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The Effects of Ocean Acidification and Eutrophication on the Growth, Lipid Composition and Toxicity of the Marine Raphidophyte Heterosigma Akashiwo.

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology
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THE EFFECTS OF OCEAN ACIDIFICATION AND EUTROPHICATION ON THE GROWTH, LIPID COMPOSITION, AND TOXICITY OF THE MARINE RAPHIDOPHYTE *HETEROSIGMA AKASHIWO*.

(Thesis format: Monograph)

by

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Abstract

Anthropogenic forcing, such as ocean acidification caused by rising carbon dioxide emissions, and eutrophication due to increased nutrient loadings in run-off, are causing major changes to the biogeochemistry of the oceans. As a consequence, coastal phytoplankton are susceptible to altered biogeochemical environments. This study examined the effect of a lower pH and increased levels of nutrients on the common coastal harmful alga, *Heterosigma akashiwo*. Growth rates, maximal cell yields, neutral lipid accumulation and toxicity of cells grown under various pH and nutrients regimes were measured. *Heterosigma akashiwo* growth was near maximal when grown at lower pH levels. There was a strong correlation between macronutrient concentration (nitrogen and phosphorus) and physiological responses such as cell yield, toxicity, and neutral lipid accumulation. Cells cultured on ammonium were less toxic that cells supplied with either nitrate or urea as a nitrogen source. Neutral lipid accumulation and cell toxicity varied under different environmental regimes but did not co-vary, indicating that polyunsaturated fatty acid production was not the mechanism of toxicity. Based on the ecophysiological profile, *H. akashiwo* will be both present and toxic in the future nutrient-rich, acidified coastal ocean waters.

Keywords

Acknowledgments

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Acronyms and Abbreviations

ANOVA	analysis of variance
CDLC	chemically defined lipid concentrate
EBUS	eastern boundary upwelling system
EDTA	Ethylenediaminetetraacetic acid
EFA	essential fatty acids
ESAW	enriched seawater artificial water
HAB	harmful algal bloom
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
L-15	Leibovitz L-15 medium
NaOH	sodium hydroxide
pH	$-\log [H^+]$
pHi	initial pH of the culture
ppm	parts per million
PUFA	polyunsaturated fatty acid
SOP	standard operating protocol
TAG	triacylglyceride

"In the end we will conserve only what we love; we will love only what we understand;
and we will understand only what we have been taught."
~ Baba Dioum, 1968

"Don't blow it – good planets are hard to find."
~ Quoted in Time Magazine, 1996

CHAPTER 1: INTRODUCTION

1.1 The anthropocene

Anthropogenic activities are having profound effects on the Earth and its systems (Steffen et al., 2011). Ecosystems are exhibiting dramatic changes on a variety of scales, from nutrient levels to population dynamics (Rockström et al., 2009; Hallegraeff, 2010). Climate patterns are becoming increasingly extreme and unpredictable (IPCC, 2012). Since this is such a recent occurrence in the Earth's history, the body of accompanying research is still in its infancy (Corfee-Morlot et al., 2007). In order to prevent further degradation and loss, there is a pressing need to understand the influence of anthropogenic activities on ecosystems (IPCC, 2007). In response, researchers have begun to answer such questions using a variety of approaches, from laboratory-based hypothesis testing to field-based observational studies, each with their own merit (Glibert and Burkehold, 2006; Hallegraeff, 2010). One major Earth system that is being negatively impacted by human activities and negatively affecting human activities as a result, is the ocean (Royal Society, 2005).

1.2 The future ocean

The oceans have a great capacity to buffer against change; as such they have remained relatively stable since the last ice age (Zeebe, 2012). The future ocean, however, may bear few similarities to the ocean of today. Industrialization, intensified agriculture practices and coastal development are directly impacting oceans via the release of nutrient-rich run-off into coastal waters (Cloern, 1999). This "cultural eutrophication" is contributing to massive algal blooms, and subsequent hypoxic "dead zones" (Rosenberg and Loo, 1988). Carbon emissions, a defining feature of the current

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anthropogenic era, have caused atmospheric levels of carbon dioxide to rise (Doney et al., 2009). The ocean serves as one of the largest carbon reserves on Earth, absorbing nearly one third of all carbon dioxide emissions (Zeebe, 2012). However, the ocean's capacity to absorb excess carbon is reducing with each increment of intensification of global carbon emissions (Royal Society, 2005). When this tipping point is breached, it would require in the order of 10,000 years or more for deep mixing to restore our oceans buffering compounds and sea surface pH to their former state (Royal Society, 2005). One of the indirect effects of increasing global carbon emissions, with its own set of accompanying problems, is ocean acidification (Doney et al., 2009).

1.2.1 Ocean carbon chemistry

Atmospheric carbon dioxide levels have risen from ~315 ppm to almost 400 ppm since 1960, and are predicted to increase to ~750 ppm by 2100 (IPCC, 2007). As atmospheric CO_2 concentrations climb, increased oceanic uptake via dissolution into seawater causes an increased concentration of CO_2 throughout much of the upper ocean (Denman et al., 2011). This results in the production of the intermediary carbonic acid (H_2CO_3) followed by bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and hydrogen ions (H^+). The overall result of this change in seawater-chemistry is a shift towards increased oceanic H^+ concentrations (lower pH) (Shi et al., 2010) (Figure 1.2.1).

$$CO_{2\,(g)}$$

$$\updownarrow$$

$$CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$$

carbonic acid   bicarbonate ion   carbonate ion

more acidic
$$pH \approx -\log_{10}[H^+]$$
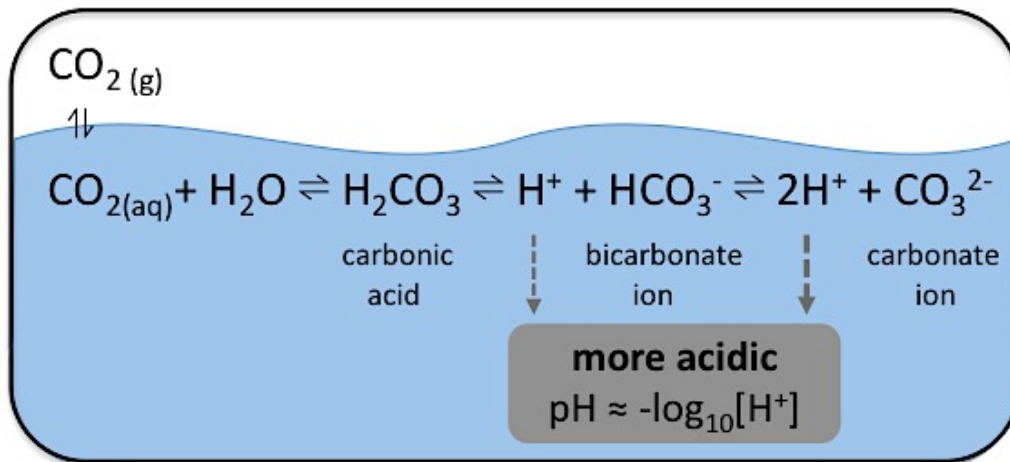
Figure 1.2.1 The ocean carbon chemistry of atmospheric carbon dioxide dissolving into sea surface waters, reacting with water to form carbonic acid, bicarbonate/carbonate ions, and hydrogen ions, resulting in lowered pH.

1.2.1.1 Ocean acidification

Since the industrial revolution, mean global surface water pH has decreased by 0.1 pH units (Denman et al., 2011). Based on various carbon dioxide emission regimes, from conservative to business-as-usual emission scenarios, pH could drop by another 0.10 to 0.35 units by the year 2100 (Orr et al., 2005). This represents a 30 to 105% increase in ocean acidity, respectively. It is important to emphasize that these declines in pH are averaged global values. Certain coastal upwelling regions, like the eastern boundary upwelling systems (EBUS) off the west coast of North America, are already experiencing levels of ocean acidification predicted for surface waters at the end of this century (Feely et al., 2008). Seasonal decreases in pH to ~ 7.7 or less have already been recorded in such CO_2–rich, nutrient-replete upwelling systems, and the pH of these upwelling regions could drop to as low as 7.4 by the end of the century (Feely et al., 2008).

Although reduced rates of calcification (shell-deterioration of calcareous marine organisms) are most commonly associated with ocean acidification, lower pH can impact other aspects of marine biogeochemistry such as the cycling of nutrients and their interaction with biological organisms. Ocean water pH influences bioavailability of macronutrients and trace metals, which impact the marine food chain (Royal Society, 2005). For instance, when pH sensitive nutrients, like iron, are limiting to most species of phytoplankton, an increase caused by acidification could have a severe impact on species composition and population dynamics.

The composition of the phytoplankton population can also change based on alterations in the pH-associated levels of inorganic carbon. Certain species may not be influenced by pH-induced carbon concentrations, should they possess a carbon concentrating mechanism (CCM)(Raven et al., 2011). CCMs allow for the concentration of carbon dioxide around the RuBisCO enzyme, allowing for higher photosynthetic efficiency (Reinfelder, 2010). CCMs also contain a putative HCO_3^- transporter, which serves as an additional source of inorganic carbon within the cell (Raven et al., 2011). More acidic ocean contains more CO_2 and HCO_3^-, so cells with a CCM are less likely to experience carbon-limitation (Reinfelder, 2010). However, recent investigation has shown a reduced affinity for carbon in CCM-containing algae grown under high CO_2 and in some cases, a loss of expression of the CCM (Raven, 2010; Raven et al., 2011). This could translate to algal species that lack a CCM being more likely to out compete those with a CCM, who could lose their competitive advantage, under high CO_2 conditions (Reinfelder, 2010).

1.2.1.1.1 Iron

Iron is required in many cellular structures that enable nitrogen use within the cell (Raven, 1988). Its oxidation-reduction properties allow iron to facilitate electron transport. Iron based enzymes (ferredoxins) are also able to reduce inorganic nitrogen species (nitrate and nitrite) to provide phytoplankton with a usable nitrogen supply (Morel *et al.*, 1991).

In most of the ocean, iron is also the micronutrient that limits the growth of phytoplankton (Boyd et al., 2007). The majority of Fe(III) are either bound by chelators or found within the highly insoluble neutral tri-hydroxy species $Fe(OH)_3$ (Shi et al., 2010). Iron uptake rates in some phytoplankton have been shown to drop by 10-20 % when the pH drops from ~8.4 to ~7.4, which could be due to natural iron scavenging ligands being less effective chelators at low pH values (Shi et al., 2010). However, for ambient organisms that do not produce iron-organic ligands, a drop in pH from 8.1 to 7.8 will increase the solubility of iron in the ocean waters, increasing bioavailability (Breitbarth et al., 2009).

1.2.2 Ocean eutrophication

The concentration of macronutrients (nitrogen and phosphorus) in the open ocean is very minimal compared to most freshwater ecosystems (Hecky et al., 1993). However, the concentration tends to increase in coastal regions, and zones of extreme upwelling (Capone and Hutchins, 2013). This phenomenon can cause coastal ocean waters to become nutrient-rich, fueling the growth of primary producers (Rosenberg and Loo, 1988).

1.2.2.1 Nitrogen

Agricultural fertilizers, effluent, and atmospheric deposition all lead to increasing levels of nitrogen in coastal waters (Davidson et al., 2012). Nitrogen has an essential role in the formation of amino acids, proteins and chlorophyll *a*, and as such, cellular requirements are also high. Although nitrogen (N_2) makes up ~78% of the atmosphere, phytoplankton are unable to fix this into a useable form, making nitrogen the most limiting macronutrient in ocean waters. Oceanic nitrogen is normally present in the form of ammonium (NH_4^+), nitrate (NO_3^-) or urea (CH_4N_2O) (Flynn and Butler, 1986). The concentration of nitrogen, found as nitrate, ammonium, and urea, in the open ocean are 0-50 μM N, 0-2 μM N, and 0-1 μM N respectively, while the concentrations that can be found in coastal waters are 500 μM N, 600 μM N, and 25 μM N respectively (Collos & Berges, 2004).

In terms of dissolved inorganic nitrogen, ammonium is readily useable by phytoplankton, however nitrate needs to be further reduced to nitrite (NO_2^-) and then ammonium before it can be assimilated into the cell. Organic nitrogen can most often be found as urea, which after being transported through the cell membrane, is divided into ammonia (NH_3) and CO_2 (Tamminen & Irmisch, 1996; Rukminasari & Redden, 2011). Approximately 70% of nitrogen fertilizers now contain urea in place of inorganic nitrogen sources (Gilbert, 2006).

In addition to increased nutrient loadings, recent studies examining the effects of ocean acidification on the oceanic nitrogen cycle have reported that a drop in pH results in lower rates of nitrification (Beman et al, 2011). Nitrification involves the oxidation of ammonium to nitrite and then nitrate by marine bacteria (Huesemann et al., 2002). Over

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30% of marine primary production is supported by nitrification that occurs within the uppermost 80 m of the water column (Yool et al., 2007). As such, when pH levels decrease, there are lower rates of nitrification, and hence more ammonium available (Beman et al, 2011). There is a subsequent need for a better understanding of the influences of changes in coastal nitrogen inputs, as well as pH and nitrification rates on the phytoplankton community.

1.2.2.2 Phosphorus

Phosphorus (P) commonly enters marine aquatic systems via sewage, phosphorus-based detergents, fertilizers and manure. Over 75% of phosphorus loadings to coastal zones have been attributed to anthropogenic sources, with the remainder being a result of natural weathering processes (Harrison et al., 2010). The average open ocean surface water concentration of inorganic phosphorus is ~0.015 μM P, while average coastal concentrations range from 0.2-2.0 μM P (Benitez-Nelson, 2000; McLaughlin et al., 2004).

Algal cells are able to attain P via the uptake of phosphate (PO_4^{3-}), which is essential for the synthesis of a wide range of macromolecules including nucleic acids, proteins and ATP. Increased P-loadings into the system can skew the ratio of N:P, which can trigger unbalanced growth within phytoplankton (Davidson et al., 2012). On the other hand, P-limitation has been proven to trigger toxin production in certain species of phytoplankton (Fu et al., 2008; Fu et al., 2010; Sun et al., 2011; Hardison et al. 2013).

1.2.2.3 The importance of the nitrogen to phosphorus (N:P) ratio

Anthropogenic inputs of nitrogen and phosphorus have been shown to "artificially" control the ratio of N to P in coastal waters. The global ocean average ratio

and cellular requirement of N:P according to Redfield (1953) should be approximately 16:1. There is, however, notable variation in elemental ratios between marine species. This means that a different phytoplankton may have the ability dominate, based on their cellular requirements and the environmental ratio of N:P. This has led many to believe that algal dominance, and toxin production of certain types of phytoplankton are governed by N:P, while levels of nitrogen are responsible for overall bloom formation.

1.2.3 Combined impacts of ocean acidification and coastal eutrophication

Ocean acidification and coastal eutrophication also exert synergistic effects on ocean biogeochemistry. Ocean water pH influences bioavailability of macronutrients and trace metals. When pH sensitive micronutrients, like iron, are currently considered limiting to most species of phytoplankton, an increased bioavailability, caused by acidification, can have a severe impact on species dynamics (Breitbarth et al., 2009; Royal Society, 2005). This change in nutrient availability can alter both organism physiology and community structure, which can allow for the proliferation of certain toxic algal species such as *H. akashiwo* (Fu et al., 2008; Fu et al., 2010; Sun et al., 2011; Hardison et al., 2013).

1.3 Harmful algal blooms (HABs)

Modern day harmful algal bloom (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 naturally occurring phytoplankton community (Smayda, 1997). The presence of HAB species may result in an alteration of the natural food chain, a change in water quality and/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 generally thought to be the result of human modification of coastal water quality (Quayle, 1969; Hallegraeff, 1993).

There is a general 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) and increased deposition of novel nitrogen to coastal waters (e.g., urea-based fertilizers) (Glibert et al., 2005; 2006). 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. Some researchers propose that the increased frequency of HAB reports is due primarily to the "observer effect" – there are more people watching the coastal waters and reporting incidents now. To illustrate this point, the 1st International Meeting of HABs in 1972 drew 15 researchers, while the 14th International HABs Meeting in 2010 had over 450 attendees. Whether there are more observers or more environmental outbreaks remains to be determined. There is, at present, no single factor that describes the increased frequency of HAB events.

1.3.1 *Heterosigma akashiwo*

Heterosigma akashiwo causes red tides throughout the world and is a grave concern for natural and penned fisheries. *Heterosigma akashiwo* can be found in temperate coastal waters worldwide, and has been observed off the coast of all continents other than Antarctica. *Heterosigma akashiwo* is classified in the class Raphidophyceae,

order Chattonellales and family Chattonellaceae (Throndsen, 1997). *Hetero* is Greek for 'different,' *sigma* is Greek for 'curves,' and *akashiwo* is Japanese for 'red sea water' (Throndsen, 1997). *Heterosigma akashiwo* is appropriately named based on its cellular appearance of variable shape, which has been described as ranging from potato-like to lumpy or cornflake-like, and its characteristic red discolouration of the coastal waters of Japan (Horner, 2002). As the cells range in size from 10-25 μm in length, 8-15 μm in width and are ca. 4 μm thick, *H. akashiwo* is a nanoplankton (Smayda, 1998). A single cell contains up to 95 chloroplasts, a high number relative to other phytoplankton species (Smayda, 1998).

Despite its fragile cell wall and ~10-20 μm diameter, *H. akashiwo* (Hada) Hada ex Y. Hara & M. Chihara is a marine raphidophyte capable of producing dense golden-brown toxic blooms (Hara & Chihara, 1987). This particular species has two heterodynamic flagella – appendages able to move independently of one another – that allow vertical migration in the water column (Band-Schmidt et al., 2004).

1.3.1.1 *Heterosigma* bloom events

H. akashiwo blooms have been associated with the mortality of cultivated fish in the coastal regions of many countries, including Canada (Haigh and Taylor, 1990), the United States of America (Horner et al., 1997), Japan (Honjo, 1993), China (Tseng et al., 1993), Australia (Ajani et a*l.*, 2001), New Zealand (Chang et al., 1993), Denmark, Sweden, Chile, and Brazil (Honjo, 1993). More recently, blooms have also been observed along the coasts of the southwestern United States of America (Herndon, 2003) and Mexico (Band-Schmidt et al., 2004). Its fish-killing, or ichthyotoxic, mechanism remains somewhat ambiguous, despite previous scientific investigations (Ono et al., 2000; Twiner

et al., 2001; Fredrickson et al., 2011).

1.3.1.2 *H. akashiwo* in the Salish Sea

In some locations, there have been well-documented increases in the negative effects of this particular HAB species. In the Salish Sea region, the species *Heterosigma akashiwo* forms nearly mono-specific blooms – meaning they are the dominant species contributing to the bloom.

The Salish Sea is an estuarine system that includes the Juan de Fuca Strait, the Strait of Georgia and Puget Sound, and is bordered by British Columbia (Canada), Washington State (United States of America), and the Pacific Ocean. The surface waters in this region span ca. 18,000 km^2 and the Salish Sea drainage basin is ca. 110,000 km^2 (Jarvis et al., 2008). *Heterosigma akashiwo* has been documented in this region since 1976, with reported increases in bloom frequency and densities beginning in the year 2000 and continuing to present (Gains and Taylor, 1986; Rensel et al., 2010). This region is part of the Eastern Boundary Upwelling System and receives nutrient-rich, acidified inputs from coastal wind-driven upwelling during summer and fall months (Feely et al., 2008). The pH in the Salish Sea varies seasonally from as low as 7.6 to 8.5. Nutrient-rich waters, in the form of run-off, also enter the system from the Fraser River, which drains into the Strait of Georgia. The seasonal drop in pH and enrichment of nutrients from deep waters make this region an ideal study zone for changes in global ocean acidification and eutrophication of coastal waters forecasted for the end of the century. It is interesting to note that blooms of *H. akashiwo* have been on the rise in this region (Rensel et al., 2010).

Deemed the most significant fish-killing HAB species in Pacific Northwest coastal waters, *H. akashiwo* has caused massive aquaculture fish kills and associated loss

in revenue (Rensel et al., 2010). *Heterosigma akashiwo* may have a direct influence on the survivorship of natural salmon populations, as there was a strong correlation between bloom events in the Strait of Georgia, BC and survival rates of Fraser River sockeye salmon (Rensel et al., 2010). Nearly 650 million Atlantic salmon have been killed by a lone harmful algal bloom event (Rensel, 1995). In terms of economic impact, the annual estimated cost of HABs to the United States of America is $50 million (Anderson et al., 2000). This is listed as moderate, and takes into account public health costs, losses to the tourism industry, monitoring and management, as well as aquaculture losses.

1.3.2 *Heterosigma akashiwo* growth

The response of raphidophyte species to increased carbon dioxide concentrations/ lower pH was the subject of a recent investigation (Fu et al., 2010). Higher CO_2 concentrations, resulting in lower pH, could increase phytoplankton growth and/or affect inter-species competition (Fu et al., 2008). *Heterosigma akashiwo* were found to have stimulated growth under elevated CO_2 concentrations alone, while the dinoflagellate *Prorocentrum minimum* experienced no significant change (Fu et al., 2008). Future ocean conditions (lowered pH and/or increased temperature) were shown to promote significant increases in growth rates of *H. akashiwo* compared to controls (Fu et al., 2008; Sun et al., 2011).

Experiments modelling future ocean conditions, with respect to both pH and temperature, show the elemental composition of *H. akashiwo* shifting towards a lower cellular P requirement (Fu et al., 2008; Sun et al., 2011). Under high temperature and low pH conditions, the elemental ratios of N:P and C:P both increased, while C:N remained relatively similar when compared to the control (Fu et al., 2008). Variation in elemental

ratios between marine species means that a *H. akashiwo* could dominate, based on their cellular requirements.

The paradox of the eutrophication situation is that, despite the large supply of nitrogen available to coastal phytoplankton, growth-limitation can occur if levels of P are unable to support growth. Phosphate (PO_4^{3-}) can effectively limit growth when nitrogen inputs are in excess (Rudek et al., 1991). Based on contemporary food production needs – requiring heavy applications of N-based fertilizers – coastal N input is increasing much faster than P (8x and 3x respectively) (Smil, 2001). Cells are able to cope with minor disruptions to phosphorus supplies by dipping into cellular reserves of polyphosphates (Harrison et al., 1990). However, if environmental P continues below cell subsistence quota for too long, growth may be negatively affected (Harrison et al., 1990).

1.3.2.1 Unbalanced growth

Balanced growth occurs when all cellular components are manufactured at a steady rate that is relative to one another allowing for maximum rates of cell division (Prescott et al., 1999) (Figure 1.3.2.1 A). The unbalanced growth model, however, is based on the comparative rates of carbon, nitrogen and phosphorus acquisition, relative to the acquisition of energy from photosynthesis. This is analogous in nature to the "Redox Balance" proposed by Maxwell *et al.* (1995). Unbalanced growth results in the differential rates of production of certain cellular components relative to others (Prescott et al., 1999). This can take place when the rate of incorporation of nutrients into the cell is less than the division rate of the cell. Unbalanced growth scenarios have been shown to result in differential cellular composition of *H. akashiwo*, specifically with regards to lipid production (Fuentes-Grünewald et al., 2012).
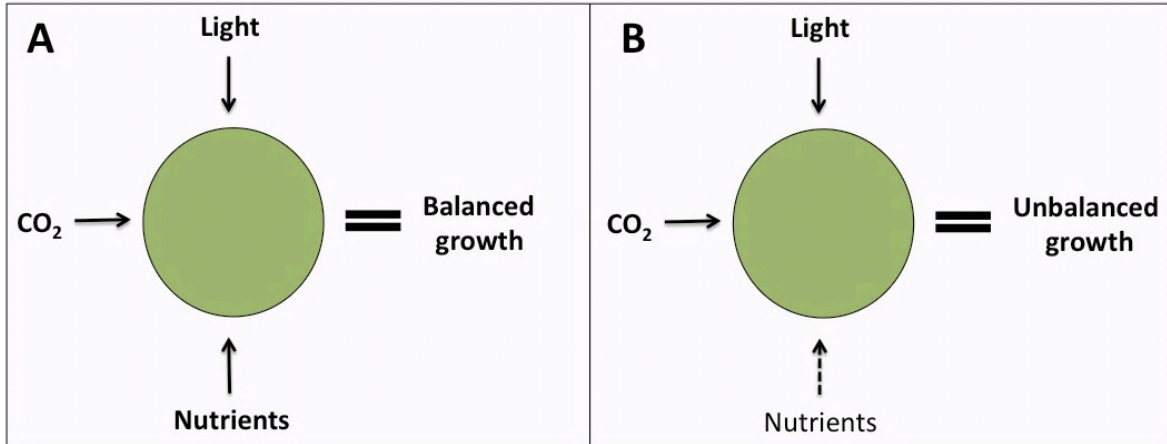
Figure 1.3.2.1 A cell receiving sufficient inputs of photosynthetic reagents (light and CO_2) and nutrients (based on the acquisition rate / cell quota) to support balanced cell growth (A) and insufficient nutrients resulting in unbalanced growth (B).

1.3.3 Lipid accumulation in *Heterosigma akashiwo*

When phytoplankton photosynthesize, the resulting cellular constituents are carbohydrates, protein, and lipids. Cells that are undergoing balanced growth tend to allocate the majority of photosynthetically derived resources towards protein (~50%), and carbohydrate (~40%) production, with lipids accounting for a relatively small proportion (~10%) (Geider & La Roche, 2002). The lipids produced in this scenario are mainly structural, membrane polar lipids, and their accumulation remains quite constant over time. If growth becomes unbalanced, however, the phytoplankton will shift production towards much greater proportions of lipids (~50%), and lesser amounts of carbohydrates (~30%), and proteins (~20%) (Geider & La Roche, 2002). Lipids in the cell undergoing unbalanced growth are primarily neutral lipids (triacylglycerol or TAG), which are highly variable in terms of accumulation (Hu, 2008). These are high carbon energy stores, within the cytoplasm, that are utilized to sustain metabolic requirements when needed (Fuentes-Grünewald et al., 2012). These shifts in cellular composition can impact other marine organisms, as they rely upon phytoplankton as a food source. Cellular

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composition also has an impact on biofuel production strategies (Sharma et al, 2012). Most recently investigated, cellular composition could also alter the production of allelopathic and/or toxic agents (Ikawa, 2004).

1.3.3.1 Quality food source

Phytoplankton account for 45% of the earth's primary production, and are the base of the oceanic food web. As such, their presence is required to provide energy that supports the proliferation of higher trophic levels (Rossoll et al., 2012). One of the unique roles of phytoplankton is the synthesis of essential fatty acids (EFAs). Marine predators are not able to generate essential fatty acids *de novo*, and rely upon bioaccumulation from their diet (Igarashi et al. 2007).

Long chain polyunsaturated fatty acids (PUFAs), such as omega-3 and -6 fatty acids, can effectively control the growth and reproductive health of organisms of a higher trophic level (Veloza, 2005). Humans can be considered part of this trophic system, and are recommended to consume fish, supplements, etc. to acquire omega-3 and -6 fatty acids. Polyunsaturated fatty acids are non-constitutive, fluctuating components of neutral lipids (Hu, 2008). Production of PUFAs varies over space and time, in that they accumulate during conditions of environmental stress (Liu et al., 2008). As such, ocean acidification and nutrient modification can lead to unbalanced growth, which impacts the production of PUFAs in phytoplankton, affecting their quality as a food source for marine organisms (Rossoll et al., 2012).

1.3.3.2 Algal biofuels

The lipid composition of phytoplankton is of particular interest to those in the field of third-generation biofuel development. Biofuels are currently harvested from food

crops such as oil palm, coconut, and rapeseed. The issues that arise with this form of biofuel are the dilemma of fuel versus food – should food crops be used for fuel for the affluent as opposed to a food source for those in need – and the large amount of resources (fertilizers, fresh water, etc.) and acreage required to grow the crops (Singh et al., 2011). Algal biofuels are unique in their ability to yield oil volumes per acre that are in the range of an order of magnitude greater than the most efficient food crops (Hu, 2008; Singh et al., 2011). Additionally, they can be grown in seawater, which reduces the strain on fresh water resources.

Heterosigma akashiwo, and other raphidophytes, are capable of producing relatively rapid yields (0.55 divisions per day) of high biomass productivity (185,000 cells mL^{-1}) (Fuentes-Grünewald et al., 2012). Studies examining *H. akashiwo* productivity against both neutral lipid content and lipid profiles justify its use in the algal biofuel industry (Doan et al., 2011; Fuentes-Grünewald, 2012). An emerging field of research has discovered that modification of abiotic parameters can enhance neutral lipid (oil) production in microalgae. Modification of nutrient inputs, light, temperature, and salinity have been shown to alter neutral lipid levels (Hu, 2008; Fuentes-Grünewald et al., 2012). Increased neutral lipid production allows for higher yields of biodiesel, and manipulation of abiotic parameters is a part of the most cost-effective and simplistic ways to accomplish this (Fuentes-Grünewald et al., 2012). The influences of pH and nutrient speciation on neutral lipid production have only just begun to be examined (Singh et al., 2011).

1.3.3.3 Toxic EFAs

Marine microalgal essential fatty acids (EFAs) have been shown to exert negative allelopathic effects on other algal species, inhibiting growth, reproductive success, and/or survival (Ikawa, 2004). In this sense, EFAs could be controlling which algal species is dominant in the marine phytoplankton community, while also exerting toxic effects on other marine organisms, notably fish species.

PUFAs in particular are known to increase phospholipid membrane permeability at concentrations of an order of magnitude lower than saturated fatty acids (Castaing et al., 1993). This may be the mechanism through which *Heterosigma akashiwo* exerts toxic effects on marine finfish and allelopathic effects on co-occurring marine organisms. *Fibrocapsa japonica*, another raphidophyte, was found to produce high concentrations of PUFAs, which caused mortality in brine shrimp (*Artemia salina*), inhibition of *Allivibrio fischeri* bioluminescence, as well as mortality in European seabass (*Dicentrarchus labrax*) (Pezzolessi, 2010). Okaichi (1989) found two PUFAs were produced corresponding to the death of yellowtail within a bloom of yet another raphidophyte, *Chattonella antiqua*. The PUFAs were described as stearidonic acid (18:4(n-3)) – an omega-3 polyunsaturated fatty acid – and hexadecatetraenoic acid (16:4(n-3)) (Okaichi, 1989). The PUFAs had an adverse interaction with the mucosal lining of the fish gill cells, which resulted in swelling, reduced gas exchange, lack of oxygen, and asphyxiation (Toyoshima et al., 1989). The proposed mechanism of toxicity – gill cell interaction – is important when selecting an appropriate bioassay to gauge algal toxicity.

1.3.4 *Heterosigma akashiwo* toxicity

The exact mechanism of *Heterosigma* ichthyotoxicity has yet to be determined. Proposed mechanisms involve the release of a brevetoxin-like compound, or more recently, the production of EFAs, specifically PUFAs (Haque & Onoue, 2002; Okaichi, 1989). For the purposes of this study, the primary focus of toxicity research will be to investigate possible regulators of the toxic mechanism in question and secondly, to determine if the toxic response correlates to PUFA accumulation.

The most common regulator of *H. akashiwo* toxicity is thought to be the disruption of cell growth caused by the depletion of a given macronutrient, in relation to the others (Twiner et al., 2001; Fu et al., 2010; Fredrickson et al., 2011). The primary macronutrients that limit the growth of the cells include nitrogen and phosphorus (Glibert & Burkholder, 2006). Over the last two decades, each element has been implicated as the toxicity inducer (Fu et al., 2010; Kudela et al., 2008). Recently, low iron availability within HAB events has been considered the stimulus of toxin production (Ling & Trick, 2010). The rationale is that the low availability of iron in coastal waters regulates the efficiency of nitrogen use, and alters the competitive ability of the species. The exact trigger(s) of toxicity has yet to be confirmed.

1.3.4.1 Toxicity & unbalanced growth

The balance between macronutrient availability and iron might suggest that toxicity is caused by an unbalanced growth scenario, rather than the absolute nutrient deficiency (Ling & Trick 2010; Powers et al. 2012). This model moves our attention away from the view that toxins are predatory molecules and towards the idea that toxins are waste products of cell growth (Trick, 2012).

Another aspect of unbalanced growth and *H. akashiwo* toxicity relates to ocean acidification. If the relative supply of N, P, and Fe control the balance of cellular metabolites, the acidification of coastal waters – and the corresponding alteration of the nutrient biogeochemical cycles discussed earlier – may strongly influence *H. akashiwo* toxicity.

Fu et al. (2010) demonstrated that a toxic species of dinoflagellate, *Karlodinium veneficum*, produced more of the toxic compound karlotoxin (KmTx-1) under lower pH conditions, especially when paired with nutrient limitation of phosphorus. Similar results were obtained for the harmful bloom diatom, *Pseudo-nitzschia multiseries*, which produced elevated levels of domoic acid under both acidified P-limited and acidified Si-limited conditions (Sun et al., 2011; Tatters et al., 2012).

1.4 Study statement

Changes to phytoplankton physiology in response to the potentially interactive effects of pH and nutrient levels have been demonstrated (Fu et al., 2010; Sun et al., 2011; Tatters et al., 2012). This thesis focused on gaining a better understanding of the effect that factors predicted for the future ocean have on a common coastal HAB genus, *Heterosigma*. The main objectives were to determine if modelled future ocean conditions of low pH, higher nitrogen, and variable iron would adversely affect the ecological importance of this genus. I used the cellular growth rate to assess if the genus has the capacity to grow under the predicted new ocean conditions. Toxicity levels indicated if there would be adverse effects of the genus in the water column. An assessment of the neutral fatty acids provided insight into two critical aspects of *Heterosigma* ecology: are neutral fatty acids the mechanism of fish-killing potential and are levels of neutral fatty

acids indicative of a species that would supply fatty acids to the fecundity of the next step in the food chain (zooplankton) (Fu et al., 2010; Sun et al., 2011; Fuentes-Grünewald et al., 2012). Results from this thesis are critical to understanding the consequences of *Heterosigma* in the water column, and will influence natural food web dynamics (including the domination of this genus as a HAB), fish-rearing aquaculture and biofuel industries.

1.4.1 Hypothesis

The main hypothesis of this study is that *Heterosigma akashiwo* will remain a potential HAB species under the new ocean conditions. To remain a potential HAB species, this genus must maintain its growth rate and nutrient-use efficiency, decrease levels of neutral lipid levels to reduce the consumption by zooplankton, and increase its toxicity under novel nutrient-rich conditions and lower pH values projected for future ocean waters.

1.4.2 Objectives

In order to test the above hypothesis, multiple objectives were established. The first objective was to measure the change in growth rate and cell yield across a suite of nutrient (nitrogen, phosphorus, and iron) concentrations and pH levels (7.4, 7.8, 8.1). This will help predict *H. akashiwo* bloom frequency and intensity in future nutrient-rich, acidic ocean waters.

The second objective was to detect how changing nutrient and pH regimes will alter the neutral lipid content of *H. akashiwo*. Cells with higher amounts of neutral lipids could be a lower quality food source and indicate an inability to support marine

ecosystem productivity, lowering predation. They also indicate PUFA production, which

has been linked to both allelopathy and ichthyotoxicity.

        The third objective was to gauge the toxicity of *H. akashiwo* under altered

nutrient and pH conditions using a RT-gillW1 assay. As this HAB species exhibits

variable toxicity, it is critical to examine under modelled future ocean conditions.

CHAPTER 2: METHODS

2.1 Culture Preparation

2.1.1 Isolate & stock culture conditions

2.1.1.1 Cell isolate

Heterosigma akashiwo isolate 513 was chosen for this study. This isolate was originally collected from the waters of Puget Sound, Washington, USA, where this species is common and has a history of finfish devastation. The isolate was maintained in the Northwest Fisheries Service Center and was given the designation NWFS 513. The alga has been maintained as a unialgal, non-axenic strain.

2.1.1.2 Culture conditions

Stock cultures were grown at 10% (v/v) inoculum in 1 L Erlenmeyer glass flasks. Flasks contained 270 mL of autoclave sterilized (to 135 ˚C and 30 psi over 60 minutes) nutrient enriched artificial seawater (ESAW) medium with specified modifications as implemented by Herndon and Cochlan (2007), and 30 mL of inoculum (Harrison *et al*. 1980; Berges *et al*. 2001). All flasks were autoclaved prior to use, after being fitted with cheesecloth-wrapped non-absorbent cotton stoppers, which were then covered with aluminium foil. Cultures were grown at 19 °C under cool white fluorescent light, at a constant irradiance of 65-80 μmol photons $m^{-2}s^{-1}$. Irradiance was measured using a Quantum Scalar Laboratory 2100 irradiance sensor (Biospherical Instruments, San Diego, CA). Stock cultures were maintained in exponential phase by subculturing every 4-5 days.

2.1.2 pH adjustment & maintenance

For all experimental treatments 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was added to the media at a final concentration of 20 mM in order to maintain desired pH (Harrison & Berges, 2005). Sodium hydroxide (2 M NaOH) and hydrochloric acid (4.1M HCl) were titrated into the media to attain the experimental pH levels of 8.1, 7.8 or 7.4, representing current average global ocean pH, global average ocean pH projected for 2100, and the pH of surface waters in the Eastern boundary upwelling system (EBUS) projected for 2100, respectively (IPCC, 2007). The flasks were swirled by hand throughout the titration process. A Thermo Scientific[TM] Orion[TM] 2-Star Benchtop pH meter was used to measure pH levels during titration. The buffered and titrated media was allowed to attain homogeneity for approximately 1 hour at 19 °C, prior to inoculation.

2.1.3 Inoculum

Stock cultures of *H. akashiwo* 513 in stationary phase were used as inoculum for all experiments. This growth phase was chosen to reduce the transfer of nutrients. Inoculation was performed under aseptic conditions in a laminar flow hood, using a serological pipette, and added until the final culture volume was inoculated to 10% (v/v). Stock cultures were gently swirled prior to inoculation, and experimental cultures were gently swirled post-inoculation.

2.1.4 Iron & pH treatments

All media stocks were chelated using ethylenediamine tetra-acetic acid (EDTA) to facilitate the modification of the original ESAW iron levels for the purposes of this experiment (Price *et al.* 1988/1989). The volume of modified ESAW salts amended with

f/2 nutrients was then divided evenly into two separate allotments and enriched with ferric chloride to attain the desired level of iron. A ferric chloride ($FeCl_3$) concentration of 0.1 μM was used for iron-deplete treatments, and 11 μM was used for iron-replete treatments. These values were selected based on preliminary work done by Ling and Trick (2010) and Bronicheski (2014) involving *Heterosigma* growth characterization over a wide range of iron concentrations. A 270 mL volume of media was added to each 1 L flask and buffered and titrated, with the exception of the controls, which remained without HEPES at the medium's ambient pH of 8.2. Flasks were then inoculated with 30 mL of culture that had been grow in either iron-replete or deplete conditions for 10+ days (at least 8 generations) to adjust to their respective treatment conditions. Each treatment was repeated 3 times, for a total of 20 samples per experiment. The experiment was conducted in triplicate, non-concurrently.

2.1.5. Nitrogen & pH treatments

Modified ESAW salts with f/2 nutrient medium were enriched with one of nitrate (as $NaNO_3$), ammonium (as NH_4Cl), or urea (as CH_4N_2O) as the source of nitrogen. Nitrogen concentrations were maintained at 10 μM N across all three nitrogen sources, in a constant 12:1 ratio of nitrogen to phosphorus. Standard ESAW + f/2 medium ferric chloride concentration of 11 μM of was used. A 270 mL volume of media was added to each 1 L flask and buffered and titrated, with the exception of the controls, which remained without HEPES at the ambient media pH of 8.2. Flasks were then inoculated with 30 mL of inoculum that had been grow in ESAW + f/2 media enriched with nitrate (as $NaNO_3$), ammonium (as NH_4Cl), or urea (as CH_4N_2O) for 3+ days (at least 6 generations) to be better conditioned to their respective treatment conditions. Each

experimental treatment was executed in triplicate, for a total of 30 samples per experiment. The experiment was also performed with non-concurrent triplicates.

2.1.6 N:P and pH treatments

Modified ESAW salts with f/2 nutrients medium was divided into seven separate Nalgene containers and enriched with nitrate ($NaNO_3$) and phosphate (as $NH_2PO_4 \cdot H_2O$) to yield N:P ratios of 1:1, 3:1, 6:1, 12:1, 15:1, 24;1 and 48:1. Nitrate and phosphate concentrations used are given in Table 2.1.6.

Table 1.1.6 Concentrations of nitrate and phosphate in batch cultures of *Heterosigma akashiwo*.

N:P Ratio	NO_3^- [μM]	PO_4^{3-} [μM]
1:1	100	100
3:1	100	33.2
6:1	100	16.7
12:1	100	8.33
15:1	125	8.33
24:1	200	8.33
48:1	400	8.33

Nitrogen levels were adjusted to maintain biomass in all treatments, and to ensure any changes were due to N:P ratios rather than nitrogen shortage (an uncommon occurrence in coastal waters). The standard media ferric chloride concentration of 11 μM was used. A 125 mL volume of media was added to each 500 mL flask and buffered and titrated to a pH of 7.4. The controls, which remained without HEPES, contained 125 mL of ambient media at a pH of 8.2. Flasks were then inoculated with 25 mL of *H. akashiwo* 513 inoculum that had been grown in ESAW + f/2 media enriched with 125 μM of nitrate and 8.33 μM phosphate (15N:1P) for 3+ days (at least 6 generations) to adjust to the treatment closest to Redfield's ratio (16N:1P). Each experimental treatment was run in

triplicate, for a total of 42 samples per experiment. The experiment was also performed with non-concurrent triplicates.

2.2 Experimentation

2.2.1 Growth

2.2.1.1 Daily measurements of cell density

Immediately following inoculation, all flasks were swirled to allow for a ubiquitous distribution of *H.* akashiwo cells, and approximately 1.5 mL was aseptically sub-cultured from each, using a serological pipette, into a 1.7 mL microcentrifuge tube. In preparation for cell counts, 0.5 mL of each sample was removed from the initial microcentrifuge tube, after being gently mixed with a pipette, and placed into a new microcentrifuge tube. This tube was subsequently loaded onto a C6 Flow Cytometer (BD Accuri^{TM}) and 50 μL of sample was run through the flow cytometer. C6 Analysis Software (BD Accuri^{TM}) allowed for gating and cell density measurements of *H. akashiwo* – through the use of quadrat lines applied to a histogram of measured chlorophyll a fluorescence versus particle size. Cell densities (given as number of cells per 50 μL) were converted to cells per millilitre of sample and recorded. This was performed for each triplicate of a given treatment, and averaged to attain daily growth values. The entire process was repeated once every 24 hours (± 2 hours) over the course of the iron & pH experiments, and once every 12 hours (± 2 hours) for the duration of nitrogen & pH and N:P & pH experiments.

2.2.1.2 Determination of growth rates

Log transformed cell density measurements were plotted against time to establish the time of maximum rate of change of cells (the exponential growth phase). The slope of

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the line during the exponential phase was used to calculate the growth rate using the following equation:

$$\mu = \frac{\ln\left(\frac{N_2}{N_1}\right)}{t_2 - t_1}$$

where μ is the specific growth rate (measured in divisions per day [d^{-1}] and N_1 and N_2 are the averaged cell counts at t_1 and t_2 (measured in days) (Fu *et al*. 2008; Guillard, 1973).

2.3 Cell assays

2.3.1 Neutral lipid analysis: Nile red assay

The Nile red assay was used to measure the level of neutral lipids produced per *H. akashiwo* cell during growth experiments. Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) selectively stains intracellular lipid droplets (neutral lipids), causing them to fluoresce (Greenspan *et al*., 1985). Fluorescence was then measured on a fluorescence spectrophotometer and used as a semi-quantitative estimate of neutral lipid production. The standard operating protocol (SOP) developed by Bjornsson (2009) was followed, with modifications outlined below.

2.3.1.1 Nile red assay: algal sample preparation

All flasks were swirled to allow for a ubiquitous distribution of *H.* akashiwo cells, and approximately 1.5 mL was aseptically subcultured from each, using a serological pipette, into a 1.7 mL microcentrifuge tube. This was repeated once every 24-hours over the course of the growth experiments.

2.3.1.2 Nile red assay: modifications to SOP

Stock aliquots of the 5% (v/v) standard solution of chemically defined lipid concentrate (CDLC) (MPN: AK8993-0100, Akron Biotechnology, LLC.) were stored in the dark at -20°C. Experimental samples were diluted 1:5 in ESAW + f/2 in the 96-well deep well plate (DWP) (MPN: 82006-448, VWR®). A final sample volume of 200 μL was transferred from the 96-well DWP to a clear 96-well plate using a mechanical multi-channel pipette. The outer wells were not used to avoid edge effect evident during preliminary tests. Plates were read on a Cary Eclipse Varian fluorescence spectrophotometer fitted with a multi-well plate attachment.

2.3.2.3 Nile red assay: analysis

Raw fluorescence units (RFUs) of the acetone and sample control wells were subtracted from the corresponding nile red acetone solution and sample wells. Values were then standardized per cell and then normalized using the 5% (v/v) CDLC standard. Although this method does not indicate total amounts of neutral lipids per cell, it is able to demonstrate relative neutral lipid accumulation.

2.3.2 Toxicity analysis: RTgill-W1 cytotoxicity assay

2.3.2.1 Rainbow trout gill cell line maintenance

The Rainbow trout gill cell line (RTgill-W1) assay was used to determine the level of toxicity expressed by *H. akashiwo* cells grown under a given treatment. Rainbow trout (*Onchorhunchus mykiss*) gill cells – from a continuous cell line – were purchased from the American Type Culture Collection (ATCC) and maintained in the dark at 18°C. Cells were grown in sterile Leibovitz's L-15 medium (MPN: 10-045-CV, Corning cellgro® Mediatech), supplemented with 10% (v/v) Fetal Bovine Serum (MPN: 1500-

100, Seradigm) and 2% (v/v) antiobiotic/antimycotic (MPN: A5955, Sigma-Aldrich) solution to form L-15 complete. Gill cells were initially grown in sterile plug 25 cm^2 treated tissue culture flasks (MPN: 353108, BD Flacon®). Cells were observed under an Axiovert 100 TV (Zeiss) inverted microscope to determine the degree of cell confluence, according to Dayeh *et al.* (2003). Once gill cells had formed a confluent monolayer, they were aseptically transferred into larger 75 cm^2 tissue culture flaks (BD Flacon® MPN: 353136).

2.3.2.2 Rainbow trout gill cell line harvesting

In preparation for the gill cell assay, confluent 75 cm^2 flasks were harvested and concentrated. Cells were first exposed to 1.5 mL of 0.53 mM EDTA Versene solution (MPN: 17-711E, BioWhittaker® Reageants Lonza) for 1 minute, which was then aspirated off and replaced with 3 mL of Trypsin solution (0.25% in Hank's balanced salt (HBS) solution) (MPN: 25-052-CI, Corning cellgro® Mediatech) for 4 minutes. Five millilitres of L-15 complete were added to the flasks, and cells were gently detached using a serological pipette. Cells were then collected in a sterile 15 mL centrifuge tube (MPN: 352196, Falcon®) and immediately centrifuged at 200xg for 4 minutes at ~12°C in a Beckman Coulter Avanti J-251 centrifuge. The supernatant was then aspirated from the tube, leaving ~0.5 mL of solution to avoid disputing the cell pellet. Cells were then resuspended in 7-10 mL of L-15 complete.

2.3.2.3 RTgill-W1 assay preparation

In order to determine cell concentrations, 0.5 mL of cellular solution was transferred to a 1.7 mL microcentrifuge tube and counted using a Haemocytometer. In

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order to run the bioassay, gill cells concentrations were adjusted to 2 x 10^6 cells mL^{-1} with the addition of L-15 complete.

Two hundred microliters of the density-adjusted gill cell solution was pipetted into a clear 96-well plate, with the exception of 3 interior wells (to serve as "no cell" controls) and all outer wells (to eliminate edge-effects) that were filled with 200 μL of L-15 complete alone. The lidded 96-well plate was then allowed to incubate for 2-3 days in the original incubation conditions, until cells were deemed confluent.

2.3.2.4 RTgill-W1 assay algal sample preparation

In order to collect algal samples for the assay, 45 mL of culture was sampled from each treatment flask, and place into a sterile 50 mL centrifuge tube (MPN: 352070, Falcon®). This was done twice for each culture, first when a given treatment entered late exponential growth phase, and then again once the cells entered early stationary growth phase. Samples were then spun down at 2000xg at 15°C for 5 minutes in a Beckman Coulter Avanti J-251 centrifuge. The majority of the supernatant was decanted from the pellet and placed in a sterile 50 mL Falcon tube and stored at -20 °C. Approximately 1mL of the supernatant was left in the original tube to allow for resuspension of the pellet. After cells were resuspended, they were transferred to a sterile 1.7 mL microcentrifuge tube and stored at -20 °C until further analysis.

While the gill cells were growing to confluence in 96-well plates, the frozen algal samples were thawed at room temperature, in order to lyse the cells. A total of two freeze-thaw cycles were performed to ensure complete cell lysis. Previous research has shown that *Heterosigma* toxicity in vitro requires both fractured cell wall and periplasmic components (Ling, 2006; Powers et al., 2012). Samples were centrifuged at 10,000xg for

3 minutes at 20°C in a bench top microcentrifuge. The majority of the supernatant was aspirated from the pellet and 1 mL of L-15 experimental (ex) was added. L-15ex contained the same nutrients, vitamins, and salts as L-15 complete, but was not amended with FBS or antibiotics. The pellet was then resuspended and diluted with more L-15ex to a concentration of 200,000 cells mL^{-1}.

2.3.2.5 RTgill-W1 assay

Procedures were followed as described by Dorantes-Aranda et al. (2011) and Dayeh et al. (2003). The 96-well microplate with a confluent monolayer of prepared RTgill-W1 cells was rinsed twice with 100 μL of L-15ex to remove any L-15 complete. The gill cells were then exposed to 200 μL of the previously prepared algal exposure solution, or a 3% hydrogen peroxide solution control (shown to elicit complete toxicity). The plate was then sealed with sterile Parafilm M$^{®}$, lidded, and placed back into the incubation area (18°C in the dark) for a 24-hour exposure period. Following exposure, all wells were rinsed twice with 100 μL, and then exposed to a 100 μL solution of 5% (v/v) PrestoBlue™ (MPN: A-13262, Life Technologies) in L-15ex. PrestoBlue™ is a fluorescent dye that contains resazurin. Plates were again sealed with sterile Parafilm M$^{®}$, lidded, and placed back into the incubation area (18°C in the dark) this time for a 2-hour exposure period.

Fluorescence readings were taken after a 2-hour incubation in the dark on a fluorescence spectrophotometer plate reader (Agilent Technologies, Santa Clara, CA). Metabolically active cells reduce resazurin to resofurin, which fluoresces (emission 590 nm) when exposed to green light (excitation 540 nm). This fluorescence measurement represents gill cell viability in the presence of *H. akashiwo*. A lack of fluorescence,

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relative to the positive control, can be used as indicator of toxicity. Raw fluorescence units (RFUs) were converted to gill cell viability in relation to controls using the following equation described by Dayeh et al. (2003):

$$\text{GC Viability (\%)} = \left(\frac{(\text{Exp. RFU} - \text{No Cell Control})}{\text{Average (Hydrogen Peroxide Control} - \text{No Cell Control})} \right)$$

2.4 Data Analysis

Two-way ANOVAs were used to detect main effects and interaction effects and post-hoc Tukey's Tests were used to determine differences between groups. Principal component analysis and Spearman's rank order correlation coefficient were used to assess correlation between dependent and independent variables across experiments.

Statistical analysis was performed using Microsoft Excel 14.3 (Microsoft, Redmond, Washington, USA), GraphPad PRISM® 6.0 (GraphPad Software, San Diego California USA), JMP 11.1 (SAS Institute Inc., Cary, NC, USA), and SPSS 21 (SPSS IBM, New York, USA).

CHAPTER 3: RESULTS

3.1 Preliminary experimentation

3.1.1 Determination of peak cell yield, neutral lipid accumulation, and toxicity

Maximal cell yield, neutral lipid accumulation, and toxicity data was selected for presentation in this thesis. Peak neutral lipid accumulation was found to occur during the transition between late exponential and stationary phase sampled (data not shown). Toxicity also peaked during this period of growth (Ling and Trick, 2010).

3.1.2 Verification of experimental pH values

Preliminary experimentation allowed for minimal testing of pH during growth experiments, in an effort to maintain culture volume needed for bioassays. Figure 3.1 illustrates that cultures of *Heterosigma akashiwo* (isolate 513), buffered and titrated to pH levels of 7.4, 7.8, and 8.1, all held $\pm$ 0.11 pH units over a 21-day period. The pH of the culture that was left at the initial ambient media of 8.2 and not treated with HEPES buffer was allowed to fluctuate naturally over the course of the growth experiment. Over the first four days, the pH in the non-buffered treatments rose above 9.0 and then dropped back down to approximately 8.7 by day 7. The pH fluctuated around 8.7 until day 12 when it dropped to 8.2 by day 16, where it remained for the remainder of the experiment.
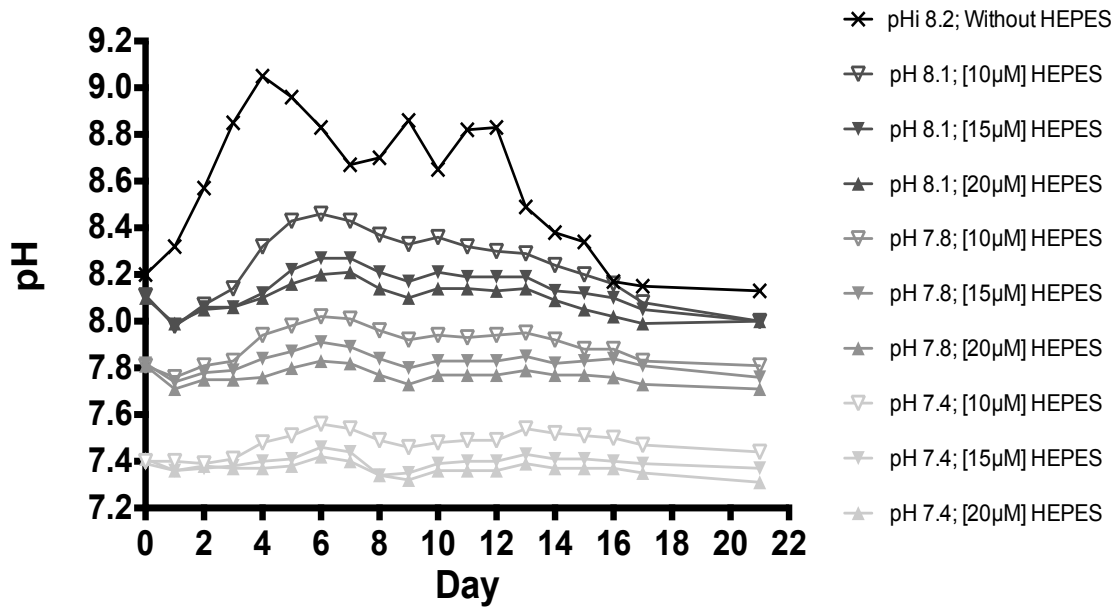
Figure 3.1.2 The pH of acid/base titrated cultures of *Heterosigma akashiwo* 513 grown in ESAW + f/2 media with the addition of HEPES buffer at concentrations of 0 mM, 10 mM, 15 mM and 20 mM over a 21-day period.

3.2 Specific growth rates of *Heterosigma akashiwo* 513

Specific growth rates were measured to gauge the success of *Heterosigma akashiwo* under different nutrient and pH regimes possible by the year 2100. Specific growth rates of *Heterosigma akashiwo* 513 grown at 880 µM N and 24:1 N:P were not altered by pH (F=0.4965, df=3,8, p=0.6948; post-hoc Tukey's test) but were influenced by iron concentration (F=107.3, df=3,8, p<0.0001; post-hoc Tukey's test), with the 11 µM FeCl$_3$ treated cultures achieving a specific growth rate almost double that of those grown at 0.1µM FeCl$_3$ (Figure 3.2 A). However, there was a significant effect of both pH (F=17.77, df=1,28, p<0.001; post-hoc Tukey's test) and N:P (F=6.897, df=6,28, p<0.0001; post-hoc Tukey's test) on the specific growth rate of *H. akashiwo* 513 grown at 100-400 µM N and 11 µM FeCl$_3$ (Figure 3.2 B). The highest growth rates were attained at an N:P ratio of 12:1 and a pH of 7.4 (4.43 d^{-1}, SD± 0.31, n=6), and the lowest

were grown at an N:P ratio of 48:1 and an initial pH of 8.2 (2.30 d^{-1}, SD± 0.35, n=6)

(Figure 3.2 B). When *H. akashiwo* 513 were grown at 10 μM N, 11 μM $FeCl_3$ and 12:1

N:P the pH did not significantly impact growth rates, but the form of nitrogen did

(F=18.94, df=2,24, p<0.0001; post-hoc Tukey's test), with nitrate yielding the highest

specific growth rates across pH treatments (Figure 3.2 C).
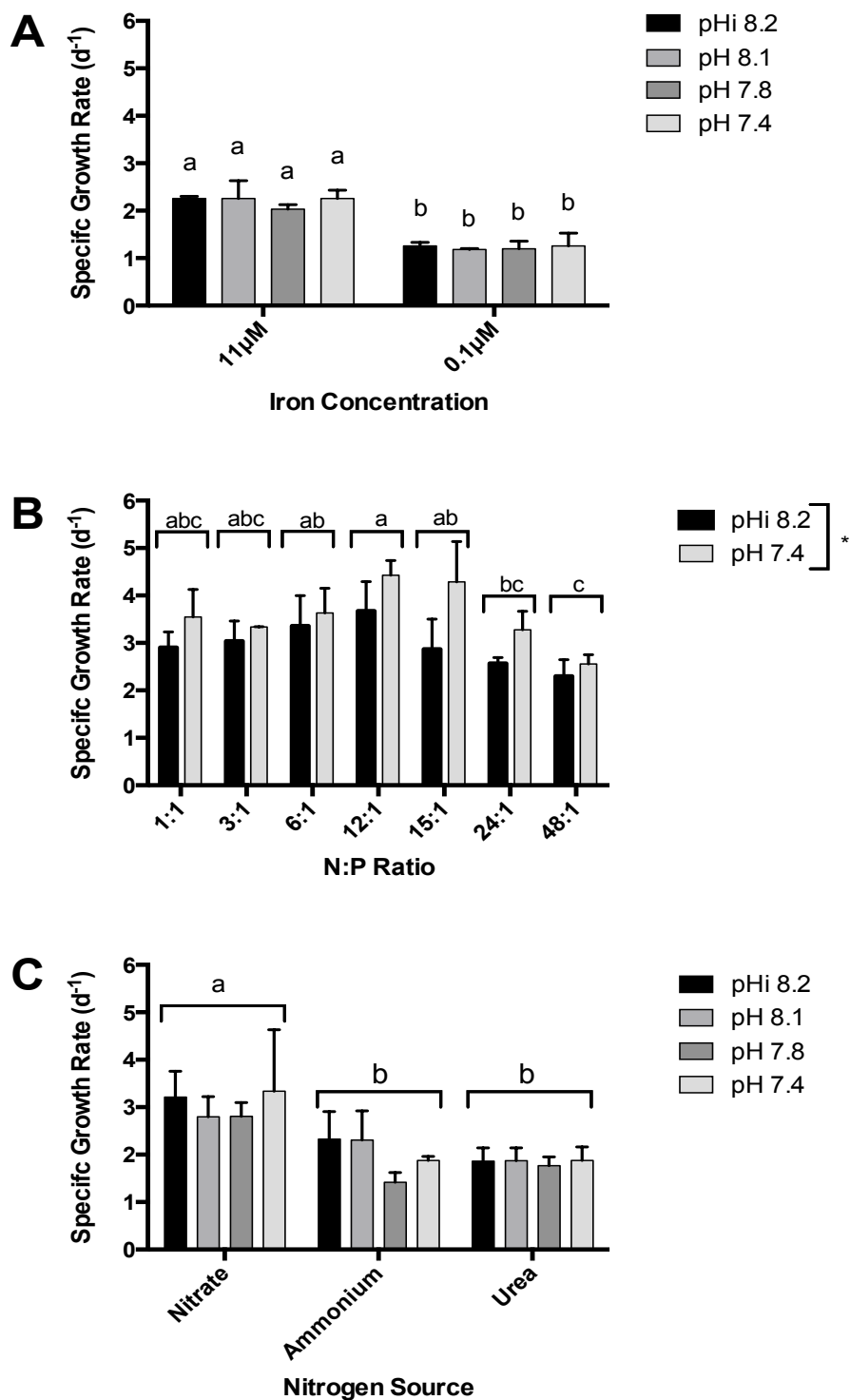
Figure 3.2 Specific growth rates of *H. akashiwo* grown in medium adjusted to a range of pH treatments (8.2 initially with no buffer; 8.1, 7.8 and 7.4), and (A) iron concentrations (11 μM and 0.1 μM), (B) nitrogen to phosphorus ratios (1:1, 3:1, 6:1, 12:1, 15:1, 24:1 and 48:1), and (C) nitrogen sources (nitrate, ammonium, and urea). Values were determined by flow cytometer during the exponential growth phase, and indicate the average of duplicated triplicate samples (n=6), with error bars indicating one standard deviation and lettering/asterisks representing statistically significant differences between treatments.

3.3 Maximal cell yields of *Heterosigma akashiwo* 513

Maximal cell yields were measured as an indicator of *Heterosigma akashiwo* densities possible in the future ocean, which could indicate the extent of a given bloom event. These were measured along with specific growth rates, in order to better theorize the future occurrence and intensity of *H. akashiwo* bloom events. Maximal cell yields of *H. akashiwo* 513 grown at 880 μM N and 24:1 N:P were significantly impacted by pH (F=10.59, df=3,31, p<0.0001; 2-way ANOVA & post-hoc Tukey's test) but were not influenced by iron concentration (F=1.656, df=1,31, p=0.2077; 2-way ANOVA & post-hoc Tukey's test), with the most acidified iron-rich cultures achieving the lowest maximal cell yield (159,584 cells mL^{-1}, SD±5023, n=6), and the most alkaline iron-rich achieving the highest maximal cell yield (339,160 cells mL^{-1}, SD± 11936, n=6) (Figure 3.3 A). There was a significant effect of pH (F=218.1, df=1,40, p<0.001; post-hoc Tukey's test), N:P (F=79.83, df=6,40, p<0.0001; post-hoc Tukey's test), as well as an interaction effect of the two (F=39.61, df=6,40, p<0.001; post-hoc Tukey's test) on the maximal cell yields of *H. akashiwo* grown at 100-400 μM N and 11 μM FeCl$_3$ (Figure 3.3 B). The highest cell yields were attained at an N:P ratio of 48:1 and an initial pH of 8.2 (103,733 cells mL^{-1}, SD± 9002, n=6), and the lowest were grown at an N:P ratio of 12:1 and a pH of 7.4 (13,097 cells/mL, SD± 3308, n=6) (Figure 3.3 B). These maximum and minimum values were attained under the differing treatments, as were the maximum and minimum specific growth rates. When *H. akashiwo* were grown at 10 μM N, 11 μM FeCl$_3$ and 12:1 N:P the main effects of pH (F=14.10, df=3,68, p<0.0001, post-hoc Tukey's test) and nitrogen source (F=7.830, df=2,68, p=0.0009, post-hoc Tukey's test) were significant. There was also a significant interaction effect (F=2.244, df=6,68,

p=0.0492, post-hoc Tukey's test) between the main effects, with a trend of increasingly acidified treatments resulting in lower maximal cell yields under each nitrogen source tested (Figure 3.3 C).

Figure 3.3 Maximal cell yields of *H. akashiwo* grown in medium adjusted to a range of pH treatments (8.2 initially with no buffer; 8.1, 7.8 and 7.4), and (A) iron concentrations (11 μM and 0.1 μM), (B) nitrogen to phosphorus ratios (1:1, 3:1, 6:1, 12:1, 15:1, 24:1 and 48:1), and (C) nitrogen sources (nitrate, ammonium, and urea). Values were determined by flow cytometry during stationary growth phase, and indicate the average of duplicated triplicate samples (n=3), with error bars indicating one standard deviation and lettering representing statistically significant differences between treatments (with asterisks denoting significant differences between pH values within a given treatment).

3.4 Peak neutral lipid accumulation in *Heterosigma akashiwo* 513

Peak neutral lipid accumulation within *Heterosigma akashiwo* was measured as an indicator of changes to essential fatty acid composition possible in the future ocean, which could alter marine ecosystems, be it negatively or positively. They could negatively impact marine organisms if they are a component of the toxic/allelopathic mechanism. As shown in Figure 3.4 A, peak neutral lipid accumulation in *Heterosigma akashiwo* 513 grown at 880 μM N and 24:1 N:P was not significantly impacted by either pH or iron concentration ($F=0.8520$, $df=3,40$, $p=0.4738$; $F=3.460$, $df=1,40$, $p=0.0702$; post-hoc Tukey's test), with all values less than 0.5%. The peak accumulation of neutral lipid increased in *H. akashiwo* 513 grown at 100-400 μM N and 11 μM $FeCl_3$, with values ranging from 0.23% to 4.05% (Figure 3.4 B). The effects of pH and N:P, and the interaction of both factors ($F=13.57$, $df=1,28$, $p=0.010$; $F=36.71$, $df=6,28$, $p<0.0001$; $F=5.068$, $df=6,28$, $p=0.0012$; post-hoc Tukey's test) significantly influenced peak neutral lipid accumulation in these cells, with the highest peak neutral lipid accumulations attained at N:P ratios of 24:1 and 48:1, indicative of P-limitation (Figure 3.4 B). When *H. akashiwo* 513 were grown at 10 μM N, 11 μM $FeCl_3$ and 12:1 N:P the effects of pH and nitrogen source were not statistically significant ($F=0.4511$, $df=3,14$, $p=0.7205$; $F=0.5888$, $df=2,14$, $p=0.5682$; post-hoc Tukey's test), with the greatest enhancement in neutral lipid accumulation shown in the most acidified culture grown with nitrate as the N-source (3.36%, SD±0.43, n=3) (Figure 3.4 C).
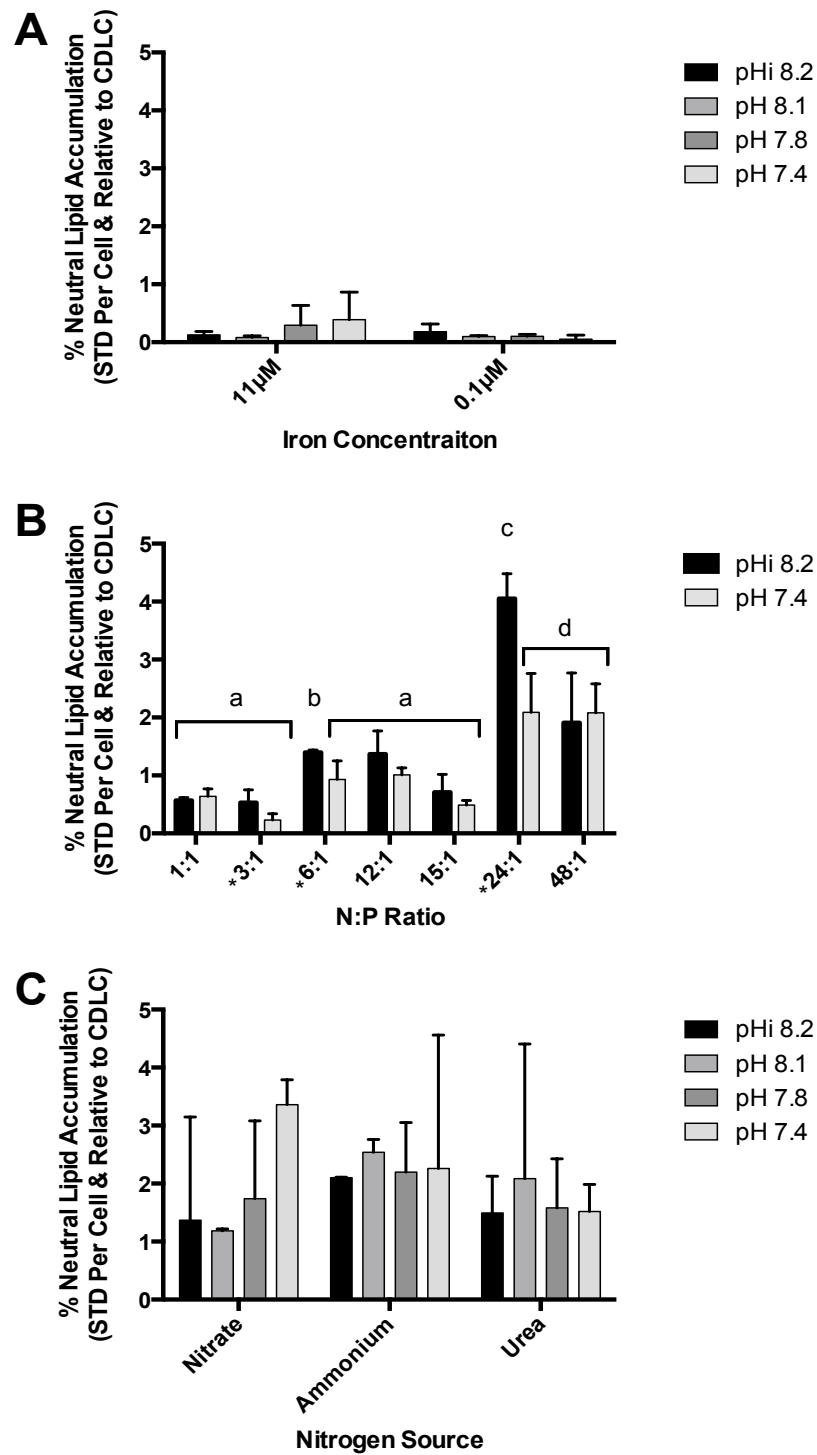
**Figure 3.4** Peak neutral lipid accumulation (as a percentage of the standard chemically defined lipid concentrate; CDLC) in *H. akashiwo* grown in medium adjusted to a range of pH treatments (8.2 initially with no buffer; 8.1, 7.8 and 7.4), and (A) iron concentrations (11 μM and 0.1 μM), (B) nitrogen to phosphorus ratios (1:1, 3:1, 6:1, 12:1, 15:1, 24:1 and 48:1), and (C) nitrogen sources (nitrate, ammonium, and urea). Values were determined by fluorescence spectrophotometry during the transition between exponential and stationary growth phase, and indicate the average of triplicate samples (n=3), with error bars indicating one standard deviation and lettering representing statistically significant differences between treatments (with asterisks denoting significant differences between pH values within a given treatment).

3.5 *Heterosigma akashiwo* 513 toxicity

The toxic effect of *Heterosigma akashiwo* on Rainbow trout gill cell lines was measured as an indicator of ichthyotoxicity under possible future ocean nutrient and pH regimes, which could greatly impact natural finfish populations as well as aquaculture facilities. As shown in Figures 3.4 A and B, *Heterosigma akashiwo* 513 toxicity from cells grown at 880 μM N (A), and 400-100 μM N (B) was above 87.7% toxicity under all iron concentrations, N:P ratios, and pH values tested, indicating that these cells were highly toxic. Toxicity was not significantly impacted by pH in either experiment, and was not impacted by iron concentration or N:P ratio (F=2.489, df=3,40, p=0.0742; F=1.444, df=1,28, p=0.2396; F=0.3192, df=1,40, p=0.5752; F=0.5088, df=6,28, p=0.7964; post-hoc Tukey's test). Again, toxicity was very high across every variable tested in experiment A and B, ranging from 87.7% to 97.7%. Toxicity was generally lower when *H. akashiwo* 513 was grown at 10 μM N, 11 μM FeCl$_3$ and 12:1 N:P (Figure 3.5 C), ranging from 43.9% to 76.4%. The effect of nitrogen source on peak toxicity measured in *H. akashiwo* 513 was statistically significant (F=10.14, df=2,60, p=0.0002; post-hoc Tukey's test), with ammonium resulting in lower toxicity than nitrate or urea (Figure 3.5 C).
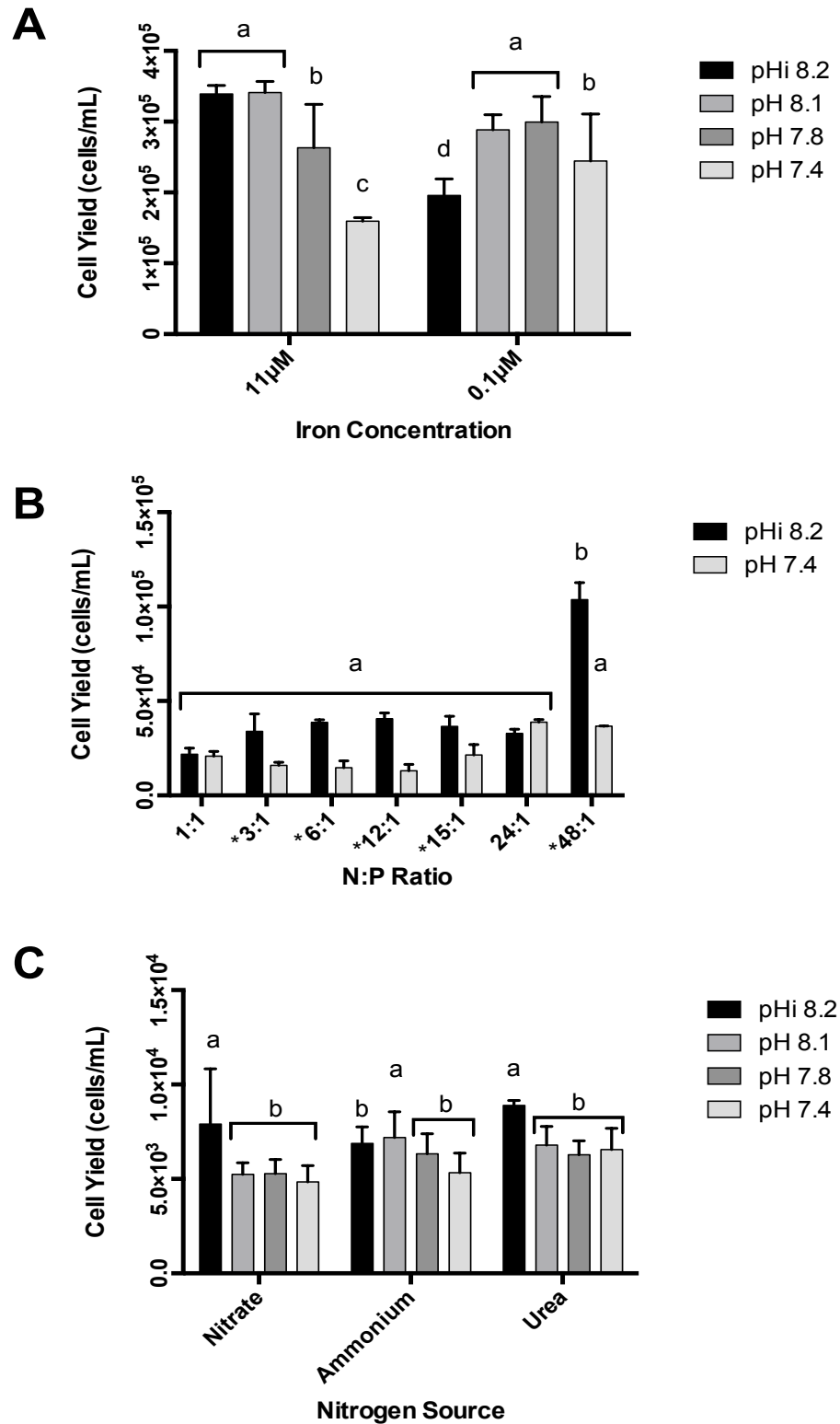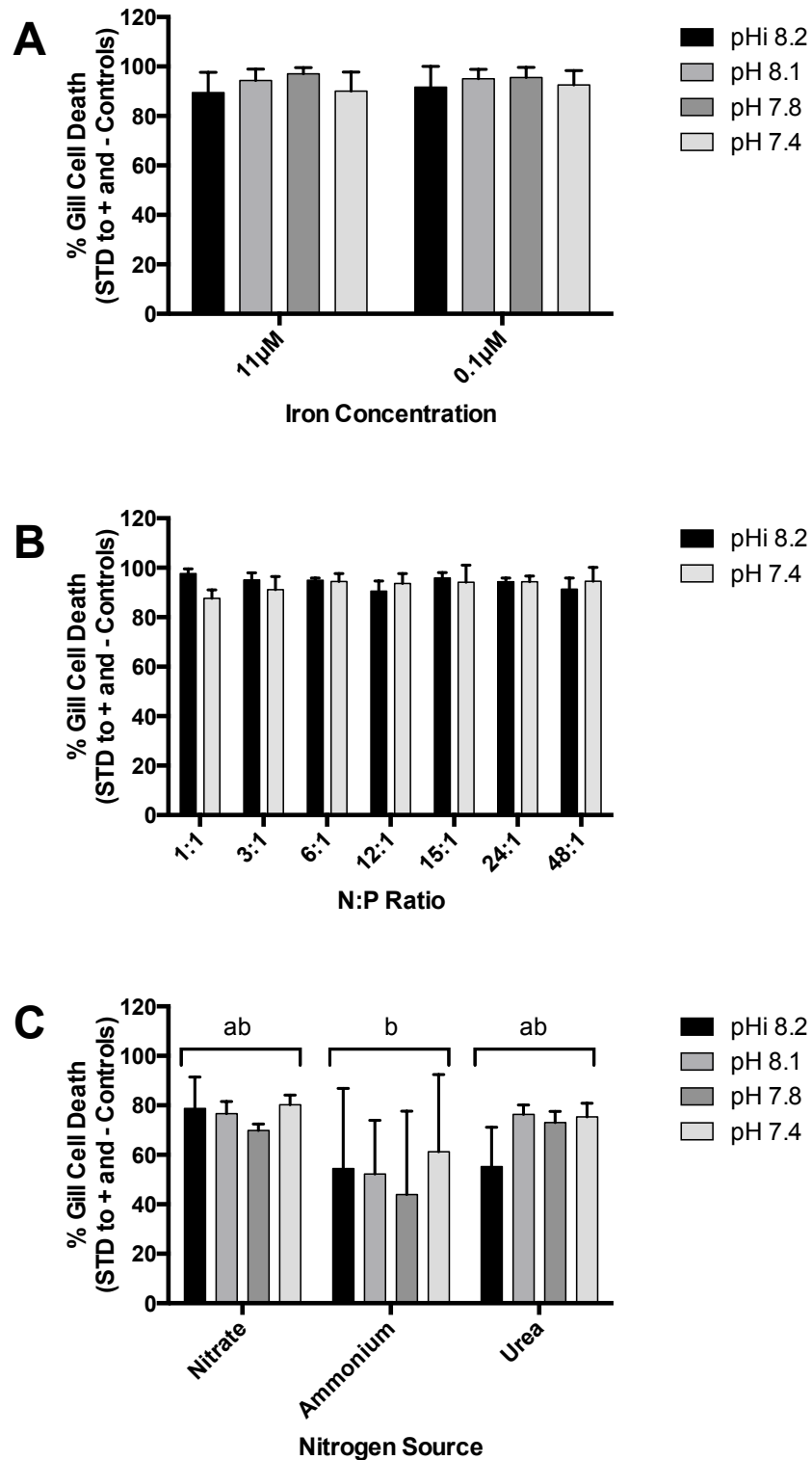
Figure 3.5 Peak toxicity in *H. akashiwo* grown in medium adjusted to a range of pH treatments (8.2 initially with no buffer; 8.1, 7.8 and 7.4), and (A) iron concentrations (11 μM and 0.1 μM), (B) nitrogen to phosphorus ratios (1:1, 3:1, 6:1, 12:1, 15:1, 24:1 and 48:1), and (C) nitrogen sources (nitrate, ammonium, and urea). Values were determined by fluorescence spectrophotometry during the transition between exponential and stationary growth phase, and indicate the average of triplicate samples (n=6), with error bars indicating one standard deviation and lettering representing statistically significant differences between treatments.

3.6 Correlations between nutrients, pH and physiological responses

In order to gain a better understanding of the relationship between nutrient concentrations (nitrogen and phosphorus) and pH, and growth rates, cell yields, peak neutral lipid content, and peak toxicity in *Heterosigma akashiwo*, correlations were first examined using a principal component analysis (Figure 3.6). The PCA was used to transform multidimensional data (many variables and interactions) to 2-D data, while retaining the majority of the data story. Figure 3.6 A shows data that was used to identify the two-principal components. Figure 3.6 B gives a clear picture of which variables are related, and the nature of the relationship. Toxicity appears to be negatively correlated with neutral lipid production (Figure 3.6 B). Macronutrient concentrations and cell yield seem to be positively correlated with toxicity, and pH does not appear to bear strong relationships with any other variables (Figure 3.6 B).

From the apparent relationships shown above, Spearman's rank correlation coefficient for non-parametric, non-linear variables was then utilized to ascribe values to these relationships (Table 3.6). As expected, the strongest relationships existed between macronutrient concentrations and cell yields (N: ρ=0.9391, p<0.0001; P: ρ=0.8114, p<0.0001; Table 3.6). There were also strong relationships between macronutrient concentrations and both neutral lipids (N: ρ= -0.6761, p<0.0001; P: ρ= -0.7900, p<0.0001) and toxicity ((N: ρ=0.7125, p<0.0001; P: ρ=0.7116, p<0.0001; Table 3.6).
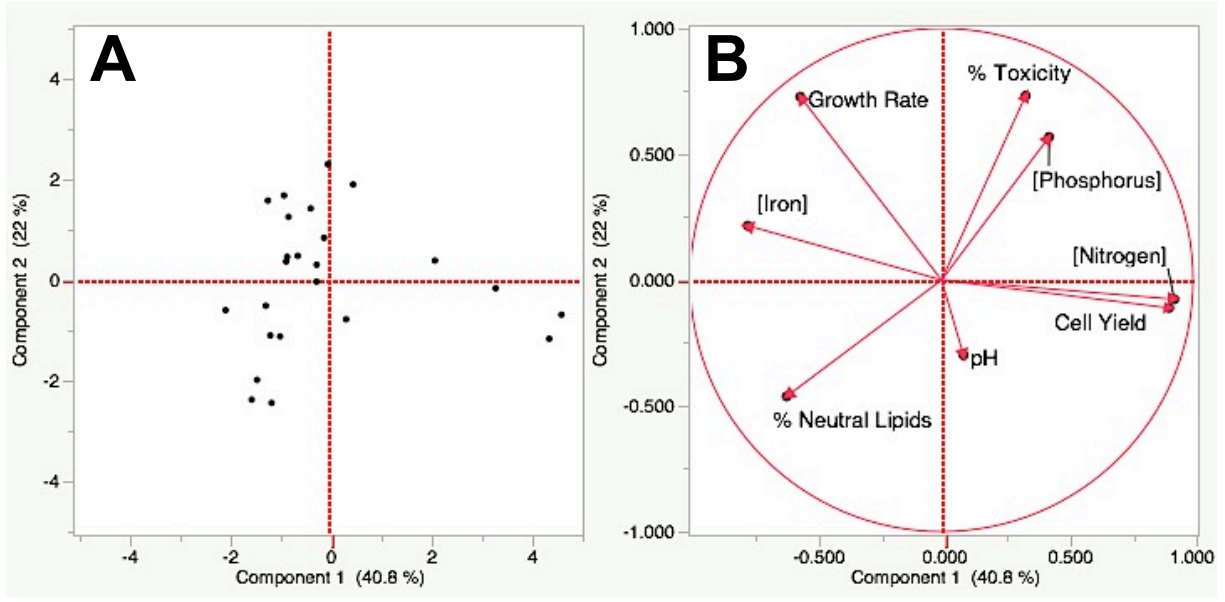
Figure 3.6 Principal component analysis of the correlations between independent (iron, nitrogen, and phosphorus concentrations and pH) and dependent variables (growth rate, cell yields, neutral lipid accumulation, and toxicity) tested on *Heterosigma akashiwo* 513. Two components were used to map correlations (eigenvalues > 1 are retained), accounting for a cumulative 62.8% of the variance. The score plot (A) is a standard interpretive aid showing raw component data, while the loading plot (B) shows normalized data.

Table 3.6 Spearman's rank correlation coefficients of dependent variables (growth rate, cell yield, neutral lipid accumulation, and toxicity) by independent (iron, nitrogen, and phosphorus concentrations, and pH) and dependent variables. Asterisks denote statistically significant correlation coefficients. Spearmans's ρ values are depicted visually in the last column.

Variable	by Variable	Spearman ρ	P Value	
Growth Rate	[Iron]	0.5586	0.0006*	
Growth Rate	[Nitrogen]	-0.1848	0.2954	
Growth Rate	[Phosphorus]	-0.0090	0.9596	
Growth Rate	pH	-0.1061	0.5502	
Cell Yield	[Iron]	-0.4652	0.0056*	
Cell Yield	[Nitrogen]	0.9391	<.0001*	
Cell Yield	[Phosphorus]	0.8114	<.0001*	
Cell Yield	pH	0.2049	0.245	
Cell Yield	Growth Rate	-0.1870	0.2897	
% Neutral Lipids	[Iron]	0.5212	0.0016*	
% Neutral Lipids	[Nitrogen]	-0.6761	<.0001*	
% Neutral Lipids	[Phosphorus]	-0.7900	<.0001*	
% Neutral Lipids	pH	0.0063	0.9716	
% Neutral Lipids	Growth Rate	0.1352	0.446	
% Neutral Lipids	Cell Yield	-0.6811	<.0001*	
% Toxicity	[Iron]	-0.2747	0.1159	
% Toxicity	[Nitrogen]	0.7125	<.0001*	
% Toxicity	[Phosphorus]	0.7116	<.0001*	
% Toxicity	pH	0.0824	0.6432	
% Toxicity	Growth Rate	0.1812	0.3052	
% Toxicity	Cell Yield	0.6843	<.0001*	
% Toxicity	% Neutral Lipids	-0.5271	0.0014*	

-1.0   -0.5   0.0   0.5   1.0

Sperman's correlation coefficient

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3.7 Nitrogen, phosphorus, neutral lipids and toxicity in *H. akashiwo* 513

Although the positive relationship between macronutrient concentration and cell yields was expected, the negative correlation with neutral lipids and the positive relationship with toxicity were not preconceived. In an attempt to gain a better understanding of the nature of these relationships, nitrogen and phosphorus concentrations were plotted separately against peak neutral lipid accumulation and peak toxicity in Figure 3.7. Figure 3.7 A indicates that peak neutral lipid accumulation is higher in more alkaline waters, between 100 and 400 μM N, with a similar less extreme trend exhibited at pH 7.4. Figure 3.7 B shows that concentrations of phosphorus lower than 1 μM result in the highest peak neutral lipid accumulation, while at pH 8.2 this value shifts to somewhere between 1 and 10 μM P. In terms of peak toxicity, there seems to be a rapid increase between 0 and 100 μM N where it remains above 90% toxicity for all other concentrations tested (Figure 3.7 C). Phosphorus presented a parallel trend between 0 and 20 μM, with a very slight dip in toxicity possible between 20 and 100 μM P (Figure 3.7 D).
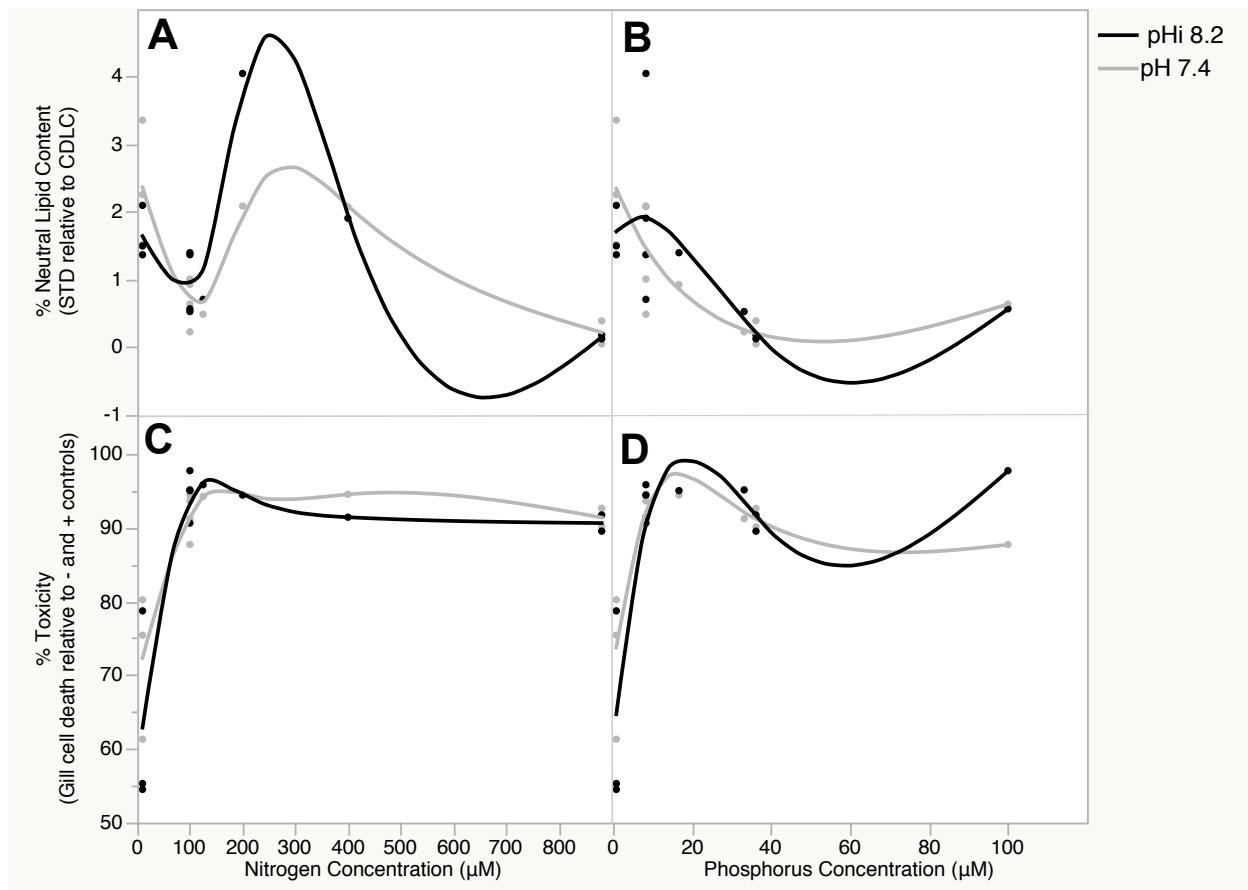
Figure 3.7 Peak neutral lipid accumulation (as a percentage of the chemically defined lipid concentrate standard) (top) and peak toxicity (bottom) in *Heterosigma akashiwo* at pH 8.2 and 7.4 across nitrogen (A&C) and phosphorus (B&C) concentrations. A cubic spline, with lambda 0.05, has been applied over individual data points.

3.8 Nitrogen concentrations and *Heterosigma akashiwo* 513

In order to better assess the impact of nitrogen concentration on *H. akashiwo* 513 physiology, experimental treatments grown under identical iron concentrations, N:P ratios, nitrogen sources, and pH values with different concentrations of nitrogen were selected across all experiments and compared against each other. Suitable treatments were grown under 11 μM FeCl$_3$, with nitrate as the N-source, at pH values of 7.4 and 8.2, with an N:P ratio of 12:1 or 24:1, with 10/100 μM N or 200/880 μM N respectively.

3.8.1 Specific growth rates

Specific growth rates were measured across nitrogen concentrations of 10 μM and 100 μM (Figure 3.8.1 A), as well as 200 μM and 880 μM (Figure 3.8.1 B), to gauge how *Heterosigma akashiwo* persistence could be altered under nutrient and pH regimes possible for the future ocean. Specific growth rates of *Heterosigma akashiwo* 513 grown at 10 μM/100 μM N and 12:1 N:P were not impacted by pH or nitrogen concentration (F=0.9706, df=1,8, p=0.5053; F=2.944, df=1,8, p=0.1245; post-hoc Tukey's test; Figure 3.8.1 A), however these were influenced by nitrogen concentration (F=17.60, df=1,6, p=0.0057; post-hoc Tukey's test) when grown at 24:1 N:P under 200 μM/880 μM N (Figure 3.8.1 B). Specific growth rates appear to decrease with increasing nitrogen concentration (Figure 3.8.1 B).
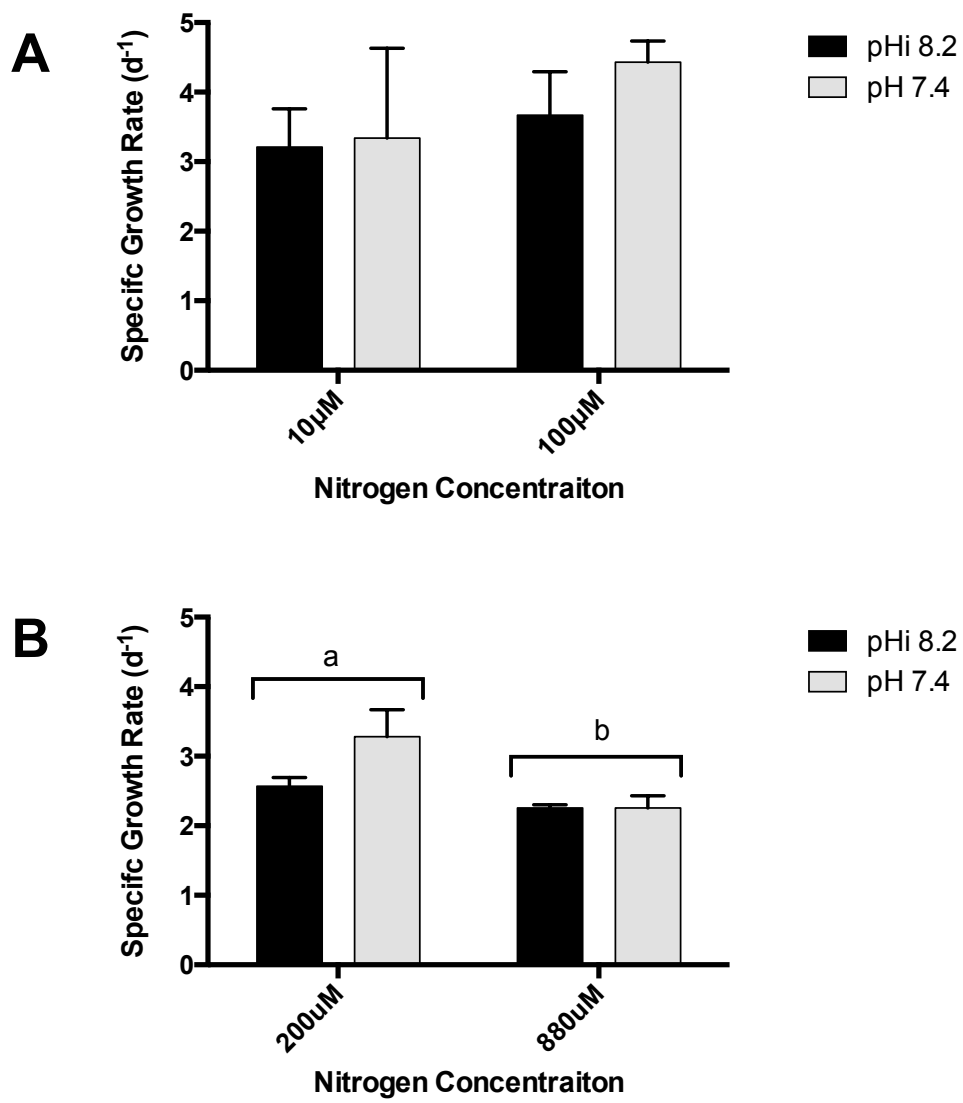
Figure 3.8.1 Specific growth rates of *H. akashiwo* grown in medium adjusted to a range of pH treatments (8.2 initially with no buffer and 7.4), and nitrogen concentrations of (A) 10 μM and 100 μM N (12:1 N:P) (B) 200 μM and 880 μM N (24:1 N:P). Values were determined by flow cytometry during the exponential growth phase, and indicate the average of triplicate samples (n=3), with error bars indicating one standard deviation and letters indicating statistically significant differences between groups (α=0.05).

3.8.2 Maximal cell yields

Maximal cell yields were measured across nitrogen concentrations of 10 μM and 100 μM (Figure 3.8.2 A), as well as 200 μM and 880 μM (Figure 3.8.2 B), in an effort to gauge how *Heterosigma akashiwo* persistence could be altered under high nutrient and low pH regimes possible for the future ocean. Cell yields of *Heterosigma akashiwo* 513 grown at 10μM/100μM N and 12:1 N:P were significantly impacted by pH and nitrogen concentration (F=138.7, df=1,15, p<0.0001; F=249.2, df=1,15, p<0.0001; post-hoc Tukey's test; Figure 3.8.2. A). Specific growth rates were also significantly influenced by pH and nitrogen concentration (F=769.8, df=1,9, p<0.0001; F=466.2, df=1.9, p<0.0001; post-hoc Tukey's test) when grown at 24:1 N:P under 200 μM/880 μM N (Figure 3.8.2 B). Maximal cell yields decrease with pH and increase with increasing nitrogen concentration (Figure 3.8.2).
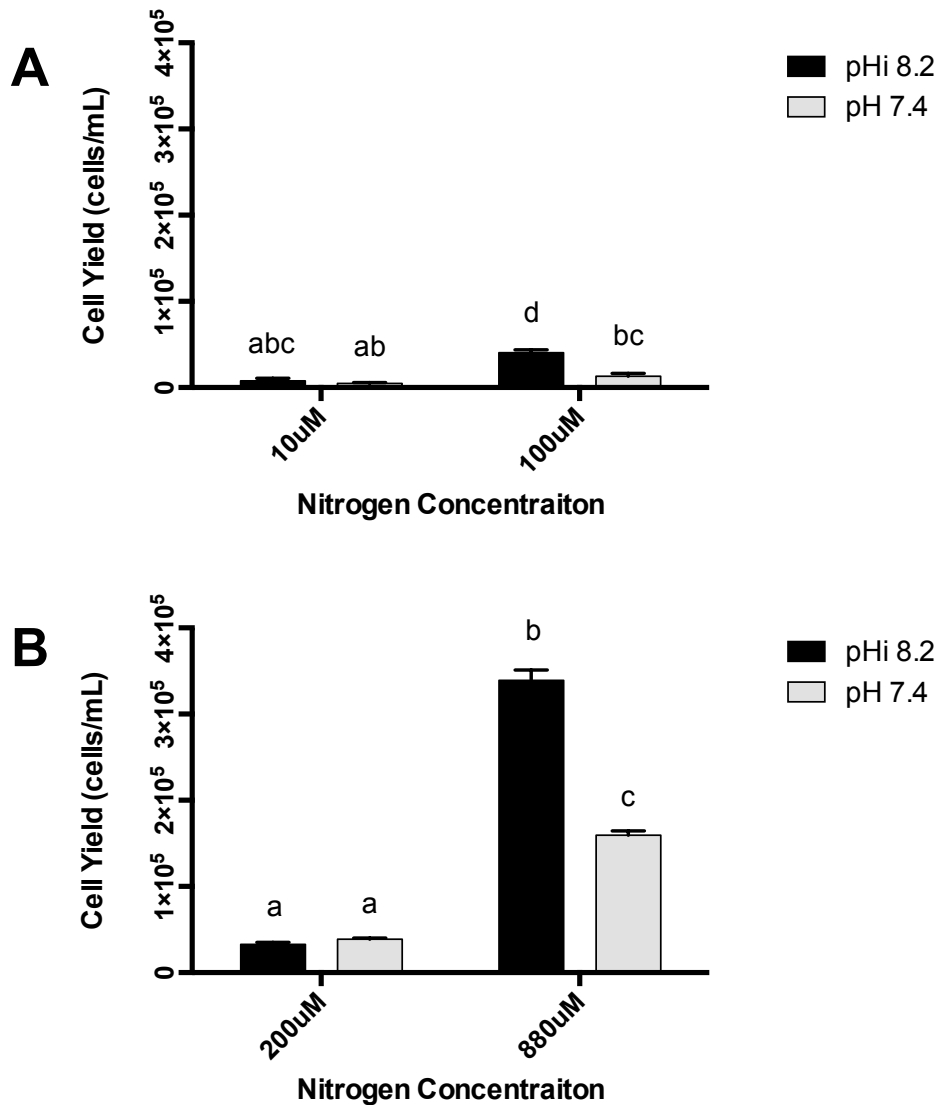
Figure 3.8.2 Maximal cell yields of *H. akashiwo* grown in medium adjusted to two different pH treatments (8.2 initially with no buffer and 7.4), and nitrogen concentrations of (A) 10 μM and 100 μM N (12:1 N:P) (B) 200 μM and 880 μM N (24:1 N:P). Values were determined by flow cytometry during stationary growth phase, and indicate the average of triplicate samples (n=3), with error bars indicating one standard deviation and letters indicating statistically significant differences between groups (α=0.05).

3.8.3 Peak neutral lipid accumulation

Neutral lipid accumulation within *Heterosigma akashiwo* was measured across nitrogen concentrations to indicate changes in essential fatty acid composition possible in the future ocean, which could alter marine ecosystem food chains. Again, although they have a positive role in the development of the food chain, these compounds could also negatively impact marine organisms if they function as toxic/allelopathic agents. Neutral lipid accumulation in *H. akashiwo* 513 grown at 10/100 μM N and 12:1 N:P were not significantly impacted by either pH or nitrogen concentrations ($F=2.595$, df=1,6, $p=0.158$; $F=5.392$, df=1,6, $p=0.0592$; post-hoc Tukey's test; Figure 3.8.3 A), with values ranging from 1.01% to 3.36% (SD ±0.12; SD ±0.43, n=3). This was not the case for *H. akashiwo* 513 grown at the higher nitrogen concentrations (200 μM/880 μM N at 24:1 N:P), which were significantly impacted by pH, nitrogen concentration, as well as the interaction of the two ($F=16.78$, df=1,14, $p=0.0011$; $F=184.6$, df=1,14, $p<0.0001$; $F=28.92$, df=1,14, $p<0.0001$; post-hoc Tukey's test) and ranged from 0.13% to 4.05% (SD ±0.06; SD ±0.43, n=3; Figure 3.8.3 B). Decreased nitrogen concentration resulted in increased neutral lipid accumulation in this experiment, which was most pronounced in the cultures at pH 8.2 (Figure 3.8.3 B).
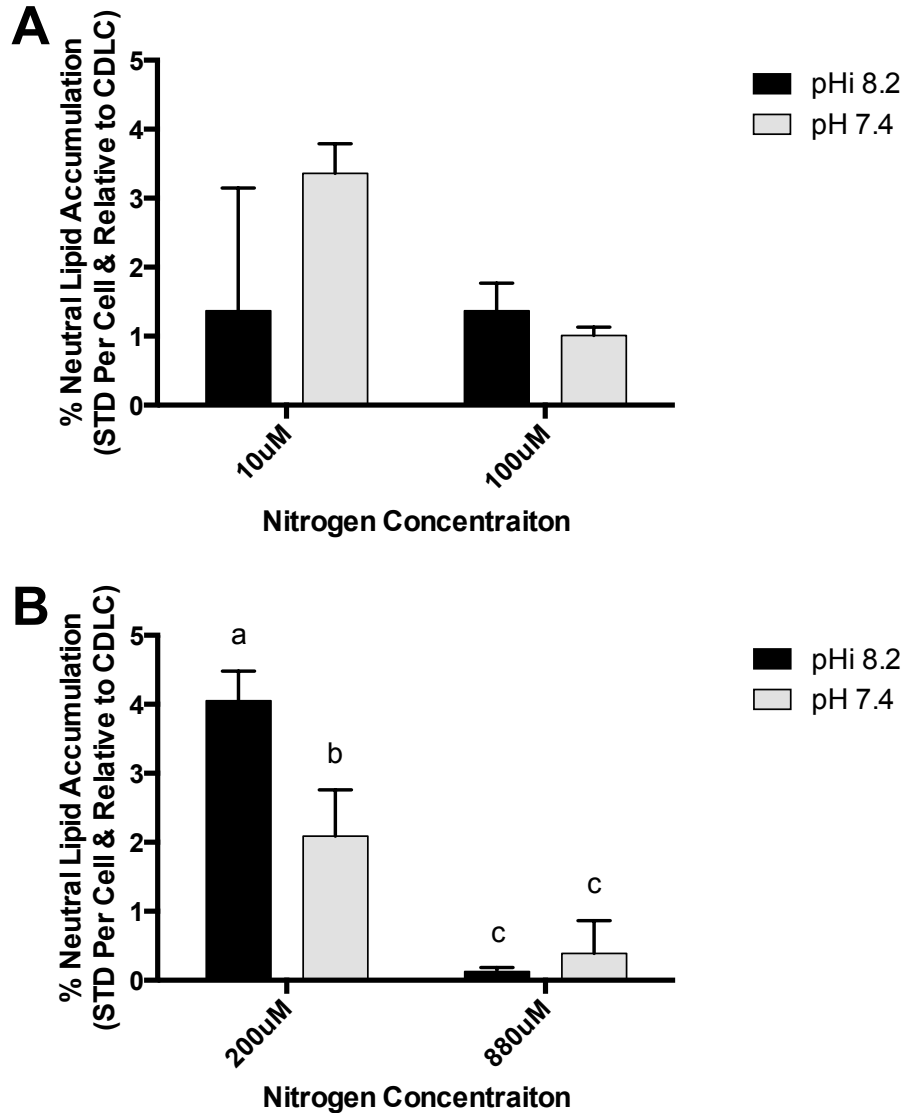
Figure 3.8.3 Peak neutral lipid accumulation (as a percentage of the standard chemically defined lipid concentrate; CDLC) in *H. akashiwo* grown in medium adjusted to two different pH treatments (8.2 initially with no buffer and 7.4), and nitrogen concentrations of (A) 10 μM and 100 μM N (12:1 N:P) (B) 200 μM and 880 μM N (24:1 N:P). Values were determined by fluorescence spectrophotometry during the transition between exponential and stationary growth phase, and indicate the average of triplicate samples (n=3), with error bars indicating one standard deviation and letters indicating statistically significant differences between groups (α=0.05).

3.8.4 Toxicity

The toxic effect of *H. akashiwo* grown on different nitrogen concentrations was measured as an indicator of ichthyotoxicity under possible future ocean nutrient-rich and low pH regimes, which could greatly impact natural finfish populations as well as aquaculture facilities. As shown in Figures 3.8.4 A and B, peak *H. akashiwo* 513 toxicity from cells grown at 10-100 μM N (A), and 200-880 μM N (B) was above 78.7% toxicity under all iron concentrations, N:P ratios, and pH values tested, indicating that these cells were highly toxic. However, after further examination, the only statistically significant differences in toxicity lie between *H. akashiwo* grown at 10 μM and 100 μM N ($F=9.370$, $df=1,14$, $p=0.0085$; Figure 3.8.4 A). This could indicate that nitrogen concentrations must reach a certain threshold (lower than 10 μM N in this case) before cells become toxic to the Rainbow trout gill cells, and then again before cells become highly toxic to the gill cells. Toxicity was not significantly impacted by pH in either experiment, and remained highly toxic across the higher nitrogen concentrations of 200 μM and 880 μM N ($F=0.3012$, $df=1,14$, $p=0.5981$; $F=0.007168$, $df=1,14$, $p=0.9337$; $F=1.837$, $df=1,14$, $p=0.1968$; post-hoc Tukey's test; Figure 3.8.4).
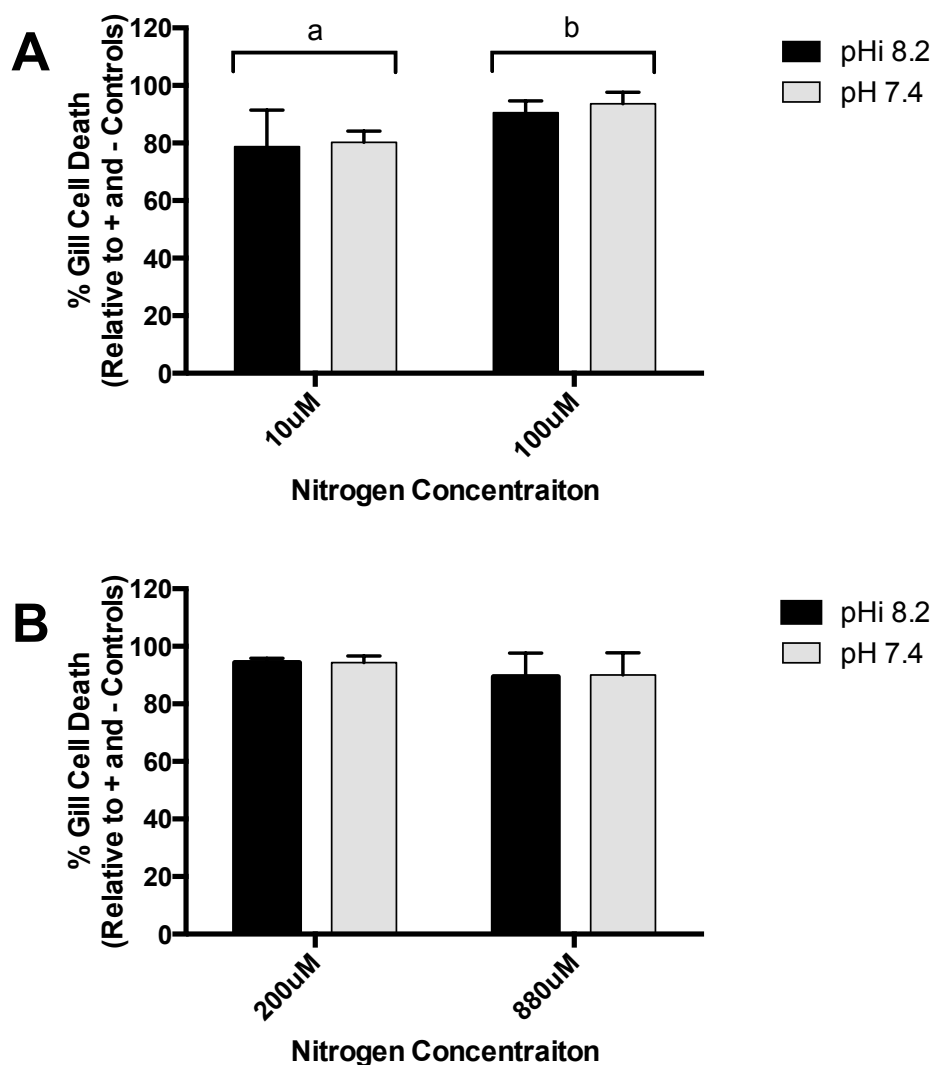
Figure 3.8.4 Peak toxicity in *H. akashiwo* grown in medium adjusted to a pH of 8.2 (initially with no buffer) and 7.4, and nitrogen concentrations of (A) 10 μM and 100 μM N (12:1 N:P) (B) 200 μM and 880 μM N (24:1 N:P). Values were determined by fluorescence spectrophotometry during the transition between exponential and stationary growth phase, and indicate the average of triplicate samples (n=3), with error bars indicating one standard deviation and letters indicating statistically significant differences between groups (α=0.05).

CHAPTER 4: DISCUSSION

4.1 Future *Heterosigma akashiwo* bloom formation

The first aim of this study was to characterize the growth of *H. akashiwo* cultured under various nutrient and pH regimes possible in the future ocean. This information can be used to help predict the frequency and intensity of potentially harmful blooms of *H. akashiwo* in the future. It was initially predicted that *H. akashiwo* would be able to maintain their presence in the future, and continue to form blooms in coastal waters.

4.1.1 Specific growth rates

In this study, it was shown that the highest specific growth rates were achieved under iron-rich treatments (11 μM FeCl₃), with nitrate as the source of nitrogen, at an N:P ratio of 12:1 and low nitrogen concentration (100 μM N) (Figure 3.2B). Specific growth rates were impeded at low iron concentrations (0.1 μM FeCl₃), with ammonium or urea as the nitrogen source, and at an N:P ratio of 48:1 (Figure 3.2). These trends can be explained by iron requirements for photosynthesis, nitrogen usage, as well as balanced versus unbalanced growth, which will be discussed further on in this section (Raven, 1988). Specific growth rates also declined with increasing nitrogen/phosphorus concentrations, which was not expected (Figure 3.2B). Even though the growth rates were slower in these cases, they remained above 2 divisions per day (except in the case of low iron), indicating that they would still proliferate (Figure 3.2). Average maximum *in situ* growth rates for phytoplankton were found to be 3.0-3.6 divisions per day (diel-adjusted) and laboratory culture average maximum growth rates are generally less than 3 divisions per day (Furnas, 1990).

Iron was also shown to exert a large control on specific growth rates, which was expected due to its classification as a micronutrient (Figure 3.2A). Cellular iron requirements are significantly higher than concentrations typically recorded in the surface ocean (concentration factor of ca. 62,000 within autotrophic phytoplankton versus ocean waters) (Twining et al., 2004). Iron enables cellular use of inorganic nitrogen in the form of nitrate, as it is an essential component of nitrate/nitrite reductase (Raven, 1988; Wells et al., 1995). A lack of iron can therefore co-limit nitrogen and cause lower growth rates (Sunda and Huntsman, 1995), which the results showed (Figure 3.2A). Continued acidification of coastal waters can increase the bioavailability of iron, which would increase specific growth rates of phytoplankton (Breitbarth et al., 2009).

It was interesting to note that nitrate promoted a higher growth rate than ammonium – the more readily useable form of nitrogen (Figure 3.2C). Previous work conducted by Herndon and Cochlan (2007) found that *H. akashiwo* grew faster on ammonium and urea compared to nitrate. It should be noted that it was a different isolate of *H. akashiwo* (CCMP 1912). Other factors such as temperature, and nitrogen concentration make comparisons ploblematic as well (Herndon and Cochlan, 2007; Miki, 1983 as cited by Okaichi, 2003). For example, a study by Miki (1983) found that temperature influenced whether phytoplankton grew faster with nitrate (>17 °C) or ammonium (<17 °C) as the nitrogen source. In a warmer future ocean, nitrate could be the preferred N-source for *H. akashiwo*.

The results of the N:P manipulation experiment suggest that heavy nitrogen inputs, which could occur as a result of anthropogenic coastal nutrient loadings, into the system can hamper growth rates (Figure 3.2B). This could be explained by excess

nitrogen fueling cell growth, but the proportionate lack of P limiting growth – unbalanced growth. When we examine resultant cell yields under the same treatment, however, they are the highest of any N:P ratio tested (Figure 3.3B). This points to nitrogen promoting high cell yields that are undergoing balanced growth until there is inadequate carbon to support such a high cell yield.

It should also be noted that because *Heterosigma akashiwo* lack a carbon concentrating mechanism (CCM), carbon-limitation is possible. Some toxic algal species have evolved CCMs that allow algae to uptake bicarbonate (HCO_3^-) otherwise, like *H. akashiwo*, they rely solely upon dissolved carbon dioxide uptake (Nimer et al. 1997). Bicarbonate is the much more abundant form of dissolved carbon in the ocean accounting for 90%, while less than 1% remains as dissolved carbon dioxide (Doney et al., 2009). Carbon-limitation due to high biomasses attained through increases in nutrient loadings or ocean acidification increasing bioavailability of micronutrients could be possible in future waters. Other algal species that have this carbon concentrating mechanism could possibly outcompete *H. akashiwo* in this scenario, albeit in future surface waters with higher $CO_{2(aq)}$ the CCM would have a diminished importance.

4.1.2 Maximal cell yields

The other component of *H. akashiwo* growth examined was maximal cell yield, or peak biomass. The finding that biomass was highest under high nutrient conditions (iron, nitrogen and phosphorus) at high N:P ratios (as discussed above) (Figure 3.3) can be explained by the balanced growth model. Increasing cell yields with increased concentrations of iron, nitrogen, and phosphorus were expected, as the lack of any of these nutrients can be extremely limiting to growth (Figure 3.3). This link between algal

blooms formation and high nutrient concentration has been clearly established (Schindler, 1974; Anderson et al., 2002; Glibert and Burkholder, 2006). There was also an interesting trend of decreased cell yields across all three nitrogen sources, as pH decreased, in the low nitrogen experiment (Figure 3.3C). This could be caused by cells experiencing N-limitation earlier under more acidic conditions that are more carbon-rich, and able to fuel faster rates of cell division. As such, more acidic open ocean waters that are N-limited could see slight drops in overall biomass into the future.

In terms of N:P ratios, higher amounts of N promoted greater cell yields (Figure 3.3B). Again, this is logical due to the abundance of N to fuel growth. These cultures eventually suffered P-limitation, which could have been delayed due to high cellular reserves of P compared to N (Harrison et al., 1990). *In situ* N:P ratios vary relatively slowly, and large changes in nutrient inputs are required to shift the N:P ratio in coastal environments (Davidson et al., 2012). Departures from Redfield's ratio have not been classed as a defining characteristic of bloom formation in natural environments (Davidson et al., 2012).

Although nitrogen source did affect growth rates, it was not found to significantly affect cell yields (Figure 3.2C; 3.3C). This can be explained by the overall nitrogen concentration being the same across the different nitrogen source treatments. They may utilize one source faster than others, but in the end they will attain the same cell yield. Future waters with the same level of fertilizer inputs as today, with more of them being urea-based, may grow more slowly but will still be able to attain similar maximal biomasses. This would impact the speed at which the bloom appears, and preventative

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measures (such as the relocation of aquacultural pens) could be taken with these slower growing blooms.

Cell yields were found to be almost inversely proportional to growth rates (Figure 3.6) in most cases. This could be due to carbon-limitation prematurely impeding growth in balanced, nutrient-replete cultures. Insufficient carbon dioxide, a necessary reactant for photosynthesis, would prevent cells from attaining higher cell yields that would normally be possible under the same N/P/Fe concentrations in the presence of dissolved carbon.

The overall finding regarding growth was that no modelled parameter negatively influenced growth to the extent that *H. akashiwo* would not be present in future coastal waters (Figure 3.3). A natural bloom population density of 1,000,000 cells mL^{-1} has been found to cause a very visible colouration of surface waters (Trainer, 2012). None of the batch cultures in this study reached similarly high bloom densities, which is considered one limitation of laboratory batch culture experiments, unable to perfectly mimic natural conditions. Similar experiments performed *in situ*, with a constant renewal of nutrients (through coastal loadings, upwelling, *H. akashiwo* movement in the water column, etc.) could very well attain densities higher than 1,000,000 cells mL^{-1}.

Species dynamics and community structure could also allow for the proliferation of *H. akashiwo* in the future ocean. The growth of other co-occurring species – like the dinoflagellate *Prorocentrum minimum* – has been shown to be negatively impacted by more acidic, warmer conditions, which was not the case with *H. akashiwo* that experienced increased growth under the same conditions (Fu et al., 2008). Population reductions in co-occurring species could reduce pressure for resources (light, nutrients, etc.) and further enhance the success of *H. akashiwo*. Despite any limitations of this

study, it is fair to predict *Heterosigma akashiwo* will have a continued presence in future ocean waters.

4.2 Impacts of *Heterosigma akashiwo* in the future ocean

Since it is likely that *H. akashiwo* will retain the ability to form blooms in the future, it is important to determine the nature of these blooms, in terms of their effect on the surrounding marine community. *H. akashiwo* could have a positive effect, via an increased production of neutral lipids (EFAs) that can accumulate under certain environmental conditions. The effect could also be negative if blooms become increasingly toxic to marine life through allelopathy or ichtyotoxicity (Ikawa, 2004). These toxic agents have been labelled as PUFAs in *Fibrocapsa japonica*, another member of the class Raphidophycaea (Pezzolessi, 2010). By monitoring neutral lipid accumulation in tandem with toxicity, we gain a better idea of whether this is also the case for *H. akashiwo*.

4.2.1 Neutral lipid accumulation in *Heterosigma akashiwo*

Results from this study showed that peak neutral lipid accumulation was not significantly affected by iron concentration or pH (Figure 3.4A). This can be explained by a high nitrogen concentration (880 μM), later found to have a dominant control on neutral lipid production, which could have masked any significant differences caused by iron availability or pH.

The highest amounts of neutral lipid accumulation occurred in the most alkaline, P-limited cultures and the lowest accumulations occurred in the most acidic, N-limited cultures (Figure 3.4B). This lower neutral lipid accumulation at a lower pH could be attributable to lower cellular P requirements, which have been documented to occur

under lower pH conditions in *H. akashiwo* (Fu et al., 2008). The fact that neutral lipid accumulation decreased with pH is concerning, as this could translate to less EFAs available to sustain marine food chains.

Nitrogen source was not found to significantly impact neutral lipid accumulation (Figure 3.4C). The key finding with respect to neutral lipid accumulation was the fact that it appears to be primarily governed by nitrogen/phosphorus concentrations. When nitrogen concentrations were examined across experiments, in treatments with other experimental variables being equal, it became apparent that lower nitrogen concentrations corresponded to higher accumulations of neutral lipids (Figure 3.8.3). A drastic decline in neutral lipid production was also evident with increasing concentration of phosphorus (Figure 3.7B).

My suggested reasoning is that high nutrient cultures experienced dissolved inorganic carbon (DIC)-limitation prior to N, P, or Fe-limitation and would not have accumulated carbon-rich energy stores in the form of TAGs, or neutral lipids, as they were undergoing balanced growth. This will be expanded upon in more detail in section 4.3.

4.2.2.1 Implications of altered neutral lipid accumulation

The implications of altered nutrient levels and pH within marine ecosystems are that *H. akashiwo* could be a less nutritious food source for higher trophic levels under increasingly eutrophic/acidic conditions, as they have been shown to produce less neutral lipids or EFAs (Figure 3.8.3).

The algal biodiesel industry could also be impacted by these findings. It seems as though adding less N to the system increases neutral lipid accumulation (a high-value

source of energy). *Heterosigma akashiwo* grown at low N (10 μM), as nitrate, accumulated nearly twice as many neutral lipids under the most acidified treatment (pH 7.4) (Figure 3.4C). This could mean that much less N would need to be added into the system to attain the same neutral lipid yields, so long as the culture was acidified (a cost-effective and simple adjustment).

4.2.2 *Heterosigma akashiwo* toxicity

An initial examination of toxicity data reveals that almost every treatment applied elicited a very strong toxic response, shown by gill cell death following a 24-hour exposure period (Figure 3.5). In order to investigate toxicity results in this study, it is important to understand that in nature blooms of *H. akashiwo* are variably toxic. Within this section, treatments that were found to elicit a variable toxic response will be examined, followed by strong correlations between variables tested/measured and toxicity, to determine what the toxic compound could be, and when *H. akashiwo* HABs are most likely to be toxic.

4.2.2.1 *Heterosigma akashiwo*: variable toxicity

The lowest peak toxicity was exerted by *H. akashiwo* grown with ammonium as the nitrogen source (Figure 3.5C). This is a particularly interesting finding, as *H. akashiwo* are toxic to fish, which excrete large amounts of ammonia. This leads to the idea of a possible interaction between finfish defences, and the ichthyotoxic *H. akashiwo*. This finding has possible implications for the aquaculture industry, in that the addition of ammonium to areas of high-density netted fish could potentially prevent the induction of toxicity in *H. akashiwo*.

4.2.2.2 *Heterosigma akashiwo*: the toxic compound

The toxic component of *H. akashiwo* has yet to be determined. There exist two streams of thought; that it is a brevetoxin-like compound, or a PUFA. Other members of the algal class Raphidophyceae (Chattonella and Fibrocapsa) have been examined, and were found to produce toxic brevetoxins in the case of certain Chattonella spp., and toxic PUFAs in the case of Fibrocapsa (Pezzolesi et al., 2010; Haque & Onoue, 2002). By examining the variable accumulation of neutral lipids in *H. akashiwo* alongside its variable toxicity, a case could be made if there was a positive correlation, for toxic PUFAs being the mechanism of ichthyotoxicity.

Since the results showed a negative correlation between peak neutral lipid accumulation and peak toxicity, this could point to the toxic component of *H. akashiwo* being a brevetoxin-like compound rather than a PUFA (Figure 3.6B & Table 3.6). It is possible that the toxic component is a metabolic derivative of EFAs, rather than the PUFA itself, and was not measured in these experiments.

Another explanation would be that a brevetoxin-like compound is being produced by *H. akashiwo*. A recent study examining toxicity within the dinoflagellate *Karenia brevis* found that brevetoxin production, and ensuing toxicity, increased dramatically during periods of nitrate and phosphate limitation (Hardison et al. 2013). This is inconsistent with the toxicity results from this study, which found increasing toxicity as concentrations of nitrate and phosphate reduced (Figures 3.7C/D). From this, it seems questionable that *H. akashiwo* are producing a brevetoxin-like compound, similar to Chattonella species (Haque & Onoue, 2002).

An alternate theory is that the toxic compound is more similar in structure to that of a karlotoxin than a brevetoxin. Karlotoxins are polar lipids, which were not measured during this study. They exert haemolytic, cytotoxic, and ichthyotoxic effects on surrounding marine life (Deeds et al., 2002).

4.3 N/P concentration: a regulator of *H. akashiwo* physiology

Cells grown under high concentrations of nitrogen/phosphorus had lower specific growth rates and attained higher cell yields (Figures 3.8.1 & 3.8.2). If we recall Redfield's ratio of 106C:16N:1P, these cells would undergo dissolved inorganic carbon (DIC) limitation prior to nitrogen/phosphorus-limitation. *H. akashiwo* have been found to fully utilize 100 μM N before DIC-limitation occurs (Cochlan, 2013). Carbon-limitation would prevent the accumulation of neutral lipids during the transition period between exponential and stationary growth phase. Cells could, however, be producing the carbon-rich brevetoxin ($C_{49}H_{70}O_{13}$) under balanced growth with high nutrient conditions, while maintaining a low level of accumulated neutral lipids. This suggests that a brevetoxin is a natural component of *H. akashiwo*. Toxicity was greater than 88% across all experiments with concentrations of nitrogen higher than 100 μM N, while neutral lipid accumulation increased with lower concentrations of nitrogen (Figures 3.8.4B & 3.8.3B). The experiment with the lowest concentration of nitrogen/phosphorus, generated some of the highest specific growth rates and lowest cell yields (Figures 3.8.1A & 3.8.2A). These cells had significantly lower toxicity, and high neutral lipid accumulation (Figures 3.8.4A & 3.8.3A). When cells experience unbalanced growth, due to nutrient-limitation, they accumulate higher amounts of neutral lipids, since they are still receiving reactants necessary for photosynthesis. Cells store this carbon as neutral lipids within the

cytoplasm. The least toxic of the treatments within this low nitrogen experiment had ammonium as the N-source (Figure 3.4C). Since *H. akashiwo* readily uptake ammonium in this nitrogen-source experiment, but require multiple enzymatic processes to convert nitrate or urea into a useable form, this could account for the differential toxicity and neutral lipid production. In all pH treatments but 7.4, ammonium-grown cells accumulated a higher amount of neutral lipids, and exhibited lower toxicity than nitrate or urea (Figures 3.3C & 3.4C). I propose that N was used at a faster rate when provided as ammonium, and thus, these cultures became nutrient-limited well before the others.

In summary, nitrogen and phosphorus concentrations were found to be a major control of specific growth rates, cell yields, neutral lipid accumulation, and toxicity in *H. akashiwo* under iron-replete conditions. Unbalanced growth due to N/P-limitation resulted in the accumulation of neutral lipids and lower toxicity. Cell undergoing balanced growth, interrupted by C-limitation, did not have a chance to accumulate neutral lipids and toxicity was higher. This points to the notion that the toxin is a natural component of *H. akashiwo* undergoing balanced growth.

4.4 *Heterosigma akashiwo* HABs in the future ocean

In the future ocean we expect to see higher amounts of nutrients due to anthropogenic nutrient-loadings and lower pH attributable to increased carbon dioxide emissions (Anderson et al., 2002; Royal Society, 2005; Doney et al., 2009; Hallegraeff, 2010; Zeebe et al., 2012). Based on the results of this study, these conditions could cause slower growth rates, yet higher cell yields of *H. akashiwo*. Cell densities over 1,000,000 cells mL^{-1} constitute a visibly dense algal bloom for this species. This points to a continued likelihood of N/P fuelled bloom events. The other important factor to examine

is the variable toxicity of *H. akashiwo*, as these bloom events can be toxic to marine life. The results of this study suggest nearly complete toxicity at concentrations of nitrogen higher than 100 μM and phosphorus greater than 8.3 μM (Figures 3.7C/D) that could very well be the case in a eutrophic coastal area of the ocean. This could mean almost exclusively toxic blooms of *H. akashiwo* in future eutrophic coastal ocean waters. Based on my proposed model of physiology and toxicity at high nutrient concentrations, I would expect to see more *H. akashiwo* toxic blooms that would be most toxic once they experience DIC-limitation, due to localised drawdown of carbon during a natural bloom event. More acidic waters could provide more DIC initially, however this would merely delay the inevitable limitation, due to a lack of a carbon concentrating mechanism within *H. akashiwo* (Nimer et al. 1997).

4.5 Implications of future HAB presence

The future ocean environment would be a suitable location for *H. akashiwo*, possibly exerting dominance over co-occurring HAB species. Not only do the cells grow exceptionally well but lower rates of EFAs may restrict their consumption by primary consumers. This study also points to *H. akashiwo* becoming increasingly toxic, with a high potential to cause devastating fish kills within areas with aquaculture operations. Industry should pair with government to create strong legislation governing allowable nutrient inputs. Excessive use of synthetic-N fertilizers should be reconsidered. If nothing is done to manage the problem, we will continue to see a loss of fish landing revenue within coastal waters, and other negative effects on coastal communities.

4.6 Conclusion: the big picture

Anthropogenic activities have large-scale effects on biogeochemical systems, such as carbon dioxide emissions driving ocean acidification, and fertilizer run-off resulting in coastal ocean eutrophication. These large-scale processes dictate a suite of interactions among living organisms, seawater chemistry, and nutrient cycles that could exert negative or positive changes as we proceed into the unknown future. Increased formation of highly toxic *H. akashiwo* HABs that are of a lesser nutritional value to the marine food chain seems to be a distinctive possibility in the future ocean. This would be controlled by increased nutrient loadings of nitrogen and phosphorus and increased bioavailability of iron through ocean acidification. In this instance, our negative impact on the Earth has come back to negatively impact our aquaculture operations, coastal communities, and health. Efforts to drastically reduce carbon dioxide emissions, such as algal biofuel production, and regulation/reduction of nutrient inputs into natural systems needs to be a top priority at all levels of governance.

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CONTRIBUTIONS
Oral Presentations
Matheson. J.R., Cochlan, W.P. & Trick, C.G. (2014) Multiple stressors on the potential toxicity of *Heterosigma akashiwo*, a fish-killing flagellate in the Salish Sea. Salish Sea Conference 2014, Seattle, Washington, USA.

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Matheson, J.R. & Trick, C.G. The fate of the marine raphidophyte *Heterosigma akashiwo* in the future ocean: How will effects of ocean acidification and eutrophication affect its growth, neutral lipid levels and toxicity? Environment & Sustainability Showcase 2014, UWO, London, ON, Canada.

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