemolytic activity occurring in cultures grown under higher light (90 and 180 µmol photons m$^{-2}$ s$^{-1}$) (Fig. 3.12). The opposite was observed for isolate 2827, with cultures grown under higher light intensities (30-180 µmol photons m$^{-2}$ s$^{-1}$) being more hemolytic (Fig. 3.14). Higher hemolytic activity was observed for cells of isolate 2797 grown at lower salinities (1-5‰) than higher salinities (10-30‰) (Fig. 3.13). Cultures grown under the lowest light intensity (10 µmol photons m$^{-2}$ s$^{-1}$) were least hemolytic and those grown under 30 and 50 µmol photons m$^{-2}$ s$^{-1}$ displayed maximal hemolysis (Fig. 3.13).

Existing studies on the effects of salinity and light on *P. parvum* toxicity show conflicting results, and Larsen and Bryant (1998) suggested that this is due to the use of different
isolates in each study. Some studies have shown that salinity and light influence the toxin production of P. parvum (Larsen et al. 1993; Shilo 1967; Shilo and Rosenberg 1960), while others have shown that salinity and light do not significantly affect toxicity (Larsen and Bryant 1998). There are also studies that have shown an inverse relationship, with toxicity decreasing with an increase in salinity (Shilo 1967). All of the aforementioned results have been observed in this study, where different P. parvum isolates were used, supporting the suggestion put forth by Larsen and Bryant (1998). This further demonstrates that there are differences between the responses of P. parvum isolates to similar environmental conditions.

This study shows that Prymnesium isolates are able to survive and maintain hemolytic activity over a range of salinities and light intensities, which provides some insight into the ability of this species to invade freshwater systems throughout North America (Table 3.1). The observation of high growth and hemolysis across the range of salinities and light intensities also demonstrates the difficulty of determining conditions which induce HABs and suggests interactions with other factors play a role in their formation.

4.3. Effect of temperature on growth and hemolytic activity

All P. parvum isolates were able to survive in the three tested temperatures (12, 18 and 23°C) (Fig. 3.15-3.16). When grown at 18 and 23°C, isolate 2797 had a significantly higher growth rate than when grown at 12°C (Fig. 3.15). However, temperature did not significantly affect the growth of isolates 995 and 2827 (Fig. 3.15). In agreement with this study, previous studies have shown that P. parvum is able to survive over a wide range of temperatures (Larsen and Bryant 1998; Larsen et al. 1993).

Tetraselmis suecica is a marine flagellate that is known to be non-toxic. This species was used as a non-toxic control for the temperature assay. T. suecica did not display hemolytic activity, while the Prymnesium isolates were hemolytic (Fig. 3.17). This is in agreement with previous studies which, even at a high biomass, did not detect hemolytic activity in
the non-toxic control (*Tetraselmis apiculata, Nannochloropsis oculata*, and *Chlamydomonas reinhardtii*) (Kuroda et al. 2005; Ling and Trick 2010).

The hemolytic activity of isolates 995 and 2827 increased with temperature (18 and 23°C) (Fig. 3.17). This suggests that these isolates do not maintain the same level of hemolytic activity at all temperatures, and may produce fewer hemolysins at lower temperatures. In contrast, the hemolytic activity of isolate 2797 remained relatively constant at all temperatures (Fig. 3.17). This suggests that isolate 2797 maintains the same level of hemolytic activity regardless of temperature. Shilo (1967), and Larsen and Bryant (1998) also showed that there was no clear correlation between the hemolytic activity of *P. parvum* and temperature.

These results show that *Prymnesium* isolates are able to maintain high growth rates, biomass and hemolytic activity at warm and cool temperatures (Table 3.1). This is also observed in *P. parvum* blooms which have been reported in the southern United States during the fall and winter months, in China during late spring and early fall, and even during the summer in Finland (Manning and La Claire 2010). These observations further demonstrate that HAB formation is regulated by more than one factor and it is difficult to determine the optimal conditions for their stimulation.

### 4.4. Effect of nitrogen source on growth and hemolytic activity

Many studies have focused on the influence of P availability on *P. parvum* toxicity and less information exists on the effects of N availability (Johansson and Granéli 1999), particularly the effects of different N sources. Most studies on the effects of N availability on *P. parvum* toxicity have focused on the effects on nitrate, and not many studies exist on the effects of urea and ammonium.

Growth of the *Prymnesium* isolates was lowest when grown in urea (Fig. 3.18-3.19). For isolate 995, growth was highest when grown in ammonium (Fig. 3.18-3.19). For isolates 2797 and 2827, cultures grown in nitrate and ammonium showed comparable growth (Fig. 3.18-3.19). These results suggest that nitrate and ammonium were good N sources
for the *Prymnesium* isolates, while urea was not. At concentrations of N lower than 100 µmol, the *Prymnesium* isolates had lower growth rates and yields due to N limitation, while at concentrations higher than 150 µmol, they had lower growth rates and yields due to P limitation.

In congruence with this study, previous studies have shown that *P. parvum* can utilize nitrate, urea and ammonium (as well as other N sources, such as amino acids) (Grover et al. 2013; Johansson and Graneli 1999; Lindehoff et al. 2010; Solomon et al. 2010). Low additions of ammonium have been found to enhance growth, while high doses can be toxic to *P. parvum*. This is due to the deprotonation of ammonium that produces unionized ammonia, which causes osmotic stress, swelling and cell lysis in *P. parvum* (Grover et al. 2013). The uptake of urea by phytoplankton can occur both passively and actively (Anita et al. 1991). Prymnesiophytes have been found to use urease as the catabolic enzyme in urea metabolism (Anita et al. 1991; Solomon et al. 2010). Since urease is a nickel-ligated metalloprotein, urea metabolism by *P. parvum* is dependent on the addition of Ni$^{2+}$ (nickel) ions (Lindehoff et al. 2010). As well, for *P. parvum* to grow well on urea, the addition of Ni$^{2+}$ ions is necessary. However, low amounts of Ni$^{2+}$ ions are sufficient for growth (Oliveira and Anita 1986). Uptake of urea can still occur even if urea metabolism is unable to occur because urea transport is not affiliated with metabolism (Lindehoff et al. 2010; Solomon et al. 2010). This may explain the unusual ability of *P. parvum* to survive in the high concentrations of urea tested in this study (>100 µmol). While Ni$^{2+}$ ions were not directly added to the growth medium used in this study, it is possible that there was some contamination when other trace metals were added.

In all concentrations of nitrate, hemolytic activity remained relatively similar for all isolates (Fig. 3.20-3.22). Cultures of isolate 995 grown in urea displayed high hemolytic activity at 25 and 100 µmol (Fig. 3.20). For cells grown in ammonium, a dramatic decrease in hemolytic activity occurred at 50 µmol (Fig. 3.20). A drastic decrease in hemolytic activity was observed at 150 µmol for intact cells of cultures of 2797 grown in urea (Fig. 3.21). However, the hemolytic activity of lysed cells of cultures grown in urea
was similar at all concentrations (Fig. 3.21). The hemolytic activity of intact cells of cultures grown in ammonium decreased dramatically at 100 μmol, while for lysed cells the decrease occurred at 150 μmol (Fig. 3.21). The hemolytic activity of intact cells of isolate 2827 decreased drastically at 100 μmol ammonium and 500 μmol urea (Fig. 3.22). However, lysed cells of cultures grown in urea and ammonium displayed similar hemolysis at all concentrations (Fig. 3.22).

This study shows that at all tested N concentrations where cells are either N or P limited, cells grown in nitrate display similar hemolytic activity. In contrast, the hemolytic activity of cells grown in both ammonium and urea decreased dramatically at higher concentrations of N. Similarly, Johansson and Granéli (1999) found that cultures grown in N or P-limited media displayed a greater amount of hemolytic activity than cultures grown in N or P-replete media. These results suggest that there is a relationship between nutrient limitation and toxicity (Johansson and Granéli 1999). Most likely N or P limitation leads to physiological stress rather than a direct link to toxicity, since neither N nor P are major constituents in the identified toxin complex (Igarashi et al. 1996) or involved in its synthesis (Johansson and Granéli 1999). However, Johansson and Granéli (1999) only examined the effect of nitrate limitation on hemolytic activity, not urea and ammonium. Grover et al. (2013) found that at low concentrations of ammonium abundance and toxicity increased but at high concentrations, abundance and toxicity decreased. In this study, only a small decrease in abundance was observed at higher concentrations of ammonium, while a large decrease in toxicity was observed. It is possible that cells grown at the higher concentrations were delegating their energy to growth rather than the synthesis of secondary metabolites. This may also be true of cells grown in higher concentrations of urea. The cells may not have enough Ni^{2+} ions for urea metabolism so most of the energy went towards growth rather than the synthesis of secondary metabolites.

This study demonstrates that *Prymnesium* isolates can utilize different N sources for growth and the production of hemolytic metabolites (Table 3.1). It also indicates that these isolates can maintain a population at high concentrations of urea and ammonium,
which are toxic to other phytoplankton species. These abilities may provide an advantage for *Prymnesium* isolates over other phytoplankton species, allowing them to form frequent monospecific blooms.

4.5. Effect of iron chelator on growth and hemolytic activity

Iron is a vital nutrient for the growth and function of marine phytoplankton (Sunda and Huntsman 1995). Iron bound in a ligand-Fe complex may or may not be accessible to phytoplankton. (Rahman et al. 2014). Under conditions with low concentrations of bioavailable Fe, phytoplankton alter their Fe acquisition strategy by up-regulating Fe transport competence, reducing Fe (III) chelates, and ingesting insoluble Fe to satisfy cellular Fe demands (Rahman et al. 2014). Fe-limitation causes decreased CO$_2$ fixation in marine phytoplankton. In response to Fe-limitation, *P. parvum* has been found to alter cellular biochemical processes through the upregulation of proteins which may be involved in Fe uptake (Rahman et al. 2014). Efficiency of uptake may be improved by extracellular Fe$_{\text{III}}$ reduction and through increasing Fe acquisition sites. During Fe limitation, RuBisCo (ribulose-1,5-biphosphate carboxylase/oxygenase) is up-regulated due to an increase in photorespiration (Rahman et al. 2014). This requires high metabolic energy and this demand is satisfied through an increase in ATP synthase. *P. parvum* also up-regulates oxidative stress response proteins, MnSOD (manganese superoxide dismutase) and STK (serine threonine kinase), potentially as a mechanism for reducing oxidative stress caused by Fe-limitation (Rahman et al. 2014). It is also possible that *P. parvum* may produce hemolysins as a mechanism for scavenging Fe from the environment (e.g. erythrocytes), particularly during Fe-limiting conditions. This mechanism was shown in *H. akashiwo* in a study conducted by Ling and Trick (2010), where hemolytic activity increased under Fe-stress and subsequently decreased when the cells recovered from this stress.

The artificial ligand DFB mimics natural ligands in seawater and binds to Fe using secondary hydroxamate groups (Ling and Trick 2010; Wells and Trick 2004). Isolates 2797 and 2827 were able to survive at moderate to high concentrations of DFB (5-30 μM).
before a decrease in growth was observed (50-100 µM) (Fig. 3.23-3.24). In contrast, isolate 995 maintained a similar growth rate and yield in all cultures (Fig. 3.23-3.24). These results suggest that *P. parvum* is able to access and utilize free and chelated Fe for growth. It also suggests that isolates 2797 and 2827 have a different mechanism for obtaining Fe than isolate 995.

An inverse relationship between the concentration of DFB and the hemolytic activity of intact cells of *P. parvum* isolates was observed. This relationship was also observed for lysed cells of isolate 995 (Fig. 3.25). Isolate 995 was directly affected by the addition of DFB, with hemolytic activity decreasing at 5-100 µM (Fig. 3.25). Intact cells of isolates 2797 and 2827 maintained hemolytic activity at moderate to high concentrations of DFB (5-10 µM) before a decrease in hemolytic activity was observed (30-100 µM) (Fig. 3.25). However, lysed cells of these isolates maintained high hemolytic activity at all concentrations of DFB. This indicates that although the hemolytic activity of the North American isolates decreased with an increase in DFB, the hemolytic compounds were still maintained within the cells. These results also suggest that *P. parvum* does not produce hemolysins in order to scavenge Fe from erythrocytes. However, it is possible that this was not observed because the cells were able to satisfy their Fe demand by using other mechanisms to access and utilize Fe from the DFB-Fe complex.

The results of this study further suggest that the European isolate is more sensitive to the removal of Fe, since growth and hemolytic activity were not maintained at high concentrations of DFB (Table 3.1). On the other hand, the North American isolates were able to survive and maintain hemolytic activity at low levels of Fe (high concentrations of DFB). Since Fe is a vital nutrient for phytoplankton, the low abundance of Fe can control the diversity of species (Sunda and Huntsman 1995). The ability of the North American isolates to survive at low levels of Fe, presumably by altering and increasing uptake strategies, may provide a competitive edge over other phytoplankton species. This may explain why they are more invasive and are able to form frequent HABs.
5. CONCLUSIONS

In this study, *Prymnesium* isolates survived over a wide range of salinities (1-30‰), light intensities (10-180 µmol photons m\(^{-2}\) s\(^{-1}\)) and temperatures (12-23°C). The isolates were also able to thrive in a range of N concentrations (25-500 µmol) and sources (nitrogen, urea and ammonium), and were able to utilize both free and chemically compounded Fe (0-100 µM DFB) (Table 3.1). This shows that *P. parvum* is able to survive in many environmental conditions and may explain why this species is so successful and able to become so widespread. Furthermore, this study demonstrates the difficulty of determining conditions which induce HABs and suggests that formation is regulated by more than one factor.
REFERENCES


Appendix A

Transmission electron microscopy (TEM) images of *Prymnesium parvum* cells fixed in glutaraldehyde and osmium tetroxide.
# Curriculum Vitae

**Name:** Christine Dulal-Whiteway  

**Post-secondary**  
The University of Guelph  

**Education and Degrees:**  
Guelph, Ontario, Canada  
2010 - 2014 B.Sc.  
The University of Western Ontario  
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**Related Work**  
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**Conferences**  
International Association for Great Lakes Research (Guelph, ON)  
June 6 - 10, 2016
Poster Presentation: The Fish-Killing Activities of *Prymnesium parvum*
Authors: Dulal-Whiteway C., Trick C.G.

Yeast Cell as a Bio-Model for Measuring the Toxicity of Harmful Algal Blooms
Authors: Mehdizadeh Allaf M., Dulal-Whiteway C., Trick C.G.