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Growth and toxicity of geographically-distinct isolates of the fish-killing phytoflagellate, *Heterosigma akashiwo*

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Abstract

Harmful Algal Blooms (HABs)—an accumulation of toxic phytoplankton—often result from environmental changes reflecting the increasing global human footprint and climate change. One HAB species drawing attention is *Heterosigma akashiwo*, a fish-killing flagellate that can cause extensive fish loss or be benign, depending on location or environmental conditions. Here, I investigate if this difference in toxicity is regulated by environmental conditions or differences in cellular physiology. Six strains were examined. Three originate from the Salish Sea, where fish-kills are common, and three from Japan, where blooms of *Heterosigma* are common but fish-kills are rare. By measuring growth and toxicity of cells grown under two environmental stressors—temperature and salinity—I concluded the low frequency of fish-kills in Japan was due to absence of environmental conditions associated with highest toxic expression. However, reduced temperature conditions, which may occur more frequently with climate change, may stimulate toxicity in Japanese strains.

Keywords

Fish-killing, gill-cell assay, harmful algal blooms, hemolytic assay, *Heterosigma akashiwo*, Japan, Salish Sea, growth, toxicity, salinity, temperature

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Preface

“The seaweed is always greener in somebody else’s lake”

— The Little Mermaid

*“My soul is full of longing
for the secret of the sea,
and the heart of the great ocean
sends a thrilling pulse through me.”*

— Henry Wadsworth Longfellow “The Secret of the Sea” 1850

1 INTRODUCTION

The ocean is teeming with organisms invisible to the naked eye. Microorganisms called phytoplankton, tiny they may be, are photosynthetic organisms that constitute the base of aquatic food webs (Falkowski et al., 1998; Field et al., 1998). Like terrestrial plants, phytoplankton contain chlorophyll to capture sunlight, and in turn use photosynthesis to convert that sunlight into chemical energy. As a consequence, phytoplankton convert carbon dioxide into sugars and release oxygen, thereby playing a crucial role in driving the global carbon cycle. Overall, phytoplankton contribute 40% of global photosynthesis (Falkowski, 1994). Unlike terrestrial plants, phytoplankton are planktonic and rely on water currents for transport—establishing their highest growth rates when encountering waters with inorganic nutrients (nitrogen, phosphorus and iron) and light (~top 100 m of the ocean) (Anderson et al., 2002).

1.1 Harmful Algal Blooms

The ocean's bounty of life has continuously provided humankind with a source of food, recreation, beauty and ecological services. Often referred to as the grass of the sea, phytoplankton serve as the basic food source upon which all other marine life depends (Guillard, 1975; Gilbert, 2013). In addition to the positive role phytoplankton play in the global carbon cycle, some play a more negative role in influencing the ecology of marine and freshwater ecosystems. Of the approximately 3500 species of phytoplankton, about 250 species are denoted as toxic or harmful based on the observable negative impacts on the food chain (Sournia et al., 1991). This distinction gave rise to the term harmful algal blooms (HABs) (Anderson, 2014). Some HAB species reproduce prolifically, often manifesting as water discoloration; whereas, other species are nearly inconspicuous if not for the environmental destruction they cause (Smayda, 1997). Nearly every coastal region worldwide is impacted by harmful algal blooms colloquially, yet erroneously, termed "red tides". Given that the latter term is a misnomer—incorrectly including blooms that discolor water but cause no harm and excluding blooms of toxic cells that are problematic

at low, and essentially invisible, cell concentrations—the term HAB is preferred among the scientific community (Anderson, 2014).

HABs are a taxonomically unrelated group of ecologically exploitative species that have one unique feature in common—they cause harm, either through the production of toxins or the accumulation of biomass impacting co-existing organisms (Anderson et al., 2002). The range of toxins produced include, but are not limited to: brevetoxins, the cause of neurotoxic shellfish poisoning (NSP); saxitoxins, the cause of paralytic shellfish poisoning (PSP); okadaic acid, the cause of diarrhetic shellfish poisoning (DSP); domoic acid, the cause of amnesic shellfish poisoning (ASP); ciguatera fish poisoning (CFP), caused by ciguatoxin and maitotoxin; and azaspiracid, the cause of azaspiracid shellfish poisoning (AZP); and various others (Van Dolah, 2000; Landsberg, 2002; Watkins et al., 2008). The toxins produced may directly result in shellfish or fish mortalities, or indirectly result in illness or death when shellfish that have accumulated the algal toxins are consumed (Smayda, 1997; Anderson et al., 2012). Additionally, high biomass accumulation may result in hypoxia from environmental oxygen depletion, anoxia and water discoloration (Hallegraeff, 2010), ultimately damaging the environment and defending the ‘harmful’ label. HABs are a natural phenomenon that have occurred throughout recorded history, however there is growing concern that they are becoming increasingly common and impacting the economy and public health (Anderson et al., 2002; Brooks et al., 2016). Although phytoplankton blooms typically fuel productive ecosystems, some blooms fashion an environment characterized by toxin production or low oxygen concentrations in bottom waters, killing or driving out marine fish. The expansion of anthropogenic activities in coastal watersheds has increased the influx of fertilizer into coastal waters, fueling unsolicited algal growth (Heisler, 2008; Hallegraeff, 2010). In combination with, or independent of, anthropogenic forces, abiotic factors such as irradiance, temperature, pH and salinity play a pivotal role in initiating, developing and maintaining HABs (Cochlan et al., 2013).

1.1.1 Fish-killing Phytoflagellates

Phytoplankton denoted as harmful algal bloom species produce ichthyotoxic agents resulting in toxigenic reactions, noxious agents that alter the quality of the water (i.e.

reduce dissolved O₂), or compounds with direct killing activities. In the latter category are the fish-killing phytoflagellates—a group of about 20 species that pose a formidable natural hazard (Smayda 1997, 1998). These phytoflagellates grow rapidly and produce metabolites that directly or indirectly damage or kill commercially valuable finfish, oysters, and sea urchins—a significant consideration given that ocean harvests are declining (Rensel et al., 2010). In comparison to non-HAB species, unique to HABs is the ability for the same species to have widely different impacts in separate geographic regions (i.e. the same species can be toxic in one location and non-toxic in another) (Anderson et al., 2002). Underlying this observation is the recognition that morphological species identifications may not offer the resolution required to explain differences between HAB events. Cells may appear morphologically identical, however genetic differences abound. Research demonstrates that high levels of genetic diversity can exist within a single species, and that only some of these genotypes will “bloom” under a given set of environmental conditions (Glibert et al., 2005). Additionally, genetic differences have been shown to exist even within a single phytoplankton-bloom population, as well as among populations that bloom at different times in the same location (Lebret et al., 2012). This diversity may be instrumental in achieving competitive success in the ecologically fluctuating Anthropocene.

Over the past several decades, the complexity of determinants regulating HABs have expanded in their scopes, giving rise to concerns regarding the potential environmental and public health impacts (Glibert et al., 2005; Brooks et al., 2016). Coastal waters throughout Canada and many regions of the world are under the emerging, ongoing and cumulative influence of HABs due to a myriad of factors ranging from eutrophication of anthropogenic origin to climatic changes in atmospheric CO₂, and subsequent changes in temperature (Anderson et al., 2002; Moore et al., 2008; Wells et al., 2015). While many different factors may contribute to HABs, how these factors interact to create an algal bloom is less understood. The dogma of their salient cosmopolitanism makes managing HABs challenging. The consensus is that the incidence and prevalence of fish-killing phytoflagellates has increased over the last few decades (Heisler et al., 2008; Wells et al., 2015). Alternatively, this increased frequency could be attributed to the “observer effect”, whereby more people are noticing and reporting incidents now than in the past. Whether

the apparent increase in the frequency of HABs is a real increase, or a consequence of increased observation efforts and advances in species identification technology, remains to be determined. Presently, no single factor can be attributed to this increased frequency. Rather, the synergistic interaction between multiple factors, such as increased eutrophication, carbon dioxide emissions, temperature, and salinity, have been implicated in HAB prevalence (Cochlan et al., 2013). When optimal, these environmental conditions may induce growth and toxicity. Conversely, even the most potentially toxic alga may not express satisfactory growth or toxicity if the environmental conditions are not conducive (Ono et al., 2000; Wells et al., 2015). The diversity of HAB species and variations in their manifestation present a significant challenge to those responsible for managing coastal resources and public health. This diversity is particularly evident in organisms belonging to the class Raphidophyceae, where little is known about the mechanisms by which they express toxicity.

1.2 Raphidophyceae

The Raphidophyceae are a small group of eukaryotic algae that inhabit diverse marine, brackish, and freshwater habitats. Presently, ten genera are recognized: *Gonyostomum*, *Merotricha*, *Vacuolaria*, *Chattonella*, *Chlorinimonas*, *Fibrocapsa*, *Haramonas*, *Heterosigma*, *Psammamonas*, and *Viridilobus*—the first three are freshwater representatives and the latter seven are marine representatives (Horiguchi, 2017). The absence of a rigid cell wall allows for flexibility in shape and size of Raphidophyceae under fluctuating environmental conditions (Cattolico et al., 1976; Hara et al., 1985). However, relative to other harmful algal species, little is known about the mechanisms by which raphidophyte species are toxic to marine invertebrates and fish (Horiguchi, 2016). The majority of marine raphidophytes are widely recognized as “nuisance” species, impacting the survival of commercially valuable aquaculture such as sea urchins, oysters and finfish (Berdalet et al., 2015). Species such as *Chattonella* spp., *Fibrocapsa japonica*, and *Heterosigma akashiwo* have been associated with finfish kills (Horiguchi, 2016).

1.3 *Heterosigma akashiwo*

Heterosigma akashiwo is a microscopic alga of the class Raphidophyceae, order Chattonellales and family Chattonellaceae. It is a globally occurring toxic genus that has revealed itself as a potent fish-killer, episodically forming HABs (Han et al., 2002; Harvey et al., 2015; Ikeda et al., 2016). *H. akashiwo* is euryhaline, indicating its unique ability to grow in a wide range of salinities (Ikeda et al., 2016), affording this species its prominent nature in HABs. Although not presently known to be toxic to humans (Fredrickson et al., 2011), cells have been indicted for extensive fish mortality, resulting in major economic losses for commercial fishing and aquaculture industries (Rensel et al., 1988; Horner et al., 1990; Rensel et al., 2010). *H. akashiwo* presents a challenging piece of the puzzle for the management of harmful algal blooms. The ecological conditions affecting its physiology remain relatively unknown due to conflicting results caused by high intraspecific variation (Harvey et al., 2015). This variability allows *H. akashiwo* to persist in rapidly changing environments and over broad ranges of environmental conditions (Fredrickson et al., 2011).

H. akashiwo is aptly named for its variable cell shape, ranging from potato to cornflake-like, and causing red tides in temperate and coastal waters worldwide (Anderson et al., 2002). *Hetero* is Greek for ‘different’, *sigma* is Greek for ‘curves’, and *akashiwo* is Japanese for ‘red sea water’ (Wikfors, 2003; Matheson, 2014). Cells range in size from 18-20 μm in length, and each cell can contain up to 95 chloroplasts—a significant number relative to other species (Hara and Chihara, 1987). This coastal raphidophyte is capable of producing dense, golden-brown toxic blooms (Cochlan et al., 2013).

These single-celled organisms are reminiscent of a “super bug”—a formidable cross that combines photosynthesis with self-propelled movement. *H. akashiwo* is a flagellate in that it possesses two heterodynamic flagella—appendages capable of moving independently of one another—that propels them through the water column in search of nutrients (Han et al., 2002). The two flagella are equal in length, with the anterior flagellum used for swimming (Matheson, 2014). It exists in two forms: the resting stage—a stage in which the cells produce cysts and sink to the sediment—and the

vegetative state, in which *H. akashiwo* is metabolically plastic and capable of adapting to environmental perturbations (Martinez et al., 2010). When a threshold is reached, these vegetative cells enter a quiescent phase, becoming immobile cysts that seed coastal sediments. These cysts can survive long-term metabolic stasis, thereby potentiating future blooms when conditions are favorable. Excystment of *Heterosigma* from shallow sediments is known to be successful above the 15 °C bottom temperature threshold (Imai and Itakura, 1999). This cyst-forming ability offers a competitive advantage for overwintering, surviving nutrient stress, or relocating to a new geographic location via ocean vessel transport or natural currents (Shikata et al., 2007).

In laboratory batch cultures, a typical growth curve of *H. akashiwo* can best be described by Figure 1.1 The earliest phase is described as the lag phase, where little increase in cell density occurs as cells adapt and begin to exploit new environmental conditions. Cells will then begin to grow rapidly and increase in population size as a function of time. This period of rapid growth is termed the exponential growth phase, where photosynthetic capacity is high and, in natural environments, likely represents their growth physiology in response to nutrients from run-off, agro-industrial waste water, or aquaculture. It is during this period of exponential growth where the growth rate (μ) can be calculated from the linear portion of the exponential phase. The growth rate provides an indicator of the organism's ability to grow under select environmental conditions and therefore its ability to be an effective competitor for nutrients. After a period of time, there is insufficient nutrient availability or space to continue growing, thereby reducing the growth rate, resulting in constant cell density—this is characterized as the stationary phase. During this stationary period, the parameter of cell yield can be obtained by averaging cell densities over this stage. This parameter is valuable as it represents the maximum biomass, or the carrying capacity, that can be supported by the available resources. In other words, it provides an indication of the organism's nutrient use efficiency and ability to successfully convert the available nutrients into biomass.

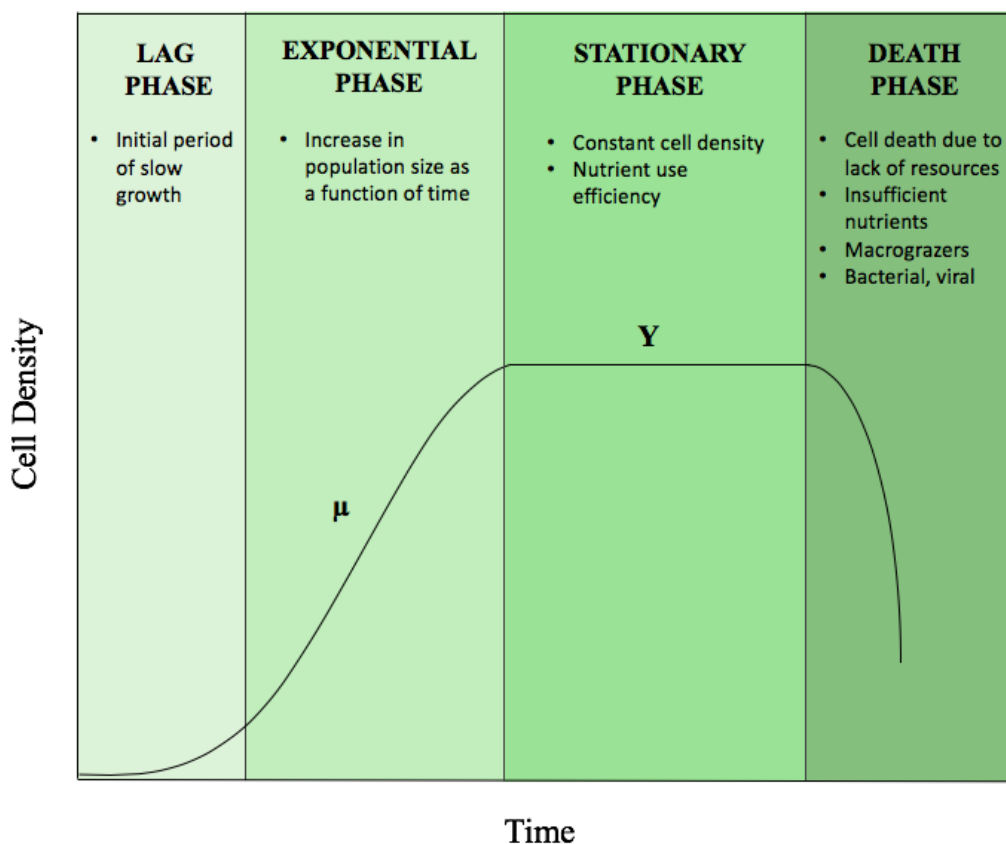


Figure 1.1 General model of the growth curve of an axenic culture of *Heterosigma akashiwo*. During the exponential growth phase, cells begin to grow rapidly and increase as a function of time as they receive nutrients from chemically conditioned waters. Growth rate (μ) can be calculated from the linear portion of the curve. During the stationary phase, the rate of growth decreases, resulting in constant cell density. Cell yield (Y), indicating the ability to convert nutrients into biomass, can be measured during this stage.

This stage is succeeded by the death phase, where nutrients are depleted to a level that is unable to sustain growth, resulting in a rapid decline in cell density and ultimately, collapse of the culture. Exponential and stationary phases have been examined extensively, as they represent the processes of cell division and cessation of division, respectively. Overall, this model suggests that bloom formation of *Heterosigma* is under multi-factorial control. Its euryhaline and eurythermal nature, in combination with its

ability to remain encysted during adverse conditions, makes it a potentially successful colonizer of coastal ecosystems, which in turn presents many challenges for the economies that depend on coastal resources.

1.4 Economic Implications

The impact of harmful microalgae is particularly evident when it affects the consumption of marine food resources. The Food and Agriculture Organization (FAO) of the United Nations estimates that over half of the world's fish production for human consumption is sourced from aquaculture (FAO, 2016). Notorious for its dramatic devastation, *Heterosigma*-mediated fish kills have resulted in multi-million-dollar losses in aquaculture and shellfish industries (Cochlan et al., 2013). Occurring globally in highly eutrophic coastal and estuarine systems (Honjo, 1993), the impression it makes on the economy can be dramatic. *H. akashiwo* outbreaks have been associated with cultivated fish mortalities in the coastal regions of many countries, including Canada (Taylor and Haigh, 1993), the United States of America (Horner et al., 1997), Japan (Honjo, 1993), China (Tseng et al., 1993), Australia (Ajani et al., 2001), New Zealand (Chang et al., 1990), Denmark, Sweden, Chile, and Brazil (Honjo, 1993). To illustrate the economic impact, between 1972 and 2012, economic losses for the finfish industries in Japan were 34.2 billion JPY (approximately \$352 million USD) (Honjo, 1993). Furthermore, Big Glory Bay, Stewart Island, New Zealand established its first free floating cages for rearing salmon in 1981, after which this bay became the main area for rearing chinook salmon in New Zealand. In 1989, Big Glory Bay experienced a loss of approximately 600 tonnes of farmed chinook salmon. Microscopic examination and histopathological changes in the affected salmon tissue implicated *H. akashiwo* as the principal offender (Chang et al., 1990). Likewise, in June 2006, a bloom of *Heterosigma* resulted in two million dollars-worth of farmed salmon that were being reared in Puget Sound—a coastal area of the Pacific Northwest in the U.S. state of Washington—followed by a subsequent bloom in 2007 that mirrored the same pattern of events (Cochlan et al., 2013). From a broader ecosystem and societal perspective, *Heterosigma* blooms transcend monetary losses and disturb the existing ecological balance—albeit the two are not mutually exclusive.

1.5 Inter-Strain Variability

1.5.1 Global Expansion: Nature vs. Nurture

Climate change is a catalyst for the global expansion of harmful algal blooms. Previous studies demonstrated that optimum temperature and salinity ranges differ between strains of *Heterosigma* species, thereby resulting in recognizably distinct growth and ichthyotoxic responses. Nielsen and Tønseth (1991) proposed that there are strain-specific preferences and requirements for ecological parameters among phytoflagellates. In May 2010, the Red Sea coastal waters off the coast of Saudi Arabia were confronted with their first HAB of *H. akashiwo*. Preceding this event, only two incidences of HABs of *Noctiluca scintillans* and *Gonyaulax sp.* occurred in 2004 (Mohamed et al., 2007). The means by which *Heterosigma* was introduced into Saudi waters is unclear but could have been transported via ship ballast water—a common avenue for the trans-oceanic dispersal by HAB taxa (Smayda, 1997; Marshall et al. 2005). This mechanism has been invoked as an effective method for the dispersal of harmful algae throughout the oceans (Smayda, 2007). However, inoculation of the seed population into new waters does not necessitate a HAB; environmental conditions must be favorable. For *H. akashiwo*, bloom development has been associated with cultural eutrophication (Anderson et al., 2002, Rensel et al., 2010), and abiotic factors such as salinity, pH, temperature, and irradiance (Mohamed et al., 2007).

1.5.2 Variation Among Geographically-Distinct Isolates

Recently, the occurrence and intensity of blooms appear to have increased, with the extension of their geographic distributions (Wells et al., 2015). Physiological data indicates that *H. akashiwo* strains vary in fitness with regards to surviving cyst formation and their ability to return to a metabolically active swimming state (Imai and Itakura, 1999). Han, Kim et al., (2002) demonstrated inter-strain variability in resting stage entrance. Strains are known to differ in their salinity tolerance, carotenoid production, pH optima, growth rate and nutrient dependence. Inter-strain differences have also been observed in several ecologically important physiological processes such as: salinity and temperature optima (Smayda, 1998), growth rate (Connell and Cattolico, 1996),

swimming speed, and ichthyotoxicity (Khan et al., 2005). Unfortunately, limited evidence exists documenting genetic differences among strains (Lebret et al., 2012; Harvey et al., 2015).

Not all blooms are constitutively toxic. Environmental conditions specific to a geographic region may trigger changes in cellular physiology that accordingly govern bloom toxicity (Twiner and Trick, 2004). This disparity leads to the idea that *H. akashiwo* comprises of a variety of ecotypes—a genetically-distinct geographic population within a species, which is genotypically adapted to specific environmental conditions (Smayda, 1998; Fredrickson, 2011). *H. akashiwo* has cemented itself as a causative organism of red tides across the globe. Despite its cosmopolitan presence, it has been shown to exhibit variation in growth and ichthyotoxic responses in different geographic locations. One salient example of this is the distinct growth and toxic responses exhibited by *H. akashiwo* originating from North America and Japan (Fig 1.2). *H. akashiwo* is a representative phytoplankton that forms red tides in the temperate coastal waters of Japan (Honjo et al., 1978). In Osaka Bay, *Heterosigma* outbreaks have typically been observed during June to July (Yamochit, 1983). Yamochit (1983) revealed the lack of stimulated growth of an axenic clone of *H. akashiwo*, isolated from Osaka Bay, following the addition of nitrogen and phosphorus—suggesting that these nutrients are already present in Osaka Bay water at sufficiently high concentrations to sustain the growth of this species. In the Salish Sea—a network of coastal waterways encompassing the southwest and northwest portion of British Columbia and the state of Washington, respectively—the occurrence of *Heterosigma*-mediated blooms has been shown to coincide with changes in salinity (Cochlan et al., 2016).

Japanese waters, where surface water temperatures are warmer (22-30 °C) and salinities are greater (32-35) than in the Salish Sea, *Heterosigma* cells proliferate yet very few fish-kills have been reported. *Heterosigma* cells found in Canadian waters mirrors this inverse relationship between growth and toxicity that exists in Japan. In Canadian waters, where surface water temperatures are cooler (15-20 °C) and salinities are lower (20-27), although the abundance of cells is considerably low, the toxic insult is substantially larger. The wider range of salinities where *Heterosigma* exists in Canadian waters is

attributed to the deposition of water from major river systems, shaping the variation in salinity. Japanese waters that contain blooms of *H. akashiwo* rarely produce concomitant fish-kills (Imai and Itakura, 1999); yet, the presence of cells as low as 1000 cells ml⁻¹ place aquaculture fish in Puget Sound at risk and require immediate mitigative procedures by fish farm managers (Cochlan et al., 2013) (Fig. 1.2). There are two potential explanations for this difference: 1) The cells from Japanese waters lack one, or more, of the key processes associated with West Coast toxicology; or 2) the cells from Japanese waters contain all the biochemical elements to be toxic but thrive under environmental conditions that are not associated with the induction of toxin formation.

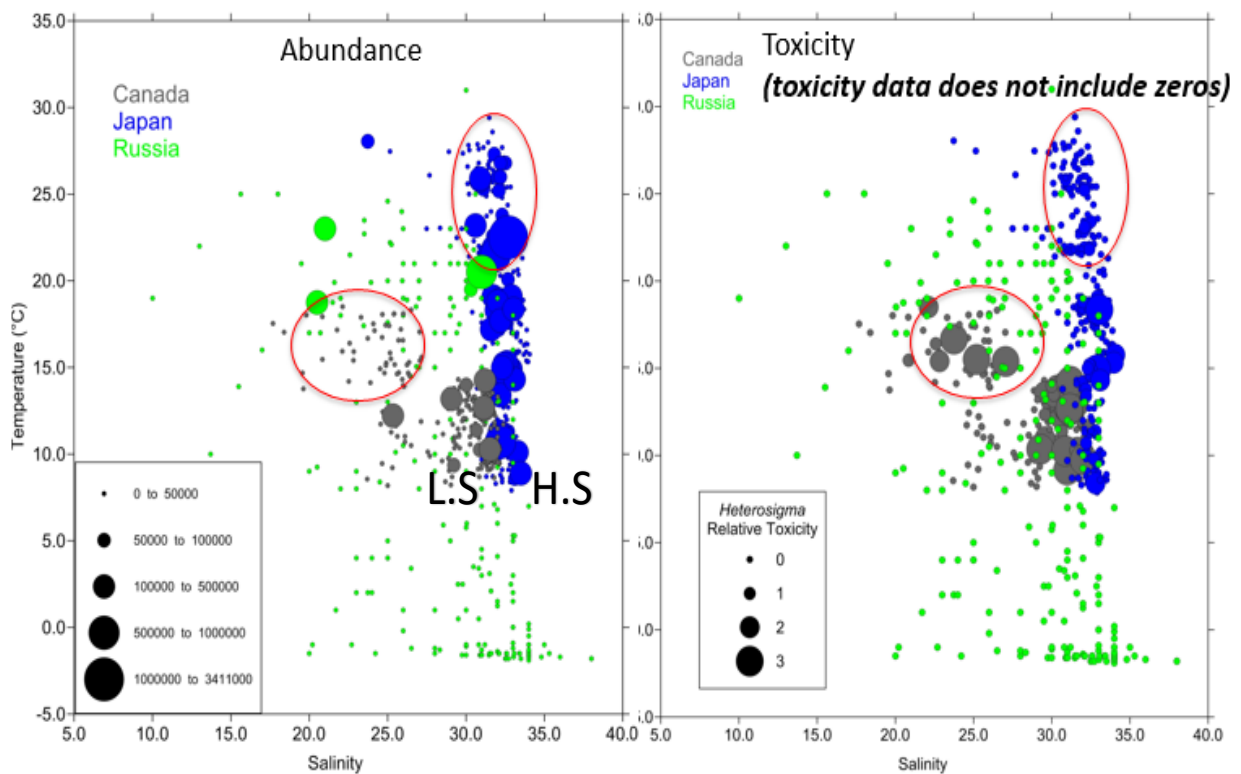


Figure 1.2 A meta-analysis of *H. akashiwo* reports from 3 countries in the North Pacific (Trainer and Trick, unpublished). Toxicity is measured as the number of fish kills. Each country has an optimal window of temperature and salinity that supports growth and toxicity. This figure illustrates the idea that at higher relative toxicity, indicated by the size of the circles, there is lower abundance. In Canada, there is high toxicity but low abundance at lower salinities (20-25). In Japan, there is low toxicity and higher abundance at higher temperature (22-30°C).

1.5.3 Wicked Problem

Management of these blooms is complicated as the exact cause of toxicity remains controversial and inconclusive. This knowledge gap is what classifies blooms of *Heterosigma* as a “wicked problem”. A wicked problem, as defined by Rittel and Weber (1973), is characterized as “a problem that is difficult to solve because of incomplete, contradictory, and changing requirements that are often difficult to recognize”. The concept of this wicked problem can be applied to harmful algal blooms, which often engender a degree of conflict due to lack of agreement with respect to problem management (Weber and Khademian, 2008). The introduction of non-native organisms into new territory can have unintended and unpredictable ramifications on the native flora and fauna.

This wicked problem, as it relates to *Heterosigma* blooms, can be further reduced to three dimensions of problems: 1) Why are there more blooms occurring now than in the past? This may be related to the increased nutrient loading and eutrophication experienced by these systems; 2) Why are there more fish kills now? This is complicated to answer because the drivers for selection of fish are still unknown; 3) Why is there such a large variation in toxicity between *Heterosigma* isolates? This is also not entirely clear as environmental conditions play a large role in influencing toxicity. Therefore, a better understanding of the role that the environment plays in influencing toxicity in geographically-distinct isolates is insightful. Emerging over the last few decades, there has been increasing recognition that many problems are not traditional and can challenge the confines of problem definition and resolution (Rittel and Weber, 1973). Efforts to manage wicked problems requires developing a comprehensive understanding of the mutable, interconnected, multidisciplinary and overlapping nature of the problem (Weber and Khademian, 2008).

1.6 Regulation of Toxicity

1.6.1 Temperature

One of the contingencies regulating the toxicity of *H. akashiwo* is the environmental parameter of surface water temperature. Surface water temperatures fluctuate over the

seasonal life history of *H. akashiwo*, and also vary according to geographic location. In literature, germination of *H. akashiwo* cysts is known to be successful above the 15 °C bottom temperature threshold (Taylor and Haigh, 1993; Smayda, 1998; Twiner and Trick, 2000), with optimum conditions for growth ranging between 15 and 25°C (Rensel et al., 2010). Several laboratory studies indicate that maximum growth occurs at 25 °C, although minimal toxicity is observed at this temperature (Ono et al., 2000). Other studies also indicate optimal growth at a temperature of 17 °C, which explains the occurrence of blooms even in coastal waters at high latitudes with maximum summer temperatures of approximately 17 °C (Connell and Jacobs, 1999). In Northern Hiroshima Bay, blooms occur in the summer coinciding with temperatures ranging from 20.7 to 23.8 °C (Tomaru et al., 2004). *Heterosigma*-laden blooms occur every spring and summer in the Pacific Northwest with association to increases in temperature and freshwater inputs (Cochlan et al., 2013). Although Fredrickson et al. (2011) identified the presence of several different ecotypes of *H. akashiwo* in this region, generally, the growth of *H. akashiwo* responds positively to increased temperature and tolerates a wide range of salinities. Despite studies revealing optimal growth over a range of temperatures, toxicity is thought to increase when the alga is exposed to temperatures that are not ideal for growth (Lewitus et al., 2012).

1.6.2 Salinity

A defining feature ascribed to *H. akashiwo* is its euryhaline nature, allowing for rapid adaptation to salinities ranging from 5 to 40 (Martinez et al., 2010). However, the mechanism that allows for rapid adaptation to salinity is poorly understood. Its broad tolerance to salinity has been suggested to play a role in bloom formation by enabling cells to avoid predation by migrating to salinity conditions that inhibit the survival of predators (Harvey and Menden-Deuer, 2012). Haque and Onoue (2002) demonstrated a negative correlation between an increase in salinity and toxin production, however lower salinities promoted an improved morphological condition and motility of cells. Minimal growth was reported below a salinity of 10, with optimal growth occurring between salinities 20 and 35 (Honjo, 1993). Given the greater depth of the channel, salinity acts as the principal factor determining water column stratification in the northern regions of

Puget Sound. However, in the shallower regions, the importance of temperature in controlling stratification outweighs salinity (Rensel, 2007).

1.6.3 Growth Phase

Phytoplankton isolated in culture generally proceed through a typical growth pattern spanning the lag phase, logarithmic (exponential) phase, stationary phase and senescence phase (Figure 1.1). During the lag phase, little increase in cell density occurs as cells use new nutrients to replenish their existing internal pools of nitrogen and phosphorus constituents. During the exponential phase, cell density increases as a function of time and the organism has a high capacity for photosynthesis (Wood et al., 2005). During stationary growth, the limiting factor and growth rate are balanced (Klausmeier et al., 2004). Comparing cells during their growth phases is insightful because cells in both stages persist and have the potential to contaminate the environment. For some marine phytoplankton, toxin production has been shown to be produced during the stationary period (Powers et al., 2012), whereas for others, toxin production coincides with the exponential growth phase (Ono et al., 2000). Toxicity of *H. akashiwo* is thought to vary with its growth phases. Haque and Onoue (2002) found that a Japanese strain from Kagoshima Bay exhibited the highest toxicity in the mid-late exponential phase at the range of salinities used. Conversely, Cochlan et al. (2013) recorded a non-toxic response from *H. akashiwo* during its nutrient-replete, exponential phase of growth, but instead demonstrated elevated toxicity during stationary growth.

1.6.4 Cellular Fragility

In natural aquatic ecosystems, the integrity of intact phytoplankton cells may be compromised at any stage of growth, resulting in lysed cells that vary in their ichthyotoxic properties. However, in contrast to most phytoplankton, *H. akashiwo* lacks a rigid cell wall, making it susceptible to lysis via movement or agitation (Smayda, 1998). The fragility of this species is often implicated in ichthyotoxicity—either directly (clogging fish gills with fractured cell remnants) or releasing intracellular toxins upon cell lysis (Ling and Trick, 2010). Mechanisms by which lysis may occur among phytoplankton populations include: predatory infection by lytic viruses, autolysis,

mechanical damage, cell age, nutrient deprivation, oxidative stress, herbivore grazing and other environmental stresses (i.e. temperature, salinity, pH) (Lawrence et al., 2001).

Therefore, comparing toxicity of cells when they are intact or lysed helps understand the location of the toxin. Specifically, if the toxin is located on the surface of intact cells, or stored within the cells—in which case, it would be released upon lysis.

1.7 Toxicology

1.7.1 Why Do Algae Produce Toxins?

Although the insult of many algal blooms is merely an aesthetic nuisance, some algal species produce toxins that are fatal to fish, shellfish, livestock, wildlife and humans. Documented instances of wild bird mortality have been associated with some toxins produced by dinoflagellates and cyanobacteria, including domoic acid, brevetoxin, saxitoxin, and other cyanobacterial toxins (i.e., microcystins, nodularins and anatoxins). Marine algal toxins—including domoic acid, brevetoxin and saxitoxin—that bioaccumulate in the food chain by fish and shellfish impair the nervous system (Smayda, 1997; Landsberg, 2002). The consequences of some harmful algae are not associated with toxin production, but rather with algal proliferation resulting in depleted dissolved oxygen concentrations, obstruction of sunlight limiting primary productivity, or physical damage to fish gills as a result of algal morphology (Hallegraeff, 2010). The wide spectrum of toxins produced begs the question: why do algae produce toxins? Surviving in a highly competitive environment necessitates the development of defense strategies by marine or freshwater algae. A product of these defense strategies is a diversity of compounds, varying in their structural-chemical profile, created from different metabolic pathways.

The products of phytoplankton photosynthesis include carbohydrates, protein, and lipids. Under scenarios of unbalanced growth, phytoplankton shift production toward increased lipids, which are mainly neutral lipids (triacylglycerol). These function as high carbon energy stores within the cytoplasm and can be leveraged to sustain metabolic requirements. Polyunsaturated fatty acids are a sub-category of neutral lipids and can accumulate during conditions of environmental stress (Castaing et al., 1993). Marine

microalgal essential fatty acids (EFAs) have been demonstrated to exert negative allelopathic effects on other algal species, thereby hindering growth and survival (Ikawa, 2004). The release of allelopathic compounds have been suggested to play an essential role in plankton succession, competition and bloom formation. In this sense, EFAs could be exercising control over which algal species dominates the phytoplankton community while simultaneously exerting toxic effects on surrounding organisms, notably fish species. The dynamics of toxin production are still being elucidated. What is clear is that the suite of toxins that are produced fashion an environment that is not always conducive to the growth and survival of co-existing species.

1.7.2 Mechanisms of Toxicity

The study of the mechanisms of toxicity in the raphidophyte, *H. akashiwo*, still remains in its infancy. Although the mode of ichthyotoxicity for this species remains misunderstood, several mechanisms aimed at identifying the toxic principle have been hypothesized (Fig. 1.3). The first mechanism postulates that extracellular production of reactive oxygen species (ROS) by *H. akashiwo* results in the suffocation of fish by means of damaging gill cells (Twiner and Trick, 2000). The second mechanism involves mucus production by fish exposed to *H. akashiwo* cells, which in turn can exacerbate ROS-mediated toxicity by increasing ROS production in *H. akashiwo* (Handy and Eddy, 1991; Twiner and Trick, 2000). The third mechanism proposes that *H. akashiwo* produces a neurotoxin that negatively affects the cardiovascular system of fish, thereby asphyxiating them (Khan et al., 1997). Lastly, hemolytic activity has been engendered as a mechanism via hemagglutinating and hemolytic compounds (Ling & Trick, 2010; Mohamed et al., 2012), such as free fatty acid production by *H. akashiwo* which can lyse fish erythrocytes (Matheson, 2014). Toxicity may not solely be the function of one of these mechanisms. Rather, it is possible that the overall toxicity of *H. akashiwo* is attributed to a combination of these factors. For instance, the combinatorial effect of superoxide and polyunsaturated fatty acids (PUFAs) on bacterial luminescence and ichthyotoxicity of *Vibrio fischeri* on damselfish has been established (Marshall et al., 2003).

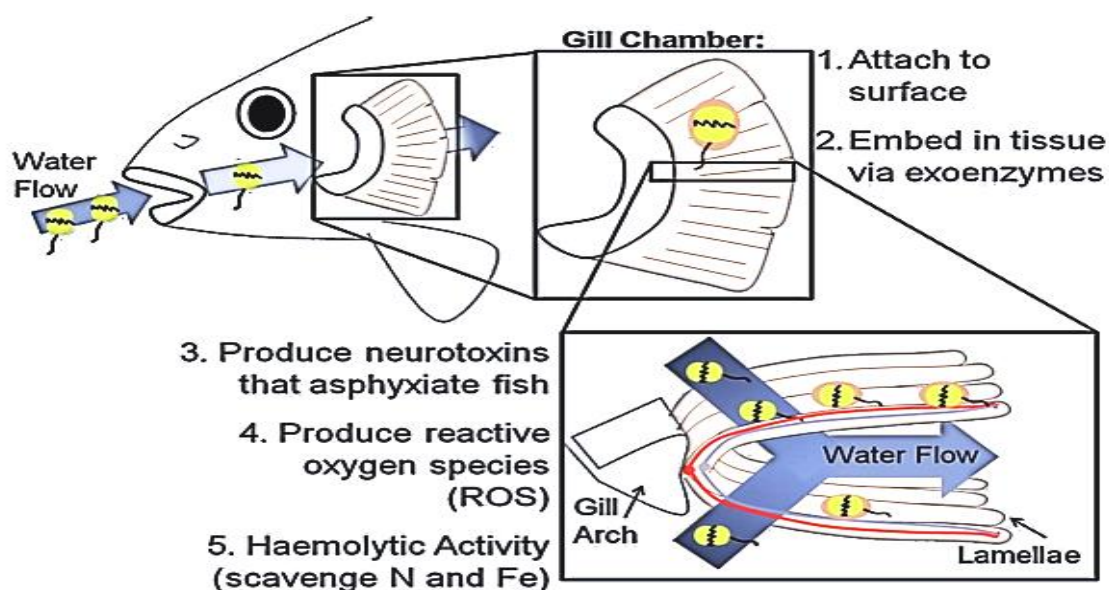


Figure 1.3 Model of ichthyotoxicity; the ability of toxic algae to kill fish.

Physiological activities associated with a generalized fish-killing phytoflagellate.

Algal cells attach to fish gills and embed themselves into the tissue via exoenzymes.

Cells produce neurotoxins and reactive oxygen species that lead to the asphyxiation

of fish. Hemolytic activity may be the final step of the model, lysing erythrocytes thereby leading to fish kills (Trick, unpublished).

Understanding the mechanism of toxicity has established itself as the Achilles heel in *H. akashiwo* research. Despite considerable efforts directed at understanding their toxicity, the toxic mechanisms of raphidophytes remains unclear. It remains enigmatic if toxicity is regulated by environmental conditions specific to a geographic region, or if it is a function of strain variability (Han et al., 2002; Martinez et al., 2010). Species that thrive in one geographic location under a given set of temperature and salinity may not reproduce the same response in a different location where the environmental conditions differ (Smayda, 1998; Trainer et al., 2014; Bronicheski, 2014). Therefore, the proposed mechanism of toxicity is an important consideration when selecting a suitable assay to assess toxicity.

1.7.3 Reactive Oxygen Species

Production of reactive oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot), can damage tissue and lead to asphyxiation (Twiner and Trick, 2000; Ling and Trick, 2010). Superoxide anion radicals and hydrogen peroxide are thought to remove mucus from fish gills, resulting in osmoregulatory stress (Tanaka et al., 1994). However, this contradicts histological studies of *Heterosigma*-inflicted salmon kills, in which the gills contain little to no mucus in under normal, unstressed conditions (Handy and Eddy, 1991). Additionally, superoxide damages gill epithelium, resulting in subsequent impairment to respiratory capacity. This toxic insult is thought to occur at the gill membrane, where contact occurs between the gill epithelia and *Heterosigma* cells (Tanaka et al., 1994). *Chattonella marina*, also a marine raphidophyte, possesses the unique ability to produce levels of reactive oxygen species 100-fold higher than most algal species. Despite these elevated levels, superoxide was not exclusively found to be the principle cause of fish mortality (Marshall, 2003), casting doubt on the postulation of reactive oxygen species as the causative agent of ichthyotoxicity. Twiner et al. (2001) further investigated the role of hydrogen peroxide on vertebrate cell lines and brine shrimp, *Artemia salina*. Results indicated that production rates of hydrogen peroxide fall short of the rate required to cause mortality, by orders of magnitude. Therefore, although substantial amounts of ROS are produced by *H. akashiwo*, the levels are below the threshold at which a pathological response is induced.

1.7.4 Mucus Production

The presence of mucus produced by fish has been implicated as a mechanism of toxicity responsible for fish mortality. In response to gill clogging, irritation, or mechanical damage to gill tissue induced by algae, the production of protective mucus—composed of glycoproteins—is stimulated by fish. Under stressed conditions, this response aids in alleviating the abrasion of the epidermis or clearing obstructed filaments (Handy and Eddy, 1991). However, this response ricochet's as the accumulation of mucus has been cited to slow gas exchange and negatively affect osmoregulation (Ling and Trick, 2010). The presence of mucus appears to be absent on the secondary lamellae in unstressed rainbow trout, *Oncorhynchus mykiss* (Handy and Eddy, 1991), suggesting the limited

physiological role mucus plays under normal conditions. A fatal flaw with this postulation is that it remains inconclusive if mucus is produced by the fish or the alga (Twiner et al., 2001), making the link between mucus production and ichthyotoxicity a tenuous one.

1.7.5 Neurotoxins

The production of intracellular brevetoxin-like compounds by *H. akashiwo* has been hypothesized as a mechanism of toxicity. This is suggested to contribute to cardiac disorder and asphyxiate fish (Endo et al., 1992; Khan et al., 1996). Khan et al. (1997) concluded that the toxin profile of *H. akashiwo* contained neurotoxic, hemolytic and hemagglutinating fractions, and reported that the neurotoxic component was more toxic to fish than the latter two components. However, several studies cast doubt on this. For instance, brevetoxins are known to bind to voltage-gated sodium channels, thereby disrupting normal neurological processes and causing the illness that has clinically been termed as neurotoxic shellfish poisoning. Despite this, studies on brevetoxin-like components have historically been based on detection through TLC and HPLC co-chromatography, as opposed to sodium channel inhibition (Ling and Trick, 2010). Additionally, unlike brevetoxin which alters the sodium balance of cells, organics isolated from *Heterosigma* have been shown to alter calcium homeostasis, suggesting that the substance is a bioactive metabolite (Cochlan et al., 2013; Trainer et al., 2014; Twiner et al. 2005). Furthermore, Twiner and Trick (2004) revealed a novel bioactive exudate produced by *H. akashiwo* that is inconsistent with, and therefore distinct from, brevetoxin.

1.7.6 Hemolytic Activity

Although the potential mechanisms described above are plausible and possess merit, none of them have undisputedly been shown to be the ichthyotoxic agent responsible for fish kills. The most likely mechanism of fish kills by *H. akashiwo* and other ichthyotoxic raphidophytes is hemolytic activity (Landsberg, 2002; Mohamed and Al-Shehri, 2012), in which hemolysins—compounds that lyse erythrocytes—are produced by the alga (Ling and Trick 2010). Being embedded in the gill tissue surface allows for close proximity to

the fish's blood supply. Under environmental stress the toxin/hemolysin produced lyses red blood cells and compromises the integrity of the endothelial layer of blood vessels to scavenge iron and nitrogen from the blood (Yasumoto et al., 1990; Ling and Trick, 2010). To illustrate this, Eschbach et al. (2001) observed significant lysis of carp (*Cyprinus carpio*) erythrocytes by *Prymnesium parvum*, another marine fish-killing flagellate, and *Alexandrium tamarense*, a marine dinoflagellate. In addition to being toxic to fish, *H. akashiwo* revealed hemolytic activity towards brine shrimp (*Artemia salina*) and rabbit erythrocytes (Landsberg, 2002; Ling and Trick, 2010). These hemolytic agents have been identified as polyunsaturated fatty acids (Marshall et al. 2003, Pezзолesi et al. 2010).

1.7.7 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids play an essential role in the human diet. Unable to be synthesized by humans, PUFAs are obtained via consumption of animals, plants, fungi and microalgae (Igarashi et al., 2007). Although essential for some organisms, elevated concentrations have been reported to have toxic effects and increase membrane permeability (Castaing et al., 1993). Upon active release of PUFAs from microalgae, induced by cell damage, fish mortality may ensue. PUFAs are thought to be a potential hemolysin, however it is unclear if they are produced in sufficient amounts to suspect a role in ichthyotoxicity. Yasumoto et al. (1990) recorded two hemolytic components from a sizeable fish kill caused by *Gyrodinium aureolum* along the Norwegian coast in 1998. The free fatty acid octadecapentaenoic acid corresponded to the component that expressed higher hemolytic activity against mouse blood cells. Furthermore, Okaichi (1989) discovered two PUFAs within a bloom of the raphidophyte, *Chattonella antiqua*, that were associated with yellowtail death. The interaction of these PUFAs with the mucosal lining of fish gill cells triggered swelling, impaired gas exchange and asphyxiation (Toyoshima et al. 1989). High concentrations of PUFAs were also found in the raphidophyte, *Fibrocapsa japonica*, resulting in mortality of European seabass (*Dicentrarchus labrax*) and brine shrimp (*Artemia salina*), and inhibiting bioluminescence of *Allivibrio fischeri* (Pezзолesi, 2010).

1.8 Measurement of Toxicity

Direct and indirect methods are commonly used for measuring toxicity. Direct measurements of the compound of interest include mass spectroscopy, chromatography and capillary electrophoresis (Holland, 2008). Although valuable in separating crude and impure samples, direct measurements often fail to provide an understanding of the mode of action or toxicity. Indirect measurements include gill cell viability assays, hemolytic assays, mammalian cell viability assays, and radio-immunoassays (Bertin et al., 2012). There is a need for rapid, consistent and economical tools to detect the toxicity of marine organisms. Research has examined the ability of cell-based assays to gauge the ichthyotoxic effects of compounds produced by marine phytoplankton (Dorantes-Aranda et al., 2011). Although whole organism bioassays offer investigators an effective way to evaluate the toxicity of a composite sample (Dayeh et al., 2003), cell-based assays offer a similar form of composite sample analysis with the added benefit of several key advantages including: low cost, low volume, high throughput, rapid response time, reproducibility, and avoiding whole organism sacrifice and confounding stress response (McLaughlin, 2013).

1.8.1 Erythrocyte Lysis Assay

Hemolysins damage the host cytoplasmic membrane, resulting in cell lysis and death. The activity of the toxins is most easily observed in assays that involve the lysis of red blood cells. The erythrocyte lysis assay (ELA) allows for the detection and quantification of hemolytic activity of chemically different substances based on the lysis of erythrocytes by these substances and subsequent photometrical determination of the released hemoglobin into solution (Eschbach et al., 2001). To determine whether geographically-distinct isolates vary in their ability to produce hemolysin, expression of hemolytic activity can be measured using this sensitive bioassay. Hemolytic activity is directly proportional to the overall toxicity of the organism. In addition to detecting hemolytic compounds in ichthyotoxic marine microalgae, it also detects hemolytic activity in bacteria, marine invertebrates, and higher plants (i.e. saponin produced from the soapbark tree, *Quillaja saponaria*) (Ling and Trick, 2010).

1.8.2 Gill Cell Assay

Two types of immortal cultures can be employed for the *in vitro* examination of animal cells, namely the primary culture and cell lines. Primary cultures originate directly from the cells, tissues or organs of fish and last a few days. Cell lines are initiated from primary cultures. Essentially, the cell line begins upon sub-culturing of the primary culture into new culture flasks. By repeating cycles of sub-cultivation, this cell line can continue to be propagated by allowing cell density to increase and dividing the cell population into new culture flasks. This cycle of growth and splitting—termed “passaging”—may be performed a limited number of times, which is a finite cell line, or indefinitely, in which case it is a continuous or immortal cell line (Dayeh et al., 2003). Despite the development of several cell viability assays, the use of an assay that focuses on the integrity of the plasma membrane and metabolism while using a fluorescent dye to visualize impairment are favorable. The gill cell assay is a fluorescent assay that makes use of the immortal cell line to assess gill cell viability following exposure to algal samples (Dayeh et al., 2003).

Derived from the gill explants of a healthy rainbow trout, the RTgill-W1 cell line has been used to test a wide range of environmental contaminants. Rainbow Trout, *Oncorhynchus mykiss*, gill cells have been widely used to improve efficiency and circumvent problems faced when using whole organisms in toxicological studies (Dayeh et al., 2003). Easy to maintain and handle, this *in vitro* assay is also ideal for examining the effect of algal metabolites on gills since fish gills are a key organ involved in several interconnected processes such as gas exchange, osmoregulation and pH regulation (Dorantes-Aranda et al., 2011). It provides advantages over mammalian cell lines including: continuous supply of cells, ease of sample preparation, low volume sample requirement, the ability to conduct experiments at room temperature (20 °C), the capacity for fish cell lines to withstand prolonged exposure to experimental samples and avoiding whole organism sacrifice (Dorantes-Aranda et al., 2011; McLaughlin, 2013). Rainbow trout is also widely available, encouraging the intensive use of this species in toxicological studies, and in some cases, being referred to as the piscine ‘white rat’ (Wolf and Rumsey, 1985). For instance, Canadian legislation mandates that effluent from pulp

and paper mills must be assessed using the 96 h rainbow trout lethality test (Environment Canada, 1989). Consequently, the wealth of knowledge regarding the toxicology of rainbow trout is likely greater than any other aquatic vertebrate. According to Segner (1998), many studies report a high correlation between data from *in vitro* cytotoxicity from fish cell lines and *in vivo* fish mortality.

1.9 Study Statement

This investigation used isolates of *H. akashiwo* with two distinct regions of origin: Japan and Salish Sea. Three strains of *H. akashiwo*, isolated from the Pacific Northwest, are compared to three strains isolated from Japanese waters. To evaluate whether toxicity is regulated by environmental conditions, or if it is an inherent, strain-specific characteristic, six strains were selected to elucidate the effect of geographic location on growth and toxicity. Here, I investigate deeper into the regulation of toxicity by evaluating across-strain differences in growth and toxicity under a given set of temperature and salinity conditions. The research question is as follows: is the reduced toxicity of Japanese strains of *H. akashiwo* the product of a limited environment or due to the lack of toxic potential of cells? Confirming this question will assist in understanding toxicity of this enigmatic species.

1.9.1 Hypothesis

Given the euryhaline and eurythermal nature of *H. akashiwo*, the principal hypothesis of this study is that reduced temperature and salinity conditions will promote toxicity in the Japanese strains of *H. akashiwo*. Given that Japanese cells have been shown to be less toxic in their native habitats, if North American water conditions do promote toxicity in these strains, it may indicate that the Japanese cells are not in an environment that supports toxicity and therefore, toxicity is indeed regulated by environmental conditions. Environmental conditions driving the growth may repress toxicity.

1.9.2 Approach

In order to test the aforementioned hypothesis, multiple objectives were established. The first objective was to identify how changing temperature and salinity affect the rates of

growth and cell yield of the six *Heterosigma* strains. Obtaining growth measurements provide an indication as to when the cells are in the exponential phase or stationary phase. The second objective was to measure the toxicity of *H. akashiwo* under altered temperature and salinity conditions using two different proxies for toxicity—the Gill Cell Assay (RT-gillW1) and Erythrocyte Lysis Assay.

2 MATERIALS AND METHODS

2.1 Algal cultures and culturing conditions

This study used six isolates of *H. akashiwo* that varied in their location of origin and time spent in culture (Table 2.1). Three non-axenic strains of *H. akashiwo* (NWFSC-512, -513, and -524), collected from the southern section of the Salish Sea (Table 2.1) were provided by the National Oceanic Atmospheric Administration (NOAA) Northwest Fisheries Science Center (NWFSC), Seattle, WA. These strains were selected following an initial investigation of the growth and toxic response of seven North American strains. Isolates were selected to cover both central waters of Puget Sound (NWFSC-513, Clam Bay) and two protected embayments of southern Puget Sound (NWFSC-512; NWFSC-524).

Three non-axenic strains of *H. akashiwo* (NIES-145, -293, and -561) were obtained from the National Institute for Environmental Studies (NIES) Microbial Culture Collection, Tsukuba, Japan. Isolates were selected to cover a range of geographic locations along the Japanese coast (Table 2.1).

Stock cultures for each isolate were grown in 50 mL batch cultures, in 125 mL Erlenmeyer flasks, without shaking. Flasks contained 45 mL of autoclave sterilized (135°C and 2.07 Bar over 60 minutes) nutrient enriched artificial seawater (ESAW) medium—supplemented with *f*/2 nutrients, metals and vitamins—and 5 mL of inoculum (Andersen, 2005). The medium was modified by excluding the addition of silica. All flasks and glassware were rinsed extensively with Milli-Q water and autoclaved prior to use—after being fitted with foam plugs, which were then covered with aluminum foil.

Stock cultures were grown at 20 °C under a continuous light flux of cool white fluorescent light, at a constant irradiance of 90 -110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Irradiance was measured using a Quantum Scalar Laboratory 2100 irradiance 4- π collector sensor (Biospherical Instruments, San Diego, CA). Stock cultures were maintained in exponential growth phase by sub-culturing every 5-6 days.

Table 2.1 Origins and isolation dates of the *Heterosigma akashiwo* strains. North American strains (shown in blue) were collected from the Northwest Fisheries Science Center (NWFSC) and Japanese strains (shown in green) were collected from the National Institute for Environmental Studies (NIES Collection, Tsukuba, Japan).

Strain	Geographic Origin	Collection Source	Isolation Date
512	North Bay, Washington, USA (47.3585°N, 122.8166°W)	NWFSC	August 2009
513	Clam Bay, Washington, USA (47.5713° N, 122.5468° W)	NWFSC	June 2010
524	Puget Sound, Washington, USA (47.7237°N, 122.4713°W)	NWFSC	January 2013
145	Kagoshima, southern Japan near Kanoya (31.5966° N, 130. 5571° E)	NIES	May 1978
293	Onagawa Bay, Miyagi, northern Japan near Sendai (38.2688° N, 140.8721° E)	NIES	August 1984
561	Mikawa Bay, central Japan near Nagoya (34.7578° N, 137.2338 °E)	NIES	N/A

2.2 Experimental Design

For all experiments, stock cultures served as inoculum for pre-experimental inoculum and were added to 125 mL Erlenmeyer flasks containing experimental culture medium. For experimental cultures, salinity was modified by manipulating the concentration of sodium chloride in the ESAW recipe. The pre-experimental inoculum cells were grown for five days to allow the cells to acclimate under experimental conditions, thereby reducing the effect of environmental stress on results. After the cultures had grown for five days at their respective experimental conditions, they were used as inoculum for experimental cultures. Experimental cultures were inoculated from the exponential growth phase of pre-experimental stock cultures into the experimental culture media, and subsequently placed in temperature regulated incubators under a continuous light flux of 90 -110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For the experiments, a multi-stressor approach was carried out (Figure

2.1), where each *Heterosigma* strain was grown at all the possible combinations of salinity (15, 20, 25, 30, 35) and temperature (15 °C, 20 °C, 25 °C, 30 °C), conditions that have been shown to support the growth of *H. akashiwo* in coastal waters. All experiments were conducted three times, with three replicate flasks per treatment.

2.3 Biomass Measurements

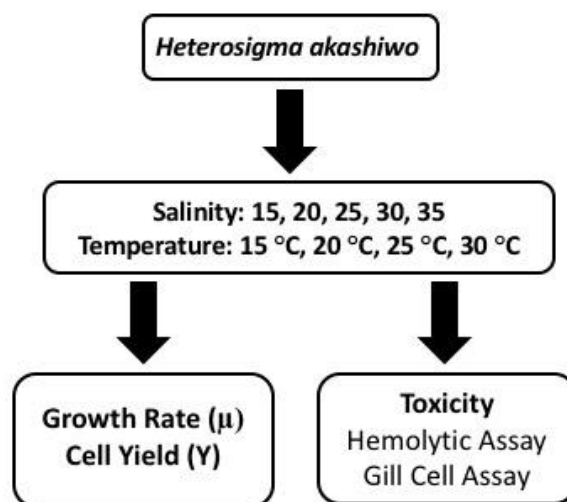


Figure 2.1 Experimental design illustrating a multi-stressor approach for measuring the growth and toxicity of *Heterosigma akashiwo* at varying temperatures and salinities.

Cell yield. For the growth rate experiments, data was collected using a Turner Designs C6 flow cytometer (Becton-Dickinson, NJ USA) to calculate cell density (cells mL⁻¹) and cell yield. Cell counts were determined using the FL-3 (signal from chlorophyll-a) and FSC (signal proportional to the disruption of the laser beam and is proportional to cell size) channel, which measure chlorophyll-a per cell and forward scatter for particle density, respectively. For each treatment, direct cell counts were performed daily—starting from the initial inoculation (Day 0) and continued until cells reached the

stationary growth (Day 10). In preparation for cell counts, immediately following inoculation of experimental cultures, flasks were gently swirled to allow for ubiquitous distribution of *H. akashiwo* cells, after which 0.5 mL from each flask was aseptically transferred into a 1.7 mL microcentrifuge tube (Axygen Scientific, Union City, CA) using a micropipette. This microcentrifuge tube was loaded onto the flow cytometer, after which 30 μL of the sample was analyzed. Cell concentrations, provided as the number of cells per 30 μL , were converted to cells mL^{-1} and recorded.

Growth rates. Log transformed cell density measurements were plotted against time, and the exponential portion of the curve was used to calculate the growth rate according to the equation:

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t_2 - t_1}$$

where μ is the specific growth rate (measured in divisions per day, d^{-1}), N_t is the population size at time point two, N_0 is the initial population size at time point one, and t_1 and t_2 is the difference, in days, between the two time points (Guillard, 1973; Ikeda et al., 2016). The cell yield (Y , cells mL^{-1}) was determined by averaging cell densities from three consecutive days once the cultures entered the stationary growth phase.

Cell size. Cellular dimensions were determined by flow cytometry. The instrument was set up to measure forward-scattered light (FSC) values, which are proportional to cellular surface area (Shapiro, 2005). To determine cell size from FSC values, non-fluorescent particle size calibration beads, ranging in size from 1-15 μm (Molecular Probes), were used to generate a standard curve (Appendix A). The average sizes, in diameter, of the *H. akashiwo* cells were determined by interpolating the measured FSC values over a standard curve spanning 1-15 μm . Cell sizes (μm) were calculated using standard linear regression.

2.4 Toxicity Assays

Two different assays were used as proxies of the fish-killing attributes of the cells. One assay, the erythrocyte lysis assay (otherwise known as the hemolytic assay), reflects on

the cell's ability to create a compound(s) that lyses or penetrates membranes that have structural lipids. The second assay, the fish gill cell cytotoxicology assay, indicates a combination of the presence of a neurotoxic compound in the algal cell or lysate.

2.4.1 Erythrocyte Lysis Assay

2.4.1.1 Preparation of algal samples

This assay examines variations in the expression of hemolytic activity among geographically distinct isolates (Table 2.1). Algal samples for the Erythrocyte Lysis Assay (ELA) were prepared following a modified protocol of Eschbach et al. (2001) and Ling & Trick (2010). Samples were prepared from cultures in exponential growth (3-4 days after initial inoculation) and late stationary growth (8-9 days after initial inoculation). Cell cultures were diluted to 1×10^5 cells mL^{-1} in Erythrocyte Lysis Assay (ELA) Buffer (150 mM NaCl, 3.2 mM KCl, 1.25 mM MgSO_4 , 12.2 mM TRIS base, and pH adjusted to 7.4). Cell densities of the algal cultures were determined using a flow cytometer and subsequently transferred to separate centrifuge tubes to prepare the following algal samples: intact cells (viable cells and extracellular material) and lysed cells (ultrasonic ruptured cells). Both intact and lysed algal cells were prepared in order to determine if the hemolytic compounds are extracellular or intracellular, respectively. Algal cells were lysed via sonication (continuous output power of 6 for 90 s on ice using a Virsonic 100 Ultrasonic Cell Disrupter (VirTis Company, Gardiner, NY). Cells were observed under a microscope to ensure complete lysis.

2.4.1.2 Preparation of erythrocytes

Rabbit blood (50% whole blood: 50% Alsever's (isotonic salt solution), (v/v)) was obtained from Aviva Systems Biology Corp. (San Diego, California) and was stored in the dark at 4-8 °C. Erythrocyte preparation followed the protocol from Eschbach et al. (2001) with two modifications: erythrocytes were centrifuged at a speed of 1000 g as opposed to 2000 g, and rabbit erythrocytes were used in place of carp erythrocytes. The rationale behind selecting rabbit erythrocytes is based on the knowledge that rabbit erythrocytes have been shown to be commonly sensitive to hemolysins produced by a number of algae. For the assay, erythrocytes were harvested from the blood by

centrifugation in a 1.7 mL microcentrifuge tube at 1000 *g* for 5 minutes at 4 °C (Eppendorf 5415D, Eppendorf North America Inc., Westbury, NY). Pelleted erythrocytes were washed twice with ELA buffer by vortexing the sample, followed by centrifugation. Erythrocyte suspensions were then diluted to 10⁷ cells mL⁻¹ in ELA buffer and adjusted to the appropriate cell density using a hemocytometer.

2.4.1.3 Erythrocyte Lysis Assay

The ELA protocol followed Eschbach et al. (2001) but was conducted using larger volumes in larger vessels instead of in 96-well microplates. Equal volume amounts of erythrocytes and intact or lysed algal cells were incubated in 1.7 mL clear, conical bottom polypropylene microcentrifuge tubes (Axygen Scientific, Union City, CA) to yield final cell densities of 5 x 10⁶ cells mL⁻¹ of erythrocytes and 5 x 10⁴ cells mL⁻¹ algae. A total volume of 1 mL was used for the assay.

Intact erythrocytes and lysed erythrocytes incubated in ELA buffer served as negative and positive controls, respectively. The positive controls—representing complete lysis—were sonicated at the same settings as the lysed algal cells, followed by microscopic observation to confirm complete lysis. Equal volume amounts of algal samples and ELA buffer were incubated and served as controls for any background absorbance of the algal cells.

Samples were incubated for six hours under experimental conditions and a continuous light flux of 90 - 110 μmol photons m⁻² s⁻¹. Following this incubation period, each sample was centrifuged at 2000 *g* for 5 minutes at 18 °C (Eppendorf 5415 D, Eppendorf North America Inc., Westbury, NY). The supernatant from each sample was then transferred to disposable polymethylmethacrylate cuvettes with a path length of 10 mm. Absorption of hemoglobin molecules released from erythrocytes was measured at a wavelength of 414 nm using a CARY 300 spectrophotometer (Agilent Technologies, Santa Clara, CA). Hemoglobin released into the solution by erythrocytes is proportional to the amount of cell lysis, and can be expressed as a percentage of hemolysis relative to the positive and negative controls, according to the following equation:

$$\% \text{ hemolysis relative to control} = \frac{E_{414} - A_{414} - N_{414}}{P_{414}} \times 100$$

where E_{414} is the absorption of experimental sample (erythrocytes incubated with *H. akashiwo*), A_{414} is the absorption of the algal sample incubated with ELA buffer, N_{414} is the absorption of the negative control (intact erythrocytes) and P_{414} is the absorption positive control (lysed erythrocytes) at 414 nm. Samples with a percentage of hemolysis above zero were considered hemolytic, and samples at or below zero were considered non-hemolytic.

2.4.1.4 Erythrocyte Fragility Test

Saponin (Sigma-Aldrich, St. Louis, MO), is a glycoside extract from the soapbark tree (*Quillaja saponaria*) that completely lyses erythrocytes and as such, was used as a positive control. It is a chemically defined hemolytic agent used to standardize the ELA. Erythrocytes were incubated with a range of saponin concentrations (0 - 20 $\mu\text{g mL}^{-1}$) under the same experimental conditions described for the ELA, after which a standard curve was obtained.

2.4.2 RTgill-W1 cytotoxicity assay

2.4.2.1 Rainbow Trout gill cell line maintenance

The Rainbow trout gill cell line (RTgill-W1) assay was performed to provide an assessment of toxicity expressed by *H. akashiwo* cells, grown under a given treatment, by measuring gill cell viability after exposure to algal samples. The protocol followed Dayeh et al. (2003). The employment of a continuous gill cell epithelial cell line provided a measure of acute toxicity. An immortal cell line of rainbow trout (*Onchorhynchus mykiss*) gill cells (RTgill-W1) was purchased from the American Type Culture Collection (CRL-2523) and routinely grown in sterile 25-cm² culture-treated flasks (MPN: 353108, BD Flacon®). 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 (FBS) (MPN: 1500-100, Seradigm) and 2% (v/v) antibiotic-antimycotic (MPN: A5955, Sigma-Aldrich) solution—containing penicillin, streptomycin and amphotericin—to yield L-15

complete medium. Cells were maintained in the dark at 20 °C (\pm 2 °C), renewed with fresh medium twice per week, and sub-cultured into new flasks on a monthly basis. As this cell line forms an adherent monolayer and grows attached to the bottom surface of the flask, gill cells were routinely observed under an Axiovert 100 TV (Zeiss) inverted microscope to evaluate the degree of cell confluence. Experiments were performed only after a confluent monolayer was formed.

2.4.2.2 Rainbow Trout gill cell line harvesting

In preparation for the gill cell assay, 25 cm² flasks with confluent gill cells were harvested and concentrated. Previous medium was aspirated from the flasks followed by the addition of 1.5 mL of 0.53 mM EDTA Versene solution (MPN: 17-711E, BioWhittaker® Reagents Lonza) into each flask for 1 minute. The Versene solution was then aspirated and replaced with an additional 1 mL of Versene solution and 1 mL of Trypsin solution (0.25% in Hank's balanced salt (HBS) solution) (MPN: 25-052-CI, Corning cellgro® Mediatech) for 3 minutes at 37 °C to facilitate cell detachment. Cells were left in the trypsin solution for no longer than 5 minutes, as the enzymes may cause cellular digestion, thereby resulting in cell death. Once the cells were detached via gently tapping the side of the flask, 3 mL of Leibovitz's L-15 complete medium was supplied to each flask, and the cell suspensions were then collectively transferred to a sterile 15 mL centrifuge tube (MPN: 352196, Falcon®). Cells were centrifuged at 200 g for 5 minutes at 12 °C in a Beckman Coulter Avanti J-251 centrifuge. Following centrifugation, the supernatant was discarded from the tube, leaving ~0.5 mL of the solution to avoid discarding the cell pellet. Cells were then resuspended in 10 mL of L-15 complete medium.

2.4.2.3 RTgill-W1 assay preparation

Prior to initiating experiments, gill cell concentrations were determined by transferring 0.5 mL of the cellular solution to a 1.7 mL microcentrifuge tube and counted using a hemocytometer. Cell concentrations were subsequently adjusted to a concentration of 2.5×10^5 cells mL⁻¹ with the addition of L-15 complete medium. Following this, 100 μ L of the density-adjusted gill cell solution was seeded into the wells of a clear 96-well flat-

bottom microplate. The plate was then enclosed with a lid, sealed with parafilm, and allowed to incubate under the original incubation conditions for 2-3 days—when cells were considered to be confluent.

2.4.2.4 RTgill-W1 assay algal sample preparation

In preparation for the bioassay, algal samples were prepared from cultures in late stationary growth (8-9 days after initial inoculation). Culture (1 mL) was collected from each treatment flask, and placed into 1.7 mL microcentrifuge tubes, after which they were centrifuged at 5000 g for 10 minutes in a bench top centrifuge. The supernatant was decanted thereafter, and the pellet was resuspended in L-15 experimental (ex) medium to yield a cell density of 1×10^5 cells mL⁻¹. L-15ex is similar to L-15 complete, with the exception of FBS and antibiotics to limit further cell growth. Algal suspensions were subsequently sonicated (continuous output power of 12 for 30 s on ice using a Virsonic 100 Ultrasonic Cell Disrupter (VirTis Company, Gardiner, NY)) and stored in a freezer at -20 °C until further analysis.

Over the course of the time period in which the gill cells were growing to form a confluent monolayer in the 96-well microplates, frozen algal cells were thawed at room temperature to lyse the cells. A total of two freeze-thaw cycles were conducted to ensure complete lysis. Lysed cells were used for this assay because previous research has shown that *Heterosigma* toxicity *in vitro* requires both fractured cell wall and periplasmic components (Ling, 2006; Powers et al., 2012). Following freeze-thaw cycles, samples were centrifuged at 3,500 g for 10 minutes at 20 °C in a bench top microcentrifuge. After discarding the supernatant, the pellet was then resuspended in L-15ex.

2.4.2.5 RTgill-W1 assay

In this assay, rainbow trout (*Onchorhynchus mykiss*) gill cells were exposed to *H. akashiwo* cells using a modified method of Dorantes-Aranda et al. (2011). Following a 2 - 3 day incubation period, and after confirming the formation of a confluent monolayer, the 96-well microplates of RTgill-W1 cells were rinsed twice with 100 µL of Phosphate-buffered saline (PBS) solution to remove any L-15 complete from the wells. The gill cells

were then exposed to 100 μ L of *H. akashiwo* extracts. The plate was subsequently lidded, sealed with Parafilm M®, and incubated under the original conditions (20 °C in the dark) for 24 hours. Following this incubation period, all wells were rinsed twice with PBS buffer and replaced with 100 μ L of L-15ex medium containing 5% Alamar Blue dye. Alamar blue is a metabolic dye which, when incorporated into viable cells, is reduced resulting in a fluorescent product which can subsequently be translated into gill cell viability. The plated samples were then sealed and incubated under the original conditions for an additional 2 hours to measure metabolic activity.

Fluorescence readings were taken after a 2-hour incubation period in the dark. The amount of reduction is proportional to gill cell viability after exposure to *H. akashiwo*. The fluorescence was detected on a fluorescence spectrophotometer plate reader (Agilent Technologies, Santa Clara, CA), using an excitation and emission wavelength of 540 nm and 590 nm, respectively (McLaughlin, 2015). Raw fluorescence units (RFU) were translated into gill cell viability using the following equation:

$$\text{Gill Cell Viability (\%)} = \frac{\text{RFU}_{\text{ex. cells}} - \text{RFU}_{\text{ex. no cells}}}{\text{Avg. (RFU}_{\text{con.}} - \text{RFU}_{\text{con. no cells}})} \times 100$$

Where $\text{RFU}_{\text{ex. cells}}$ is the fluorescence of the gill cells after exposure to *H. akashiwo*, $\text{RFU}_{\text{ex. no cells}}$ is the fluorescence of the *H. akashiwo* extracts in L-15ex, $\text{RFU}_{\text{con.}}$ is the fluorescence of the gill cells in the L-15ex medium and $\text{RFU}_{\text{con. no cells}}$ is the fluorescence of the L-15ex medium in the absence of any cells.

2.5 Data Analysis

Statistical analyses were performed using OriginPro (2017) Software (OriginLab Corporation, Northampton, MA, USA). One-way or two-way ANOVAs ($\alpha \leq 0.05$) were used for growth experiments to compare the growth rates and cell yields. Two-way ANOVAs were used to detect the main effects and interaction effects, and all significant ANOVAs were then followed by post-hoc Tukey's Tests ($\alpha \leq 0.05$) to determine

differences between groups. All graphs were generated using Microsoft Excel 16.15 (Microsoft, Redmond, Washington, USA).

3 RESULTS

3.1 Strain Selection

3.1.1 Growth Kinetics of North American Strains

To determine how *H. akashiwo* isolates of similar geographic origin (Pacific Northwest) respond to culturing conditions, physiological characteristics of growth and cell yield of seven isolates were examined (Fig. 3.1; Fig. 3.2). An initial survey of seven North American strains—all originally isolated from the coasts of Washington and British Columbia—were examined for their growth and toxic response. Of the seven North American strains, the three representative strains selected for this investigation were NWFSC-512, -513 and -524. Prior to selecting these strains, growth rates of all seven strains were measured to gauge the success of *H. akashiwo* grown in artificial seawater medium. Under these culturing conditions, although no significant differences in growth rates were observed among the seven strains (Fig. 3.1; one-way ANOVA, $F_{6,12} = 2.5$, $p > 0.05$), cell yield varied among the isolates tested. Cell yield for strain 510 was significantly different from the remaining strains, with a yield of 3.6×10^5 cells mL^{-1} (Fig. 3.2; one-way ANOVA, $F_{6,14} = 15.12$, $p < 0.05$). Cell yield for strain 513 was not significantly different from the other strains, with the exception of strain 510 (Fig. 3.2; one-way ANOVA, $F_{6,14} = 15.12$, $p > 0.05$). For this reason, 510 was excluded as a potential strain, and 513 was selected as one of the three comparison strains, as it was more representative of the cell yield produced by North American strains.

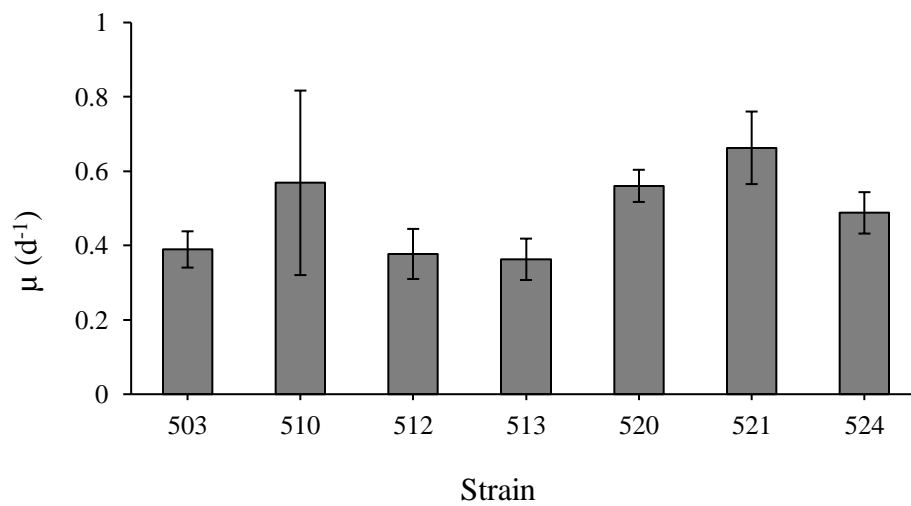


Figure 3.1 Growth rates (μ) of seven North American *H. akashiwo* strains grown in f/20 enriched ESAW medium. Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation ($n=3$). No significant differences in growth rates were observed ($p > 0.05$).

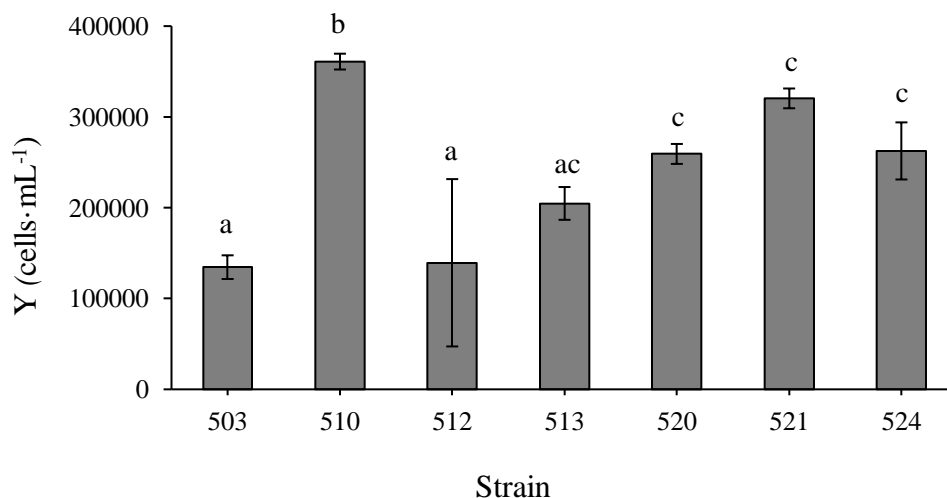


Figure 3.2 Yield (Y) of seven North American *H. akashiwo* strains grown in f/20 enriched ESAW medium. Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation ($n=3$). Same lowercase letters represent no significant difference in cell yield between strains according to Tukey's multiple comparison test. Significance tested at $p < 0.05$ level.

3.1.2 Hemolytic Activity of North American Strains

Following growth and yield measurements, hemolytic activity was assessed among the seven North American strains during exponential and stationary growth (Fig. 3.3) in an effort to select comparison strains that elicited a toxic phenotype. This initial investigation revealed that all North American strains expressed a toxic response, measured by hemolytic activity. During exponential growth, cell lysis did not significantly affect hemolytic activity in strains 512, 513 and 524 (Fig. 3.3A; two-way ANOVA, $F_{1,6} = 54.18$, $p > 0.05$). Additionally, no significant difference in hemolytic activity was observed between intact strains 512, 513 and 524. Hemolytic activity of intact strains of 512, 513 and 524 was also significantly greater compared to strains 503, 510, 520 and 521 (Fig. 3.3A; one-way ANOVA, $F_{6,14} = 13.15$, $p < 0.05$), whereas upon rupture, all strains showed no significant difference. During stationary growth, cell lysis had a significant effect on hemolysis in strains 503, 510, 512, and 520 (Fig 3.3B; one-way ANOVA, $F_{6,14} = 0.23$, $p < 0.05$). However, both intact and ruptured cells behaved similarly among the strains during this stage of growth. For these collective reasons, strains 512, 513 and 524 were selected as the North American representatives.

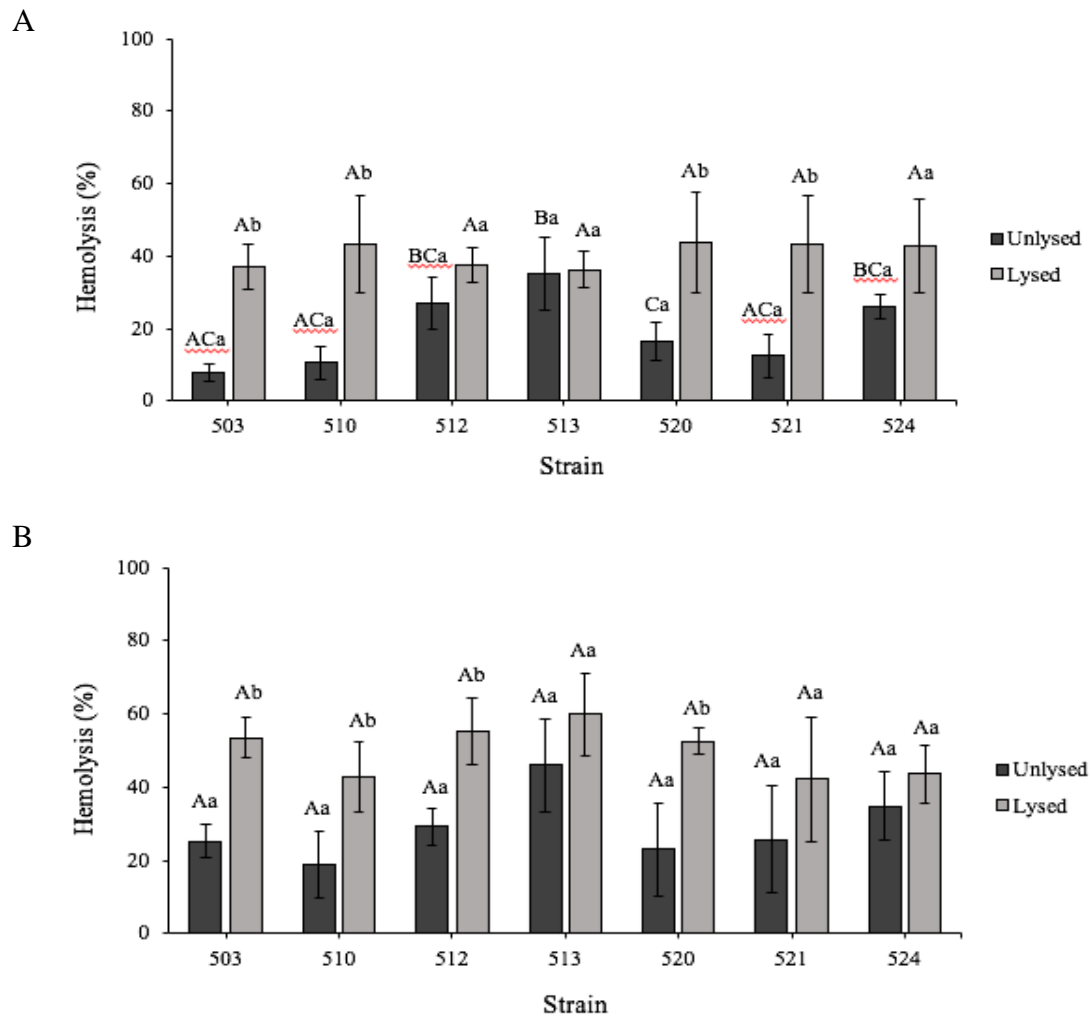


Figure 3.3 Hemolytic activity of seven North American *H. akashiwo* strains grown in f/20 enriched ESAW medium during exponential (A) and stationary (B) growth. Differences in bar colour assess the hemolytic effect of lysed and unlysed *Heterosigma* cells. Values represent the average of three assays (each performed in triplicate) \pm standard deviation (n=3). Same lowercase letters represent no significant effect of cell lysis on hemolysis within the same strain. Same uppercase letters represent no significant difference in hemolysis between strains in the same treatment, according to Tukey's multiple comparison test. Significance tested at $p < 0.05$ level.

3.1.3 Growth Kinetics of Japanese strains

The three strains isolated from Japan were NIES-145, NIES-293 and NIES-561. The Japanese strains were selected from a range of geographic locations along the Japanese coast (Table 2.1) and were measured for their growth characteristics under culturing conditions. Growth measurements revealed that strain 145, originally isolated from Kagoshima, southern Japan, exhibited a significantly slower growth rate of ca. 0.41 day⁻¹ than strain 561 (isolated from Mikawa Bay in central Japan), growing at a rate of ca. 0.53 day⁻¹ (Fig. 3.4; one-way ANOVA, $F_{2,6} = 6.78$, $p < 0.05$). Interestingly, despite a slower growth rate, strain 145 produced a significantly higher yield than strain 561 (Fig. 3.5; one-way ANOVA, $F_{2,6} = 20.0$, $p < 0.05$). Although the growth rate for strain 293, isolated from Onagawa Bay in northern Japan, was not significantly different from the other two Japanese strains (Fig. 3.4; one-way ANOVA, $F_{2,6} = 6.78$, $p > 0.05$), it was significantly more efficient at using nutrients than strain 561, as indicated by its greater cell yield (Fig. 3.5; one-way ANOVA, $F_{2,6} = 20.0$, $p < 0.05$).

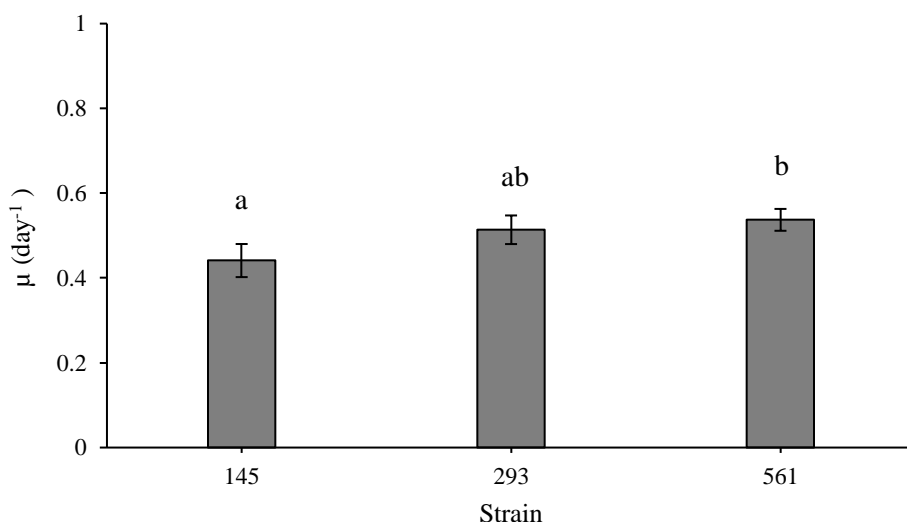


Figure 3.4 Growth rates (μ) of Japanese *H. akashiwo* strains grown in f/20 enriched ESAW medium. Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation ($n=3$). Same lowercase letters represent no significant difference in growth rate between strains according to Tukey's multiple comparison test. Significance tested at $p < 0.05$ level.

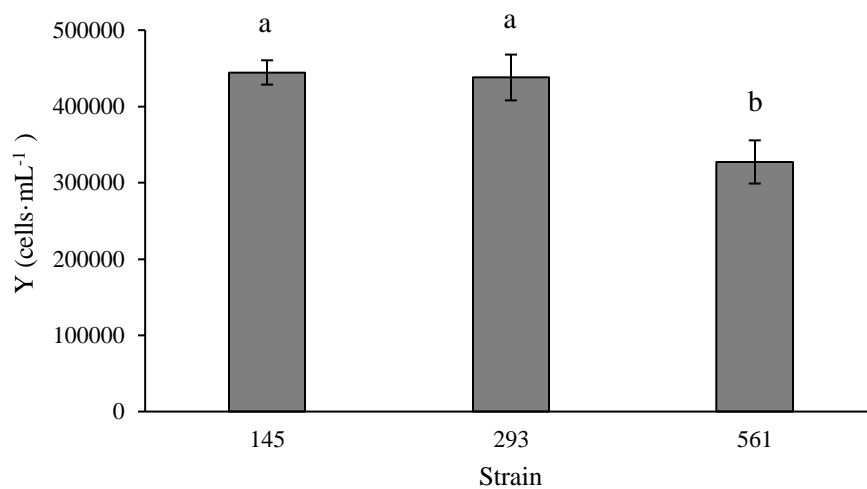


Figure 3.5 Yield (Y) of Japanese *H. akashiwo* strains grown in f/20 enriched ESAW medium. Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation (n=3). Same lowercase letters represent no significant difference in cell yield between strains according to Tukey's multiple comparison test. Significance tested at $p < 0.05$ level.

3.2 Growth variation among isolates

Exponential growth alone does not reflect the growth behavior and, as a result, growth curves were constructed along the time course at which cells reached stationary growth, where strains produced their maximum cell yield. Representative growth curves of *H. akashiwo* isolates (Fig. 3.6) grown in f/20 enriched ESAW illustrates similarities and differences in growth between the geographically-distinct and geographically-similar isolates. Growth rates were calculated from the linear portion of exponential growth and varied among the isolates, ranging from ca. 0.36 to 0.53 day⁻¹. Cell yield was obtained by averaging cell densities in the stationary phase—a phase in which the cell density remains constant as a result of limited nutrient availability—and ranged from 13.9 x 10⁴ to 44.7 x 10⁴ cells mL⁻¹. For all ELA experiments, samples were prepared from cultures in both exponential (days 3-4) and stationary (days 8-9) growth, whereas for the gill cell assay, samples were obtained from cells in stationary growth. Stationary growth for strains 512 and 524 occurred after ca. 6 days of growth, and after 7 days of growth for strain 513 (Fig. 3.6A). Although stationary growth for the Japanese strains occurred after ca. 8 days of growth, strain 561 displayed reduced cell yield compared to strains 145 and 293 (Fig. 3.6B).

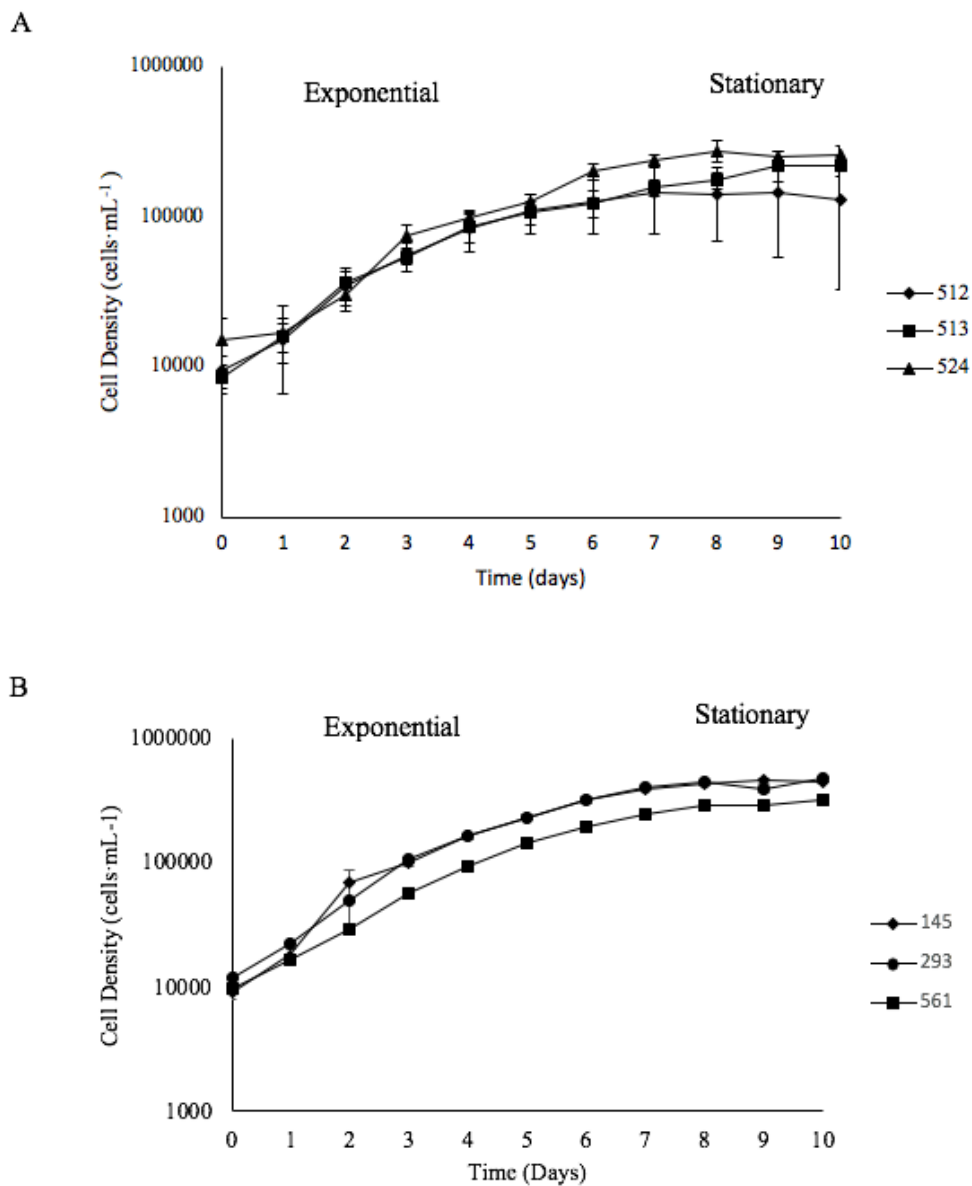


Figure 3.6 Representative growth curves of North American (A) and Japanese (B) *H. akashiwo* strains grown in f/20 enriched ESAW medium. Growth is expressed as the log₁₀ of cell density. Values represent the average of three growth experiments (each performed in triplicate) ± standard deviation (n=3). Exponential and stationary phases are labelled.

An initial assessment of the 6 *H. akashiwo* strains selected to represent North American and Japanese populations revealed inter-strain differences in physiological parameters during exponential (Table 3.1) and stationary (Table 3.2) growth. During exponential growth, the average diameter of the Japanese strains was less than the North American strains. This pattern is mirrored in stationary growth, however the size of the cells increased during this stage—with the exception of strain 524, which decreased in size. Interestingly, strain 145 exhibited growth rates comparable to its North American counterparts—particularly strain 512—and yet, produced yields two to three folds greater. In contrast, despite being one of the fastest-growing strains, strain 561 produced the lowest yield compared to its Japanese equivalents. This inverse relationship between growth and yield has previously been demonstrated (Yoshida et al., 2004), and is thought to be a product of the energetically expensive nature of fast-growing cells, thereby resulting in reduced nutrient efficiency.

Table 3.1 Comparison of six *Heterosigma akashiwo* strains under standard conditions of temperature (20 °C), salinity (30), light (90-100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) during exponential growth. (* Estimate of the volume if the cell is a perfect sphere)

<i>Heterosigma akashiwo</i> strain	Mean Diameter (μm)	Volume (μm^3) *	Mean chlorophyll a fluorescence (RFU)	Growth Rate μ (d^{-1})
NWFSC-512	14.3 \pm 0.2	1538.3	12.0 x 10 ⁶ \pm 15.3 x 10 ⁴	0.38 \pm 0.07
NWFSC-513	16.4 \pm 0.5	2313.4	13.1 x 10 ⁶ \pm 1.9 x 10 ⁴	0.36 \pm 0.06
NWFSC-524	12.9 \pm 0.2	1136.0	12.5 x 10 ⁶ \pm 3.6 x 10 ⁴	0.49 \pm 0.06
NIES-145	13.5 \pm 0.1	1296.9	10.3 x 10 ⁶ \pm 61.5 x 10 ⁴	0.41 \pm 0.04
NIES-293	13.5 \pm 0.2	1303.2	10.3 x 10 ⁶ \pm 57.4 x 10 ⁴	0.53 \pm 0.03
NIES-561	10.8 \pm 0.2	671.4	10.2 x 10 ⁶ \pm 14 x 10 ⁴	0.53 \pm 0.03

Table 3.2 Comparison of six *H. akashiwo* strains under standard conditions of temperature (20 °C), salinity (30), light (90-100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) during stationary growth. (* Estimate of the volume if the cell is a perfect sphere)

<i>Heterosigma akashiwo</i> strain	Mean Diameter (μm)	Volume (μm^3) *	Mean chlorophyll a fluorescence (RFU)	Cell Yield Y ($\text{cells}\cdot\text{mL}^{-1}$)
NWFSC-512	17.2 \pm 0.2	2678.6	9.5 x 10 ⁶ \pm 40.4 x 10 ⁴	13.9 x 10 ⁴ \pm 9.2 x 10 ⁴
NWFSC-513	17.4 \pm 0.1	2778.2	11.7 x 10 ⁶ \pm 20.1 x 10 ⁴	20.4 x 10 ⁴ \pm 1.8 x 10 ⁴
NWFSC-524	11.2 \pm 0.2	738.6	8.9 x 10 ⁶ \pm 11.1 x 10 ⁴	26.5 x 10 ⁴ \pm 3.1 x 10 ⁴
NIES-145	15.9 \pm 0.2	2105.3	8.6 x 10 ⁶ \pm 7.7 x 10 ⁴	44.7 x 10 ⁴ \pm 1.6 x 10 ⁴
NIES-293	15.9 \pm 0.3	2112.1	8.5 x 10 ⁶ \pm 6.0 x 10 ⁴	43.6 x 10 ⁴ \pm 3.0 x 10 ⁴
NIES-561	13.6 \pm 0.1	1315.5	11.5 x 10 ⁶ \pm 17.8 x 10 ⁴	29.8 x 10 ⁴ \pm 2.8 x 10 ⁴

3.3 Erythrocyte Fragility Test

Saponin, a glycoside extract from the soapbark tree, *Quillaja saponaria*, was used as a positive control for the ELA, indicating maximum hemolysis. It is a chemically-defined compound that has been demonstrated as an effective hemolyzing agent. The relatively higher sensitivity of rainbow trout red blood cells to membrane attack by saponin is useful for the detection of small amounts of hemolytic substances. The fragility of cells during the ELA was determined by measuring percent erythrocyte lysis relative to saponin concentration and was tested prior to each experiment to confirm the integrity of the erythrocytes. A spiked increase in the degree of hemolysis was observed at a saponin concentration of 1 $\mu\text{g mL}^{-1}$. Saponin had a significant effect on hemolysis (Fig. 3.7; one-way ANOVA, $F_{7,16} = 56.91$, $p < 0.001$), with maximum hemolysis occurring at a concentration of 2 $\mu\text{g mL}^{-1}$. Beyond this concentration, hemolysis remained constant and exhibited a steady plateau (Fig. 3.7).

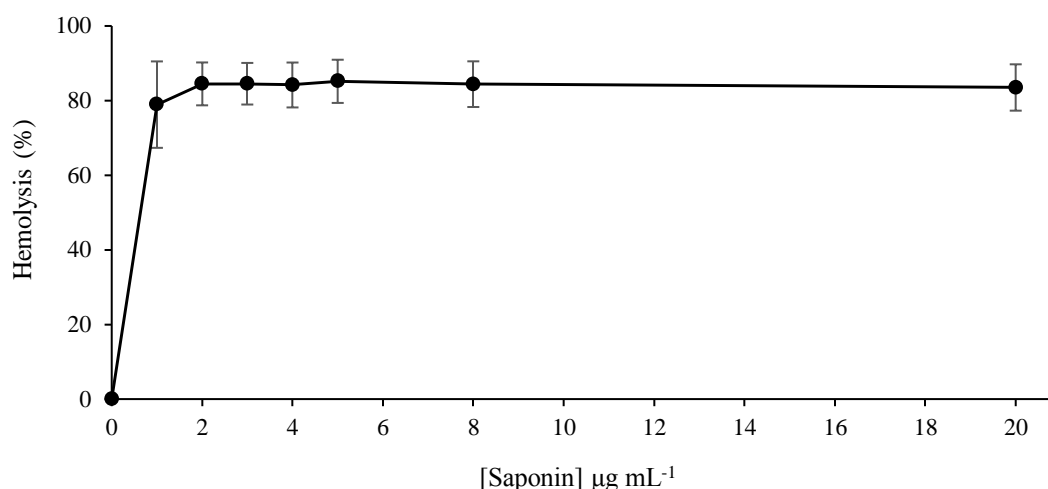


Figure 3.7 Effect of saponin on the lysis of rabbit erythrocytes. Erythrocytes were exposed to different concentrations of saponin. Values represent the average of three assays (each performed in triplicate) \pm standard deviation ($n = 3$). All values are statistically significant ($p < 0.001$) from the control ($0 \mu\text{g mL}^{-1}$) according to Tukey's multiple comparison test.

3.4 Growth

3.4.1 Growth of North American strains

At intermediate temperatures, salinity did not have a significant effect on growth rate within the same strain at 20°C (Fig. 3.8; one-way ANOVA, $F_{2,6} = 3.4$, $p > 0.05$) and 25°C (Fig. 3.8; one-way ANOVA, $F_{2,6} = 1.73$, $p > 0.05$). At the minimum temperature of 15°C , growth rate of strain 512 was significantly reduced at an elevated salinity of 30 (Fig. 3.8; one-way ANOVA, $F_{4,10} = 25.94$, $p < 0.05$), but increased at a salinity of 35 (Fig. 3.8; one-way ANOVA, $F_{4,10} = 25.94$, $p > 0.05$). This pattern was mirrored by strain 513, where growth rate was significantly reduced at a salinity of 30 but increased at a salinity of 35 (Fig. 3.8; one-way ANOVA, $F_{2,6} = 20.51$, $p < 0.05$). At an elevated temperature of 20°C , the growth of strains was insensitive to salinity, and grew equally well across the range of salinities tested.

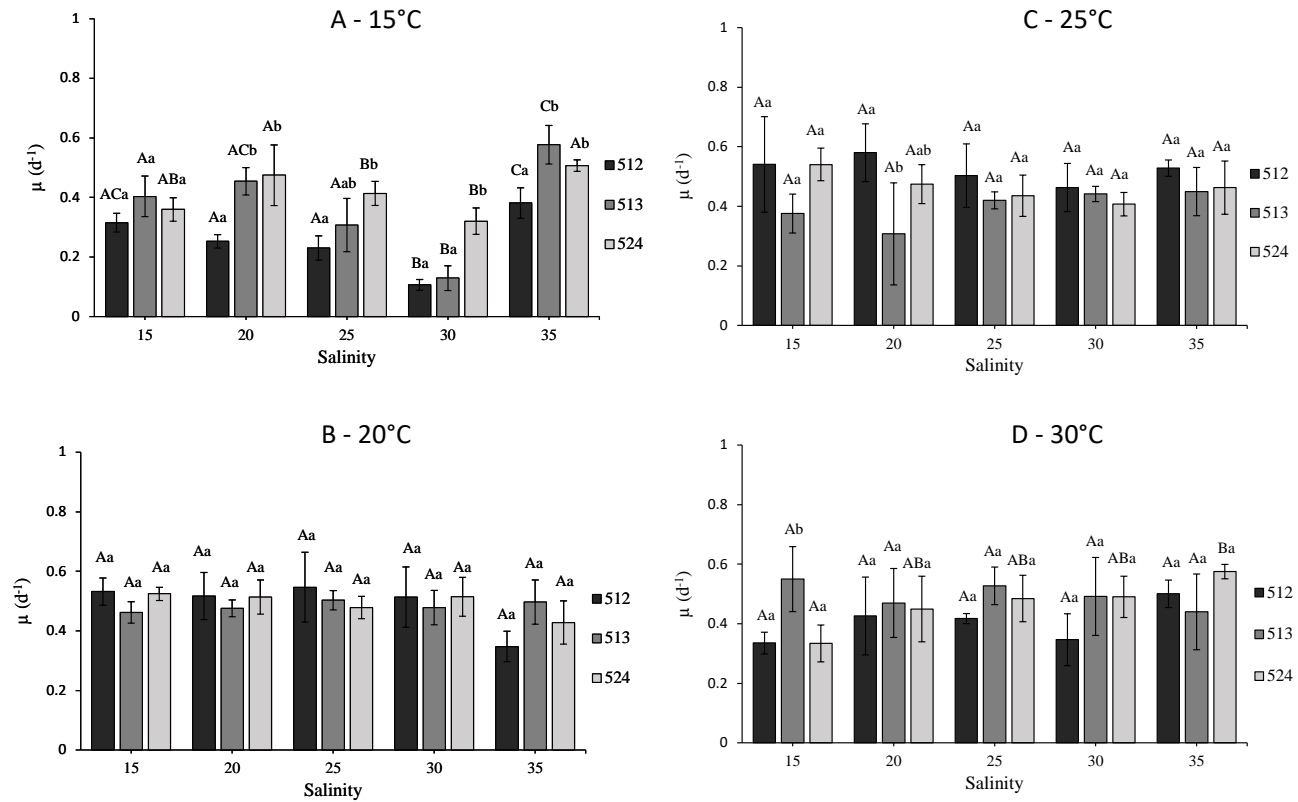


Figure 3.8 Growth rates (μ) of North American strains of *Heterosigma akashiwo* at a range of salinities (15-35) and temperatures (15 °C-30 °C). Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in growth rates of strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.4.2 Growth of Japanese Strains

The growth rate of Japanese strains mimicked the North American strains at 15°C in that at a salinity of 30, strain 145 was significantly reduced (Fig. 3.9; one-way ANOVA, $F_{4,10} = 9.91$, $p < 0.05$). As temperatures increased to 25°C and 30°C, salinity had no significant effect on the growth rate within the same strain (Fig. 3.9; two-way ANOVA, $F_{2,4} = 4.31$, $p > 0.05$).

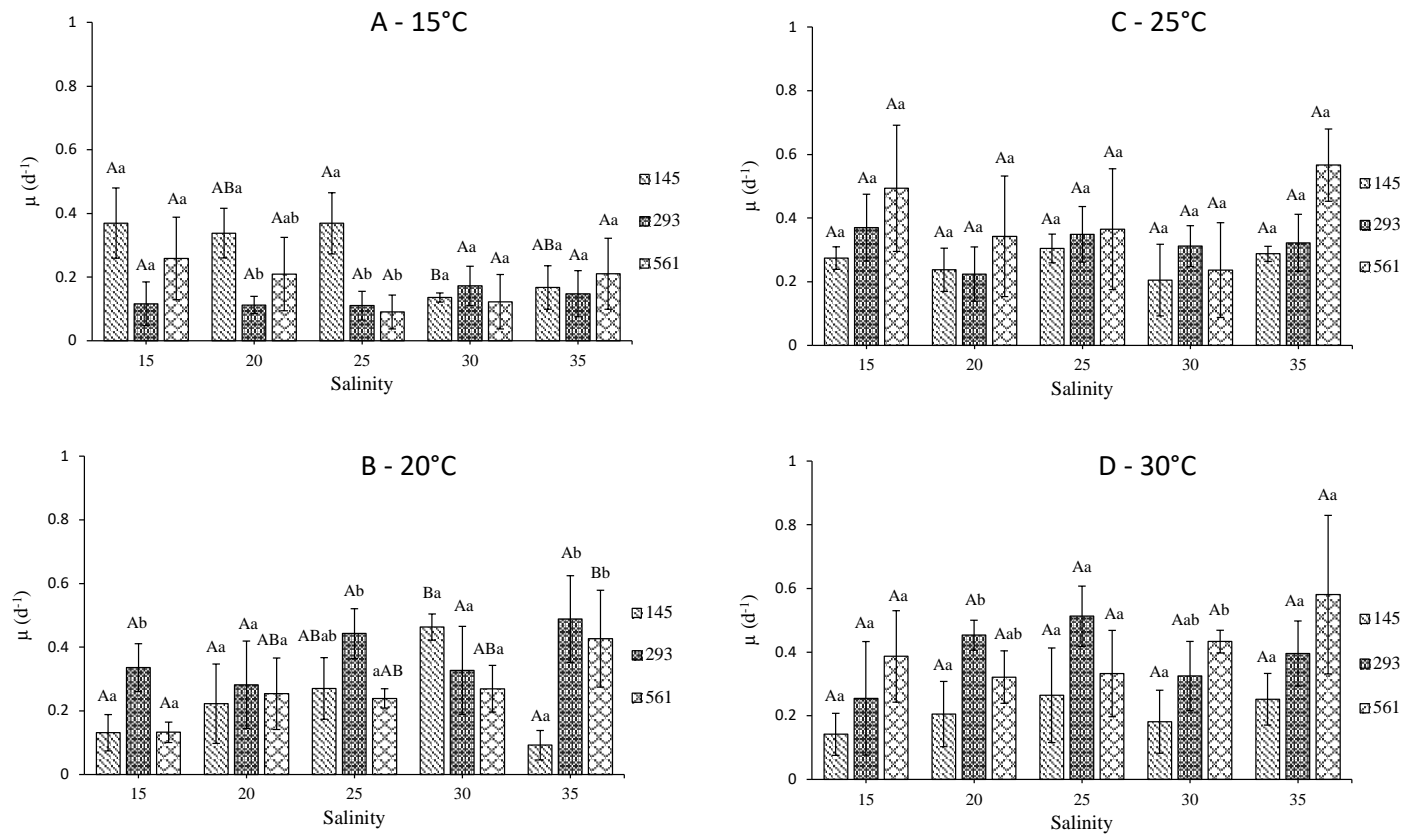


Figure 3.9 Growth rates (μ) of Japanese strains of *Heterosigma akashiwo* at a range of salinities (15-35) and temperatures (15 °C – 30 °C). Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation ($n = 3$). Same lowercase letter(s) represent no significant difference in growth rates of strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.4.3 Yield of North American strains

At 15°C, yield of North American strains is considerably less than the yield observed at higher temperatures. Yield of strain 512 is significantly reduced at a salinity of 30 (Fig. 3.10; one-way ANOVA, $F_{2,4} = 12.53$, $p < 0.05$), but was observed to be the greatest at a salinity of 35 (Fig. 3.10; two-way ANOVA, $F_{2,4} = 4.7$, $p < 0.05$). This pattern is mirrored by strain 513, exhibiting a significant reduction in cell yield at a salinity of 30 (Fig. 3.10; two-way ANOVA, $F_{2,4} = 4.7$, $p < 0.05$), but producing the highest yield at a salinity of 35. As temperature is increased to 20°C, yield produced by strain 512 is significantly reduced and the lowest at a salinity of 35 (Fig. 3.10; two-way ANOVA, $F_{2,4} = 4.7$, $p < 0.05$), indicating the depression of increased temperature on cell yield at elevated salinity in this strain.

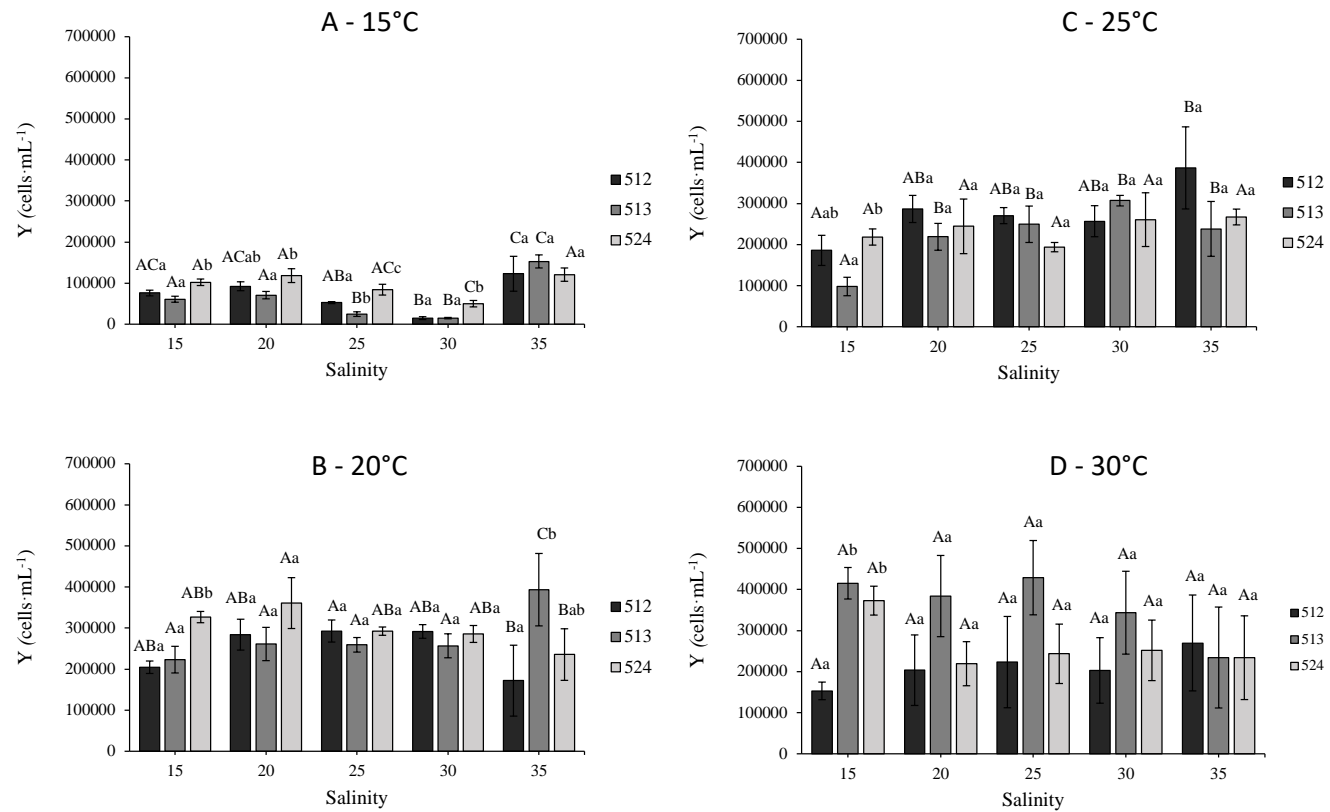


Figure 3.10 Yields (Y) of North American strains of *Heterosigma akashiwo* at a range of salinities (15-35) and temperatures (15 °C – 30 °C). Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in yield of strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.4.4 Yield of Japanese strains

Yields produced by the Japanese strains at 15°C are reduced relative to the other temperatures. In comparison to the yield of the North American strains, (Fig. 3.10), strain 145 exhibited a significant reduction in cell yield at a salinity of 30 at 15 °C (Fig. 3.11; one-way ANOVA, $F_{4,10} = 5.0$, $p < 0.05$). Yields produced by strain 293 were consistently significantly greater than the yields produced by the other two strains across all salinities (Fig. 3.11; one-way ANOVA, $F_{2,6} = 30.44$, $p < 0.05$). However, at elevated temperatures of 25 °C and 30 °C, salinity did not significantly affect the yields produced by strain 293 (Fig. 3.11 two-way ANOVA, $F_{2,4} = 4.7$, $p > 0.05$). This pattern is mimicked by strain 561 at 20 °C, 25 °C and 30 °C. Interestingly, strain 145 produced yields comparable to the yields produced at 15 °C, suggesting its inability to efficiently convert the available nutrients into biomass at extreme temperatures.

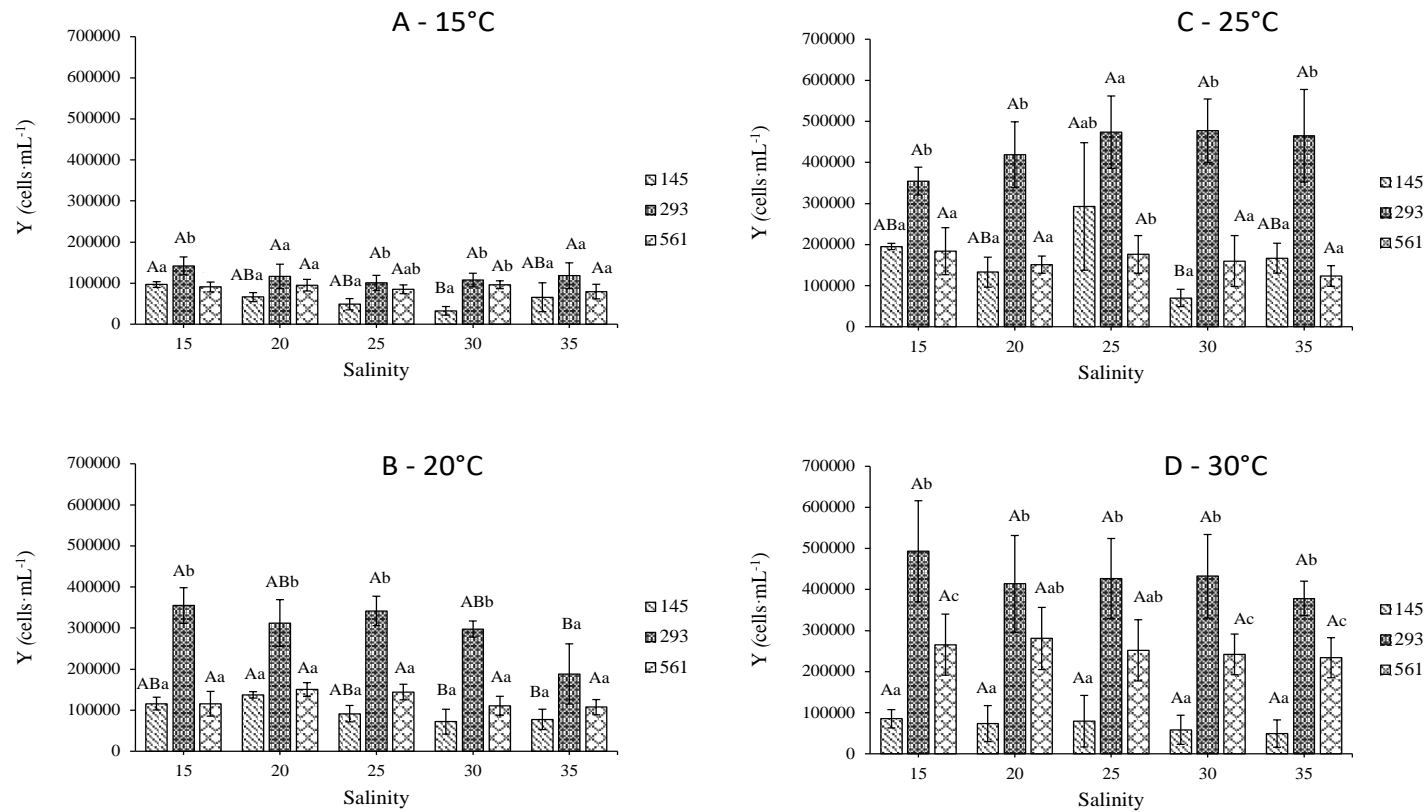


Figure 3.11 Yields (Y) of Japanese strains of *Heterosigma akashiwo* at a range of salinities (15-35) and temperatures (15 °C – 30 °C). Values represent the average of three growth experiments (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in yield of strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5 Erythrocyte Lysis Assay

3.5.1 Hemolytic activity of unlysed North American strains during exponential growth

When North American cells are intact and retain their cellular integrity during exponential growth at 15°C, toxicity of strains 512 and 513 is significantly greater at a salinity of 30 (Fig. 3.12; one-way ANOVA, $F_{4,10} = 4.22$, $p < 0.05$). —this is noteworthy given that these were the slowest growing strains at this temperature. Additionally, toxicity was observed to be highest at this reduced temperature, relative to the other temperatures tested. The lowest toxic response was observed at 20 °C.

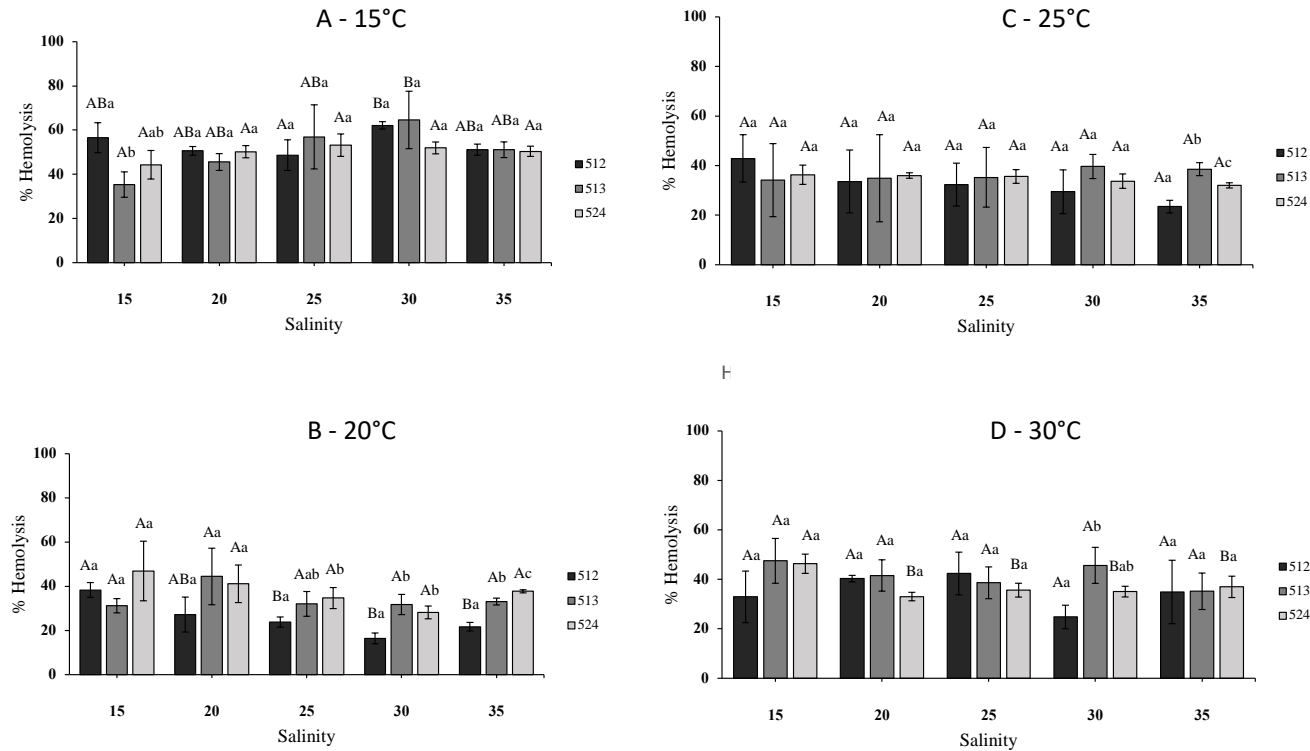


Figure 3.12 Hemolytic activity of unlysed North American strains of *Heterosigma akashiwo* during exponential growth at a range of salinities (15-35) and temperatures (15 °C-30 °C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation ($n = 3$). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5.2 Hemolytic activity of unlysed Japanese strains during exponential growth

During exponential growth, unlysed Japanese strains were not toxic at 15 °C, 20 °C and 30 °C across all salinities (Fig. 3.13; two-way ANOVA, $F_{2,4} = 0.91$, $p > 0.05$). At 25 °C, strain 145 exhibited minimal toxicity at a salinity of 15 and strain 561 exhibited negligible toxicity at a salinity of 15 and 20.

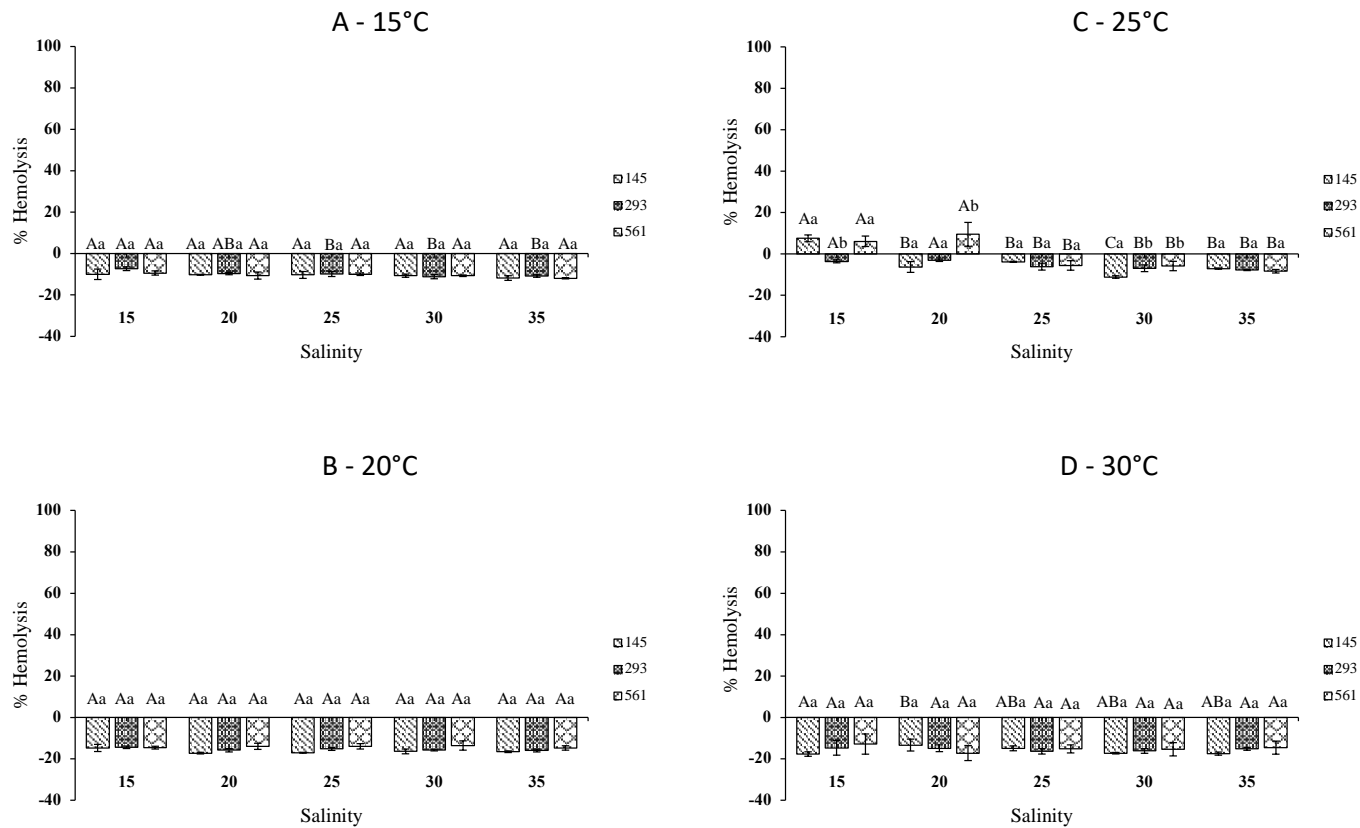


Figure 3.13 Hemolytic activity of unlysed Japanese strains of *Heterosigma akashiwo* during exponential growth at a range of salinities (15-35) and temperatures (15°C-30°C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5.3 Hemolytic activity of lysed North American strains during exponential growth

During exponential growth, lysed cells of North American strains exhibited greater toxicity relative to unlysed cells. In congruence with unlysed strains (Fig. 3.12), toxicity of all North American strains was significantly higher at the lowest tested temperature of 15 °C (Fig. 3.14; one-way ANOVA, $F_{2,6} = 5.95$, $p < 0.05$), despite having the lowest growth rate at this temperature. Additionally, the hemolytic activity for each strain was not significantly affected by salinity at this temperature (Fig. 3.14; one-way ANOVA, $F_{4,10} = 1.68$, $p > 0.05$). A similar pattern was seen as temperature was increased to 20 °C. However, as temperature was further increased to 25 °C, reduced toxicity was observed, relative to the cooler temperatures.

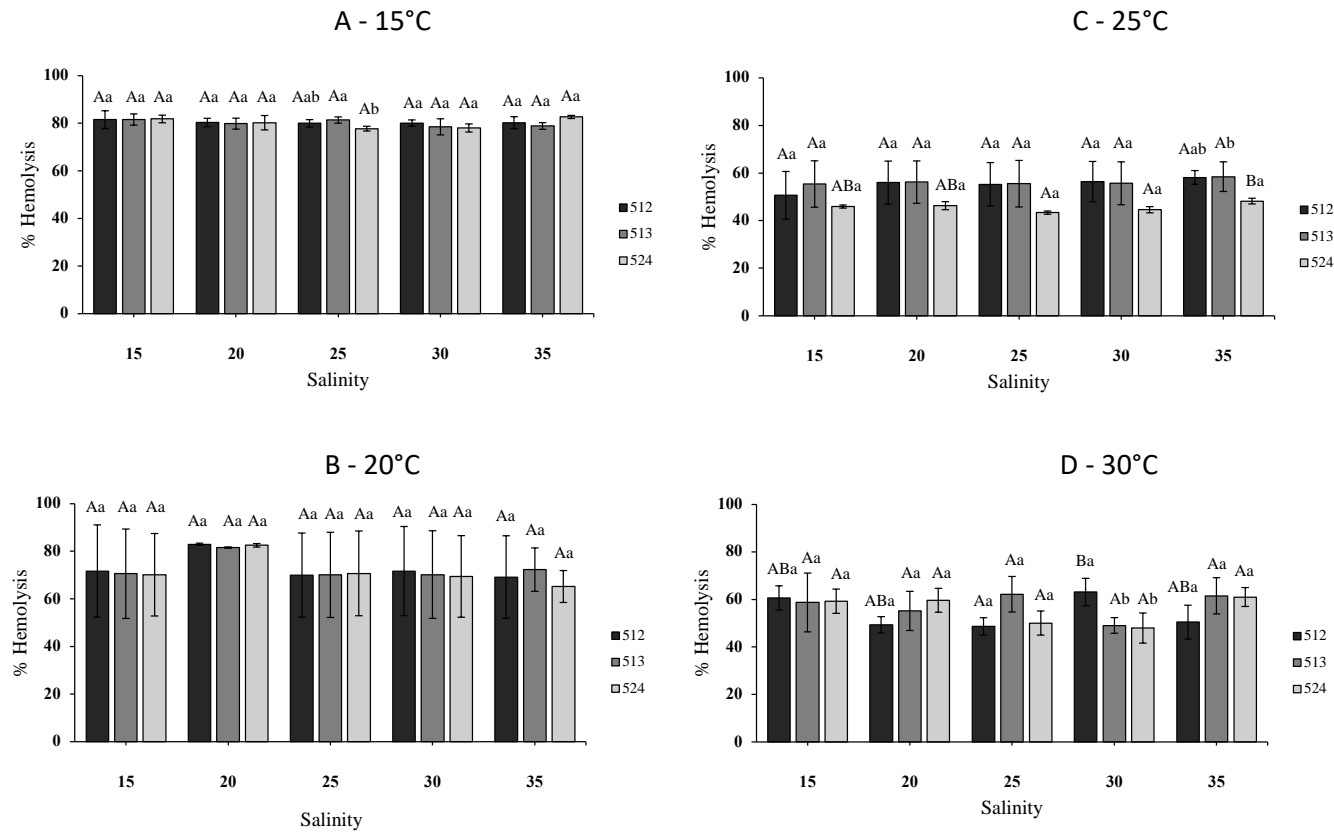


Figure 3.14 Hemolytic activity of lysed North American strains of *Heterosigma akashiwo* during exponential growth at a range of salinities (15-35) and temperatures (15°C-30°C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation ($n = 3$). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5.4 Hemolytic activity of lysed Japanese strains during exponential growth

When Japanese cells are lysed during exponential growth, toxicity is evident under certain conditions in some strains. At 15 °C and 20 °C, strains exhibit negligible toxicity. However, as temperature is increased to 25 °C, significant toxicity is observed from all three strains at a salinity of 15 (Fig 3.15; one-way ANOVA, $F_{5,12} = 14.13$, $p < 0.05$). Strains 145 and 561 retain their toxicity at a salinity of 20. Interestingly, toxicity is induced in strain 293 at the maximum salinity of 35, suggesting the role of environmental stress on inducing toxicity.

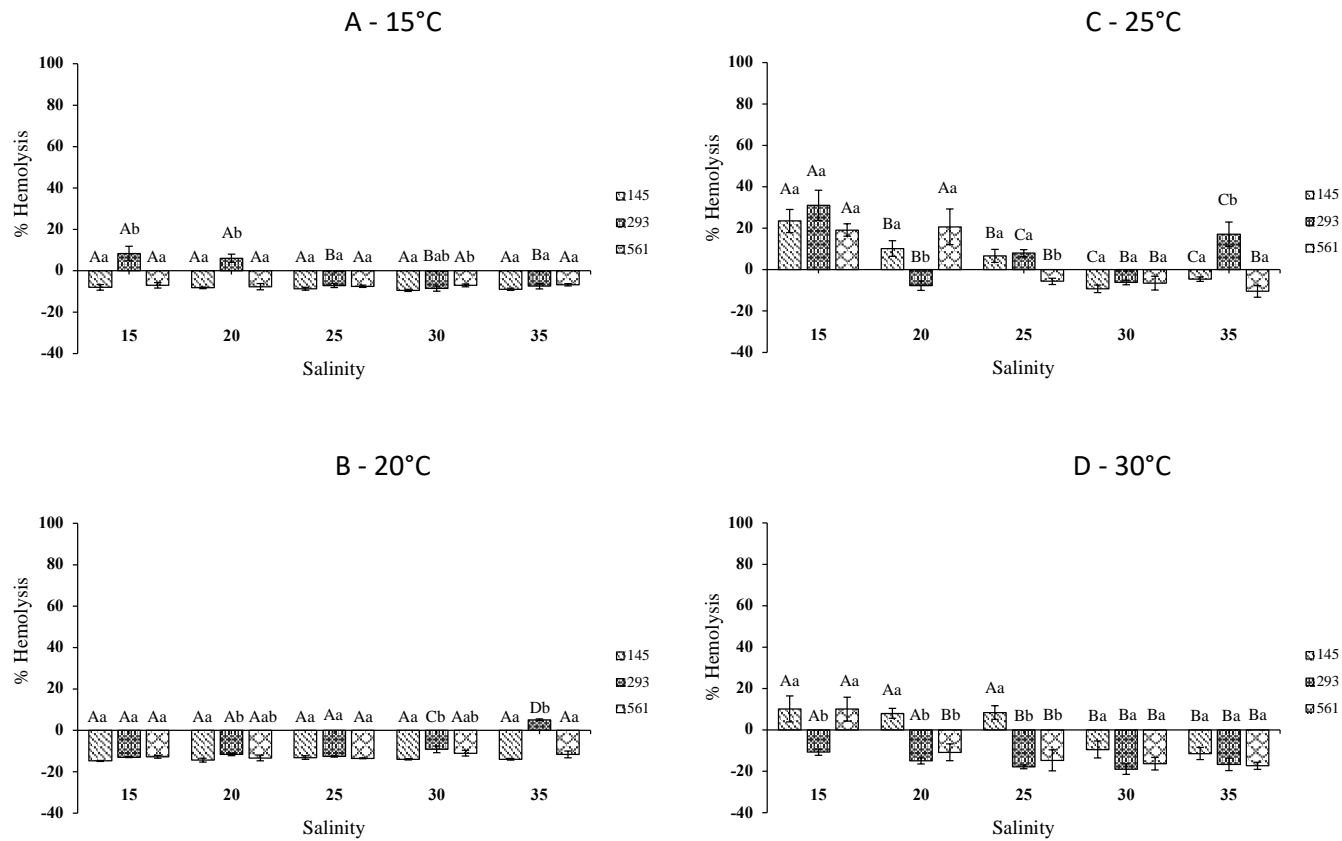


Figure 3.15 Hemolytic activity of lysed Japanese strains of *Heterosigma akashiwo* during exponential growth at a range of salinities (15-35) and temperatures (15 °C – 30 °C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5.5 Hemolytic activity of unlysed North American strains during stationary growth

Unlysed North American strains during stationary growth all expressed a toxic response, with the highest toxicity observed at the lowest tested temperature of 15 °C, and reduced toxicity observed at 30 °C, relative to 15 °C. At 15 °C and 20 °C, strain 145 exhibited significantly greater (Fig. 3.16, two-way ANOVA, $F_{2,4} = 11.05$, $p < 0.05$) toxicity at reduced salinities (15, 20, 25), whereas as temperatures increased, toxicity was no longer significantly affected by salinity in this strain ((Fig. 3.16, two-way ANOVA, $F_{2,4} = 11.05$, $p > 0.05$).

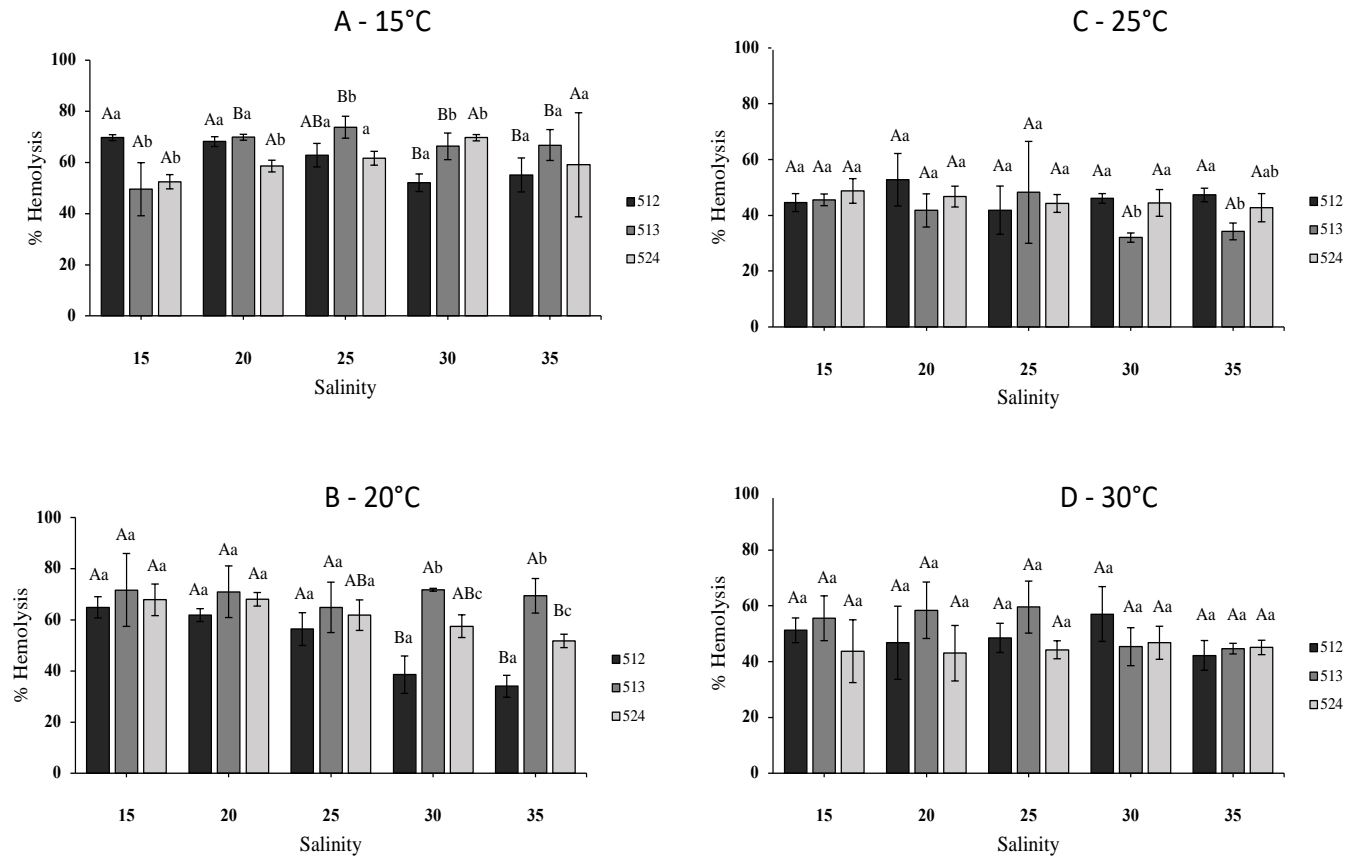


Figure 3.16 Hemolytic activity of unlysed North American strains of *Heterosigma akashiwo* during stationary growth at a range of salinities (15-35) and temperatures (15 °C – 30 °C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5.6 Hemolytic activity of unlysed Japanese strains during stationary growth

During stationary growth, unlysed Japanese strains were not toxic under all the conditions (salinities and temperature) tested (Fig. 3.17; two-way ANOVA, $F_{2,4} = 1.65$, $p > 0.05$). Negative hemolytic values indicate interference from algal samples or intact erythrocytes.

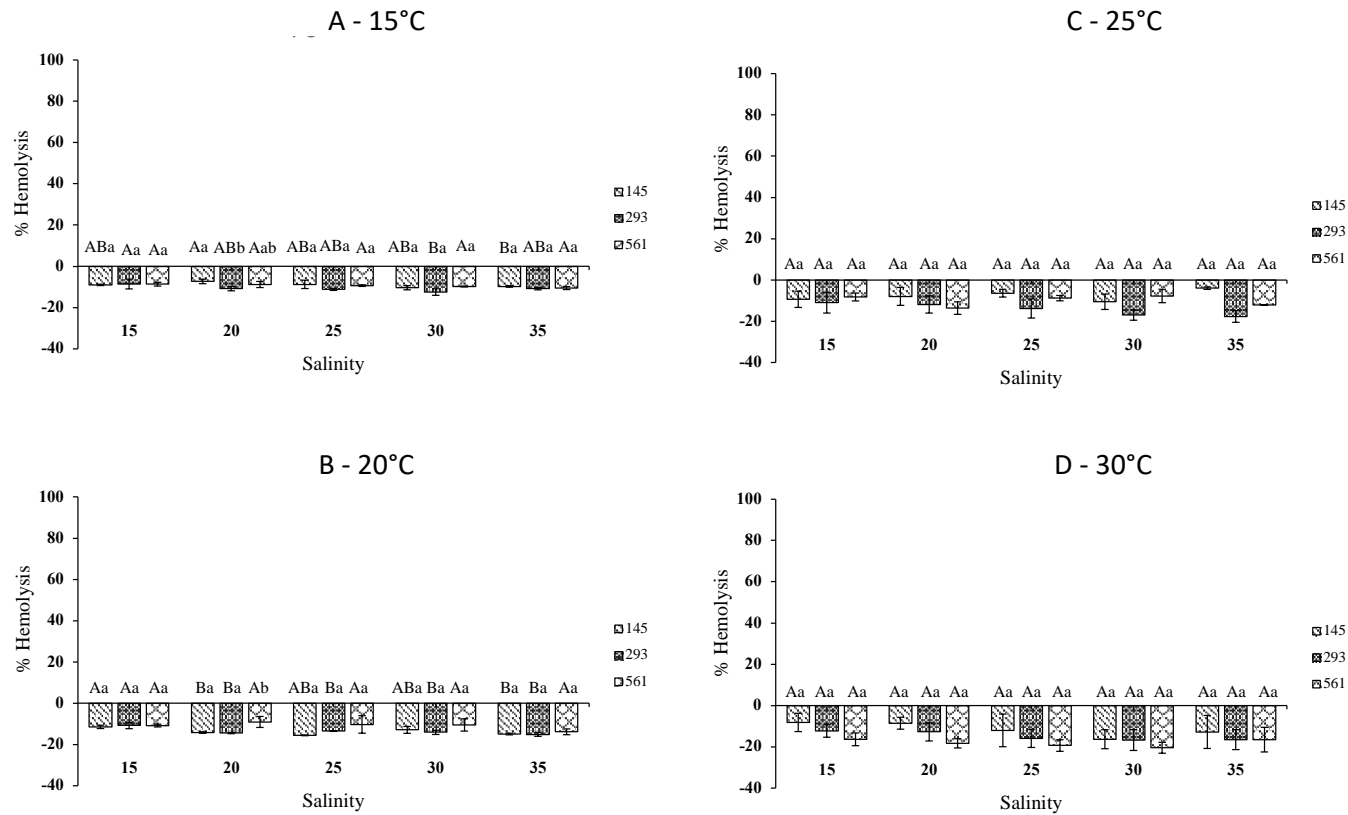


Figure 3.17 Hemolytic activity of unlysed Japanese strains of *Heterosigma akashiwo* during stationary growth at a range of salinities (15 - 35) and temperatures (15 °C – 30 °C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation ($n = 3$). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5.7 Hemolytic activity of lysed North American strains during stationary growth

Hemolytic activity of lysed North American strains during stationary growth is noteworthy because at 15 °C, North American strains had relatively lower growth rates than the other three temperatures. Inversely, hemolytic activity is highest at 15 °C during stationary growth and when cells are lysed.

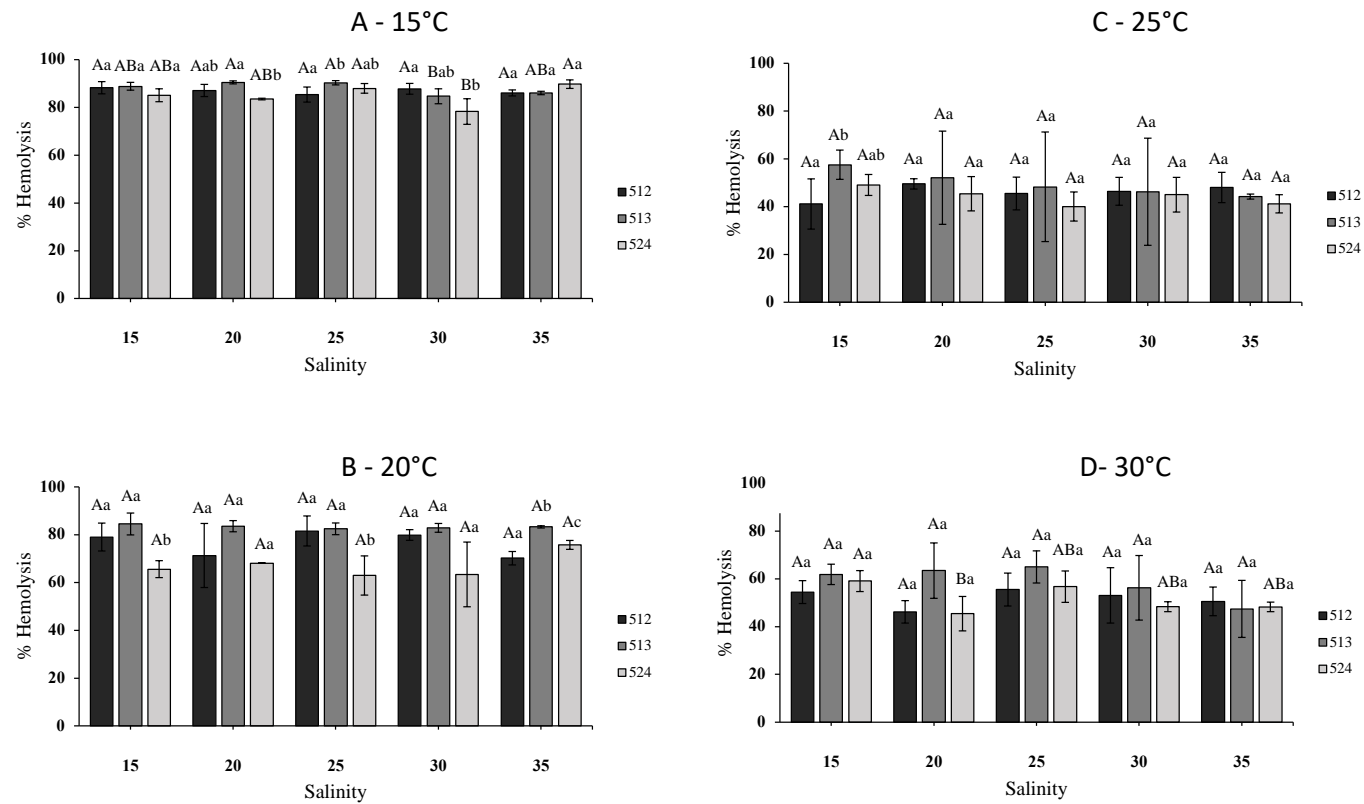


Figure 3.18 Hemolytic activity of lysed North American strains of *Heterosigma akashiwo* during stationary growth at a range of salinities (15-35) and temperatures (15°C-30°C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation ($n = 3$). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.5.8 Hemolytic activity of lysed Japanese strains during stationary growth

During stationary growth, lysed Japanese strains showed variable toxicity. At a temperature of 20°C, toxicity of strain 145 increases with salinity, with significantly higher toxicity at a salinity of 35 (Fig. 3.19; one-way ANOVA, $F_{2,6} = 14.98$, $p < 0.05$). As temperature is increased to 25 °C, this toxic potential is reduced in strain 145. However, at this temperature toxicity in strain 561 is induced at the lowest and highest salinity. As temperature is increased to 30 °C, strain 145 produces significant toxicity across all salinities relative to the other two strains (Fig. 3.19; one-way ANOVA, $F_{2,6} = 15.56$, $p < 0.05$).

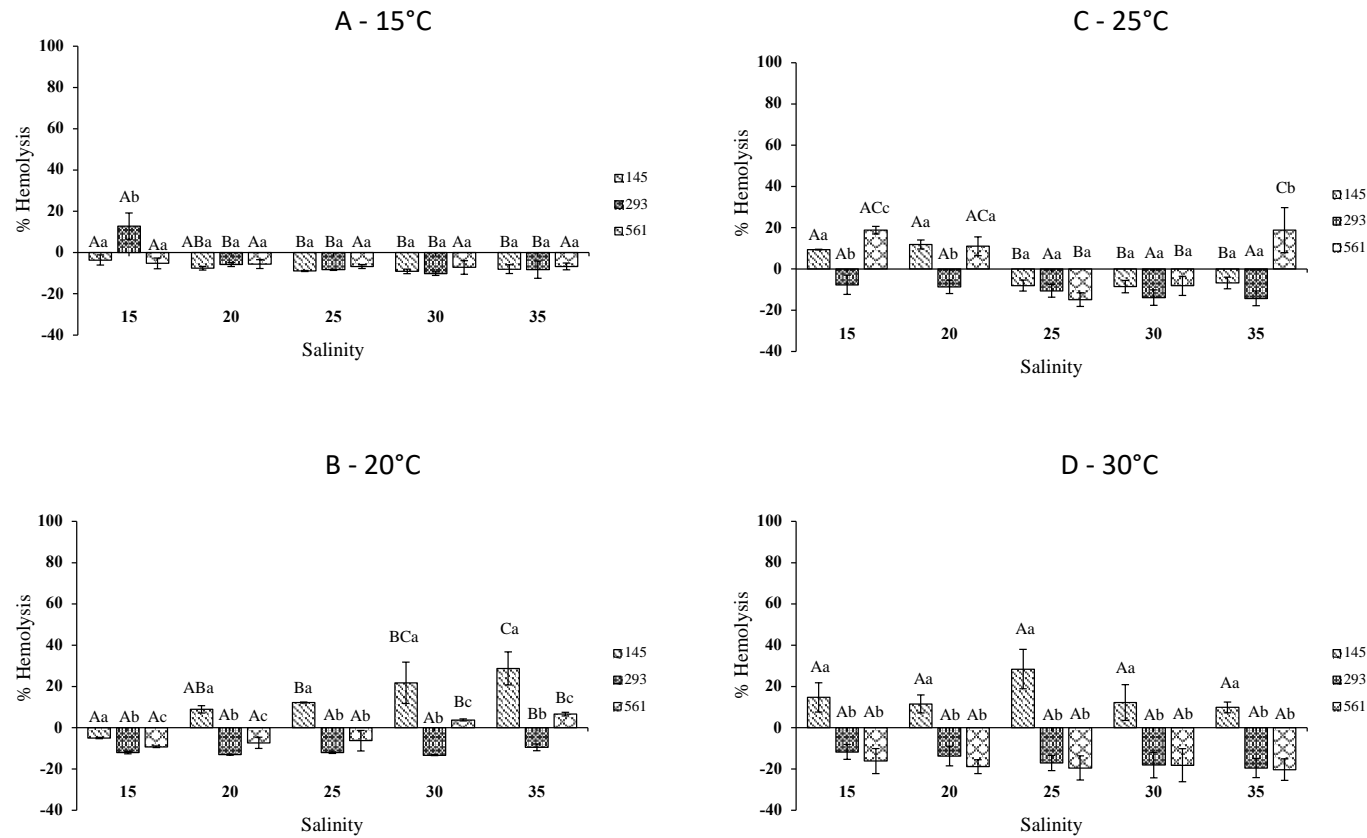


Figure 3.19 Hemolytic activity of lysed Japanese strains of *Heterosigma akashiwo* during stationary growth at a range of salinities (15-35) and temperatures (15°C-30°C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation ($n = 3$). Same lowercase letter(s) represent no significant difference in hemolytic activity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on hemolytic activity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.6 Gill Cell Assay (RTgill-W1)

3.6.1 Toxicity of North American strains

Toxicity, as measured by the gill cell assay, in the North American strains demonstrated varying degrees of toxicity. Most noteworthy is that at 25 °C, as salinity increases, the toxicity of strain 524 is significantly reduced (Fig. 3.20; one-way ANOVA, $F_{4,10} = 37.5$, $p < 0.05$). However, as temperature is increased to 30 °C, cells express toxicity.

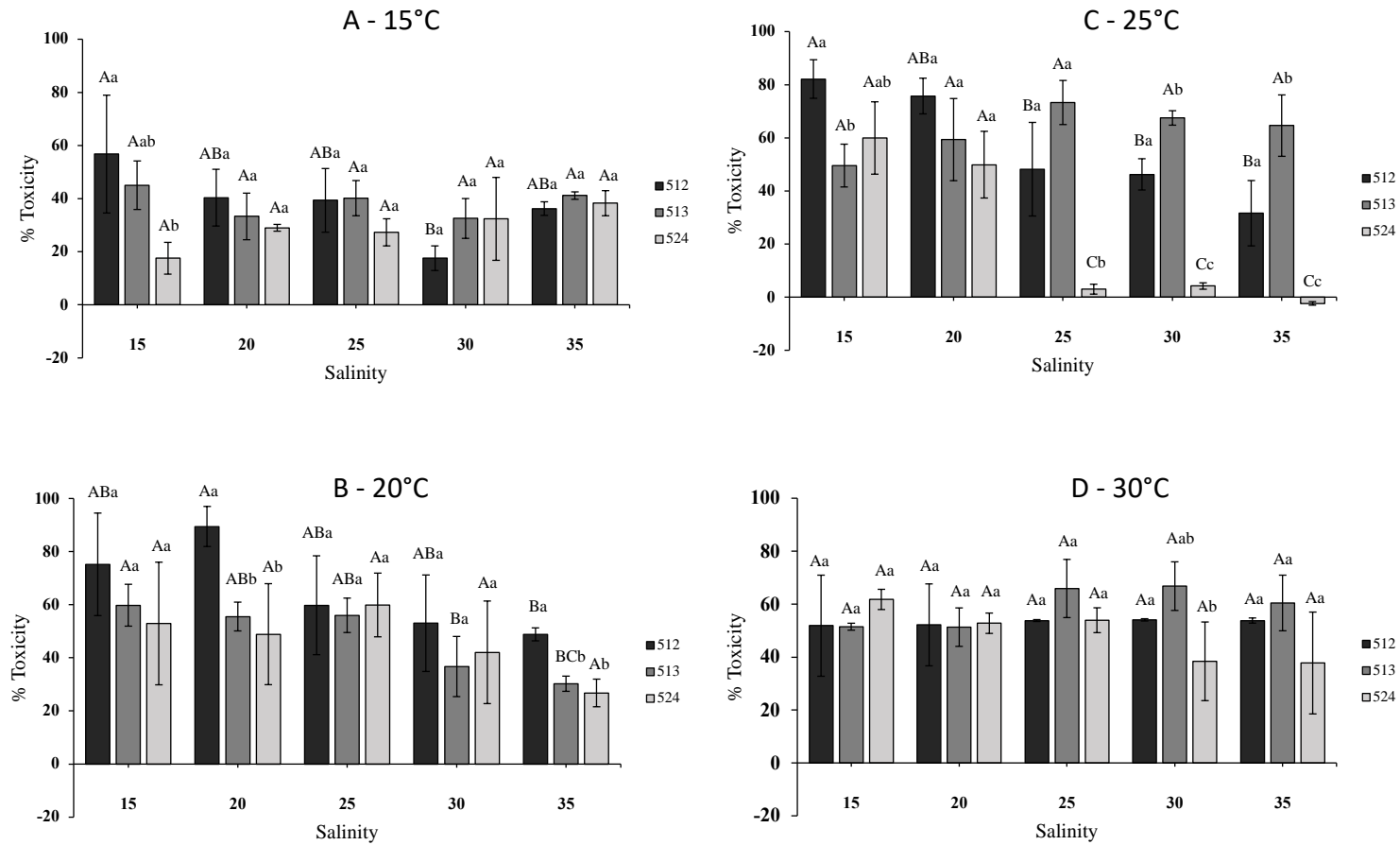


Figure 3.20 Toxicity of lysed North American strains of *Heterosigma akashiwo* during stationary growth at a range of salinities (15 - 35) and temperatures (15 °C – 30 °C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in toxicity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on toxicity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

3.6.2 Toxicity of Japanese strains

Toxicity—measured by the gill cell assay—exhibited by the Japanese strains illustrate that some strains will be toxic, primarily if they are in the right conditions. At 15 °C, strain 293 is significantly toxic across all salinities (Fig. 3.21; two-way ANOVA, $F_{2,4} = 2.8$, $p < 0.05$) whereas 561 is non-toxic across all salinities (Fig. 3.21; two-way ANOVA, $F_{2,4} = 2.8$, $p > 0.05$). Toxicity at 15 °C also reveals some notable details: 1) the growth rate for 293 at this reduced temperature was the slowest across all salinities, whereas an inverse relationship is seen with toxicity, exhibiting a significant toxic response; and 2) the growth rates for 293 and 561 were comparable at 15°C, and yet there is a significant difference in their toxic expression (Fig. 3.21; two-way ANOVA, $F_{2,4} = 2.8$, $p > 0.05$). As temperature increases to 20 °C, there is a reduction in toxicity for strain 293 across all salinities, which is interesting because at 20 °C, the growth rate for this strain began to increase. At 25 °C, none of the three strains exhibited toxicity across any salinity. Interestingly, at 30 °C, strain 561, which was non-toxic at 15 °C, 20 °C and 25 °C across all salinities, exhibits significant toxicity at this elevated temperature between salinities 15-30 illustrating that, for this particular strain isolated from central Japan, toxicity is induced at an increased temperature of 30 °C. Likewise, toxicity of strain 293 was negligible at 25 °C, but returned at 30 °C.

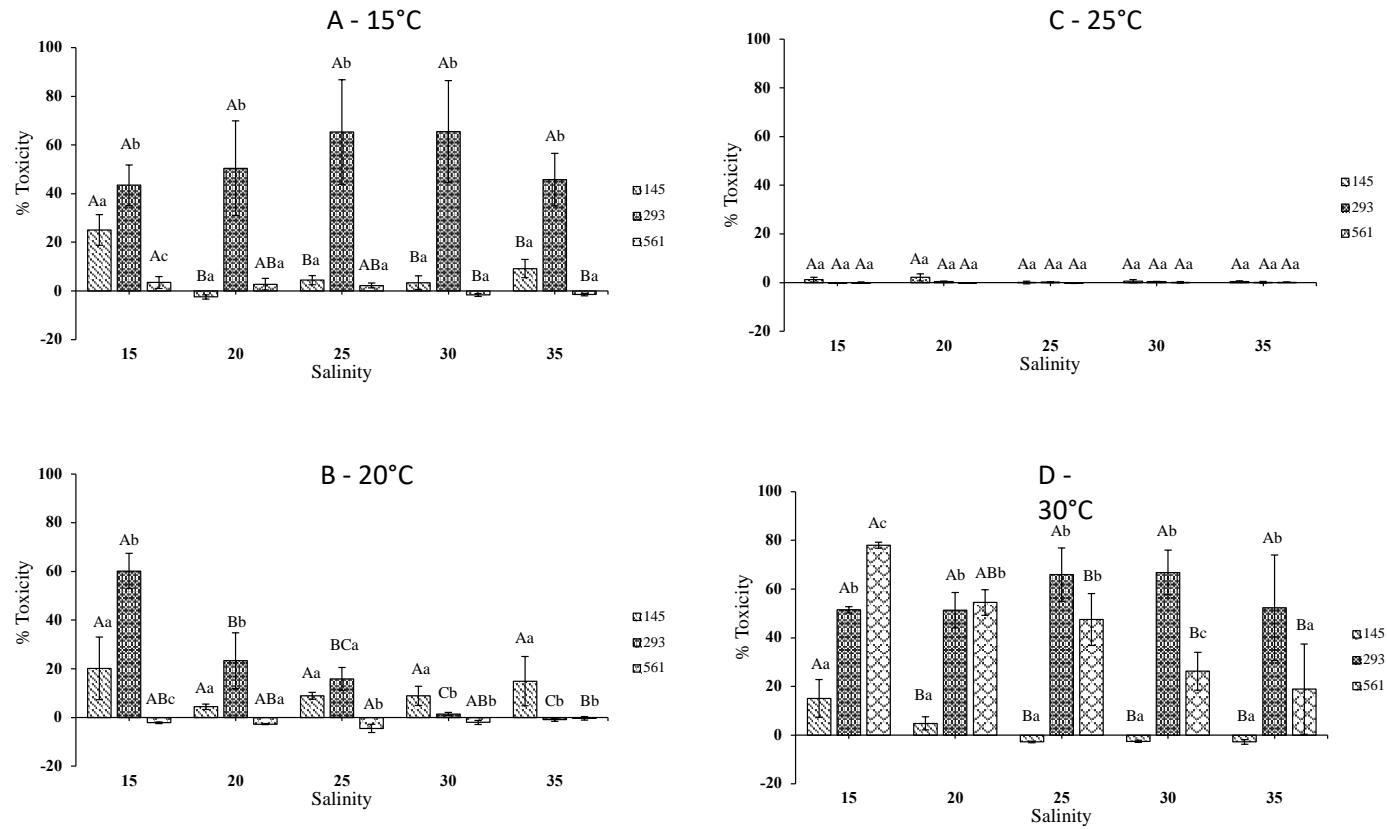


Figure 3.21 Toxicity of lysed Japanese strains of *Heterosigma akashiwo* during stationary growth at a range of salinities (15 - 35) and temperatures (15 °C – 30 °C). Values represent the average of three assays (each performed in triplicate) \pm standard deviation (n =3). Same lowercase letter(s) represent no significant difference in toxicity between strains within the same salinity. Same uppercase letter(s) represent no significant effect of salinity on toxicity within the same strain. Significance tested at $p < 0.05$ level according to Tukey's multiple comparison test.

4 DISCUSSION

Considerable phenotypic variation in growth and toxicity among the three Pacific Northwest and three Japanese *H. akashiwo* strains was found. Notable differences in several eco-physiological properties, such as cell size, growth rate, yield, salinity and temperature tolerance, and toxicity, were discovered. Previous studies have investigated strain differences in the aforementioned parameters (Connell and Cattolico, 1996; Ling & Trick, 2010; Martinez et al., 2010). However, few studies have simultaneously examined inter-strain variation in a spectrum of characteristics that is necessary to assess ecotype and relative competitive ability. Smayda (1998) documented the global temperate distribution of *H. akashiwo*, while remarking on the diversity of environments supporting blooms. These environments were categorized into three types—physically, chemically and biologically dominated—with the notion that distinct ecotypes of *H. akashiwo* flourish in each habitat. The spectrum of eco-physiological characteristics recorded (Table 3.1; Table 3.2) supports the notion that even within a similar geographic region, distinct ecotypes with different competitive strategies are present within the *H. akashiwo* population. The two cell-based assays employed to assess toxicity used different cell types—the rainbow trout gill cell line and rabbit erythrocytes. These two assays are complementary and provide an opportunity to examine the effect of samples on multiple cell structures.

4.1 Standardization of ELA

The hemolytic agents produced by *H. akashiwo* are relatively unknown in structure. Therefore, detection and quantification of these compounds is achieved by *in vitro* bioassays, such as the ELA. This assay leverages the mode of action—namely the ability to lyse erythrocytes—as a way to detect hemolytic activity. Employment of the ELA as a proxy for toxicity required the selection of optimal incubation conditions.

The conditions used in this investigation parallel those used in previous studies. An incubation time of 6 h at 100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ was used to reduce the enhanced erythrocyte autolysis and variable hemolytic activity that occurs at higher and lower

values, respectively (Ling and Trick, 2010). Measurement of hemolysis at an absorption of 414 nm demonstrated an absorption maximum at this wavelength. Additionally, a study by Ling and Trick (2010) illustrated that absorption at 414 nm was linearly correlated to erythrocyte lysis up to 5×10^6 cells mL^{-1} , prompting the selection of this wavelength and standard erythrocyte concentration for the ELA.

Detection of hemolytic activity of *H. akashiwo* resulted in some interference from the samples, yielding negative hemolysis values for some strains (Fig. 3.17). To account for interference from the background absorbance of algal samples, the absorbance of algal samples alone and erythrocytes—serving as a negative control—was measured. The negative control also accounted for erythrocyte autolysis—the lysis of erythrocytes as a result of assay conditions, including incubation temperature, irradiance and time, instead of the hemolytic agents themselves. To further reduce interference with hemolytic activity detection, intact erythrocytes were removed from the supernatant, via centrifugation, prior to measurement of absorbance at 414 nm. To enhance the pelleting of erythrocytes, small volume microcentrifuge tubes were employed in the ELA. A potential source of interference in the ELA stems from the variability in the fragility of erythrocytes. Erythrocyte fragility was tested with saponin prior to each experiment to ensure that blood age and batch variation did not affect erythrocyte fragility.

4.2 Growth and cell yield

Cell yields were measured as an indicator of potential *H. akashiwo* densities in the ocean, which could reveal the extent of a given bloom event. This parameter was measured in addition to growth rates in order to better theorize the occurrence and magnitude of *H. akashiwo* bloom events.

The Pacific Northwest isolates illustrated the broad salinity tolerance that is characteristic of this species (Haque and Onoue, 2002). Growth rates of North American strains (Fig. 3.8) at temperatures of 20 °C, 25 °C, and 30 °C are comparable and strains grew relatively well across all salinities at these temperatures. At 15 °C, growth rates were slightly reduced. The reduced growth at 15 °C is consistent given that 15 °C is the minimum surface water temperature considered to stimulate growth of *H. akashiwo*

growth. Cell yields were found to be almost inversely proportional to growth rates at 15 °C. At 15 °C, the cell yield of the North American strains (Fig. 3.10) was the lowest of the yields produced at the other three temperatures tested. Despite considerable growth of strains at 15 °C, the reduced yield indicates their inability to convert available nutrients into biomass effectively across all salinities. When North American cells are intact and retain their cellular integrity during exponential growth at 15 °C, toxicity of strains 512 and 513 was significantly greater at a salinity of 30—this is noteworthy given that these were the slowest growing strains at this temperature. However, as temperatures increased above 15 °C, the cell yield increased correspondingly—particularly for strain 513 at 30 °C, which is notable given that temperatures of 30 °C are not typical of North American waters. Contrastingly, as temperature is increased to 20 °C, yield produced by strain 512 was the lowest at a salinity of 35, indicating the depressive effect of increased temperature on cell yield at elevated salinity in this strain.

Growth rates for Japanese strains (Fig. 3.9) mirrored a similar pattern as the North American strains at this temperature, whereby growth was slightly reduced in comparison to elevated temperatures. Cell yield for the Japanese strains (Fig. 3.11) were also reduced at 15 °C despite having comparable growth rates relative to the remaining temperatures. Strains were insensitive to salinity at 30°C, with the highest yield produced by strain 293.

Growth rate and efficiency are fundamental characteristics of cellular economics that significantly influence how communities and ecosystems function. Studies of bacterial growth efficiency in marine ecosystems reveal slow growth rates are associated with inefficient growth (Pirt, 1965). However, a growing body of literature illustrates an apparent contradiction in the relationship between growth rate and yield—defined as the proportion of consumed nutrients that is converted into biomass. Studies find that this rate-yield tradeoff, which was observed in both North American and Japanese isolates at 15 °C, is central in species evolution. A biochemical perspective explains that faster growth rates result in unbalanced anabolism and catabolism, dissipating energy through energy-spilling reactions involving futile cycles or the secretion of excess metabolites (Carlson et al., 2007). Additionally, because of the energetic cost of producing protein, higher rates of protein synthesis in fast-growing cells can lead to lower efficiency relative

to a state with lower growth rate in which yield is maximized (Molenaar et al., 2009). The cellular trade-off tends to create two divergent ecological strategies: fast-growing but inefficient cells or slow-growing but efficient. Selection of which strategy will manifest can be dependent on the availability of resources. Fast-growth is energetically costly and therefore may only be used under conditions of high resource availability, whereas a metabolically-efficient, high yield strategy is employed when resources are insufficient. There are examples of strains that vary phenotypically in their position along a trade-off curve between growth and nutrient use efficiency (Yoshida et al., 2004). This perspective may help to explain the inverse relationship that was observed between growth and yield at a temperature of 15 °C for both the North American and Japanese strains.

The overall finding regarding growth was that no modelled parameter negatively influenced growth to the extent that *H. akashiwo* strains of Japanese origin could not be present in temperature and salinity conditions that characterize North American coastal waters. A natural bloom population of *H. akashiwo* has been shown to manifest as visible water discoloration at a density of 1,000,000 cells mL⁻¹ (Matheson, 2014). None of the batch cultures in this investigation reached comparable bloom densities, with the highest yield achieved by strain 293 at salinity of 25 at 30 °C, producing a cell yield just shy of 500,000 cells mL⁻¹. This infers one of the limitations of laboratory batch culture experiments—the inability to flawlessly mimic natural conditions. Despite this limitation, the results indicate that the growth of Japanese strains of *H. akashiwo* is possible at cooler surface water temperatures and lower salinities that characterize North American waters. Inversely, the growth of North American strains is also possible at increased surface water temperatures and salinities, raising concerns about its potential presence in future coastal waters, where surface water temperatures will likely be elevated as a result of climate change.

4.3 Erythrocyte Lysis Assay

4.3.1 Effect of cell lysis on hemolytic activity

The most consistent result observed was the lack of toxic response seen from unlysed algal samples relative to lysed cells (Fig. 3.12, 3.14). The results in this study illustrate the propensity for lysed samples (intracellular and extracellular material) to result in greater hemolytic activity relative to unlysed samples (extracellular fractions only). This observation is supported by previous studies (Ling & Trick, 2010) demonstrating greater hemolytic activity of lysed *H. akashiwo* strains, suggesting that the majority of toxic agents that are retained inside the cell over the course of its growth is greater than the fraction released into the extracellular environment, prior to senescence (McLaughlin, 2013). This may also explain the differences observed in the exponential and stationary phase tests, as the accumulation of intracellular compounds would increase proportionally with the age of the cells (Park et al., 1998).

Toxicity of both intact and ruptured cells were compared in the ELA. Intact cells were used to represent the presence of extracellular hemolysins located on the surface of *H. akashiwo* cells, whereas lysed cells were used to represent the release of all hemolytic compounds within the algal cells in addition to the toxicity of membranous agents. Potent hemolytic activity was detected in ultrasonic-ruptured *H. akashiwo* cells. This may be explained by the hypothesis that the hemolytic agents of raphidophytes are located in certain intracellular compartments, and leakage or release of these hemolytic agents from algal cells occurs only as a consequence of cell damage and does not take place during normal growth (Ling & Trick 2010). This hypothesis is also supported by a study performed by Mohamed & Al-Shehri (2012), indicating that the hemolytic activity of a cell-free suspension of bloom samples increased with decreasing *Heterosigma* cell numbers in the bloom, reaching its maximum when the bloom began to collapse.

The most hemolytic algal samples tested was the ruptured cell suspensions, indicating that hemolytic agents are released upon cell damage. This necessitated the standardization of the quality of cells when being lysed via sonication.

4.4 Effect of growth phase on hemolytic activity

Studies that examine the effects of nutrient limitation on toxin production indicate that phosphorus limitation increases the cellular toxin content. Johanson & Granéli (1999) also revealed that nitrogen limitation results in an increase in cellular toxin content in *Chrysochromulina polyepsis*. This raises questions regarding the effect of nutrient exhaustion that occurs as cells age and enter stationary growth, and how this influences the toxicity. A noteworthy observation in this study was that toxicity during stationary growth was greater than the toxicity produced during exponential growth, or similar to the toxicity of lysed strains at exponential growth. For example, when comparing unlysed North American strains, the toxicity is almost two-folds greater at stationary growth (Fig. 3.16) than exponential growth (Fig. 3.12)—a pattern that is observed across all salinities and temperatures at this stage. Interestingly, lysed North American strains growing exponentially (Fig. 3.14) showed a similar toxic response lysed strains in stationary growth (Fig. 3.18). This indicates that the hemolytic agents are contained within the cell, and that upon cell rupture, these agents are released. During stationary growth, due to the nature of cell age and nutrient scarcity, autolysis of algal cells often occurs, releasing the retained ichthyotoxic agents, explaining the similarity in toxicity between lysed cells at exponential growth.

With regards to lysed Japanese strains, although toxicity of cells during exponential growth (Fig. 3.15) is not considerably greater than stationary growth (Fig. 3.19), toxicity is observed for strain 145 at a temperature of 20 °C and increases with increasing salinity. This suggests a temperature and salinity optimum of 20 °C and 35, respectively, for this specific strain—isolated from southern Japan—to produce toxicity.

The reduced toxicity observed during the exponential growth phase relative to stationary growth suggests that either hemolytic agents are not being produced in the exponential growth phase or alternatively, they are being produced, but not at sufficiently high quantities to elicit a significant toxic response.

4.5 Effect of salinity and temperature on growth and hemolytic activity

The strains demonstrated the broad salinity tolerance that is characteristic of this species (Haque and Onoue, 2002). This agrees with previous studies that have demonstrated that *H. akashiwo* is euryhaline and as a result, can thrive over a wide range of salinities (Smayda, 1997). All strains of *H. akashiwo* tested were observed to grow at all salinities (15 - 35), with lower growth rates at lower temperatures (15 °C), and maximal growth rates occurring at intermediate temperatures. However, not all strains exhibited this broad salinity tolerance. At 15 °C, the North American and Japanese strains exhibited relatively slower growth, whereas upon increases in temperature, growth rates increased. The role of broad halotolerance and reduced salinity has not completely been elucidated, partially because inter-strain variability has not always been accounted for in laboratory experiments or interpreting field results (Fredrickson et al., 2011). The formation of blooms, indicated by cell yield, is influenced by salinity in several ways. Firstly, the ability of *H. akashiwo* to grow at high rates across a wide range of salinities may offer this species a competitive advantage in estuaries with strong salinity gradients. For instance, *H. akashiwo* blooms have been shown to coincide with episodes of high runoff from the Fraser River in the Strait of Georgia (Taylor and Haigh, 1993). Additionally, the ability of cells to form dense accumulations in surface waters has been associated with the presence of low salinities (Fredrickson et al., 2011). This is consistent with our findings, where North American cells grew at near-maximal growth rates at low salinities.

Inter-strain differences in toxicity were observed across the salinities tested. At a temperature of 15 °C, despite having the lowest growth rates at a salinity of 30 (Fig. 3.8), when North American strains were unlysed and growing exponentially, all three strains exhibited the highest toxicity at this salinity (Fig. 3.12). The Japanese strains behaved in the opposite manner and presenting no toxic response at this salinity and temperature (Fig. 3.13). Several existing studies of the effects of salinity and temperature on *H. akashiwo* toxicity show conflicting results, suggesting this may be due to the use of different isolates used in the study. This further reinforces the idea that even among

strains of similar geographic origin, the responses to environmental conditions may manifest differently.

4.6 Gill Cell Assay (RTgill-W1)

4.6.1 Effect of salinity and temperature on growth and gill cell toxicity

The gill cell assay proved to be more sensitive to metabolites produced by the Japanese algal strains tested. Whereas, the ELA was shown to be insensitive to the compounds produced by some of the Japanese isolates tested. The negative ELA results do not necessarily indicate that the algal strains were unable to produce hemolytic compounds, but rather that they were likely produced at concentrations too low to induce toxicity.

Varying degrees in the loss of gill cell viability was observed among the isolates tested. For the gill cell assay, the ruptured cell suspension was prepared from cultures in stationary growth since hemolytic activity was maximal during this stage. Most noteworthy was that at a temperature of 25 °C, the toxicity of North American strain 524 was significantly reduced compared to the other two strains at elevated salinities. This reduction was not observed in the ELA (Fig 3.18), potentially indicating that a temperature of 25 °C and elevated salinities is not conducive to toxic potential in strain 524. The toxicity of Japanese strains was found to be the highest at the minimum and maximum temperature tested (15 °C and 30 °C, respectively), shown by gill cell death following a 24-hour exposure period (Fig. 3.21). The consistently elevated toxicity elicited by strains 293 and 561 at 30 °C, and strain 293 at 15 °C suggest that at these temperature extremes, cells may be stressed, resulting in ichthyotoxic effects as a defense mechanism. Declines in gill cell viability were observed at intermediate temperatures of 20 °C and 25 °C, which may be a product of the cells growing in conditions that support optimal growth and performance, and therefore negates the need to allocate resources to produce ichthyotoxic agents.

What is interesting to note in the Japanese strains is that toxicity as a function of gill cell viability was greater than the toxicity that was elicited by the ELA. Given that these two assays assess toxicity directed at different targets—erythrocytes and gill cells—these results suggest that the mode of toxicity in the Japanese strains may differ from the North American strains, where significant hemolytic activity was observed. The toxic agent of *H. akashiwo* remains under investigation. However, engendered mechanisms include a brevetoxin-like compound or a PUFA. Studies investigating other members of the class Raphidophyceae (*Chattonella* and *Fibrocapsa*) found the production of brevetoxins, and in certain *Chattonella* spp., toxic PUFA (Haque & Onoue, 2002). Previous studies have also shown a negative correlation between neutral lipid accumulation and toxicity (Matheson, 2014), pointing to brevetoxin-like compounds as the toxic component. The elevated toxicity exhibited with the gill cell assay implies that toxic agents were more toxic than the hemolytic fractions, which is consistent with Onoue and Nozawa (1989), who reported that the neurotoxic fractions of *H. akashiwo* were more toxic to fish than the hemolytic and hemagglutinating fractions.

4.7 Are *Heterosigma akashiwo* blooms a wicked problem?

As previously stated, a wicked problem, as defined by Rittel and Weber (1973), is characterized as “a problem that is difficult to solve because of incomplete, contradictory, and changing requirements that are often difficult to recognize”. What is the problem? With respect to *H. akashiwo*, the ‘problem’ is that within the species, variability in growth and toxicity is observed in different geographic regions, which poses challenges to manage blooms that result in fish kills. This ‘problem’ is incomplete because the mechanisms of toxicity are largely unknown; contradictory because of inter-strain variations in growth and toxicity that manifest in different ways; and difficult to recognize because of environmental conditions that are constantly fluctuating as a result of anthropogenic and climatic forces. The findings in this study suggest that the problem is less “wicked”, but instead more complex in such a way as to have unintended consequences, and therefore requires a novel way to approach the problem. Approaching this problem necessitates that role players on several levels be informed and contribute.

5 CONCLUSION

5.1 Main Findings

The toxic profile of *H. akashiwo* has been shown to vary across geographic regions. High levels of intraspecific variability are often associated with HAB species, and this variability extends to the ichthyotoxic raphidophyte, *H. akashiwo*. In an effort to better understand if growth and toxicity are regulated by environmental conditions specific to a geographic region, or if they are a function of strain variability, the combinatorial effect of salinity and temperature on growth, hemolytic activity and gill cell interaction was assessed. The two aims of this thesis were to (1) investigate how a range of temperature and salinity conditions dictate the growth characteristics of geographically-distinct isolates of *H. akashiwo* and (2) assess the toxicity of these strains using two cell-based assays designed to evaluate the cytotoxic effects of these compounds.

This thesis examined if the growth and toxicity of strains isolated from Japanese waters—shaped by elevated surface water temperatures and salinity—exhibit a toxic phenotype when introduced into the reduced surface water temperature and salinity conditions that characterize North American waters. It also examined the toxic response over two different growth stages when cells maintained their intact form and upon cell rupture. Strains were isolated from two geographic locations—North America and Japan. Selection of strains from these specific regions was motivated by previous research that demonstrated the diametrically opposing responses in growth and toxicity that *H. akashiwo* exhibits in either region. Specifically, despite the abundance of *H. akashiwo* blooms in Japan, toxicity by the cells is inconsequential. In contrast, a low biovolume of cells originating from North American waters rendered the waters toxic.

The lack of toxicity in the Japanese strains may be attributed to inhabiting an environment that does not support a high expression of toxicity. Upon the presence of specific surface water temperatures and salinities, toxicity was induced. This toxic response will be exacerbated by factors such as cell rupture, thereby releasing ichthyotoxic agents, and stage of growth, whereby later stages of growth—characterized by nutrient limitation—result in cell lysis. This study adds to a growing body of research

that suggests the primary role of environmental factors in regulating toxicity in *H. akashiwo*. This understanding may be instrumental in the management of HABs in a dynamic era of climatic and environmental change.

5.2 Significance

There are several implications to this work. First, the Japanese aquaculture industry is protected from raphidophyte fish kills simply because the salinity of the waters remains high. Since climate changes often indicate an increased frequency in episodic rain events than the enclosed waters of Japan may have ephemeral salinity reductions that would initial a fish kill. Alternatively, the influence of the Kuroshio Current may serve to protect the coastal embayments from these reductions in salinity. The Japanese word “Kuro-shio” means ‘black stream’ reflecting on the deep intense colour of the warm, high salinity water which is found flowing north on the eastern shore of Japan (Pickard and Emory, 1990). Should however the strong Kuroshi Current deviate from the Japanese coast and allow the intrusion of the southern flowing cold, nutrient-rich, lower salinity waters of the Oyashio Current, then the extant blooms of *H. akashiwo* should evoke changes to be highly toxic.

Further investigations into the use of a variety of cell-based toxicity assays to evaluate the toxic compounds produced by geographically-distinct isolates of *H. akashiwo* would provide insight into the mechanisms of toxicity employed by this species. Additionally, examination of a larger sub-set of strains spanning a wider geographic distribution is necessary to extrapolate the data collected in this study to different geographic locations.

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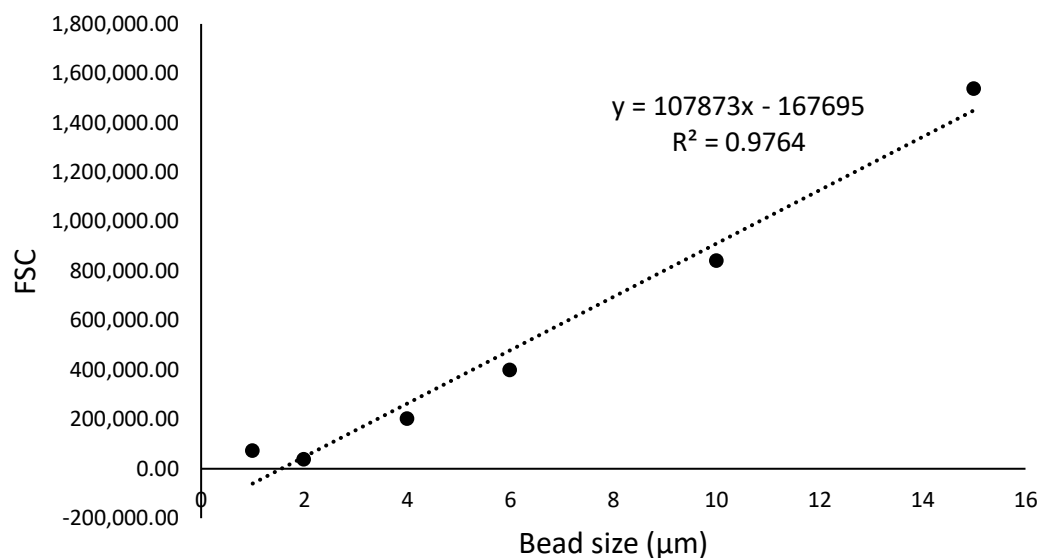
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Appendix



Appendix A: Standard curve of FSC values of beads ranging in size from 1-15 µm (Molecular Probes). The average sizes of the *H. akashiwo* cells were determined by interpolating the measured FSC values. Cell sizes (µm) were calculated using standard linear regression.

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