DIFFERENCES IN GROWTH AND TOXICITY OF *KARENIA*

A Thesis

by

TATUM ELIZABETH NEELY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Oceanography

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Approved by:

Chair of Committee,	Lisa Campbell
Committee Members,	John Gold
	Robin Autenrieth
	Richard Long
Head of Department,	Wilford Gardner

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ABSTRACT

Differences in Growth and Toxicity of *Karenia.* (May 2006)
Tatum Elizabeth Neely, B.S., University of North Carolina-Wilmington
Chair of Advisory Committee: Dr. Lisa Campbell

Harmful algal blooms (HABs) in the Gulf of Mexico are primarily caused by dense aggregations of the dinoflagellate species, *Karenia brevis*. *Karenia brevis* produces a highly toxic neurotoxin, brevetoxin which has been shown to cause Neurotoxic Shellfish Poisoning (NSP) and respiratory distress in humans in addition to a wide range of negative impacts upon natural ecosystems. *Karenia mikimotoi* is a co-existing species present during *K. brevis* blooms. *K. mikimotoi* has caused major HAB events in other parts of the ocean, but has not been recognized as a major contributor to toxicity of blooms in the Gulf of Mexico.

K. brevis and *K. mikimotoi* have both been associated with the presence of unidentified hemolytic toxins. Production of hemolysins has not previously been investigated for either species to date in the Gulf of Mexico. Presence of hemolysins may affect toxicity and the overall impact of HABs. Therefore, detection of hemolysins is imperative for accurate identification of potential harmful impacts of such blooms. The primary goal of this research is to define whether either species is capable of producing hemolytic activity independent of brevetoxin activity; and to identify if there is significant differentiation between a variety of clonal isolates regarding toxicity and growth rate when subjected to variable experimental conditions.

DEDICATION

To my Grandma,

Miss June Campbell

ACKNOWLEDGEMENTS

I would like to extend my gratitude my committee chair, Dr. Lisa Campbell for all of her support and energetic direction throughout the course of my research. I also wish to thank my committee members, Dr. John Gold, Dr. Richard Long, and Dr. Robin Autenrieth, for their support and guidance. Thanks so very much to Dr. Duncan MacKenzie for his insight and expertise in developing methodology.

I would like to sincerely thank my friends, colleagues and the oceanography faculty and staff for making my time here at Texas A&M University an incredible experience. I would also like to extend my gratitude to David Abrego, Rodney Gamez, and Nadia Ramirez at Texas Parks & Wildlife Department for providing their time, facilities and for extending their kindness to me throughout the course of my research.

Finally, I would like to earnestly thank Andrew Millar for all of his encouragement and support, Katie Neely for keeping me laughing, Kerry Stout for always being there and to my mother Elizabeth Millar, for reminding me of what is truly vital in life, to always follow your heart.

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CHAPTER I

INTRODUCTION: HARMFUL ALGAL BLOOMS IN THE GULF OF MEXICO

Karenia brevis

Phytoplankton blooms provide an essential source of nutrition for a variety of marine filter feeding organisms. Several species however have been shown to cause substantial damage to natural resources. In the Gulf of Mexico approximately 44 species of phytoplankton have been identified as either harmful, nuisance or toxic (Steidinger and Penta, 1999). Harmful algal blooms (HABs) occur when these species grow significantly above natural background levels and negatively impact natural resources and/or humans. HABs in the Gulf of Mexico have caused massive mortality in finfish, contamination of local shellfish stocks, death of marine mammals and seabirds, and include shellfish poisoning and respiratory distress in humans (Table 1). Fifty million dollars is a conservative estimate of the annual economic loss to tourism, coastal recreational and commercial fisheries in the United States as a consequence of HAB events (Evans and Jones, 2001) .[1]

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This thesis follows the format of *Harmful Algae*.

Table 1. Frequently occurring HAB species in the Gulf of Mexico and impact of respective blooms.

Species		Associated Toxins
Alexandrium monilatum	PSP*	Saxitoxin and Hemolysins
Chattonella subsalsa	NSP*, Fish kills	Brevetoxin
Dinophysis spp.	DSP*	Okadaic Acid
Gambierdiscus toxicus	CFP*	Ciguatera
Karenia brevis	NSP, Respiratory distress, Fish kills	Brevetoxin
Karenia mikimotoi	Fish kills	Cytotoxins and Hemolysins
Karenia pulchellum	NSP	Brevetoxin
Prorocentrum spp.	DSP, CFP	Okadaic Acid
Psuedo-Nitzchia spp.	ASP*	Domoic Acid

*PSP = Paralytic Shellfish Poisoning; NSP = Neurotoxic Shellfish Poisoning; DSP = Diuretic Shellfish Poisoning; CFP = Ciguatera Fish Poisoning.

Karenia brevis, formerly *Ptychodiscus brevis* (Steidinger, 1979) and *Gymnodinium breve* (Daugbjerg, et al. 2000), is a dinoflagellate which occurs at background levels of approximately 1 to 10 cells L^{-1} in the Gulf of Mexico (Geesey and Tester, 1993). *K. brevis* blooms occur annually off the western coast of Florida and more recently with increasing frequency along the coasts of Texas and eastern Mexico (Villareal et al., 2001; Mee et al., 1986). Initiating mechanisms of these blooms are not yet conclusive but several hypotheses include: fluctuation in salinity or nutrient concentration, wind and current direction, atmospheric input of Sahara dust, bacterial interactions, as well as anthropogenic influences such as increased coastal development and elevated nutrient runoff (Epstein, 1998; Tester and Steidinger, 1997; Ishida et al., 1996;

Cortes-Altamirano et al., 1995; Kin-Chung and Hodgkiss, 1991; Morris et al., 1991; Tester et al., 1991; Smayda and White, 1990).

During episodic *K. brevis* blooms, cell concentrations can reach upwards of 10^6 cells mL^{-1} causing dark green to brown water discoloration (Geesey & Tester, 1993). Blooms have been linked with massive fish kills, marine mammal deaths, contamination of shellfish beds and respiratory distress and Neurotoxic Shellfish Poisoning (NSP) in humans (reviewed in Kirkpatrick et al., 2004). A variety of natural compounds have been isolated from *K. brevis* blooms including the potent neurotoxin brevetoxin, identified as the most toxic marine biotoxin in the Gulf of Mexico to date (Mazumder et al., 1997; Baden and Trainer, 1993).

K. brevis has been shown to produce up to 14 derivatives of brevetoxin, each with variable toxicity and retention time in the water column (Bourdelais et al., 2005). Brevetoxin is a lipid soluble, cyclic polyether which selectively binds to receptor site 5 of voltage-gated Na^+ channels. Upon binding brevetoxin inhibits inactivation, locking Na^+ channels open and allows the unregulated influx of ions into the cell, resulting in cell lysis and death (Purkerson et al., 1999; Baden, 1983).

Brevetoxin can affect a wide range of marine organisms. In finfish, brevetoxin affects the central nervous system, causing lack of muscle coordination and eventual respiratory failure and death (Kennedy et al., 1992). Deaths of bottlenose dolphin, endangered Florida manatees and sea turtles have been linked to prolonged exposure to aerosolized brevetoxin and ingestion of contaminated water or vegetation (Bossart et al., 1998; Trainer and Baden, 1999). Brevetoxin is bioaccumulated in filter feeding shellfish and benthic fauna. Upon consumption of shellfish, higher trophic levels are then exposed to much higher toxin concentrations (Cummins et al., 1971; Sakamoto et al., 1987.)

Human consumption of contaminated shellfish can result in Neurotoxic Shellfish Poisoning. Symptoms of NSP include nausea, diarrhea, hot and cold reversal and cramping. Respiratory distress, asthmatic symptoms and skin

irritation occur following contact with aerosolized toxin along beaches during blooms (Asai et al., 1984; Baden et al., 1995; Fleming and Baden, 1988).

Historically *K. brevis* blooms were believed to be monospecific (Buskey et al.,1996; Steidinger and Penta,1999). Recent studies however, have shown several morphologically similar dinoflagellate species of unknown toxicity frequently co-occur with *K. brevis* during blooms (Haywood et al., 1996). If bloom dynamics have a higher complexity than originally observed, the implication is that bloom toxicity may fluctuate with species composition.

Karenia mikimotoi

Karenia mikimotoi is one particular species most frequently associated with *K. brevis* (Steidinger et al., 1998; Parrish et al., 1997; Kirkpatrick et al., 2004; Mayali and Doucette, 2002; Mazumder et al., 1997; Baden and Trainer, 1993). Morphological similarities between *K. brevis* and *K. mikimotoi* make microscopic identification extremely difficult, often resulting in the misidentification of species (Steidinger et al., 1998).

In contrast to *K. brevis* which is restricted to the Gulf of Mexico, *K. mikimotoi* occurs in temperate oceans worldwide. *K. mikimotoi* is not known to produce brevetoxin, but has been linked with fish kills in native populations and aquaculture farms off the coasts of New Zealand, Korea, Scotland and Australia (Arzul et al., 1994; Seki et al., 1995; Yamasaki et al., 2004; Zingone et al., 2000). Mortality of shellfish and benthic marine fauna has additionally been linked with *K. mikimotoi* (Dragunow et al., 2005).

Studies show *K. mikimotoi* produces toxic glycolipids along with a suite of other unidentified compounds (Yamamoto et al., 1990; Parrish et al., 1997). Uncharacterized hemolysins from *K. mikimotoi* blooms have caused extensive

damage to gill epithelia of finfish and can lead to mortality of a variety of benthic invertebrates (Seki et al., 1995; Yamasaki et al., 2004). Even though *K. mikimotoi* frequently coincides with *K. brevis*, it has not been recognized as a primary contributor in overall toxicity of blooms in the Gulf of Mexico (Kirkpatrick et al., 2004).

Harmful Bloom Monitoring

Current HAB monitoring within the Gulf of Mexico is primarily focused on detection of brevetoxin. In Florida where blooms occur almost annually, weekly regulatory cell counts are conducted along the western coast by the Florida Marine Research Institute (Kirkpatrick et al., 2004). In Texas, blooms have not historically been observed with such a high frequency, and therefore cell counts are generally conducted only after fish kills have been reported (Texas Parks & Wildlife Department; TPWD). Fish kills are generally observed when cell concentrations exceed 5,000 to 10,000 cells L^{-1} (*www.floridamarine.org*), levels which greatly inhibit the possibility for preventative measures.

When cell counts from western Florida exceed 5,000 cells L^{-1} within close proximity to shellfish beds, all shellfish harvesting is prohibited. Brevetoxin concentration in shellfish meat is then analyzed using the American Public Health Association (APHA, 1985) mouse bioassay protocol. Concentration levels which exceed 0.17 mg kg^{-1} body weight, will sustain harvesting closure until concentration levels drop below these set limits for a period of two weeks (Viviani, 1992; Park, 1995; Baden, 1983; Baden et al., 1995; ILO, 1984).

There are several complications with the mouse bioassay including expense, accurate administration of dosage and the frequent unreliability of results (Trainer and Poli, 2000). In attempts to more accurately define toxicity levels, optimized testing methods have since been introduced. Tests which

specifically target the detection of brevetoxin include a competitive ELISA (Bourdelais et al., 2000), the radioimmunoassay (RIA), neuroblastoma cytotoxicity assay and chemical analysis using High Performance Liquid Chromatography (HPLC) (Trainer and Poli, 2000). Methods which are highly sensitive to brevetoxin may exclude detection of additional harmful compounds, and ultimately may result in underestimating total bloom toxicity.

Unidentified Toxic Compounds

Several fish kills off the Texas coast have coincided with low *K. brevis* cell counts and/or low brevetoxin concentration in shellfish tissue (Villareal et al., 2001; TPWD data). The high toxicity of these particular incidents suggests the presence of unidentified toxins which may have been overlooked by current methodology (TPWD data; Steidinger et al., 1998). Algicides and cardiotoxic anti-cholinesterases have been linked to recent Gulf of Mexico blooms and large fish kills (e.g, Mayali and Doucette, 2002; Mazumder et al., 1997; Baden and Trainer, 1993). Many of these compounds have unknown toxicities and environmental impacts have not yet been fully assessed (Steidinger and Penta, 1999).

Hemolysins from *K. mikimotoi* blooms were shown to rupture gill epithelia in finfish stocks (Kennedy et al., 1992). Damaged gill tissue can cause severe respiratory distress and lead to bacterial infections, both of which are leading causes of fatality during blooms (Kennedy et al., 1992). Unsaturated glycolipids have been isolated from laboratory cultures of *K. mikimotoi* and were shown to cause hemolysis of horse and tilapia erythrocytes (Parrish et al., 1997).

Several morphologically similar species are additionally known to produce a variety of harmfully active compounds. Off the coast of New Zealand in 1994,

a bloom of *K. mikimotoi*-like cells caused widespread fish kills. Shellfish extracts were subsequently found to be highly toxic to mice when injected intraperitoneally (MacKenzie et al., 1996). Upon further investigation a new species *Karenia selliformis,* was identified as the causative agent (Haywood et al., 2004).

A new toxin gymnodimine was identified from cultures of *K. selliformis* isolated from the 1994 bloom (Seki et al., 1995). While gymnodimine was classified as a neurotoxin, it did not elicit symptoms or dose responses similar to other known marine toxins; nor cause mortality when orally administered, and was not detectable from NSP tests (MacKenzie et al., 1996). Gymnodimine did however cause mortality in fish bioassays at levels of 0.1 ppm and residual toxin was found in the water column days after bloom degeneration (Seki et al., 1995). The effects of gymnodimine on human health are still under investigation (Haywood et al., 2004).

Since the New Zealand bloom, *K. selliformis* has been identified in the Gulf of Mexico and now is also known to co-occur with *K. brevis* (Dr. A. Haywood, pers. comm., 2005). The discovery of gymnodimine provides further evidence of the threat of newly emerging harmful compounds which may be overlooked with traditional monitoring protocols (Seki et al., 1995; Zingone and Enevoldsen, 2000).

Challenges in HAB Monitoring

Presently, HAB monitoring is complicated by a variety of factors which make accurate toxicity assessment exceedingly difficult. Foremost, concentration levels of *K. brevis* cannot be directly correlated to brevetoxin concentration (Kirkpatrick et al., 2004; Baden et al., 1995; Baden and Trainer, 1993). Brevetoxin levels can fluctuate according to stages of bloom

development in addition to changes in environmental conditions (Scholin et al., 1999; Baden and Tomas, 1988). Therefore regulatory cell counts, while beneficial in detecting presence of blooms, unfortunately alone do not provide accurate toxicity analysis. In addition, brevetoxin is a particularly complex compound, with 14 recently identified derivatives (Bourdelais et al., 2005). Fernandez et al. (2003) illustrated the high variability of potency between congeners of the same toxin family. As individual brevetoxin derivatives are found in a range of concentrations and vary under given conditions; the overall concentration of a particular derivative will therefore impact toxicity levels (Baden et al., 2005).

More recently, population dynamics of blooms are becoming increasingly imperative in defining toxicity. The co-occurrence of harmful species creates a unique challenge in HAB management (Zingone and Enevoldsen, 2000). Potential emergence of new toxins may result from inter/intraspecific competition and/or synergy between compounds (Mayali and Doucette, 2002; Haywood et al., 2004). If current monitoring continues to rely primarily upon the detection of a narrow range of compounds (Baden and Trainer, 1993; Casper et al., 2004; Fleming et al., 1988), there is the potential for miscalculation of total toxicity by ignoring the presence of other potentially harmful compounds (Park et al., 1995; Zingone and Enevoldsen, 2000).

Recent discoveries of new HAB species and toxins including gymnodimine, hemolytic amphidinol analogs and ichthyotoxins, combined with increase in frequency and duration of blooms, establishes the need for further development of methods to universally screen for the presence of a wide variety of toxins (Echigoya, et al., 2005; Yang et al., 2001; Zingone and Enevoldsen, 2000; Smayda and White, 1990; Rossini, 2005; Van Dolah, 2000). The importance of accurately quantifying bloom toxicity is essential in defining potential emerging risks to marine organism and human health (Smayda and White, 1990; Zingone and Enevoldsen, 2000).

Goals of Research

The goals of this study were to 1) develop a sensitive, reliable and inexpensive method to test whether Gulf of Mexico *Karenia* isolates are capable of producing hemolytic compounds in addition to brevetoxin; 2) determine the toxicity of *Karenia* clones isolated from selected blooms in the Gulf of Mexico using a new assay for hemolytic activity; and 3) assess whether changes in salinity or temperature affect growth rate or toxicity in *Karenia*. The results from this project were used to test the following hypotheses.

Hypothesis 1: *K. brevis* and *K. mikimotoi* produce one or more hemolytic compounds

Null hypothesis: There is no production of hemolytic compounds by either *Karenia* species as detectable using the Red Drum bioassay.

Hypothesis 2: Distinct clonal isolates of *K. brevis* and *K. mikimotoi* have variable levels of toxin production

Null hypothesis: Hemolytic activity is the same among all clonal isolates.

Initial growth rates were assessed from *Karenia* clones grown within a matrix of three salinities and three temperatures to provide evidence of clonal diversity. Differences in growth rate provided further evidence of variability in toxin production (Wang and Hsieh, 2004). Toxicity was then assessed with a modified bioassay using Red drum (*Sciaenops ocellatus*) erythrocytes to detect hemolytic activity in crude algal pellets collected at specific temperature and salinity. Conclusively these results will either support the hypothesis that there is considerable variation in growth and toxicity of *Karenia* in the Gulf of Mexico; or support the null hypothesis that there are not observable differences among individual *Karenia* clones.

Results will provide further evidence that changes in population dynamics as a result of environmental conditions can have a measurable impact in overall bloom toxicity. Since hemolytic activity is not currently incorporated into current HAB monitoring, determination of activity levels from monoclonal algal cultures will help to enhance understanding of the toxic impact of particular species.

By defining differences among various clonal isolates, these results will expand upon previous studies conducted by Baden and Tomas (1988) which show differences in brevetoxin production among six *K. brevis* clones isolated from Florida coast blooms. Overall, these results will both enhance understanding of toxin variability within a single clone when subjected to changing environmental conditions, and help elucidate potential variation among geographically distinct clones.

CHAPTER II

MATERIALS AND METHODS

Karenia Clones

A total of twelve *Karenia* clones isolated from various geographical regions in the Gulf of Mexico and one clone from the coast of England were used in growth rate experiments (Table 2). Eight clones of *K. brevis* included five clones from a Brownsville, Texas bloom in 1999 (SP1, SP2, SP3, TxB3 and TxB4) and three clones obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP 718, CCMP 2228 and CCMP 2229). All *K. brevis* CCMP clones were originally isolated from various blooms off the western coast of Florida. CCMP 718, the classic Wilson's clone isolated in 1953, has been the primary clone in *K. brevis* research for over 50 years. CCMP 2228 was isolated off Sarasota in 2001 and CCMP 2229 was isolated from Manasota Key in 2001 (*www.ccmp.bigelow.org*).

Five clones of *K. mikimotoi* included: three clones from a Corpus Christi bloom in 2002 (B1, C5 and C9), one Florida clone (NOAA2) isolated off Sarasota in 2001 and one clone from the coast of England in 1980 (CCMP 429).

Table 2. *Karenia* clones selected for growth rate and toxin comparison.

Species	Strain	Source
Karenia brevis	CCMP 718	Florida, 1953 Wilson
K. brevis	CCMP 2228	Florida, 2001 C. Higham-Mote Marine Lab
K. brevis	CCMP 2229	Florida, 2001 C. Higham-Mote Marine Lab
K. brevis	SP1	Texas, 1999 T. Villareal - UTMSI
K. brevis	SP2	Texas, 1999 T. Villareal - UTMSI
K. brevis	SP3	Texas, 1999 T. Villareal - UTMSI
K. brevis	TxB3	Texas, 1999 K. Steidinger - FMRI
K. brevis	TxB4	Texas, 1999 K. Steidinger - FMRI
Karenia mikimotoi	CCMP 429	England, 1980 D.Harbor
K. mikimotoi	B1	Texas, 2002 L. Campbell - TAMU
K. mikimotoi	C5	Texas, 2002 L. Campbell - TAMU
K. mikimotoi	C9	Texas, 2002 L. Campbell - TAMU
K. mikimotoi	NOAA2	Florida, 1999 NOAA Laboratory

All clones were grown in enriched natural seawater at a salinity of 35. Seawater was collected on an incoming tide from the University of Texas Marine Science Institute's pier in Port Aransas, TX and kept in the dark at 4° C prior to use. Seawater was then filtered using 0.2 µm Supor® filters and autoclaved for sterility. To prepare L1 growth medium, sterile filtered nutrients were added to seawater to provide constant nutrient availability during experimentation (Guillard & Hargraves, 1993).

Growth Rate Experiments

All clones were maintained under light levels of 70 µEin m^{-2} sec^{-1} over a 12:12 hour light: dark cycle throughout experimentation with Phillips® Cool White bulbs. All stock cultures were maintained at 20° C. Experimental cultures grown at 20° C were then acclimated to temperatures of 22 and 25° C over a minimum period of ten generations between acclimation. Once acclimated to temperature variables, salinity range was altered from 35 to 27 with addition of appropriate amounts of sterile MilliQ® water. Each decrease in salinity was additionally maintained for ten generations for acclimation.

All clones were grown to approximately 10^3 - 10^4 cells mL^{-1} before start of each experiment (in Tube 1). Triplicate 50 mL glass Pyrex® tubes (Tubes A, B and C) were pre-filled with 40 mL of L1 medium at experimental salinity. Tubes A, B and C were then inoculated with ~2 mL of well-mixed culture from Tube 1. Directly following inoculation, *in vivo* chlorophyll *a* fluorescence readings were measured with a Turner Model 700 Fluorometer. Prior experimentation has shown a linear relationship in chlorophyll *a* fluorescence and cell counts with Sedgwick-Rafter chambers over the range of cell concentrations used in experiments (Figure 1).

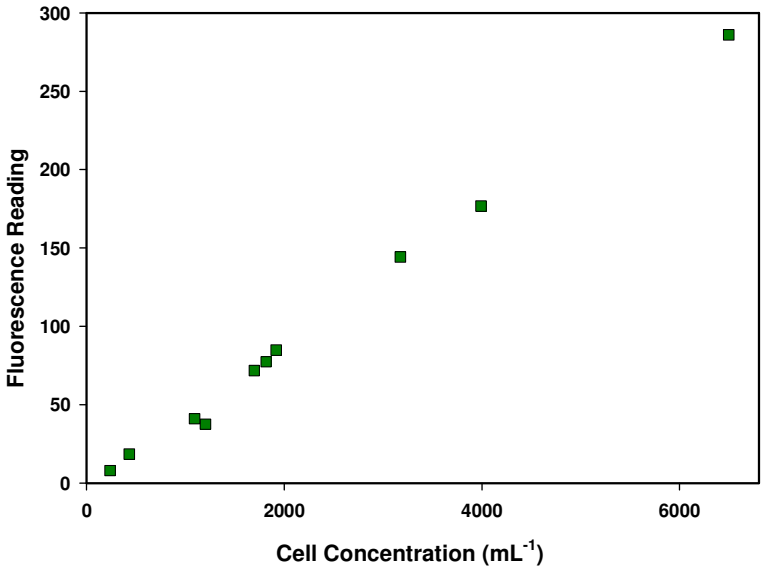


Figure 1. Linear correlation between chlorophyll *a* fluorescence and increasing *Karenia* cell concentrations. (Turner 700 Fluorometer)

Tubes A, B and C were gently mixed before taking triplicate fluorescence readings per tube. Tubes were then placed into incubators and maintained at experimental temperature with fluorometric readings taken at intervals of 24 hours. Experiments continued until cultures reached late exponential phase and sustained stationary growth for a minimum of two days (Figure 2).

Upon termination of growth experiments, replicate tubes were gently combined in a 200 mL flask and final cell density determined. Flasks were then divided into four, 50 mL centrifuge tubes and centrifuged at 4º C, 3200 g for 15 min. Replicate cell pellets were then transferred to 1.5 mL microcentrifuge tubes and centrifuged again at 3200 g for 15 min. Cell pellets were then washed in buffer solution (150 mM NaCl, 3.2 mM KCl, 1.25 mM $MgSO_4$, 3.75 mM $CaCl_2$, and 12.2 mM TRIS base; pH adjusted to 7.4 with HCl) twice to dispose of any cellular debris associated with cell pellet. Cell lysis did not occur during washing, as confirmed by microscopic examination of pellets. Washing was preformed to reduce presence of extracellular compounds which could potentially bias results.

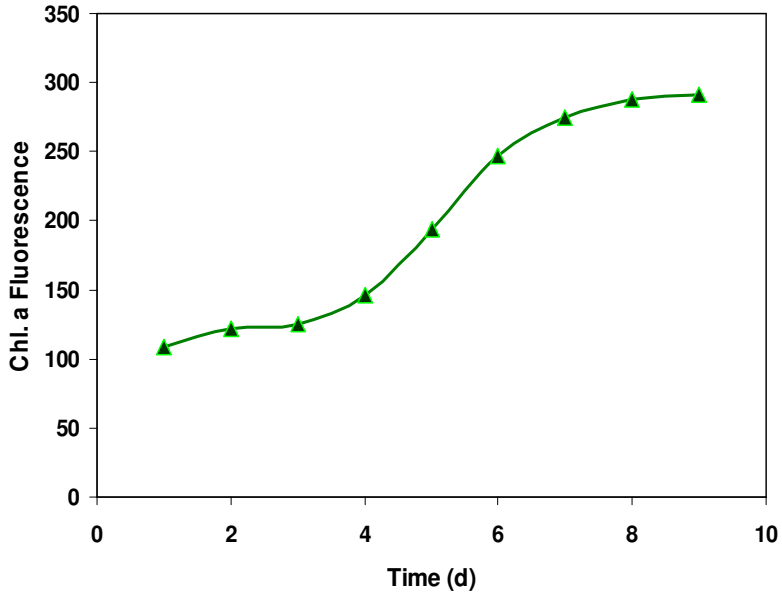


Figure 2. Daily growth measurements. (Log phase of growth exhibited on days 4-6 and stationary phase evident after day 6).

Final cell counts were typically between 1-6 x 10^6 cells mL^{-1}. Dilution of cell pellets with buffer solution yielded 1-5 x 10^5 cells mL^{-1} for toxicity assays. Pellets were concentrated by centrifugation, buffer removed by aspiration and replicate aliquots of identical cell density stored at -80º C up to 3 months for toxicity assessment.

Studies by Gentien and Arzul (1990) have shown natural variability in brevetoxin concentration of *Karenia* cultures after 18-20 days, resulting in artificial variability in toxicity. To avoid similar variation in hemolytic activity, all cell pellets were collected within 15-17 days of initial inoculation.

Red Drum Erythrocyte Lysis Assay (RDA)

Red drum (*Sciaenops ocellatus*) were obtained from SeaCenter Aquaculture Facility in Lake Jackson, TX. Individuals between 1-3 kg were carefully handled and 5-10 mL of whole blood was extracted from the caudal vein using 22 cc syringes pre-loaded with 50 IU of sodium heparin diluted in RPMI 1640 (both Sigma Scientific) according to procedures outlined in Stoskopf (1993). RPMI 1640 media was used in an effort to reduce osmotic shock of blood cells and to decrease the possibility of lysis during collection and transport. No Red drum were killed during sampling and all were directly returned to ponds to minimize shock.

Whole blood collected in syringes was then dispensed into 200 mL tissue culture flasks and kept on ice for direct transport to laboratory. Red blood cells (RBCs) were separated from blood serum with gentle centrifugation at 800 g and then diluted to 1-5 x 10^6 cells mL^{-1} in RPMI 1640 media with 50 IU of heparin to prevent coagulation. Previous attempts using only 22.5 IU proved insufficient in preventing clotting. RBCs were kept at 4º C and resuspended with daily gentle rolling of containers.

Frozen algal pellets were thawed and resuspended to appropriate (1-5 x 10^5 cells mL^{-1}) concentration in buffer solution prior to assay. Crude extracts were prepared with 30 sec / 30 sec / 60 sec pulses with a wand sonicator at 50% amplitude to ensure full lysis of algal cells. Crude extracts were kept in light-tight boxes at -20º C and used within 48 hours to minimize loss of activity over time. Studies suggest degradation in activity may result from exposure to direct light (Eschbach et al., 2001 Edvardsen et al., 1990), and therefore all assay procedures were conducted under dimmed light settings.

For control purposes, cultures of *Dunaliella tertiolecta* and *Chattonella subsalsa* were grown at 20° C in f/2 medium (Guillard and Hargraves, 1993). *Dunaliella*, a non-toxic chlorophyte provided a negative control, while

Chattonella, an estuarine species noted to produce hemolytic toxins, served as a positive control. Cultures were centrifuged after 15-17 days and concentrations adjusted to 1 x 10^5 cells mL^{-1}.

Clean 96-welled V-bottom plates (Evergreen Scientific) were pre-filled with 50 µL of assay buffer. Fifty µL of crude algal extract was then added in triplicate to top wells and serially diluted two-fold down the plate using a multi-channel pipette. Concentration of algal cells ranged from 10^5 cells mL^{-1} in top wells down to 10^3 cells mL^{-1}. This was in an attempt to observe range in activity dependant upon cell concentration.

Fifty µL of erythrocyte solution (1-5 x 10^6 cells mL^{-1} washed in buffer solution immediately prior to assay) was then added to all wells. Plates were covered and incubated in complete darkness for 10 h at 25^o C. For standardization a known hemolytic agent, Saponin (Sigma), was used at concentrations of 2 µg mL^{-1} and serially diluted along with crude extracts in each assay. Wells inoculated solely with buffer solution served as negative controls and positive controls of fully lysed erythrocytes at identical concentration as unknowns (1-5 x 10^6 cells mL^{-1}) (Figure 3).

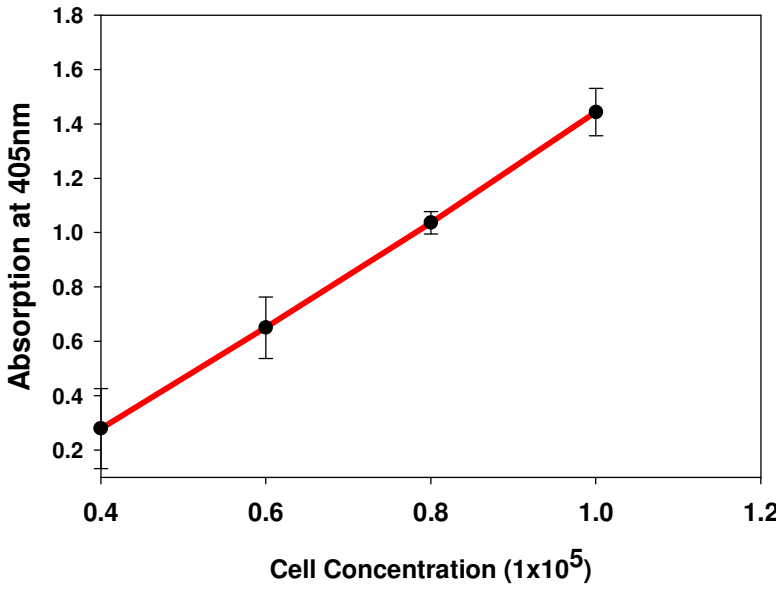


Figure 3. Linear correlation between increasing concentration of Red drum erythrocytes and absorbance (405 nm). Detection limits were 1 x 10^2 cells mL^{-1}.

After incubation, plates were centrifuged at 3600 g for 15 min. Seventy-five µL of supernatant was then gently pipetted from all wells and transferred into corresponding wells of a clean, flat bottom 96-welled plate (Evergreen Scientific). Transfer plates were read with a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA) at 405, 455, and 519 nm to determine optimal wavelength. Hemoglobin absorption was highest at 405 nm and significantly lower at other wavelengths (data not shown). Crude algal extracts read at three wavelengths showed lowest absorption at 405 nm, presenting no interference with hemoglobin absorption. 405 nm was determined an optimal wavelength as this provided highest sensitivity for detection of hemoglobin and therefore hemolytic activity. Plate reader results were exported into Microsoft Excel for all data analyses.

Time Course Experiments

Time course experiments were conducted using the two clones with the highest and lowest activities based on results of the Red drum assay. Isolates were grown up to 50 mL cultures at experimental salinity and 25° C. Four, 200 mL glass flasks per isolate were filled with 150 mL of L1 medium at appropriate salinity. 10 mL taken from a dense 50 mL culture was inoculated into each of the four flasks. Flasks were then labeled with incubation times of 4, 6, 8 or 10 days and placed in incubator. All flasks were exposed to 70±5 µEin m^{-2} sec^{-1} over a 12:12 hour light: dark cycle using Phillips® Cool White bulbs.

Flasks were removed at 4, 6, 8 and 10 days and cell pellets harvested by centrifugation (3200 g / 15 min). A total of three replicate pellets per clone and condition were then stored at -80° C. Toxicity levels were determined with the Red drum assay to reflect hemolytic activity as a function of time in culture.

***Gambusia* Bioassay**

The mosquito fish *Gambusia affinis,* was used to determine whole fish toxicity of crude extracts for further confirmation of toxicity as established by the Red drum bioassay. Standard LD_{50} for *Gambusia* assays are typically 0.01 µg pure compound L^{-1} (ILO, 1984). Due to unknown concentration of specific hemolytic compounds, positive results were determined with endpoint mortality within 1 h.

Small mosquito fish were maintained in 5 gallon aquariums kept at room temperature. Bioassays were run in 200 mL of sterile filtered ocean water adjusted to a salinity of 5 by addition of sterile MilliQ® water, in four plastic 500 mL beakers. Crude algal extracts were then diluted into beakers representative of cell concentration of 10^4, 10^3, 10^2 and 10 cells mL^{-1} (conducted

under dimmed light conditions). Triplicate mosquito fish were added to each experimental dilution and incubated for a total of 1 h.

Data Analysis

Averaged triplicate daily fluorescence (n=3) was plotted onto log graphs. Growth rates (d^{-1}) were determined from regressions of natural log of fluorescence data vs time (Figure 2). Significant differences among clones were calculated from growth rates using Wilcoxon tests and paired t-tests.

Toxicity results from the Red drum assay were averaged (n=3) from crude extract activity and corrected by subtracting negative background (buffer absorbance only). Activity for each *Karenia* clone was defined as a percentage of Saponin standards.

Statistical analysis was performed using SysStat 8.0. All data was log transformed and used in paired t-test analysis for determination of significant differences among growth rates and in toxicity of pairs. Non-parametric analysis using Wilcoxon tests were used to determine differences among all groups.

CHAPTER III

RESULTS

Growth Rate Results

Differences in growth among *Karenia* clones were determined by comparison of individual intrinsic growth rate (d^{-1}). Comparisons examined differences among clones from distinct geographic regions (Florida vs. Texas clones) as well as interspecific differences (*K. brevis* vs. *K. mikimotoi*). (Complete set of experimental results are provided in Appendix A). Growth rate as a function of temperature and salinity was first compared among *K. brevis* clones from within similar geographic regions.

Results for Florida *K. brevis* Clones

Growth exhibited by the Wilson's clone (CCMP 718) was used as a reference point to previously published studies (Baden and Tomas, 1988). Reported growth of CCMP 718 ranged from 0.2 to 1.0 d^{-1} (Aldrich and Wilson, 1968; Shanley and Vargo, 1993). CCMP 718 was among the slowest growing clones over all experimental conditions with lowest rate measured at 0.11 d^{-1} (20° C, salinity 27).

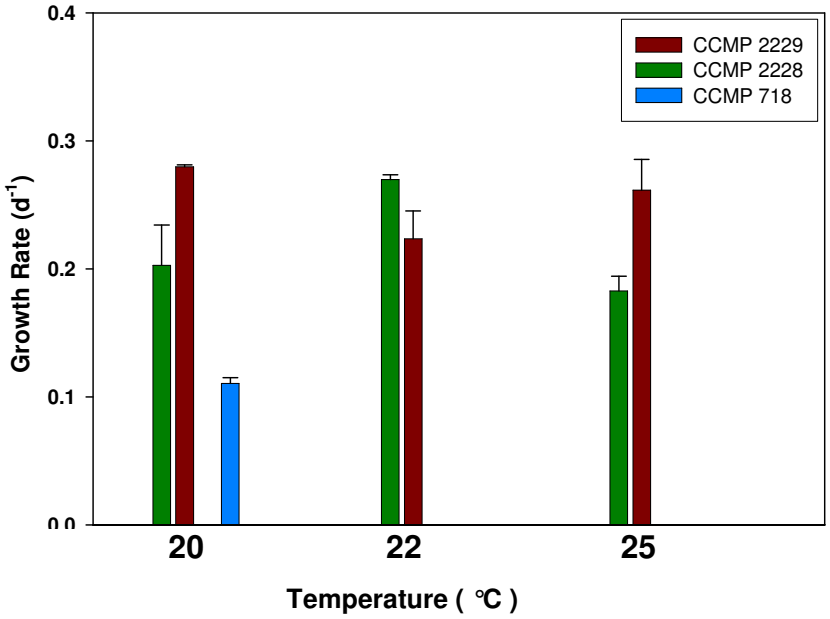


Figure 4. Growth rates of Florida *Karenia* clones grown at salinity of 27 and variable temperature.

Growth experiments were difficult at best with CCMP 718 as this clone was easily stressed from daily mixing and routinely crashed during first days of experiments. Cultures were also prone to failure when cell densities exceeded 6 x 10^4 cells mL^{-1}, requiring close monitoring to simply maintain cultures. Due to these difficulties, CCMP 718 was the sole *K. brevis* clone in which data points could not be attained for a full matrix of salinity and temperature variables.

In comparison to CCMP 718, CCMP 2228 was not as easily stressed during experimentation. CCMP 2228 exhibited optimal growth of 0.37 d^{-1} at 20° C and a salinity of 35 (Appendix A) and lowest growth at 0.18 d^{-1} at 25° C and a salinity of 27. Growth trends observed in CCMP 2228 for all three temperatures show slightly increased growth with increased salinity. Statistically the greatest difference in growth between salinities was observed at 20° C and were not as apparent at higher temperatures (Appendix A) ($p = 0.03$).

The last Florida *K. brevis* clone CCMP 2229, attained the greatest cell density of all *K. brevis* clonal cultures (up to 5 x 10^5 cells mL^{-1}). Optimal growth of CCMP 2229 was similar to other Florida clones at 0.32 d^{-1}(Figure 4). Temperature did not significantly affect CCMP 2229 ($p < 0.01$), but increased salinity improved growth from 0.27 d^{-1} to 0.32 d^{-1}.

Overall similar optimal growth rates were observed between Florida clones CCMP 2228 and CCMP 2229. Salinity changes seemed to elicit the highest degree of variability in growth among clones instead of temperature change.

Results of Texas *K. brevis* Clones

All experimental Texas *K. brevis* clones originated from a single bloom event. This provided a unique opportunity for comparison. Significant differences in growth among the Texas clones provided solid support for differences among *Karenia* clones within similar geographical locations.

As observed among the Florida clones, SP1 clones also had a limited range in growth during experimental procedures (Figure 5). SP1 was easily stressed from daily mixing in tubes, and like CCMP 718 was subject to frequent culture failure during experimentation. Growth was so significantly reduced at 20° C, that rates could not be calculated due to extended lag phase and absence of logarithmic growth at this temperature variable. Both CCMP 718 and SP1 clones succumbed under suboptimal temperature conditions, suggesting that temperature is a limiting factor in growth.

SP1 favorably responded to increased temperature and optimal growth was observed at 0.34 d^{-1} at 25° C. At both 22 and 25° C, increased salinity favored highest growth rates in SP1 (Appendix A). Growth was almost identical in SP1 cultures grown at salinities of 27 in both temperatures (0.18d^{-1}) and increased significantly when salinity was increased to 35 ($p =< 0.001$).

A dramatically different trend was observed in SP2 clones which favored lowered temperatures and significantly improved at mid salinity variables ($p =< 0.01$). The most significant differences in growth of SP2 were observed with fluctuation in salinity ($p =< 0.001$). SP2 responded negatively to increased salinity, dropping from 0.52 d^{-1} to only 0.17 d^{-1} (salinity of 27 vs 35).

In SP3 clones, a greater tolerance level to a wide variety of experimental variables was evident (Appendix A). SP3 could be maintained at all conditions with only a slight but insignificant decrease in averaged growth at increased salinity. Optimal growth was similar to previous clones at 0.37 d^{-1}. Greatest difference in growth was observed between SP3 cultures at 20° C with change in salinity. Growth significantly dropped from 0.36 to 0.17 d^{-1} when salinity was increased from 27 to 35 (20° C).

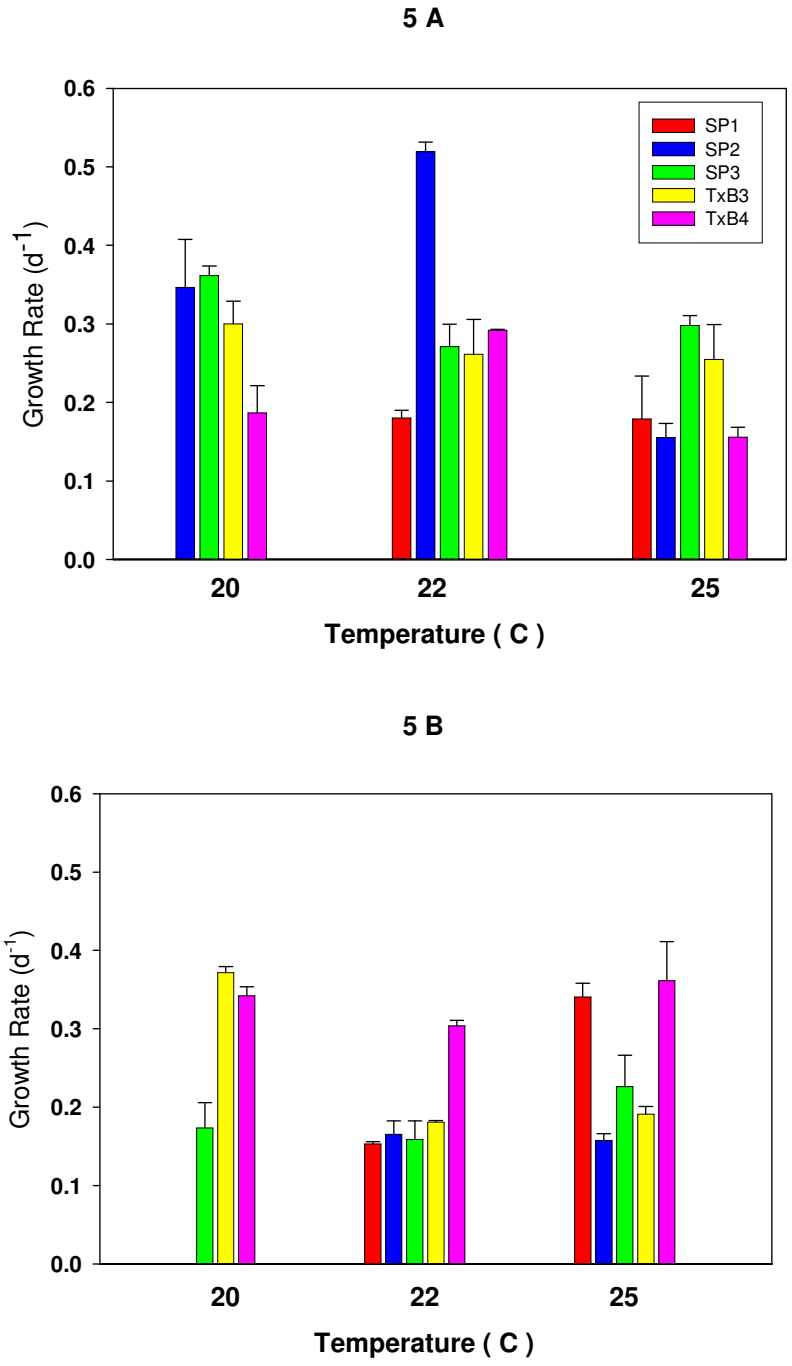


Figure 5. Growth rate for Texas *K. brevis* clones grown at A) Salinity of 27 (with variable temperature) and B) Salinity of 35 (variable temperature).

Optimal growth in TxB3 clones was 0.37 d^{-1} at low temperature and high salinity. On average, TxB3 favored mid salinity (Appendix A), growing at a steady rate of 0.33 d^{-1} at all three temperatures at a salinity of 30. Salinity also significantly impacted growth in TxB4 clones ($p =< 0.01$). Growth in TxB4 increased from 0.16 to 0.36 d^{-1} with an increase in salinity from 27 to 35. Temperature did not appear to have an impact on growth in TxB4 (Appendix A, Figure 5).

Results for *K. mikimotoi* Clones

Texas *K. mikimotoi* clone C5 grew consistently over the range of experimental temperatures, with most favorable growth observed at lower salinities (0.27 d^{-1}). Growth in C5 significantly decreased with increasing salinity ($p =< 0.001$), slowing from 0.30 to only 0.15 d^{-1} as salinity increased from 27 to 35 (25° C).

Most favorable growth in C9 was observed at highest salinity and temperature (0.55 d^{-1}). Optimal growth of C9 was significantly higher than C5 ($p =< 0.01$). Growth in C9 was significantly affected by variation in salinity; growth rate increased from 0.14 d^{-1} at low salinity increased to 0.55 d^{-1} at salinities of 35.

The highest growth rate among all five *K. mikimotoi* clones was observed in Texas B1 clone (0.61 d^{-1}) at 25° C and a salinity of 35. The B1 clone exhibited a wide range in growth over varying experimental conditions (0.10 d^{-1} to 0.61 d^{-1}). There was a significant difference in growth observed at high and low salinity; growth rate increased from 0.36 to 0.61 d^{-1} as salinity increased from 27 to 35.

NOAA2 was the only Florida *K. mikimotoi* clone available for comparison with Texas *K. mikimotoi.* Highest growth in NOAA2 (0.46 d^{-1}) was measured at

high salinity and low temperatures. Growth in NOAA2 was significantly impacted by changes in salinity, ranging from 0.31 to 0.46 d^{-1} as salinity decreased from 35 to 27 ($p =< 0.01$).

CCMP 429 was a difficult clone to acclimate to laboratory conditions. CCMP 429 originally isolated from cold temperatures in the English Channel, had been grown in K media which differs slightly from L1 (Guillard and Hargraves, 1993). Once CCMP 429 was established in a separate incubator at 17° C, it was then slowly acclimated to 20 and 22° C for growth rate analysis. Ultimately CCMP 429 could not be acclimated to 25° C. Optimal growth in CCMP 429 of 0.25 d^{-1} was observed at 22° C and a salinity of 35. Interestingly, lowest growth was observed at 17° C (0.08 d^{-1}).

On average, *K. mikimotoi* was a dynamic species with the ability to tolerate a wide range in salinity and temperature (Figure 6). *K. mikimotoi* was frequently observed to attain cell densities of over 3 x 10^6 cells mL^{-1}.

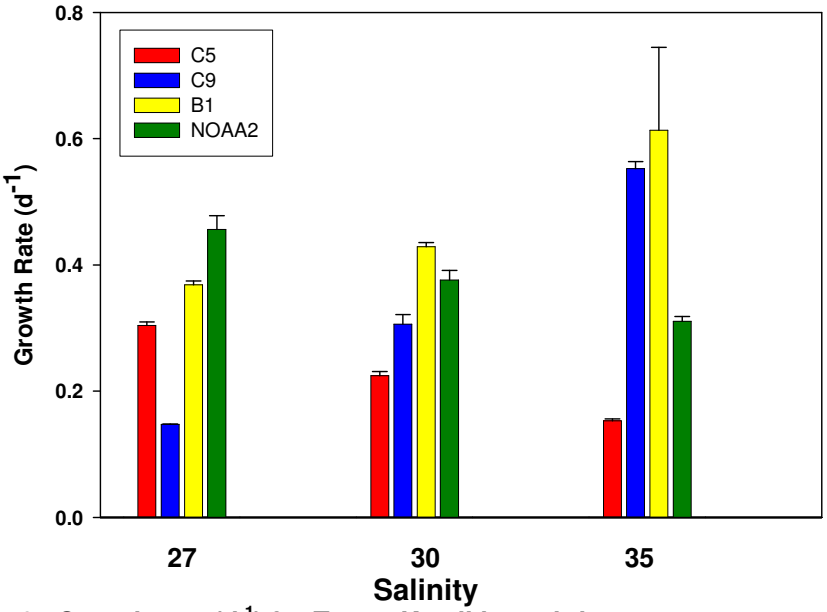


Figure 6. Growth rate (d^{-1}) for Texas *K. mikimotoi* clones grown over variable salinity at constant temperature of 25° C.

Comparison of Texas vs. Florida Clones

Texas *K. brevis* clones grew optimally over a wider range of salinity and temperature compared with Florida *K. brevis* clones. Texas *K. brevis* clones also exhibited higher average growth rates compared to Florida *K. brevis*. In Texas SP3 clone, the lowest observed growth rate was equivalent to highest observed growth rate of Florida Wilson's clone, CCMP 718.

Red Drum Assay Results

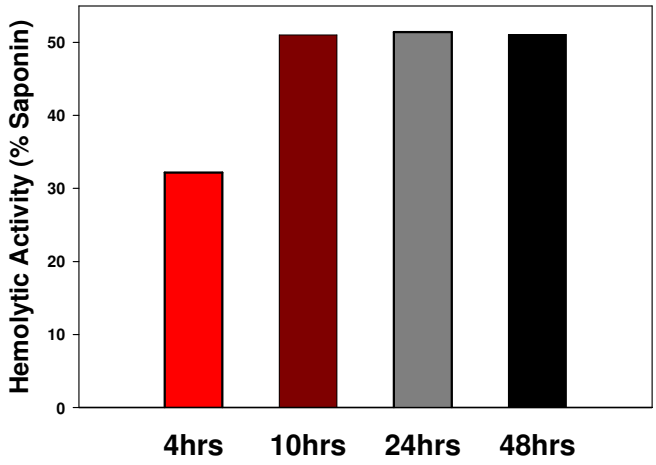
The Red drum erythrocyte assay was capable of detecting a wide variety of hemolytic activity among *Karenia* clones. Toxicity results averaged (n=3) from crude extract activity was defined as a percentage of Saponin standard. Highest hemolytic activity recorded among all *Karenia* clones was 85% of Saponin activity. Lowest observable activity was 12% of standard.

Saponin standards were run at concentrations of 2 mg mL^{-1}. 100% hemolysis was observed at concentrations of 4.5 µg mL^{-1} with lowest detectable activity observed at 1 µg mL^{-1}. Using averaged (n=3) absorption values from 100% lysis via Saponin, all subsequent hemolytic results were standardized to reflect percentages of Saponin activity.

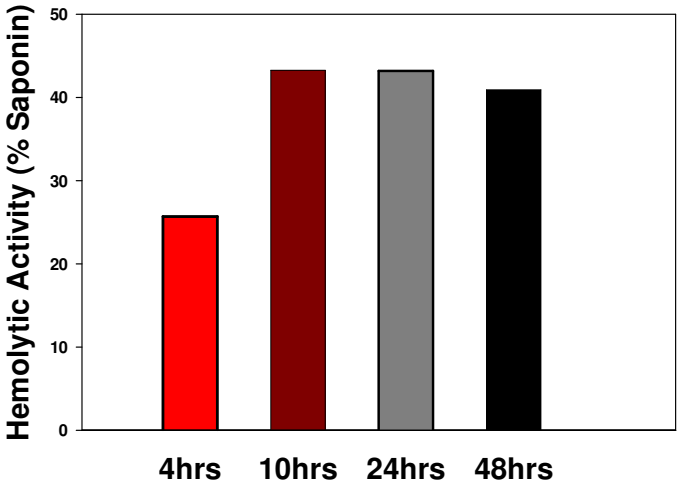
Preliminary Assay Results

Preliminary assays run with brevetoxin standard PbTx-3 (CalBioChem), confirmed that brevetoxin did not cause hemolysis in RBCs above background levels. PbTx-3 is the most common occurring brevetoxin derivative in Gulf of Mexico blooms and is regarded as the most highly toxic (Baden et al., 1995) Negative results with PbTx-3 show brevetoxin production in cultures does not affect hemolytic assay results.

To determine optimal incubation times, time course experiments were conducted to measure activity after 4, 10, 24 and 48 h incubations. Activity was examined in *K. mikimotoi* clone B1 and in *K. brevis* clone SP2. Short (4 h) incubations were not sufficient to detect the full range in activity. Ten hour incubations did not vary significantly from activity observed at 24 h (Figure 7). A slight decline in activity was observed from 24 to 48 h. Ten hour incubations were selected as optimal for detecting full activity in crude extracts (Figure 7). Hemolytic activity for all *Karenia* isolates was first examined in crude extracts from cultures grown at 25° C to determine if there were significant differences.



7 A



7 B

Figure 7. Time course incubation experiments of B1 (A) and SP2 (B). (Results are averaged hemolytic activity (n=3) at set times of 4 – 48 hours.)

Hemolytic Results of *K. brevis*

Hemolytic results for CCMP 718 ranged from 25 to 28% hemolysis from high to low salinity (25° C) (Figure 8). There was no statistical difference in activity between conditions ($t = 0.38$, $p = 0.72$). Growth rates and initial assay results indicate there was not significant variation in toxicity in CCMP 718 due to changes in salinity or temperature. No further toxicity assays were conducted as a result.

No significant difference was observed in activity with variable salinity or temperature in CCMP 2228 ($p = 0.03$). Activity in CCMP 2228 at 25° C averaged 44 to 46% with a slight decrease in activity from a salinity of 35 to 27. At 20° C activity did not significantly change averaging 41 to 43% hemolysis between high and low salinity ($p < 0.001$).

Hemolytic activity in CCMP 2229 averaged 60% at both high and low salinity (25° C). Highest activity in CCMP 2229 of 64% was exhibited in cultures grown in a salinity of 30, which was not significantly different with changes in salinity ($t = 0.28$, $p = 0.79$).

For Texas *K. brevis* clones, a wide range in activity was observed as individual clones were subjected to variable conditions. SP3 clones were the most prominent example with activity dropping significantly from 77 to 30% with a decrease in salinity from 30 to 27. Other Texas clones also exhibited a wide range in activity compared among each when grown within the same experimental conditions. Activity observed in SP3 at 25° C at 77% was more than two fold higher than activity of SP2 at the same temperature at activity less than 12% (Appendix B).

SP1 clones did not produce significantly different activity over variable salinity conditions ($p =< 0.001$). SP1 did significantly vary from Florida clones grown at identical conditions however.

In general, activity in SP2 clones decreased with increased salinity. Significant range in activity from 12 to 43% was observed with increase in salinity from 27 to 35 (25° C, p =< 0.001). Experiments conducted at 20° C did not show such strong trends as noticed in SP2 at 25° C as activity only varied from 20 to 23% with decrease in salinity.

SP3 clones exhibited the widest range in hemolytic activity among all *K. brevis* clones. A significant increase in activity from 25 to 78% was observed in clones grown in salinities from of low to mid salinity. Comparison between highest and lowest temperatures ranged from 32 to 42%. Overall, significant differences were observed in SP3 activity between all three salinities (p =< 0.001).

TxB3 similarly showed increased activity at mid salinities ranging from 33 to 59% from low to mid-salinity, and then dropped to 21% at highest salinity. These results were significant enough to continue assays at 20° C, where activity was similar observed at 25° C. TxB3 produced 32% activity at low salinity and increased to 43% at mid salinity before dropping once more to 30% with highest salinity.

TxB4 activity at 25° C ranged from 32 to 55% with decrease in salinity from 35 to 27, a significant difference (p =< 0.001). In 20° C, TxB4 was significantly more active salinity of 27 (39%) than higher salinity of 35 (28%, p =< 0.001). Overall, TxB4 increased toxicity as salinity decreased.

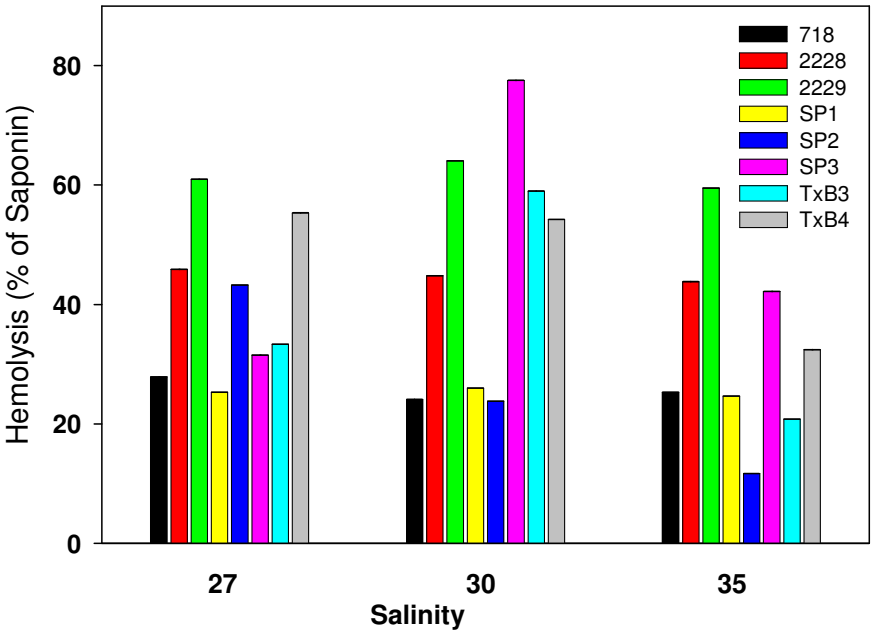


Figure 8. Hemolytic activities of *K. brevis* isolates over variable salinity (25° C).

Hemolytic Results of *K. mikimotoi*

On average hemolytic activity in *K. mikimotoi* was slightly higher than observed in *K. brevis* (46% versus 41%). Greatest differences between species were evident in cultures grown at salinities of 30 even throughout all temperature variables. Highest activity in *K. mikimotoi* clones was observed in B1 at 85% (35 salinity, 25° C) (Figure 9). Lowest activity was found in NOAA2 of only 18% (27 salinity). NOAA2 did increase to 40-42% with increased salinity at both 20 and25° C (Appendix B).

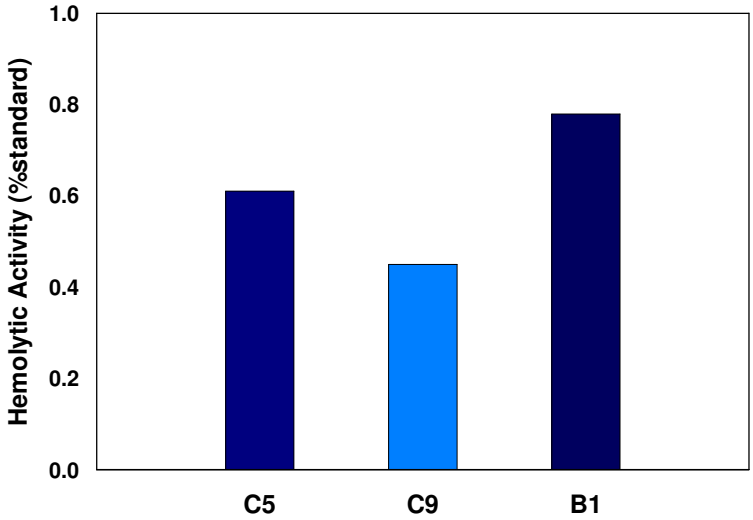


Figure 9. Hemolytic activity of *K. mikimotoi* clonal isolates grown at a salinity of 27 (25° C).

CCMP 429 could not tolerate salinity over 30 nor temperature exceeding 22° C. Throughout the limited range of growth conditions in CCMP 429, hemolysis activity remained relatively constant from 32 to 35% (Appendix B).

At 25° C similar trends were observed between Texas *K. mikimotoi* clones C5 and C9. C9 clones had significantly lower activity (57%) at low salinity which increased to 62% at salinity of 35. Increase in activity with increased salinity was also observed in C9 clones grown at low temperature, ranging from 56 to 60% ($p =< 0.001$). C5 clones also increased in activity with increased salinity ranging from 57 to 60%.

B1 clones displayed the highest hemolytic activity among all *Karenia* clonal isolates. At 25° C, activity was 51% (salinity 27) and increased to 85% with increased salinity (35) (Appendix B). The difference in activity among salinities at 25° C was statistically significant, but was not as varied when compared at 20° C.

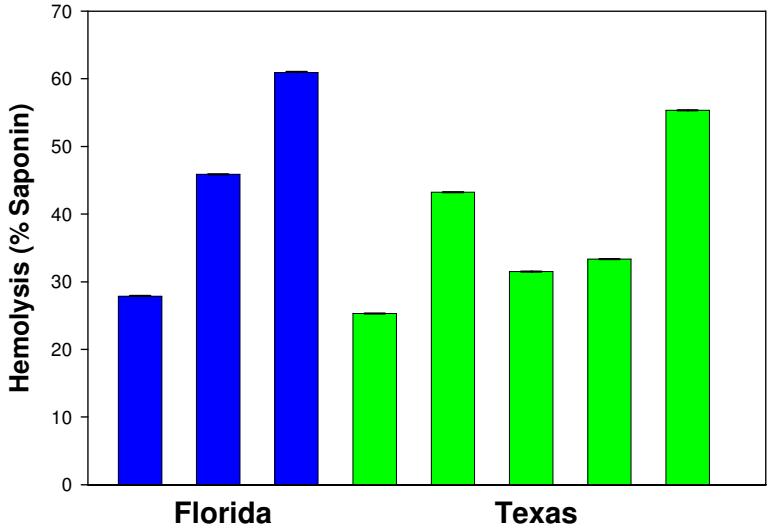


Figure 10. Hemolytic activities of *K. brevis* at 25° C and salinity of 30. (Florida clones are in blue, and Texas clones in green).

Toxicity over Time in Culture

To examine hemolytic activity of *K. mikimotoi* and *K. brevis* as cultures aged from time of inoculation, two clones of each species with the highest (B1 and SP3) and lowest activity (NOAA2 and SP2) were compared. All cultures were grown at 25° C and salinity of 30. At 4 days, B1 was the only clone which showed detectable activity at 32% (Figure 10, n=3). By 6 days, activity for B1 increased two-fold (64%), and activity was first evident for the other three strains. At 8 days, activity continued to increase for all strains. Finally at 10 days, hemolytic activity of B1 continued to increase (80%), but activity in the other three strains appeared to have leveled off. Activity for NOAA2 was 10%, SP2 was 20%, and SP3 was 67% (Figure 11).

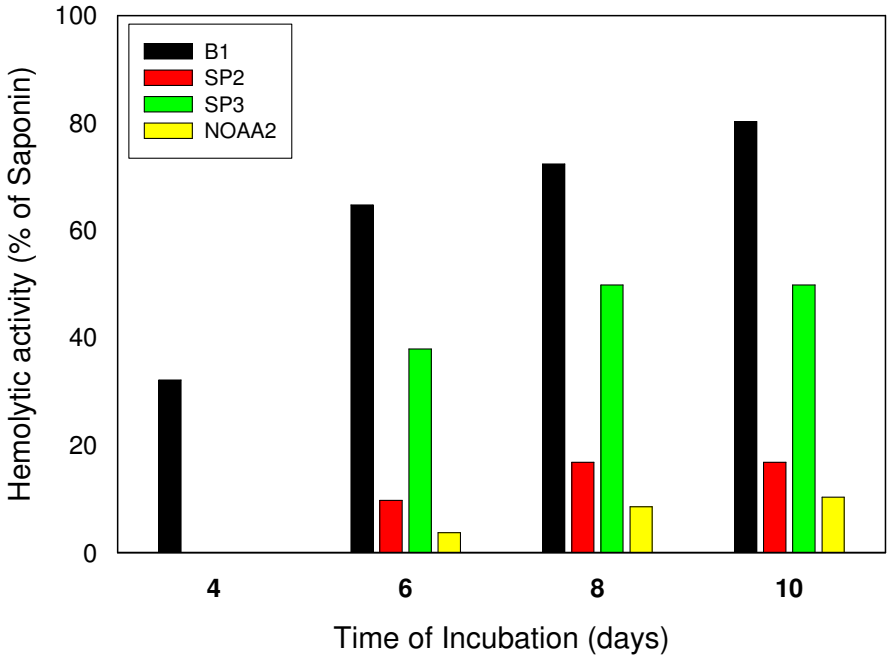


Figure 11. Time course of hemolytic activity in cultures of *K. brevis* (SP2 and SP3) and *K. mikimotoi* (B1 and NOAA2).

Gambusia Bioassay

All clones which exhibited hemolytic activity at salinity of 30 and 25° C were selected for the *Gambusia* bioassay. Florida *K. brevis* clones were all found to cause death usually within 10-15 min. Texas *K. brevis* clones caused death on average between 15-20 min. *K. mikimotoi* clones caused death with averages closer to 30-45 min., with the exception of CCMP 429 which was not found to cause death at any concentration within 1 h. All other clones were shown to cause death at cell concentrations as low as 5 x 10^3 cells mL^{-1}.

CHAPTER IV

DISCUSSION

Implications of Growth Rate Results

Results confirm previous observations of variability in growth rate among clones when grown under identical conditions (Aldrich and Wilson, 1960; Shanley and Vargo, 1993; Loret et al., 2002). Several experiments were repeated within this study for all experimental isolates over a two year span, with repeated results reliably within standard margin of error (CV < 0.05). This further demonstrated how growth rate can be a distinctive characteristic of particular clonal isolates within a set range of variables. If reported growth characteristics of the Wilson's clone have not significantly shifted in 50 years of culture, comparisons in growth rate could offer a valid factor in assessing variability among *Karenia* clones. Growth rates have been calculated using both direct cell counts and/or fluorescence measurements which can lead to discrepancies in reported growth rates. Fluorometric observation may ultimately underestimate total growth rates compared with direct cell counts, but offers a faster method in observing differences among strains with experiments conducted at multiple variables.

Growth results indicate individual *Karenia* clones are likely to have variable tolerances to changing environmental conditions. Among all *Karenia* clones examined, *K. mikimotoi* was able to grow at the widest range in salinity and temperature. Most *K. mikimotoi* clones were able to adapt to all conditions with the exception of CCMP 429.

Conversely, experiments with *K. brevis* indicated a more restricted range in growth conditions, with a majority of clones demonstrating stenohaline preference. On average, temperature did not seem to impact growth as significantly as salinity in most *K. brevis* clones.

K. brevis required longer acclimation period between conditions than *K. mikimotoi* before detectable (~1 x 10^3 cells mL^{-1}) cell density could be observed and growth experiments conducted (data not shown). *K. mikimotoi* clones generally could be expanded more rapidly with less lag time in growth when exposed to new conditions.

Given the high variability in estuaries and coastal waters in which *Karenia* blooms are frequently found, salinity and temperature may be important limiting factors in determining bloom and population dynamics. Genetically diverse phytoplankton blooms exposed to rapid environmental shifts, could potentially respond with the propagation of one species over the other (Steidinger and Penta, 1999). Enhanced phytoplankton biomass has frequently occurred along regions of strong environmental gradients. Our results further demonstrate salinity as a strong limiting factor in the geographic distribution of *K. brevis*. These results provide evidence to suggest that changes in salinity may ultimately shift phytoplankton populations from less tolerable *K. brevis* clones towards more quickly adaptable *K. mikimotoi* clones.

This hypothesis is further supported by the geographic distribution of both species. Currently *K. brevis* is only known to occur in the Gulf of Mexico (Steidinger and Penta, 1999; Geesey and Tester, 1993). *K. mikimotoi,* however, is known worldwide and is reported from regions with a wide range of salinity, temperature and nutrient concentrations (Haywood et al., 1996; Haywood et al, 2004; ILO, 1984; Ishida et al., 1996; Kin-Chung et al., 1991). Growth rate results from this study lend further evidence to suggest *K. mikimotoi* may be a more highly dynamic species than *K. brevis*, particularly in its ability to adapt to fluctuation in salinity or temperature.

Validity of Red Drum Assay

The purpose in developing an erythrocyte bioassay was the need for a simple, rapid and inexpensive method to use as a preliminary screen for

hemolytic activity among various clonal isolates. The advantage in using fish erythrocytes was of an environmentally relevant method with the capability of screening a wide range of activity levels. Bioassays are particularly advantageous as they are capable of detecting unknown and poorly defined compounds. Using the Red drum assay, we were able to detect hemolytic activity induced by *Karenia* cultures down to levels of 1 x 10^3 cells mL^{-1}. The Red drum assay was thus determined to be a valid method in identifying the presence of hemolytic activity of crude algal extracts.

Red drum RBCs were found to have high sensitivity to Saponin, a widely used standard hemolytic compound. Sensitivity of Red drum RBCs to Saponin was detectible over a measurable range in concentrations. This allowed for more sensitive comparisons to be made between clonal isolates relative to Saponin standards.

Control experiments with *Dunaliella* and *Chattonella* cultures further demonstrate the applicability of the Red drum assay. As expected, no hemolysis was induced by the non-toxic *Dunaliella,* nor was there any interference in absorption. *Chattonella* was used to provide a positive control to ensure hemolytic activity was detectable (Yamamoto and Tanaka, 1990; Haque and Onoue, 2002). *Chattonella* was found to induce 52% hemolysis of RBCs. These results supported the applicability of the Red drum assay as a valid procedure for a variety of HAB species.

Implications of Hemolytic Activity

Based on the Red drum assay, both *Karenia* species produce detectible levels of hemolytic activity. Activity among *Karenia* clones ranged from 11 to 85%. On average *K. brevis* induced less hemolysis compared with *K. mikimotoi* clones. Activity in *K. brevis* ranged from 11 to 77%. *K. mikimotoi* clones frequently induced hemolysis well above 50% and were responsible for highest detected activity of over 80%.

Growth conditions, particularly salinity of medium, had significant effects on hemolytic activity in both *K. brevis* and *K. mikimotoi*. Previous studies conducted with *Alexandrium* spp. also indicated PSP toxicity levels fluctuated with salinity changes (Frangopulos, et al., 2004; Anderson 1990). Lowest growth rates in *Alexandrium* were found to correlate with decreased salinity and increased toxicity (Hamasaki, et al., 2001). Our results for hemolytic activity indicate SP2 follows a similar trend of increased toxicity at lower growth rates.SP2 may produce higher levels of hemolytic toxin as a stress response as indicated by decreased growth. Conversely, increased growth rate in TxB3 and TxB4 coincided with increased hemolytic activity. Both TxB3 and TxB4 were significantly more toxic at higher salinities when compared with lower salinities ($p =< 0.001$); a unique response compared with other clonal isolates with the exception of B1.

Wright and Cembella (1998) comment on the significant variation in total cellular toxin content among monoclonal cultures, and suggest increased toxicity may be a direct result of stress due to environmental conditions. Our results offer further support that toxin variability may result from environmental fluctuation. Salinity change in particular was found to enhance toxicity in several *K. brevis* and *K. mikimotoi* clones.

There have been several studies which have indicated variability of brevetoxin production among *Karenia* isolates. Baden and Tomas (1988) were among the first to report substantial differences in brevetoxin profiles among six clonal *Karenia* isolates from Florida. Loret et al. (2002) reported similar differences in brevetoxin production among three *Karenia* isolates from the Texas coast. Attempts were made to incorporate as many different *Karenia* clones from the Gulf of Mexico as possible. By examining toxicity of clonal isolates taken from a single bloom off Texas, we were able to conclude there are significant differences among strains within a single bloom event.

In addition to comparisons among thirteen different clonal isolates, this study also provides results to reflect variation within a single clone when subjected to a range of environmental variables. These results show both

presence and variation of hemolytic activity in addition to brevetoxin, and can further aid in defining variability of total toxicity. Overall toxicity of a particular clone including assessment of both brevetoxin and hemolysin concentrations, may be significantly higher than a single-target toxin bioassay would reflect. Therefore the potential for hemolysin contribution in overall bloom toxicity is an important factor which should be included in future studies and bloom assessment.

The surprising results of *K. mikimotoi* inducing higher hemolysis than *K. brevis* has many implications. Since little is known regarding *K. mikimotoi* toxicity in the Gulf of Mexico, its contribution to overall bloom toxicity is not currently addressed in most monitoring programs. These results show both the ability of *K. mikimotoi* to tolerate much wider variation in environmental conditions, and to produce higher levels of hemolytic compounds compared with *K. brevis*.

K. mikimotoi clones NOAA2, C5 and B1 all exhibited increased hemolytic activity following increase in salinity. *K. mikimotoi* clones show a wide variety of trends in toxicity according to results of the Red drum assay. C9 was the only clone in which increased activity was exhibited at lower salinity. While CCMP 429 remained stable over changes in salinity, toxin production increased with increase in temperature. An inverse relationship was discovered between growth rate and toxin production most *K. mikimotoi* isolates except for B1. At high salinity, increased growth rate in B1 was accompanied by increased toxicity. B1 was additionally unique as it was found to induce the highest level of hemolytic activity among all *Karenia* clones.

Experiments conducted with two most highly active *Karenia* isolates B1 and CCMP 2229, concluded that levels of toxicity peaked in *K. brevis* (CCMP 2229) flasks at lower cell density and overall toxicity than *K. mikimotoi (*B1). This was consistent with the observation that *K. mikimotoi* could sustain dense cultures for longer periods of time. Increased biomass could potentially concentrate progressively higher toxin levels than *K. brevis* flasks which rapidly declined after surpassing particular levels in cell density.

Accurate detection of species abundance and respective toxin concentration is essential in monitoring HABs in the Gulf of Mexico (Wang et al., 2004). Population dynamics in taxonomically diverse blooms may potentially shift following environmental fluctuation. Synergism of toxic effects could also occur with presence of multiple toxins during blooms, which may be overlooked by more highly specified assays (Haywood et al., 2004). Overall, this research has demonstrated that *K. mikimotoi* is a toxic species in the Gulf of Mexico and its presence during "red tide" blooms is an essential component of the resulting toxicity. Further studies are needed to investigate the propagation of toxicity to higher trophic levels.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

In summary we found that there are significant differences observed in growth rates among clonal isolates of *Karenia*. There can additionally be significant differences in growth within a single clone when subjected to variable experimental conditions.

We found that the Red drum Assay is a valid assessment of presence of hemolytic compounds which is not sensitive to the presence of brevetoxin compounds. We found both species of *Karenia* capable of producing detectable levels of hemolytic activity. There were some observable differences in hemolytic activity when compared among clonal isolates collected from a single Texas HAB event. We were not however able to determine if there was a significant difference in hemolytic activity compared between Florida and Texas isolates, as clonal isolates varied too greatly in overall time in culture.

Conclusions

These results support the hypothesis that both *K. brevis* and *K. mikimotoi* produce hemolytic compounds which may contribute to overall toxicity of blooms in the Gulf of Mexico. Comparisons among twelve different *Karenia* isolates from various geographical areas throughout the Gulf of Mexico show significant differences in both growth rate and hemolytic activity. This supports the hypothesis that *Karenia* is a widely diverse genus and differences can be detected from individual clonal isolates from various bloom events. By testing the effects of salinity and temperature upon growth and toxicity in *Karenia*, we have provided further evidence that environmental factors can affect population dynamics and toxicity of blooms.

With development and use of the Red drum erythrocyte assay, we were able to examine differences in *Karenia* beyond variable growth and brevetoxin production to the additional variability of hemolytic activity among isolates. This research helps to define overall variability within the *Karenia* genus. *Karenia* is therefore further represented as a genus which requires additional study to achieve a better understanding of the dynamics of harmful algal blooms in the Gulf of Mexico.

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APPENDIX A

Growth rate data of all *Karenia* clonal isolates for each experimental condition.
(ng = no growth observed at this condition; nd = no data could be determined)

Strain	Temperature	Salinity	Growth	St.dev
718	17	27	*ng*	
		30	*ng*	
		35	*ng*	
	20	27	0.11	0
		30	0.17	0.01
		35	*nd*	
	22	27	*nd*	
		30	*nd*	
		35	*nd*	
	25	27	0.16	0.01
		30	*nd*	
		35	*nd*	
2228	17	27	*ng*	
		30	*ng*	
		35	*ng*	
	20	27	0.2	0.03
		30	0.17	0
		35	0.37	0.05
	22	27	0.27	0
		30	0.19	0.02
		35	0.26	0.01
	25	27	0.18	0.01
		30	0.21	0
		35	0.21	0.03
2229	17	27	*ng*	
		30	*ng*	
		35	*ng*	
	20	27	0.28	0
		30	0.32	0.04
		35	0.32	0.01
	22	27	0.22	0.02
		30	0.24	0.04
		35	0.19	0.01
	25	27	0.26	0.02
		30	0.27	0.07
		35	0.29	0.01

Strain	Temperature	Salinity	Growth	St.dev
SP1	17	27	*ng*	
		30	*ng*	
		35	*ng*	
	20	27	*nd*	
		30	*nd*	
		35	*nd*	
	22	27	0.18	0.01
		30	0.15	0.06
		35	0.24	0
	25	27	0.18	0.05
		30	0.19	0
		35	0.34	0.02
SP2	17	27	*nd*	
		30	*nd*	
		35	*nd*	
	20	27	0.35	0.06
		30	0.27	0.01
		35	*nd*	
	22	27	0.52	0.01
		30	0.3	0.02
		35	0.17	0.02
	25	27	0.16	0.02
		30	0.18	0.09
		35	0.16	0.01
SP3	17	27	*nd*	
		30	*nd*	
		35	*nd*	
	20	27	0.36	0.01
		30	0.36	0.04
		35	0.17	0.03
	22	27	0.27	0.03
		30	0.32	0
		35	0.16	0.02
	25	27	0.3	0.01
		30	0.37	0.08
		35	0.23	0.04

Strain	Temperature	Salinity	Growth	St.dev
TxB3	17	27	*nd*	
		30	*nd*	
		35	*nd*	
	20	27	0.3	0.03
		30	0.33	0.02
		35	0.37	0.01
	22	27	0.26	0.04
		30	0.33	0.06
		35	0.18	0
	25	27	0.25	0.04
		30	0.33	0.03
		35	0.19	0.01
TxB4	17	27	*nd*	
		30	*nd*	
		35	*nd*	
	20	27	0.19	0.03
		30	0.31	0.01
		35	0.34	0.01
	22	27	0.29	0
		30	0.3	0
		35	0.3	0.01
	25	27	0.16	0.01
		30	0.28	0.01
		35	0.36	0.05
NOAA2	17	27	*ng*	
		30	*ng*	
		35	*ng*	
	20	27	*nd*	
		30	*nd*	
		35	*nd*	
	22	27	*nd*	
		30	*nd*	
		35	*nd*	
	25	27	0.46	0.02
		30	0.38	0.02
		35	0.31	0.01

Strain	Temperature	Salinity	Growth	St.dev
429	17	27	0.08	0
		30	*nd*	
		35	*nd*	
	20	27	0.14	0
		30	*nd*	
		35	*nd*	
	22	27	*nd*	
		30	*nd*	
		35	0.25	0.01
	25	27	*ng*	
		30	*ng*	
		35	*ng*	
C5	17	27	*nd*	
		30	*nd*	
		35	*nd*	
	20	27	0.28	0.01
		30	0.22	0.02
		35	0.2	0.01
	22	27	0.23	0.01
		30	0.17	0.01
		35	0.15	0
	25	27	0.3	0.01
		30	0.22	0.01
		35	0.15	0
C9	17	27	*nd*	
		30	*nd*	
		35	*nd*	
	20	27	0.44	0.02
		30	0.22	0
		35	0.34	0.01
	22	27	0.22	0.01
		30	0.22	0.01
		35	0.38	0.02
	25	27	0.15	0
		30	0.31	0.02
		35	0.55	0.01

APPENDIX B

Hemolytic activities of *Karenia* clonal isolates grown under a range of temperatures and salinities (n=3).

Strain	Temperature	Salinity	Hem. Activity	SD	*Gambusia*
718	20	27	27%	0.104	+
		30	26%	0.009	+
		35	24%	0.03	+
	25	27	28%	0.034	+
		30	24%	0.018	+
		35	25%	0.006	+
2228	20	27	43%	0.004	+
		30	42%	0.003	+
		35	41%	0.013	+
	25	27	46%	0.008	+
		30	45%	0.01	+
		35	44%	0.008	+
2229	25	27	61%	0.087	+
		30	64%	0.008	+
		35	59%	0.007	+
SP1	25	27	25%	0.007	+
		30	26%	0.009	+
		35	25%	0.016	+
SP2	20	27	23%	0.007	+
		30	23%	0.029	+
		35	20%	0.013	+
	25	27	43%	0.002	+
		30	24%	0.005	+
		35	12%	0.003	+
SP3	20	27	30%	0.007	+
		30	38%	0.009	+
		35	32%	0.007	+
	25	27	31%	0.012	+
		30	77%	0.048	+
		35	42%	0.035	+

Strain	Temperature	Salinity	Hem. Activity	SD	*Gambusia*
TxB3	20	27	32%	0.007	+
		30	43%	0.026	+
		35	29%	0.042	+
	25	27	33%	0.004	+
		30	59%	0.016	+
		35	21%	0.01	+
TxB4	20	27	39%	0.048	+
		30	33%	0.008	+
		35	27%	0.02	+
	25	27	55%	0.01	+
		30	54%	0.005	+
		35	32%	0.023	+
NOAA2	20	27	19%	0.016	-
		30	39%	0.018	+
		35	40%	0.01	+
	25	27	18%	0.003	-
		30	37%	0.01	+
		35	42%	0.006	+
429	17	27	32%	0.011	-
		30	32%	0.006	-
	20	27	35%	0.007	+
		30	32%	0.002	+
C5	20	27	57%	0.011	+
		30	58%	0.007	+
		35	60%	0.02	+
	25	27	57%	0.02	+
		30	59%	0.015	+
		35	62%	0.01	+

Strain	Temperature	Salinity	Hem. Activity	SD	*Gambusia*
C9	20	27	29%	0.078	-
		30	38%	0.007	+
		35	44%	0.003	+
	25	27	42%	0.003	+
		30	32%	0.006	+
		35	23%	0.006	+
B1	20	27	37%	0.015	+
		30	32%	0.116	-
		35	38%	0.003	+
	25	27	51%	0.007	+
		30	80%	0.01	+
		35	85%	0.028	+

VITA

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