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The University of Southern Mississippi

STRESSED INDUCED CHANGES IN  
*KARENIA BREVIS* RIBOSOMAL RNA

by

David Scott Jayroe

A Thesis

Submitted to the Graduate School  
of The University of Southern Mississippi  
in Partial Fulfillment of the Requirements  
for the Degree of Master of Science

Approved:

Dr. Shahid Karim  
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May 2015

ABSTRACT  
STRESSED INDUCED CHANGES IN  
*KARENIA BREVIS* RIBOSOMAL RNA

by David Scott Jayroe

May 2015

*Karenia brevis* is a toxic marine dinoflagellate that causes harmful algal blooms (HABs), also known as red tides, in the Gulf of Mexico. These blooms are responsible for massive fish kills, shellfish bed contaminations, adverse human health effects, and vast economic loss. For these reasons, extensive research has gone into understanding the mechanisms and dynamics of bloom behavior by studying *K. brevis* in the field and in the lab. In order to understand higher order bloom behavior and dynamics it is imperative to understand *K. brevis* at the cellular level. In growing *K. brevis* in vitro under a variety of conditions, we have noted a distinct shift in the size of both ribosomal RNAs upon culturing cells under “stress” conditions, namely nutritional stress, cold shock, and decreased salinity. When the total RNA is extracted we have detected aberrant rRNA bands on the microcapillary electrophoresis BioAnalyzer (Agilent, Inc.). Interestingly when stressed, the large ribosomal subunit (LSU) becomes larger in size, and the small ribosomal subunit (SSU) becomes smaller. The initial hypothesis was that these aberrant bands were from some intracellular organism which was escaping a dying host. Through microscopy we were not able to support this hypothesis. Subsequently we also considered this response to be something *K. brevis* does in response to stress. This response by *K. brevis* had not been previously described in the literature. RNAs, pre- and post-stress, are being fully sequenced to determine how they are different, and what mechanisms may be responsible for producing them: alternative splicing, different transcriptional initiation or

termination sites, or different loci? These results will help us understand the molecular events surrounding *K. brevis* survival under certain environmental conditions, which may have implications regarding *K. brevis* biogeographical distribution and bloom termination.

## DEDICATION

I would like to thank and dedicate this thesis to my family, including my grandmother Thelma Jayroe who pushed me to pursue what I love. Huge thanks go to my parents Tom and Lynn Jayroe for constant support in all facets. My fiancé/wife Jessica Long always believed in my abilities, and kept me going through this whole process with encouragement. My brothers Tom and Pat Jayroe were instrumental in keeping me focused and reminding me this will result in a better future, and for that I thank them. To my grandparents who have passed, thanks for everything you have instilled in me. I am grateful to all of the friends I have made while living in Hattiesburg, MS, who welcomed me with open arms.

## ACKNOWLEDGMENTS

Many people have contributed to this thesis, including the effort it took to gather data, and support away from the laboratory. None of this was possible without my committee members Dr. Tim McLean, Dr. Shahid Karim, and Dr. Glen Shearer. Many thanks to several fellow graduate students along the way including, Helen Namataka, Scott Anglin, Cybil Covic, and Issac Akogwu. Special thanks to Cynthia Littlejohn for being a great microbiology lab teaching supervisor. To everybody involved I want to say thank you for your persistence, and patience.

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## CHAPTER I

### INTRODUCTION

Dinoflagellates are a diverse group of single-celled eukaryotic organisms inhabiting freshwater and marine environments. They vary in survival from photosynthetic, parasitic, symbiotic, predacious, or a combination of two or more, making some mixotrophic organisms. Dinoflagellates can serve as hosts for a variety of intracellular symbionts, including viruses, bacteria, fungi, and protists (Coats and Parks, 2002). The most notable dinoflagellate relationship is in symbiosis with corals, in which the coral polyp serves as a housing structure, and the dinoflagellate utilizes photosynthesis for the production of carbohydrates and oxygen which is used by both organisms. Not all dinoflagellate interactions with other organisms are symbiotic; some partners can actually prey on the photosynthetic capabilities of dinoflagellates. Bacteria and dinoflagellates also have close interactions, and it is generally believed that bacteria inhabiting cytoplasm and/or the nucleus of dinoflagellates have commensal or mutualistic relationships with their hosts (Coats and Parks, 2002). Approximately 45 dinoflagellate species are considered harmful algal bloom (HAB) species because of their detrimental environmental and human health impacts (Monroe and Van Dolah, 2008). Several human illnesses stem from consuming seafood contaminated with dinoflagellate produced toxins, including paralytic shellfish poisoning (PSP), azaspiracid poisoning (AZP), ciguatera fish poisoning (CFP), diarrhetic shellfish poisoning (DSP), and neurotoxic shellfish poisoning (NSP) (Monroe and Van Dolah, 2008).

*Karenia brevis* is a microscopic marine dinoflagellate that primarily occurs in the Gulf of Mexico. It usually is found blooming off the coast of Florida, Texas, and

occasionally the south east coast of Mexico. *K. brevis*, is the organism responsible for Florida red tides (MacLean, 1979). Florida red tide is a common name for *K. brevis* blooms. *K. brevis*, which is always present at background levels in the Gulf of Mexico, undergoes a population increase (Pierce et al., 1989). This population increase is called a bloom. Although atypical, some blooms occurring off the west coast of Florida have persisted up to 2 years (Walsh et al., 2008). Typically *K. brevis* blooms begin to form in autotrophic waters during the fall. As waters warm into early summer, cells grow and divide causing the bloom to intensify. Blooms can be concentrated or transported by physical features such as meteorological density fronts or ocean currents (Redalje et al., 2008). When a bloom is harmful to fish, mammals, or humans it is called a harmful algae bloom, or HAB. If a bloom is visible to the naked eye it is called a “red tide.”

Interestingly *K. brevis* is not red, but actually a green-yellow color when densities are high enough to be seen in the ocean. Most red tides (98%) are harmless, and just discolor water. Only a fraction (2%) are harmful due to physical damage, nutrient unsustainability, or the production of biotoxins, as in the case of *K. brevis*.

Dinoflagellates stand out from other known HAB species, comprising 75% of all HABs (Symayda, 1997). *K. brevis* makes a suite of toxins called brevetoxins which are intracellular until the cell is broken open. A toxic aerosol is created when the unarmored dinoflagellate is broken apart by wave action releasing the toxin into the water where it is free to combine with the salt spray produced by the wind and waves (Pierce et al., 1989). When brevetoxins are inhaled it causes acute upper respiratory irritation in healthy humans and more severe irritation in humans with chronic lung problems, such as asthma, emphysema, or cystic fibrosis. Brevetoxins are what make this dinoflagellate so

distinguishable. Brevetoxins produced by *K. brevis* can kill marine mammals, fish, and other marine creatures. Brevetoxins are tasteless, odorless, and very stable, making them nearly impossible to identify without the proper assay (Kirkpatrick et al., 2004).

Brevetoxin is also known as PbTx, and there are many derivatives (Brown et al., 2008).

There are three effects of the brevetoxins that commonly associate with *K. brevis*. The first is massive accumulations of dead fish washing up on the shore. The massive fish kills associated with Florida red tides result from the neurotoxin exposure to the fish in the environment (Kirkpatrick et al., 2004). The second is a contaminated shellfish poisoning syndrome called neurotoxic shellfish poisoning (NSP). When humans consume shellfish contaminated with brevetoxins they will most likely suffer from NSP

(Kirkpatrick et al., 2004). The brevetoxins bind to voltage-gated sodium channels leading to depolarization of nerve cells which disrupt respiratory and cardiac function. NSP causes tingling of the lips, dizziness, coordination problems, and gastrointestinal symptoms like diarrhea, nausea, and vomiting. One particularly interesting effect is the reversal of temperature sensations (Kirkpatrick et al., 2004). The third is inhalation of airborne toxin causing respiratory irritation, stinging eyes and nose, and a choking cough (Magana et al., 2003). Brevetoxins can be transferred from water to air in wind-powered white-capped waves. Inhalation exposure to marine aerosol containing brevetoxins causes upper respiratory symptoms, including an unproductive cough. (Cheng et al., 2005).

These brevetoxins are depolarizing substances that cause voltage gated sodium ( $\text{Na}^+$ ) ion channels to open in cell membranes, resulting in uncontrolled  $\text{Na}^+$  influx into the cell, (Kirkpatrick et al., 2004). This alters the membrane properties of excitable cell types in ways that enhance the inward flow of  $\text{Na}^+$  ions into the cell (Kirkpatrick et al., 2004).

The respiratory problems associated with the inhalation of aerosolized Florida red tide toxins are believed to result from the opening of sodium channels of nerve cell membranes by the brevetoxins (Kirkpatrick et al., 2004).

Although rare, occasionally blooms can be detected in the Northern Gulf of Mexico (NGOMEX). Brown et al. (2006) stated that the first recorded bloom of *K. brevis* in the low salinity waters of the (NGOMEX) occurred in November 1996. Data suggest that this bloom started on the northwest Florida Shelf where salinity is lower than the rest of Florida, and was transported by an unusual westward surface current from Tropical Storm Josephine. *K. brevis* blooms in the U.S. have been characterized by high salinities: early studies showed quite clearly that *K. brevis* was not present, nor could not grow at low salinities (Brown et al., 2006). Due to the drainage of the Mississippi river into the NGOMEX, salinity is quite lower than the east and west Gulf of Mexico. Salinities in the NGOMEX can range from zero to greater than 25 practical salinity units or PSU depending on location and time of year, and *K. brevis*' optimal salinity range is between 20 and 45 PSU (Brown et al., 2006). Interestingly Brown et al. (2006) mention that 24 PSU was thought to be the salinity barrier for this organism. Prior to this isolated bloom, and subsequent studies, *K. brevis* was believed to not be able to tolerate the NGOMEX waters, and therefore, Alabama, Mississippi, and Louisiana were purportedly out of threat.

Parasitism amongst dinoflagellates is a widespread phenomenon, and parasitic infections among HAB species is being investigated to be used as a biological control tool in the managing of HABs. It has been shown that parasite-induced death or decreased growth rate have strong influences on dinoflagellate population dynamics

(Coats and Park, 2002). Dinoflagellates can often have parasitic or symbiotic relationships with intracellular organisms, many of which are other species of dinoflagellate. Some dinoflagellate species are known to be parasites of other dinoflagellates (Erard-Le Denn et al., 2000). Dinoflagellates are part of a group of unicellular organisms including apicomplexans and ciliates that are capable of intracellular phototrophy parasitism, and predation, suggested to be extremely dissimilar capabilities amongst eukaryotes (Kuvardina et al., 2002). Parasites of phytoplankton belong to several taxonomic levels including viral, fungal, protists, and bacteria (Erard-Le Denn et al., 2000). It should also be noted that there is currently a single strain of *K. brevis* which is axenic, and very recently researchers have shown that not a single lab strain or environmental isolate has the same bacterial community associated with them (unpublished data, 7th Symposium on Harmful Algae in the U.S., Sarasota, FL, 2013). This observation supports the long-held belief that some mechanisms of *K. brevis* blooms, specifically growth, toxicity and dynamics, are intertwined or dependent upon the bacteria in the oceanic environment, and/or those living with, on, or around *K. brevis* cells. Bacterial diversity between blooms is adding to the challenge of forecasting and predicting aspects of bloom dynamics and bloom toxicity.

*K. brevis* blooms are intense, and they have the ability to completely deplete an area of nutrients. The question comes to mind as to where these blooms are getting these nutrients. Source of major nutrients nitrogen & phosphorus required to maintain blooms, some up to 18-24 months, in an area has not been identified. Research suggests sources of nutrients include aerial deposition, estuarine flux, benthic flux, zooplankton excretion, N<sub>2</sub>-fixation, and subsequent release of organic and inorganic nitrogen by *Trichodesmium*

spp., (Vargo et al., 2007). Essentials to population maintenance can also come from remineralization of fish and other vertebrates, and invertebrates killed by brevetoxins produced during *K. brevis* blooms. It is not just one phenomenon that supplies nutrients to *K. brevis*, it is a combination of sources needed to maintain a population (Vargo et al., 2007). A source of nitrogen needed by *K. brevis*, comes as organic nitrogen after a species of cyanobacterium called *Trichodesmium* fixes the inorganic nitrogen in the sea water. *Trichodesmium* takes advantage of Saharan dust that are blown from the west coast of Africa and deposited on the west side of Florida. *Trichodesmium* is able to utilize the iron in the dust to make an iron complex that helps to fix the nitrogen (Walsh et al., 2008). Besides estuarine flux no other sources of phosphorus have been identified (Vargo et al., 2007). Nitrate uptake is another biologically interesting factor about *K. brevis*. Because of its ability to move vertically within the water column, *K. brevis* it can get nutrients from the sediment-water interface, and migrate up during the day to maximize photosynthesis (Sinclair et al., 2006). Being mobile allows *K. brevis* to get its nitrates from other sources than those that deliver nutrients into the environment, which is suggested to be the strongest influence on nutrient availability to *K. brevis*. This adaptation allows *K. brevis* to thrive in places where it gets blown, or transported by water currents (Sinclair et al., 2006).

*K. brevis* also maintains its blooms by allelopathic mechanisms. Allelopathy is a process by which organisms release compounds to inhibit the growth of competitors (Prince et al., 2008). During *K. brevis* blooms it exudes potent allelopathic compounds. Phytoplankton species vary in their susceptibility to *K. brevis* allelopathy, but many *K. brevis* blooms usually become monospecific. The mechanism acts by lowering the

photosynthetic efficiency of competitors and increasing their membrane permeability resulting in competitor death (Prince et al., 2008). Prince et al., (2008) acknowledge that our understanding of allelopathy is still in its early stages, and the compounds remain mostly unidentified. Conducting field studies remains a challenge

*Karenia brevis* has now been studied for almost 60 years. Research underlying its molecular biology has only begun in the last 15 years, enabled by the availability of rapidly evolving molecular technologies from the biomedical field (Van Dolah et al., 2009). Bloom dynamics from initiation, maintenance, and termination processes remain poorly understood but are critical for the prediction and management of bloom impacts. Lack of information on these processes may in large part be because they require a more thorough understanding of the molecular and cellular nature of dinoflagellates (Van Dolah et al., 2009). Like many dinoflagellates, *K. brevis* possesses a large haploid genome of 100 pg of DNA per single cell, or approximately 30 times larger than the human genome (Van Dolah et al., 2009). Due to the enormity of the *K. brevis* genome, it has not been fully sequenced and made available to the public, mainly due to the cost of such an undertaking. Unique among eukaryotes, dinoflagellates have permanently condensed chromatin which lacks nucleosomes typically involved in regulating chromosome condensation and eukaryotic gene expression (Van Dolah et al., 2009). Due to these impediments, construction and screening of cDNA libraries is an appropriate alternative, and bioinformatics techniques, such as sequence analysis, are needed to elucidate *K. brevis* gene expression (Monroe and Van Dolah, 2008). Another interesting aspect about dinoflagellates is their ribosomal composition is unlike either prokaryotes

(23S and 16S) or, eukaryotes (28S and 18S). These cells contain a combination of a prokaryote like 23S and a eukaryote like 18 subunits.

## CHAPTER II

### PROJECT OBJECTIVE

Organisms respond to abiotic stress in a variety of ways. When *Karenia brevis* is grown in stressed conditions we have noticed that its ribosomal RNA becomes affected. When RNA is isolated from stressed and healthy/normal culture conditions, and the two are compared, we have been able to detect and reproduce differences in their banding patterns. Three types of stress we used include cold shock, nutrient depletion, and low salinity. Healthy *K. brevis* cultures are grown in an incubator allowing 12 hour light/dark diel cycle, and kept at a constant 21°C temperature. Cold shock requires 5-6 days of growing *K. brevis* in a 17°C water bath. Nutrient depletion stress takes 4 weeks, and was achieved by simply growing *K. brevis* without the nutrients added to the sea water based L1 media made without the silica component (Guillared and Hargraves, 1993). L1 media is a general purpose marine medium for growing coastal algae. The silica component is not added because *K. brevis* is an unarmored dinoflagellate, and it is debated that the silica can puncture the relatively soft cell, causing some inhibition of growth. Low salinity shock was achieved by decreasing the salinity in the *K. brevis* culture media gradually and allowing cultures to acclimate for one week at a time. Low salinity shock takes approximately 4 weeks. Once we had determined that we could achieve the cold shock stress in less than a week, instead of four weeks (nutrient depletion and low salinity), we cold shocked when we want stressed *K. Brevis* RNA that has an abnormal rRNA banding pattern. Each of the stressed conditions showed the abnormal rRNA banding pattern. We have seen this using a chip based micro-capillary electrophoresis system when RNA is run on a BioAnalyzer (Agilent, Inc). This abnormal banding pattern was also observed on a denaturing formaldehyde agarose gel electrophoresis system. This

observation was discovered, when we were attempting to do gene expression studies using a *K. brevis* specific microarray. Before the total RNA could be reverse transcribed it first had to be checked for quality. The BioAnalyzer can show degradation of total RNA due to, for example, ribonucleases which hydrolyze RNA into smaller components. Our data did not show significant degradation, but it did show us, in the case of the large ribosomal subunit (LSU), that the molecule was becoming larger. Subsequently this finding put a halt on the gene expression microarray study we wanted to conduct because it seemed like the abnormal banding pattern a possible novel finding. We could not explain the atypical banding pattern, especially when it appears that the LSU gets bigger in the stressed cultures, and thus set out to describe what was occurring.

#### Endosymbiont/Parasite

Initially we hypothesized that the banding pattern may be due to another organism entirely. Under light microscopy (including video), some of the large (30-40um) *K. brevis* cells could be seen with particles moving inside the cells. This gave us the notion that there may be an intracellular organism in our cultures. This could be the explanation of the banding pattern seen in the BioAnalyzer data. It seemed reasonable, and we wanted to know for sure because, if it was indeed a parasite of *K. brevis*, it could possibly be used as a tool to mitigate the HABs. Using parasites to control HABs has been of growing interest because to date other methods like algicidal bacteria and clay flocculation have not had much success (Lewis et al., 2003). The devastating effects economically and ecologically along with adverse human health effects has driven researchers to look for different ways of controlling HABs.

Immuno-staining, and subsequent viewing under confocal microscopy of stressed and healthy *K. brevis* culture samples stained with DAPI (fluorescent DNA stain) did not show a difference between the two cultures' cells when examined. Along with the cells, the culture water from our wet mounts of the *K. brevis* cells did not appear different either. We would expect to see an increase in DAPI staining in the stress cultures' cells, and perhaps in the stressed culture water as well if, according to our hypothesis, an intracellular organism was proliferating whence its host/symbiont began to die from stress. Upon exposure to ultra-violet wavelengths of light, chlorophyll is autofluorescent. This autofluorescence allowed us to also look in our cells, and culture media for the presence of chloroplasts. Upon the merging of chlorophyll autofluorescence, and DAPI staining it did not show the presence of another organism. Wet mounts of our cultures were also screened specifically under confocal microscopy for cyanobacteria, which have different autofluorescent pigments than chlorophyll, but no evidence of cyanobacteria was observed in our cultures.

Scanning Electron Microscopy (SEM) was also used to determine if there was another organism in our cultures. Comparisons of SEM pictures from both stressed and healthy cultures did not support the notion of a parasite or symbiont present. In addition to directly filtering, and viewing via SEM our cultures, we also employed differential centrifugation to remove the bulky *K. brevis* cells in an attempt to then ultra-centrifuge, and collect what could possibly be pico-dinoflagellates (very small dinoflagellate known to parasitize bigger photosynthetic dinoflagellates), or other presumably small eukaryote. Douncing (glass tube with a tight fitting glass pestle) was conducted in an attempt to

break open the *K. brevis* cells, exposing any potential intracellular organism prior to microscopy.

Attempts were made to isolate the LSU rRNA from a stressed culture of *K. brevis*, and sequence it. Alignments of the subsequent sequences showed near perfect agreement to *K. brevis* LSU rRNA sequence in Genbank. The LSU primers used were designed based on conserved regions of eukaryotic LSU rRNA. There is currently only a single lab strain which has been isolated, and is able to grow in axenic conditions (no bacteria present). To confirm that the aberrant rRNA bands were not from a bacterial origin, a suite of antibiotics was administered to the stressed cultures resulting in no change in banding pattern previously mentioned.

In total none of our efforts including immuno-staining confocal microscopy, scanning electron microscopy, differential centrifugation, and molecular strategies were able to detect another organism thus we conclude that the aberrant rRNA bands are not from an intracellular eukaryote.

#### Endogenous Stress Response

Efforts are underway to sequence and characterize the normal and stressed rRNAs, and determine what their role is in abiotic stress response. I propose that using high throughput Illumina RNA-seq technology to gather the full length sequences of stressed, and healthy *K. brevis* LSU and SSU rRNA will be the best option to elucidate what we are observing as a stress response in *K. brevis* that has not been characterized before. Our approach was to cold stress *K. brevis* for 5-7 days in a 1 L bottle placed into a 17°C water bath, spin down the cells via centrifugation, and extract total RNA using a GE kit. The RNA extraction procedure was repeated with a sample of a healthy *K. brevis*

culture. Upon isolation, the total RNA was incubated with DNase the total RNA. Then analyze with BioAnalyzer to check the RNA quality and to ensure the banding pattern is observed. After passing these checks, the stressed and healthy total RNA samples were ready to be sent off for RNA-Seq sequencing. Using bioinformatics assembly software, full length rRNA sequences is to be assembled to find full length rRNAs, and compare healthy versus stressed cultures. This will offer insights into *K. brevis* coping mechanisms caused by stress, and understandings of bloom termination. The coping mechanisms which allow *K. brevis* to tolerate parasitism, nutrient depletion, or low salinity, are particularly important because *K. brevis* has shown to, in some cases, persists when it was believed to not be possible.

## CHAPTER III

### MATERIALS AND METHODS

#### Stressing cells

*K. brevis* strain (CCMP2229) cells are kept in an illuminating incubator at a constant 21°C under 12 hour light/dark cycle (light between 06:00-18:00). A parent culture grown in a 15 L glass carboy in L1 media minus the silica component (Guillard and Hargraves, 1993) is used to seed all other culture bottles that may be used for experimentation. This is considered our healthy growing conditions compared to the following stressed conditions including cold shock, nutrient depletion, and low salinity. Cold shock is achieved when 600 mL *K. brevis* culture in a 1 L glass bottle and is placed in a 17°C water bath kept in a refrigerator which has glass doors allowing light to the culture. After 5-7 days the culture noticeably begins to decrease in density, indicating a stressed culture. Nutrient depletion is achieved when 600 mL of *K. brevis* culture in a 1 L glass bottle is kept in the incubator under normal healthy conditions but does not receive L1 media and thus is not split to make room for new media. After four weeks the density of the culture begins to decrease. Low salinity is achieved when 600 mL of *K. brevis* culture in a 1 L glass bottle is kept in the incubator under normal healthy conditions only receives 18 PSU seawater based L1 media instead of the normal 32 PSU seawater based L1 media. After four weeks of receiving 300 mL of low salinity media the culture is near its lowest salinity growing range. In order to show that the abnormal bands were not from bacterial origin *K. brevis* cultures were grown in the presence of an antibiotic cocktail in both cold shock conditions and healthy conditions. The antibiotic cocktail consisted of kanamycin 50 µg/mL, carbenicillin 100 µg/mL, and benzylpenicillin 10 units/mL from a

1596 units/mL stock. Kanamycin disrupts protein synthesis in bacteria, carbenicillin and benzylpenicillin prevent bacterial cell wall synthesis.

#### BioAnalyzer data

Total RNA was extracted from the 600 mL culture bottles by decanting up to 500 mL of culture avoiding the dead material on the bottom of the culture bottle into a plastic 500 mL conical spinning bottle. The spinning bottle was placed into a centrifuge to pellet the living cells. Pellets were collected from cold shock, nutrient depletion, salinity shock, and a healthy culture (control) by spinning at 1400 rcf for five minutes. These pellets went into the Qiagen RNeasy mini kit which yielded total RNA. In order to show that the abnormal rRNA bands were not from bacterial origin total RNA were collected and extracted from a five day cold shock + antibiotic cocktail culture, and a healthy + antibiotic cocktail culture. The RNA collected was run on the BioAnalyzer (Agilent Technologies) according to the Agilent RNA 6000 nano kit quick start guide, edition April 2007.

#### Microscopy

Extensive microscopy was conducted to ensure that the abnormal rRNA band was not from an endosymbiont/parasite commonly known to join/parasitize photosynthetic dinoflagellates. Microscopy included videography that led to a common belief that there were particles thought to be other organismal cells moving inside the *K. brevis* cell. This was investigated though different microscopy approaches. The methods used were, differential centrifugation of both cold shocked cultures and healthy cultures followed by DAPI immunofluorescent confocal microscopy. This approach was employed due to the fact that the movement was coming from much smaller objects than the *K. brevis* cell

itself. Through subsequent spinning cycles we broke open and spun down the much bulkier *K. brevis* cells, in order to concentrate the smaller possible intracellular organisms. Douncing of cell pellets was again conducted to break open the *K. brevis* cells followed by DAPI staining immunofluorescent confocal microscopy. Scanning electron microscopy (SEM) was also conducted on a cold shock culture sample from supernatant which was differentially centrifuged. To rule out the possibility that the intracellular organism was not a cyanobacteria, cold shock and healthy cultures were examined through fluorescent microscopy by cyanobacteria specialist Dr. Karen Orcutt (Department of Marine Sciences, University of Southern Mississippi, Stennis Space Center). All methods of microscopy were conducted in hopes of finding an intracellular organism.

*Differential centrifugation.* Cells from both five day healthy and cold shock cultures were pelleted in a 500 mL conical spinning bottle and spun at a low speed of 1900 rcf for five minutes. The supernatant was aspirated into another conical spinning bottle and spun at 4000 rcf for five minutes yielding pellets of cellular material and smaller floating particles to be placed on a wet mount slide for DAPI staining or a SEM mount. Wet mounts were created for DAPI staining by achieving a final concentration of 20 µg per mL of culture. For best results the DAPI was added right before viewing.

*Douncing.* Cells from both healthy and five day cold shock cultures were pelleted in a 500 mL conical spinning bottle at 1500 rcf for five minutes. The supernatant was poured off and one mL of pellet and residual supernatant was homogenized and was placed into a 10 mL douncing glass. After 20 strokes with the douncing pistil this mixture was placed into a microcentrifuge tube and was spun down at 2000 rcf for 10 minutes.

100  $\mu$ l of each the homogenized mixture and the microcentrifuge supernatant was examined under 400x light microscopy looking for an intracellular organism.

*DAPI*. Immunoflorescent DNA stain DAPI (4',6-diamidino-2-phenylindole) was mixed at the final concentration of 20  $\mu$ l /mL DAPI (500  $\mu$ l /mL stock) to a cellular pellet mixture to be used for wet mounts. Wet mounts were made from both healthy and three day cold shock cultures which underwent differential centrifugation, and duplicates which did not undergo differential centrifugation. Each was viewed and photographed under confocal microscopy. Confocal wavelengths of DAPI (358 nm absorption – 461 nm emission) and autoflorescent chlorophyll (565 nm absorption – 690 nm emission) were applied, overlaid and combined in order to better see smaller objects believed to be an intracellular organism. If the potential intracellular organism contained chlorophyll this method should allow visualization of the organism. Samples from both healthy and five day cold shock were spun down via differential centrifugation previously described and prepared for scanning electron microscopy (SEM). Methods used were adapted from (Garces et al., 2006). The pellets were fixed in 0.5% glutaraldehyde, and filtered through a 0.2  $\mu$ m millipore filter. The filter was then allowed to dry overnight. The filter was then adhered to the aluminum stub using double sided tape. The stub was sent off to a facility at Mississippi State University for one minute of gold sputter coating. The samples were then viewed at the Polymer Science Research Center at the University of Southern Mississippi.

#### Sequencing of *K.brevis* DNA

DNA was extracted from a cold shock stressed *K. brevis* culture in order to PCR (polymerase chain reaction) amplify, and sequence, the large ribosomal subunit gene of a

possible intracellular organism. Cold shocked stressed *K. brevis* DNA was extracted using a DNeasy blood & tissue kit, Qiagen (Valencia, CA). As a control we used a true eukaryotic organisms DNA (*Histoplasma capsulatum* strain G186AS) due to the fact that *K. brevis* has a 23S like large subunit compared to a normal eukaryotic 28S large ribosomal subunit. Primers were designed biased on the conserved eukaryotic rRNA gene found in all eukaryotes. Six gene specific primer sets were used in hopes of PCR amplifying anything eukaryotic that may be growing in the *K. brevis* culture, which could be the causative agent of the aberrant rRNA bands seen on the BioAnalyzer. Duplicates for both control and stressed DNA of each of the primer sets were made as follows. 10X buffer (Sigma-Aldrich): 2.5  $\mu$ l, 100  $\mu$ M dNTP (New England BioLabs): 0.2  $\mu$ l, Taq polymerase (isolated in lab): 0.25  $\mu$ l, 10  $\mu$ M forward primer: 0.5  $\mu$ l, 10  $\mu$ M reverse primer: 0.5  $\mu$ l, autoclaved reverse osmosis water: 19.05  $\mu$ l, sample DNA: 2  $\mu$ l. The PCR program was 94°C for 30 seconds (denaturing), 51°C for 30 seconds (annealing), and 72°C for 90 seconds (extension), cycling those three temperatures and times was 30 cycles followed by a 72°C for 10 minutes (final extension). These PCR products were analyzed using gel electrophoresis on an agarose gel, and bands from the cold shock stressed gel were aseptically removed, and gel purified using a Clontech NucleoTrap gel extraction kit, (Mountain View, CA). The purified PCR products were ligated into the pUC19 plasmid vector from Clontech, (Mountain View, CA), according to the recommended plasmid to insert ratios. The ligase and buffer used was T4 ligase Promega Corporation, (Madison, WI). The ligated DNA was then transformed into *E. coli* cells according to Zymo Research Corporation premade *mix & go* competent *E. coli* cells instruction manual, (Irvine, CA). The cells were then plated on 37 °C LB agar +

carbenicillin (1 mL LB agar: 100 µg carbenicillin) plates and placed in a 37 °C incubator overnight (~16 hours) to grow. Only the cells which took up the vector should be able to grow in the presence of the antibiotic carbenicillin. Colonies which grew well and were not touching other colonies, were picked and grown in 1 mL LB broth + carbenicillin (same concentration as the plates) in 96 well plates. A single colony per each well was picked using a 20-200 µl micropipette tip and left in the well. Once the plate was filled containing some colonies from all of the cut out bands that were transformed, the plate was covered with aluminum foil, and placed in a 37°C incubator overnight to grow. To ensure that the vector insert, and the transformation was successful, another PCR was conducted. Wells the highest densities of cells were selected to go into the PCR. The PCR was assembled the same as previously described, except SP6 and T7 promoter primers were used instead of the forward and reverse primers. These PCR reactions were analyzed through gel electrophoresis, and if the bands were still the correct size they were selected to be sent off for sequencing. Prior to sequencing the vectors had to be removed from the bacterial cells. This was done using the Zymo Research Corporation Zippy plasmid mini prep kit according to the instruction manual. Once the plasmid mini prep was completed the DNA samples were adjusted to the correct concentration to be sequenced. The samples were prepared according to Eurofins MWG Operon LLC (Huntsville, AL) instructions manual.

#### Denaturing agarose gel and RACE

Two 600 mL cultures of *K. brevis*, seeded from the same culture, were put into 1 L bottles. One was kept at normal growing conditions, and one was kept in cold shock conditions. 200 mL of the healthy culture bottle was poured into a conical spinning

bottle. 500 mL of the cold shock culture bottle was poured into a conical spinning bottle, avoiding the dead algae on the bottom. Both the healthy and the cold shock cultures were pelleted by centrifugation at 1500 rcf for 5 minutes. These pellets were resuspended in remaining supernatant and transferred to a 1.5 mL microcentrifuge tube. These were pelleted again at 1000 x g for five minutes, and the supernatant was removed. The pellets were resuspended in 1 mL of sterile water causing partial lysis, and spun at 1000 x g for five minutes. The supernatant was removed and the RNA extraction mixture was added. The RNA extraction mixture consists of 400 ul extraction buffer solution (0.1 M sodium acetate, 0.2 sodium chloride, 0.2% SDS, at a pH of 5), 400 ul acid phenol:chloroform (P:C 5:1, pH 4.5), and 0.5 mm glass beads. The pellet with the mixture was placed on ice for one minute. The mixture was vortexed using a fast prep-24 tissue and cell homogenizer at 4.0 m/s for 40 seconds, and placed on ice for 1 minute. The mixture was again vortexed and homogenized at 4.0 m/s for 40 seconds to ensure that all the cells were lysed and placed on ice. The lysed mixture was spun in a microcentrifuge at 12,000 x g for five minutes. The aqueous phase was removed being sure not to contact the lower phase, and added to a clean microcentrifuge tube. Ice cold 100% ethanol was added to the aqueous RNA in the microcentrifuge tube to precipitate the RNA. If RNA was to be used right away the mixture was spun at 15,000 x g for five minutes to pellet the RNA. Excess ethanol is removed and the microcentrifuge tube should be placed under a Laminar flow hood to completely allow the ethanol to evaporate. 10 µl of sterile water was added to resuspend the pellet for immediate use. If the RNA was to be used for a later time place the mixture in a -20°C freezer, and later the ethanol could be removed and pellet suspended as described above. The denaturing gel to run the RNA was prepared as follows; 1.2 g of

agarose was added to 90 mL DI water, melted in a microwave, and allowed to cool to ~50°C. Then 10 mL 10 X MOPS buffer was added to the mixture. The prepared solution was poured into a sterile gel setting box with a comb to make loading wells. The RNA was prepared for loading into the gel wells as follows; 1-10 µg (highest possible) of RNA was added to RNase-treated water to a maximum volume 4.5 µl. 2 µl of 10X 3-propanesulfonic acid (MOPS) buffer was included and mixed. 3.5 µl 37% formaldehyde and pipette to mix. Add 10 µl deionized formamide was included and mixed. 1 µl of 1mg/mL ethidium bromide (EtBr) was included and mixed. Heat this mixture to 55°C for 15 minutes to ensure that the RNA is denatured, and immediately chill on ice. Place gel into electrophoresis box containing 1X MOPS buffer, and load 11 µl of each healthy, and cold shock RNA mixtures into the wells. The gel was ran at 5 V/cm until the EtBr was halfway down the gel. The gel was viewed over an ultraviolet illuminator to view the rRNA bands. A picture was taken using a view finder and camera. Attempts were made to extract the rRNA bands from the gel. Once the bands were cut out, they were melted and the rRNA was put through the Clontec Smarter RACE PCR (polymerase chain reaction) kit. The kit included random primers which was used in hopes of PCR amplifying the rRNA bands which were previously cut out.

#### RNA extraction and preparation for RNA-seq

Two 600 mL cultures of *K. brevis*, seeded from the same culture, were put into 1L bottles. One was kept at normal growing conditions, and one was kept in cold shock conditions. Total RNA was extracted using a GE spin total RNA extraction kit, GE Healthcare Bio-Sciences (Pittsburg, PA), utilizing an on column DNase I digestion. These total RNA samples were run on the BioAnalyzer (Agilent Technologies), and

measured for quantity via the (Nanodrop). The total RNA was labeled, packaged, and shipped to Otogenetics Corporation (Norcross, GA) according to RNA preparation, quality control, and shipping for RNA-Seq applications guidebook. Samples will be assessed for quality control, and subsequent sequencing of RNA. Raw RNA-Seq data from the healthy conditions RNA and the cold shock (stressed) conditions RNA was sent to Contig Express (New York, NY) for bioinformatic analysis.

#### RNA-Seq conditions and parameters

RNA-seq data was generated from total RNA to enable capturing of a wider range of gene expression changes and detect novel transcripts in both coding and non-coding RNA. For this study, rRNAs were not removed from the total RNA. Fragmentation was performed so as to generate fragments of appropriate size for sequencing through shearing of RNA, not cDNA fragmentation, before reverse transcription step. Random priming was used in reverse transcribing the first cDNA strand by using random primers. The resulting cDNA is then converted into double-stranded cDNA by a DNA polymerase. Quality control was done at each of the above steps using an Agilent BioAnalyzer to ensure that the samples were still of high enough quality to proceed. Upon completion of that last step, the sequencing process was then started. The Illumina technology used for the sequencing was the HiSeq2000/2500. It has the advantage of rapid turnaround run mode, leading to cycle time reduction. Automated on-instrument cluster generation, and paired-end sequencing is conducted without user intervention. We generated paired-end reads that are 2 X 100 base pairs (bp) long with a fragment size of 300 bp using the HiSeq Illumina machine. At least 80 million reads was generated for both the healthy and the stressed total RNA, and was prepared for downstream analysis.

## Bioinformatics of RNA-Seq data

Paired-end Illumina raw reads are quality checked using FastQC, version 0.10.1, (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). The raw reads are then subjected to quality-based trimming and correction using Quake, version 0.3.4 (Kelley et al., 2010). The trimmed and corrected reads are quality checked again using FastQC. The paired-end reads after trimming and correction are down sampled (~25%) to about 1 million read pairs, and then subjected to de novo transcriptome assembly using SOAPDenovo-Trans, version 1.03 (Xie et al., 2014) with Kmer at 31. The assembled contigs are clustered (similarity cutoff at 90%) using CD-HIT, version 4.6.1 (Li and Godzik, 2006) and transcripts shorter than 300 bp are excluded. The resulting transcript collection is used as the master transcriptome set (unigenes.fa) for downstream analysis. Read mapping and expression quantification is conducted by mapping raw paired-end reads to the master transcriptome using Bowtie2, version 2.1.0 (Langmead and Salzberg, 2012). The option of “--very sensitive” is activated in the mapping step to maximize mapping accuracy and rate. Expression quantification is measured by counting the number of reads mapped to each transcript, which is calculated using HTSeq, version 0.5.4p3 (Anders et al., 2014). Differential gene expression analysis is performed using DESeq, version 1.16.0 (Anders and Huber, 2010). To determine which genes were significantly differentially expressed, the adjusted p-value cutoff was set to 0.10 (Anders and Huber, 2010). In order to identify the top 100 highly expressed transcripts, raw counts are converted to FPKM (Fragments Per Kilo base per Million mapped reads) to normalized for transcript length and total read counts. The top 100 highly expressed transcripts are identified using the average FPKM of both healthy and cold shocked

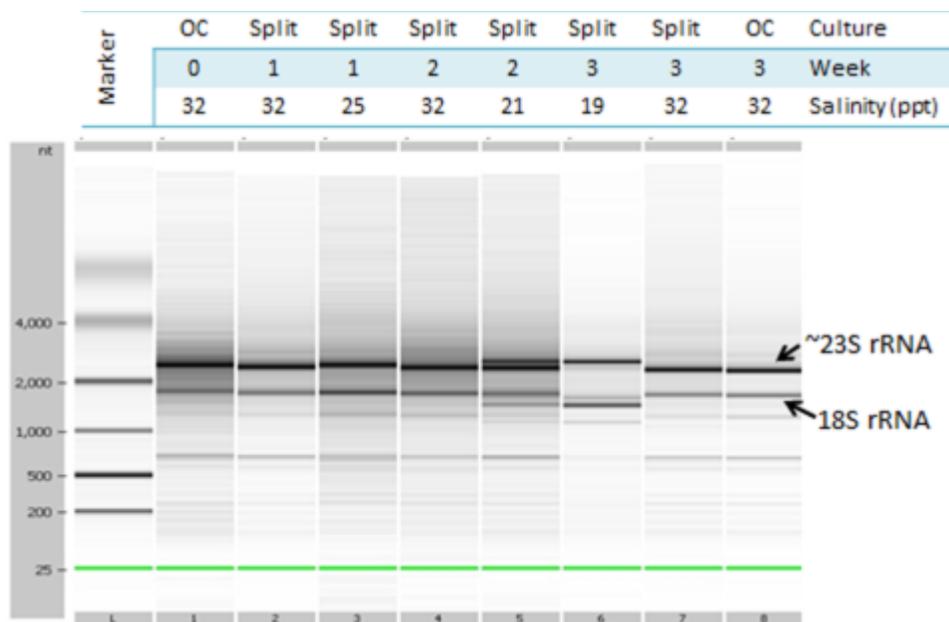
samples, and they are compared against the latest NCBI nucleotide database (nr/nt) using blastn, version 2.2.28, E-value cutoff of 1E-5 is applied (Camacho et al.,2009).

## CHAPTER IV

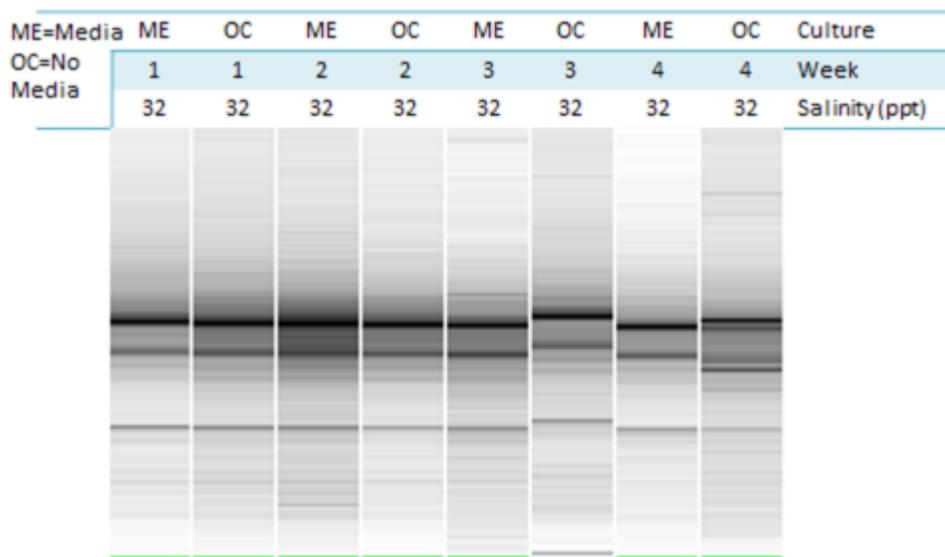
### RESULTS

#### BioAnalyzer data

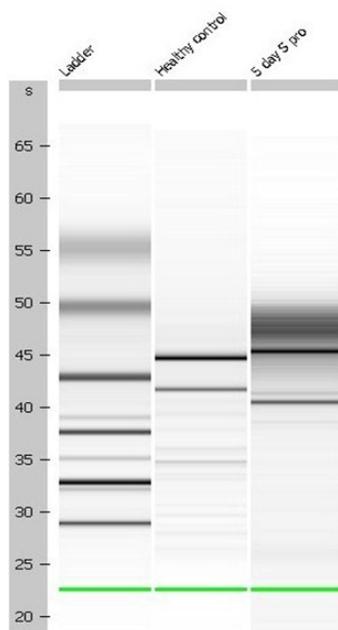
The first experiments were designed to conduct a microarray gene expression study, looking into gene expression of *K. brevis* grown in lowering salinity environments, nutrient depleted environments, and a cold shock environment. Once total RNA was extracted it was checked for quality on the BioAnalyzer. During this step it was observed that the rRNA was changing in size (Figures 1-3). High quality RNA is needed to do gene expression studies. Without an explanation of what was occurring, we wanted to investigate the abnormality to be sure it was not attributed to low quality RNA caused by breakdown. Observations were made of particles moving inside and outside of cells while doing routine culture maintenance. It was hypothesized that the BioAnalyzer data and the particles moving were actually related, not just coincidental. To rule out the possibility that the abnormal rRNA bands were somehow coming from a bacterial origin, an antibiotic cocktail was added to healthy and cold shock stressed cultures. The antibiotic cocktail did not affect the outcome of the BioAnalyzer data.



*Figure 1.* BioAnalyser data showing total RNA of two cultures. The original culture (OC) was split into two equal cultures of 600 mL and total RNA was extracted each week. One of the cultures received normal salinity media each week (32 ppt), the other received low salinity media (18 ppt) each week. By week 2 the low salinity culture had reached 21 ppt, and the rRNA bands have begun to differ from the normal salinity media culture rRNA. Weeks 1-2 the rRNA signature bands are identical. By week 3 the low salinity culture had reached 19 PSU, and the rRNA bands are completely different from the normal media culture rRNA. The far right lane has examples of the healthy cultures large (23s) ribosomal subunit RNA band, and the small (18s) ribosomal subunit RNA band.



*Figure 2.* BioAnalyzer data showing total RNA of two cultures ME and OC. The original culture was split into two equal cultures of 600 mL and total RNA was extracted each week. One of the cultures (ME) was given media each week, and the other (OC) was not given media. Weeks 1-3 the rRNA signature bands are identical. At week 4 the rRNA bands of culture OC becomes different from the rRNA bands of culture ME.



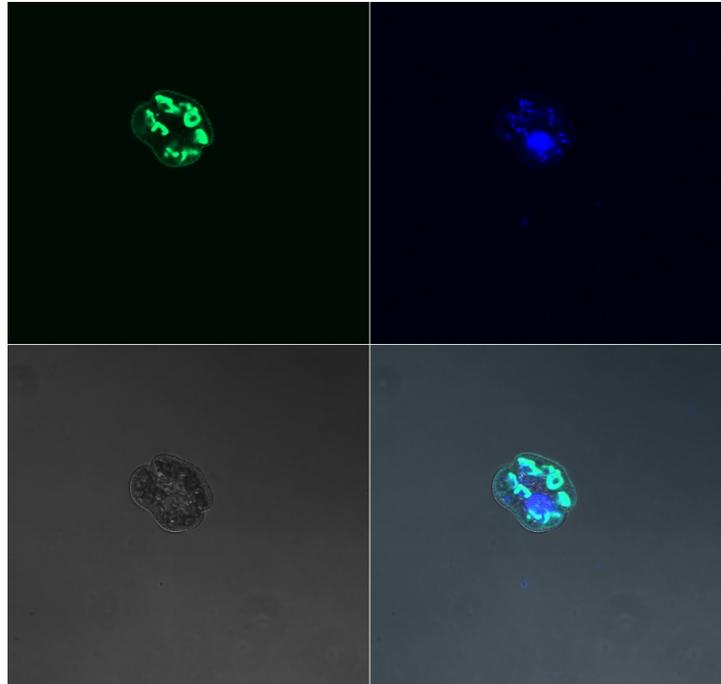
*Figure 3.* BioAnalyzer data of total RNA extracted from a healthy culture and from a five day cold shocked stressed culture. The left lane is the ladder, the middle lane is the healthy culture RNA, and the right lane is the cold shock stressed culture RNA. The cold shock stressed culture rRNA signature bands are different from the rRNA bands in the healthy lane.

## Microscopy

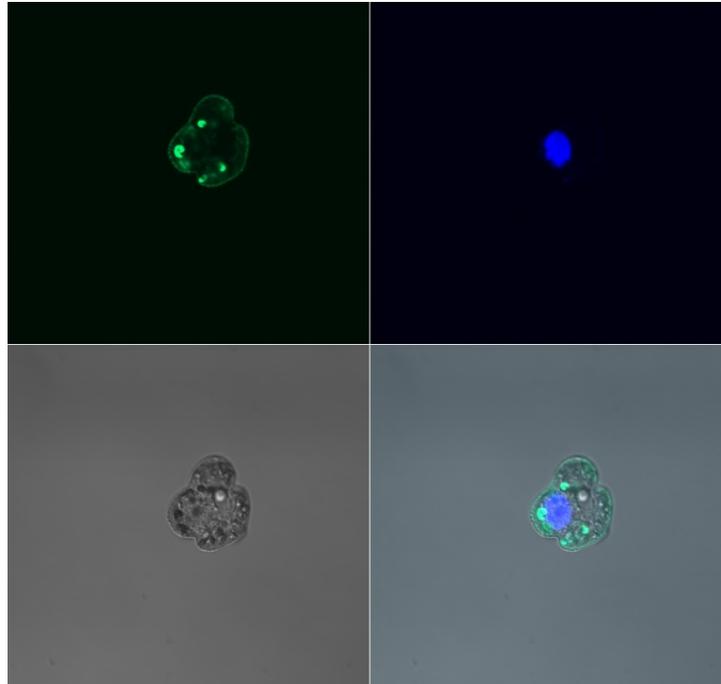
When *K. brevis* cells were fixed in 0.5% glutaraldehyde, some of the cells would exhibit a membrane bubble. Inside this bubble particles could be observed, some of them moving. Still photographs and video were recorded of particles inside the bubble moving. Upon closer inspection particles inside healthy or stressed cells could be observed to have some particles moving around. Figure 4 shows a fixed cell with a bubble formed extending from the cell membrane with a bubble with particles moving inside. These particles were believed to be an intracellular organism. There was also speculation that the movement of particles was attributed to Brownian motion. Without a definitive answer, the next step was to investigate using a more robust microscopy method. *K. brevis* cells were stained with DAPI during a healthy (Fig. 5), and stressed state (Fig. 6), with, and without a differential centrifugation step. *K. brevis* cells were also disrupted via douncing and scanned for an intracellular organism. None of the microscopic efforts to find an intracellular organism were successful.



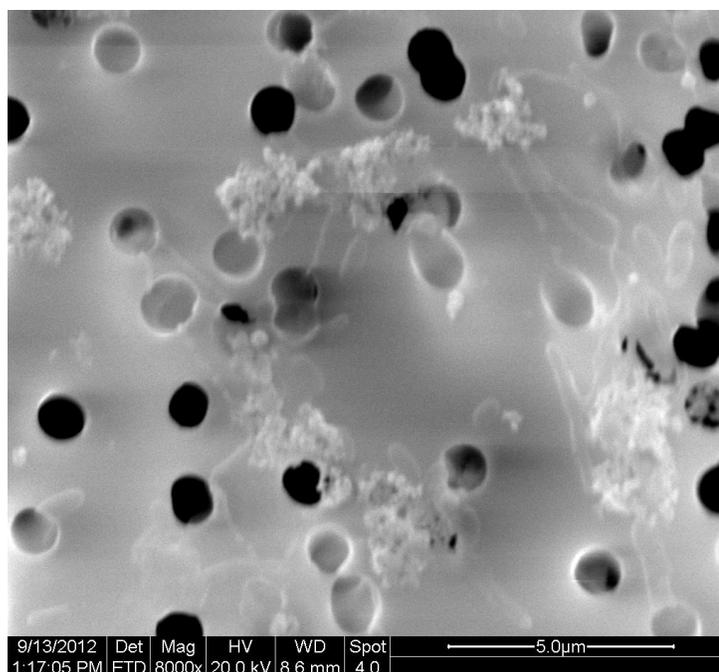
*Figure 4. K.brevis* cells from a salinity shocked culture under 600X, one of the cells has a membrane bubble with particles moving inside of it. These particles were thought to be from an intracellular organism.



*Figure 5.* Overlapping confocal microscopy image (600X) of a healthy *K. brevis* cell (bottom right), stained with immunofluorescent DNA stain DAPI (top right, blue), and the autofluorescence of chlorophyll (top left, green). Bottom left has no laser light applied. In this image there is a defined nuclei of the *K. brevis* cell, and several blue fluorescent particles in the cell presumed to be plastid genomes.



*Figure 6:* Overlapping confocal microscopy image (600X) of a three day cold shock stressed *K. brevis* cell (bottom right), stained with immunofluorescent DNA stain DAPI (top right, blue), and the autofluorescence of chlorophyll (top left, green). Bottom left has no laser light applied. In this image there is a defined nuclei of the *K. brevis* cell, and there are few fluorescent particles inside the cell presumed to be plastid genomes.

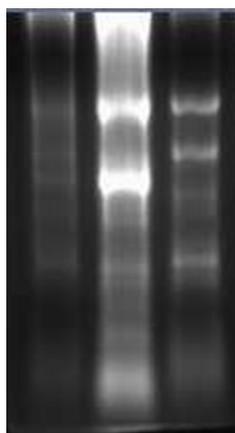


*Figure 7:* SEM image from a cold shock *K.brevis* culture with no detectable organisms observable, just debris. The black and grey roundish shapes seen are pores, some not aligned with the camera, from which the samples were filtered through.

#### DNA Sequencing, Denaturing Gel, and RACE

With no decisive evidence from microscopy, DNA was sequenced from cold shock stressed cultures. Primers used in the sequencing were from a conserved region of the eukaryotic large ribosomal subunit gene in efforts to detect any other eukaryotic organism in the culture. Nine DNA sequences were aligned using BLASTn (basic local alignment search tool for nucleotide sequences) to GenBank (a public repository of DNA sequences built from community data submissions) found at the NCBI (National Center for Biotechnology Information). Three of the sequences aligned to *Karenia brevis*, four aligned to *Karenia mikimotoi*, and two aligned to *Akashino sanguinea*. *Karenia mikimotoi* is the same genus, and *Akashino sanguinea* is a similar unarmored dinoflagellate known to cause harmful algal blooms. None of the sequences aligned to a possible intracellular organism that was in GenBank. When the healthy and the stressed

total RNA is run out on a denaturing formaldehyde gel, differences can be observed (Fig. 8). These rRNA bands were then extracted from the gel, and attempts were made to reverse transcribe and amplify them using random primers. All attempts using RACE (rapid amplification of complementary DNA ends) to directly amplify the rRNA bands directly were not successful. Thus these rRNA bands could not be sequenced individually using this method.

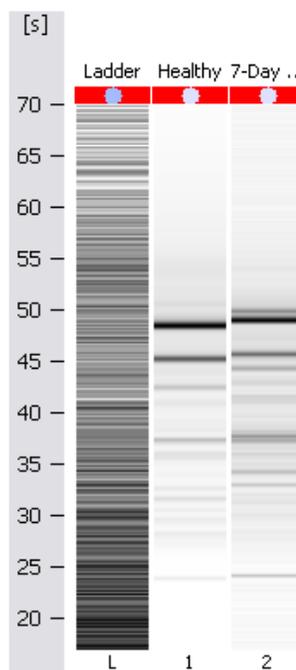


*Figure 8.* *K. brevis* total RNA run out on a denaturing formaldehyde gel. The lane on the left is RNA from a five day cold shock stressed culture. The middle lane is RNA from a four week nutrient depletion stressed culture, and the lane on the right is RNA from a healthy culture. The rRNA bands are the top two bands in each lane. The center lane, nutrient depletion had more total RNA and is more fluorescent. Comparing the healthy culture RNA (right) to the other two stressed RNA (middle and left), they do not look the same. A noticeable different sized small ribosomal subunit band is seen in the two stressed culture lanes.

#### RNA-seq Data and Bioinformatic Analysis

To better understand what is occurring to the rRNA at the transcriptomic level, total RNA was sequenced using Illumina technology (RNA-seq), from a healthy culture and a cold shock stressed culture. This technology is expensive, at \$1,000 per sample library, so no duplicates were made. Each sample library contains a guaranteed minimum amount of 80 million reads. The most abundant species of RNA is rRNA, so we felt that

one library per sample would provide good coverage of rRNA. This sequence data also ruled out the presence of an intracellular organism being in our cultures.



*Figure 9.* BioAnalyzer data of the total RNA which was used for RNA-seq. The first lane on the left is the ladder, which had some error. The second lane (1) is of healthy culture total RNA. The third lane (2) is of a seven day cold shock stressed culture total RNA. Notice that the third lane is beginning to change into the different rRNA signature banding pattern seen before.

Table 1

*Initial quality control before RNA-Seq*

Sample Name	Otogenectis Sample Name	Nanodrop Concentration (ng/ul)	Optical Density Absorbance (260nm/280nm)	BioAnalyzer Quality Control Result
OtA5930	Healthy	327	2.13	Pass
OtA5931	Cold Shock Stressed	87	2.12	Pass

Initial sample quality control report prior to RNA-seq. Optical density absorbance ratio of 260nm/280nm is a measure of contamination in RNA sample, ~2 is a pure RNA.

Table 2

*Total amount of bases and reads for healthy versus stressed*

Sample Name	Otogenetics Sample Name	Total Bases	Total Reads
Healthy	Index23_OtA5930	11,020,389,628	104,437,638
Cold Shock Stressed	Index24_OtA5931	10,328,110,424	97,435,004

Compilation of raw RNA-seq data generated for the healthy and stressed libraries.

Once the total RNA was sequenced from both the healthy and the stressed cultures, 25% the raw sequence data was assembled to make a master transcriptome (Table 3). The paired end reads were down sampled to 25% due to the cost of the assembly. Being that rRNA is very abundant in total RNA, and what we wanted to investigate, the down sampling should still give good coverage of rRNA. Raw paired-end reads are mapped to the master transcriptome to understand the expression quantification. Expression quantification is the number of reads which mapped to a given transcript (unigene).

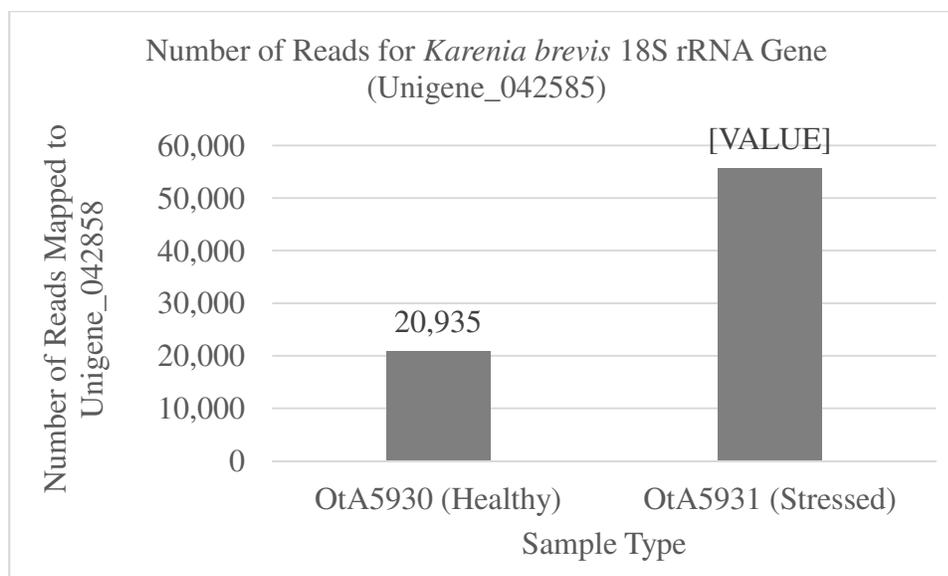
Table 3

*Summary of master transcriptome*

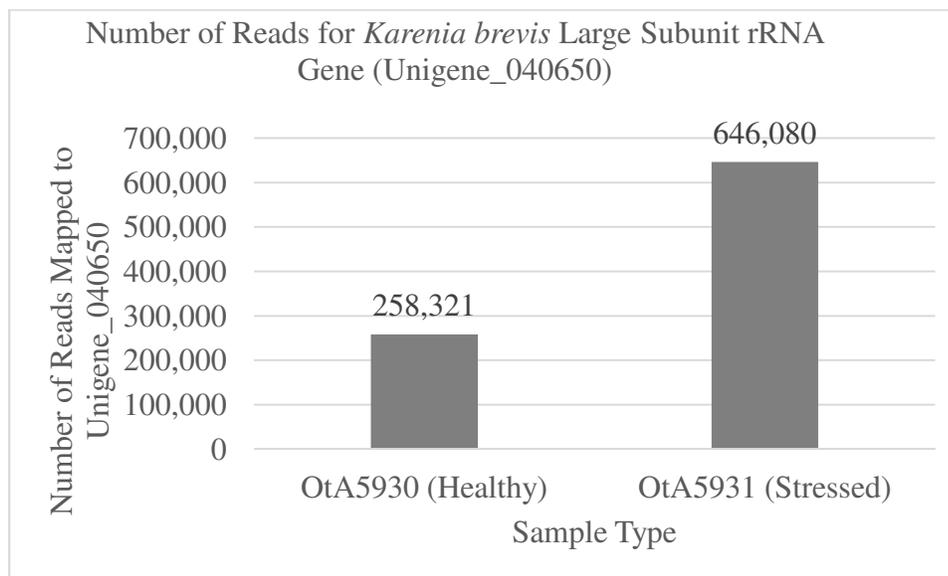
File Name	Total Base Pairs (bp)	Number of sequences	Max Length bp	Min Length bp	Average Length bp	N50	N90
Unigenes.fa	44,811,452	64,983	23,934	300	689	775	356

Summary of the master transcriptome assembly for the raw paired-end reads to be mapped to. The N50 represents the length of the contigs (unigenes) of a de novo assembly where 50% of the unigenes are that size or larger. The larger the N50 score of an assembly, equates to larger overall unigenes lengths. Similarly, N90 represents the lengths of contigs (unigenes) of a de novo assembly where 90% of the unigenes are that size or larger.

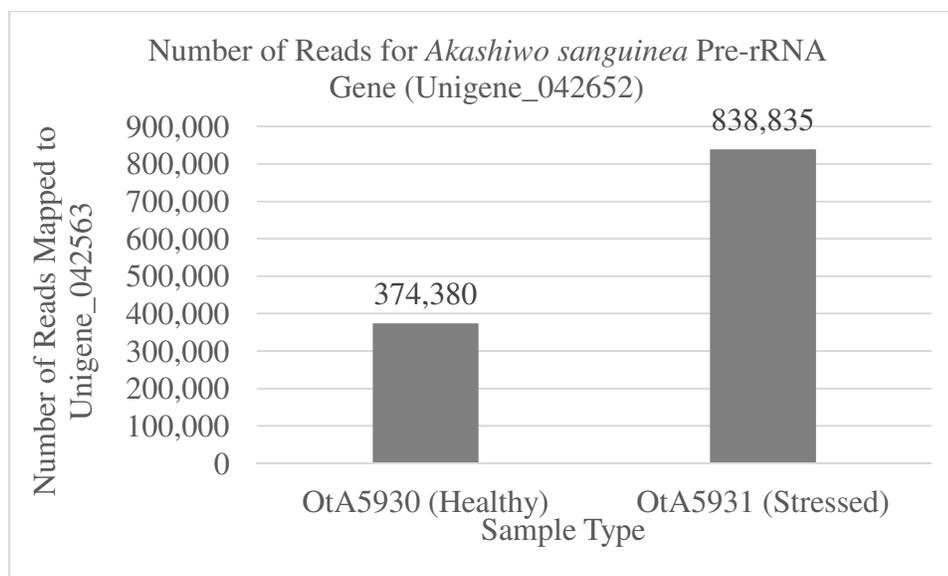
Three Unigenes from the rRNA family were significantly differentially expressed. Unigene\_042585 is a *K. brevis* 18S small ribosomal subunit (Fig. 10). Unigene\_040650 is a *K. brevis* large ribosomal subunit (Fig. 11). Unigene\_042652 is a pre-rRNA molecule matching the very similar brevetoxin producing dinoflagellate *Akashiwo sanguinea* (Fig. 12) which contains the five prime external transcribed spacer (5'ETS), 18S ribosomal subunit, internal transcribed spacer one (ITS1), 5.8S ribosomal subunit, internal transcribed spacer two (ITS2), 24S ribosomal subunit, and the three prime external transcribed spacer (3'ETS). Three other unigenes of interest were from mRNA families belonging to non-nuclear genes described in the BLASTn annotation. Unigene\_022074 is a *Karenia brevis* photosystem II CP43 chlorophyll apoprotein (psbC) gene, from chloroplast DNA (Fig. 13). Unigene\_031812 is a *Karenia brevis* ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo, or rbcL) gene, from chloroplast DNA (Fig. 14). Unigene\_047770 is a *Karenia brevis* cytochrome oxidase subunit 1 (cox1) gene, from mitochondrial DNA (Fig. 15). Unigene\_022074 and Unigene\_031812 were significantly differentially expressed, and unigene\_047770 was differentially expressed, but it was not significantly differentially expressed. Raw RNA-seq reads are submitted to NCBI under the name "non axenic culture of *Karenia brevis*, raw RNA-seq reads" BioProject PRJNA278874. The healthy culture total RNA is BioSample accession number SAMN03434847, and the stressed culture is under total RNA BioSample number SAMN03435826.



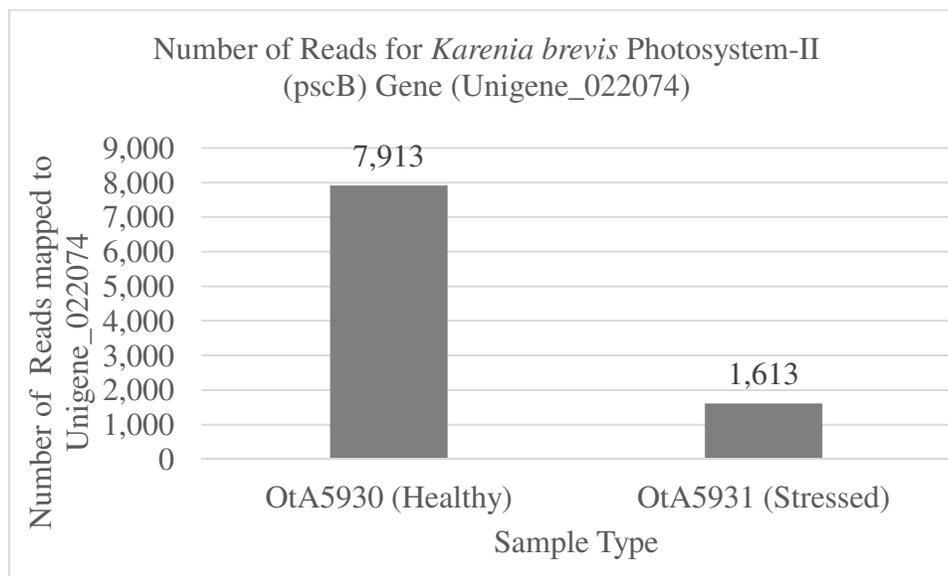
*Figure 10.* Significant differential expression of reads mapped to 18S small subunit rRNA gene (unigene\_042585). Healthy sample total RNA had 20,935 reads, and stressed total RNA had 55,686.



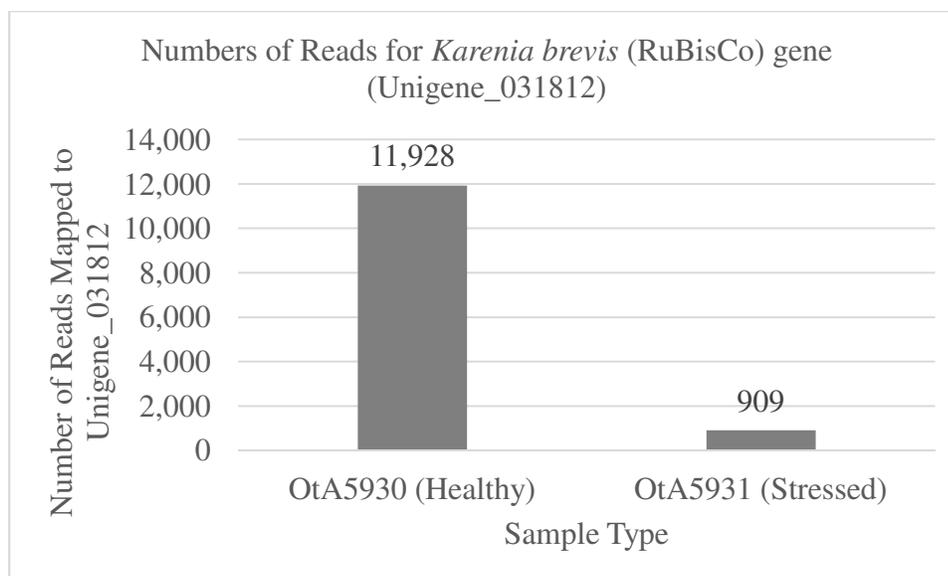
*Figure 11.* Significant differential expression of reads mapped to large subunit rRNA gene (unigene\_040650). Healthy sample total RNA had 258,321 reads, and stressed total RNA had 646,080 reads.



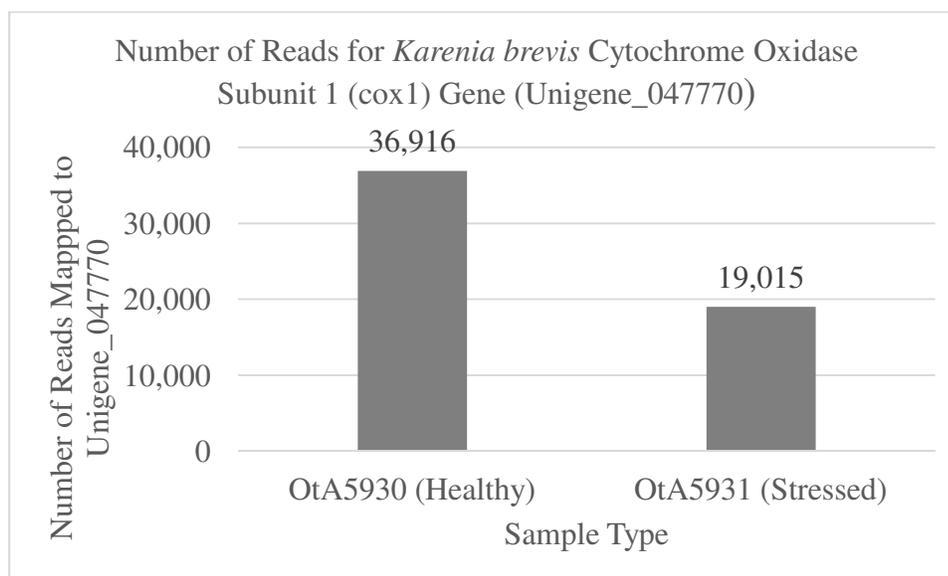
*Figure 12.* Significant differential expression of reads mapped to pre-rRNA gene product (unigene\_042563). Healthy sample total RNA had 374,380 reads, and stressed total RNA had 838,835 reads.



*Figure 13.* Significant differential expression of reads mapped to photosystem- II mRNA gene (unigene\_022074). Healthy sample total RNA had 7,913 reads, and stressed total RNA had 1,613 reads.



*Figure 14.* Significant differential expression of reads mapped to RuBisCo mRNA gene (unigene\_031812). Healthy sample total RNA had 11,928 reads, and stressed total RNA had 909 reads.



*Figure 15.* Non-significant differential expression of reads mapped to cox1 mRNA gene (unigene\_047770). Healthy sample total RNA had 36,916 reads, and stressed total RNA had 19,015 reads.

## CHAPTER V

## DISCUSSION AND CONCLUSION

The possibility of an intracellular organism had been ruled out by microscopy, DNA sequencing, and RNA-seq data. The membrane bubble with particles moving in it was in fact a phenomenon called membrane blebbing (Franklin and Berges, 2004). In the dinoflagellate *Amphidinium carterae* membrane blebbing is a type of cell morphology observed during apoptosis or programmed cell death described as loss of plasma membrane integrity (Franklin and Berges, 2004). Observations of membrane blebbing in *K. brevis* cells, would show cytoplasmic material particles moving inside the bubble or bleb which was touching the cell. Franklin and Gurges (2004) stresses that care must be taken when observing different morphologies of dinoflagellates, as to not be misled. Another explanation for the membrane blebbing could be attributed to just fixing with protein cross-linking glutaraldehyde. Some methods suggest a second fixative of osmium tetroxide to crosslink and stabilize cellular membrane lipids (Porter and Kallman, 1953), preventing loss of plasma membrane integrity. Brownian motion is the random movement of water molecules causing particles observed under magnification to move. Brownian motion likely led us to believe that particles moving under wet mount *K. brevis* microscopy was an organism moving on its own. Confusion also arose when viewing the healthy and stressed DAPI stained *K. brevis* cells under confocal microscopy because we believed that the stressed culture would have more intracellular organisms. Particles other than the nucleus that were DAPI fluorescent can only be described as plastid genomes throughout the cell, not intracellular organisms. More plastid genomes were observed in

the healthy culture, implying that cold shock stress may have some effect on plastid genomes.

Dinoflagellates are quite the unique group of organisms, typically being considered an exception to generally accepted beliefs in biology. Dinoflagellates have been described as the only living example of a histone knockout and thus do not possess nucleosomes (Espina et al., 2005). Dinoflagellates also possess extremely large genomes and have their own set of rRNAs different from all other eukaryotes. Most of the unique features can be attributed to three separate events of symbiogenesis, where in each event two existing species merge to result in the genesis of a new species. The result is the modern dinoflagellate. These events shaped dinoflagellate evolution (Yoon et al., 2005). Dinoflagellates can be great adaptors to their environment, shown by their global distribution, which is common to abundant in marine and fresh waters (Taylor et al., 2008). Part of dinoflagellates ability to adapt would seem likely explained by their tertiary endosymbiosis driven genome. Their large genomes offer an array of useful, perhaps adaptable genes to increase survival. For example transcriptomic data of the dinoflagellate *Lingulodinium polyedrum*, contains all of the core mRNA transcripts to make nucleosomes from histone proteins, yet no detectable histone protein accumulation could be found (Roy and Morse, 2012). This also supports the belief that dinoflagellate gene regulation is controlled at the post transcriptional level (Roy and Morse, 2013). Genomic sequence data from the toxic dinoflagellate *Alexandrium fundyense* show that there are two distinct small subunit rRNA genes found in the same clonal isolate, and during nutrient depletion stress one of them is not expressed (Scholin, Anderson, and Sogin. 1993). Additionally Bachvaroff and Place, 2008 described the first glimpse of a

dinoflagellate genome in *Amphidinium carterae*, finding that there are two general classes of genes, a highly expressed class composed of mostly tandem repeats, and a less expressed class described as intron rich. Gene expression and regulation in dinoflagellates is largely undescribed (Morey et al., 2011), partially due to not having a completely sequenced genome to speculate from. Dinoflagellates ability to respond/adapt to changing conditions, perhaps explains why they have been able to persist since the Triassic period, and why they represent one of the most widely diverse phyla (Taylor et al., 2008).

Alternative splicing is common in higher eukaryotes and well described as pre-mRNA being spliced alternatively into slightly different mRNA molecules that are translated into different protein. The most common is exon skipping resulting in a protein that differs in sequence and function, effectively increasing the transcriptome and proteome of the organism. Alternative splicing in crop plants has been well studied, mainly because this can lead to new pathogen resistance genes, or genotypes which can be selected for resistance to environmental stress (Mastrangelo et al., 2012). The onset of next-generation sequencing, and databases has led to better understanding of the associations, extent, and diversity of alternative splicing. Abiotic stress response in plants leads to post-transcriptional alternative splicing (Nakaminami et al., 2012). Alternative splicing in many cases is conserved across plants and even kingdoms, especially those related to stress response. Alternative splicing is a gene regulation strategy that give the transcriptome plasticity, offering new gene function, sometimes a superior performing genotype under adverse environmental conditions (Mastrangelo et al., 2012). Alternative splicing in dinoflagellates is not well understood, with the exception of a spliced leader

being spliced to all nuclear encoded mRNAs as a post transcriptional maturation leading to translation (Lidie and Van Dolah, 2007), which is atypical for eukaryotes. Alternative splicing has probably contributed to dinoflagellates ability to adapt to their changing environments. In *K. brevis* or dinoflagellates in general there has been little studied on alternative splicing in stressed conditions. Alternative splicing is also a plausible explanation for the change in sizes of the *K. brevis*' large and small rRNA subunits, observed in BioAnalyzer data of three different stressed conditions. Additional explanations are that dinoflagellates can possess different genes at different loci coding for the same rRNA products, and these are expressed differently during stressed conditions (Scholin, Anderson, and Sogin, 1993). Stress in *K. brevis* may also induce a different class of rRNA genes as described in (Bachvaroff and Place, 2008). No attention has been given to rRNA being alternatively spliced in dinoflagellates resulting in slightly different sizes of rRNA subunit products. The up regulation observed in the rRNA genes could be a result of the stressed culture expressing all the different variations of rRNA genes.

Next generation sequencing of RNA has led to a deeper understanding of cellular transcriptomic ability. Most RNA-seq projects are aimed at understanding all other RNA species except rRNA, so rRNA is almost always removed from the total RNA. rRNA deletion is done because rRNA is the most abundant species of RNA, and thus most of the reads from sequencing will be rRNA. This experiment was aimed at understanding the stressed induced changes in *K. brevis* rRNA, so the rRNA was not removed before sequencing. Specifically we wanted to compare full length large and small rRNAs of healthy vs. stressed *K. brevis* cultures. Unfortunately, we overlooked that fact that RNA-

seq fragments all of the RNA in a total RNA sample before it sequences it. The result is that minor sequence difference between the healthy and the stressed rRNA will likely be placed in the same contig (unigenes) during a de-novo transcriptome assembly. Fidelity was lost for specific sequence differences during our bioinformatic analysis. Nonetheless we found that stress response causes significant differential expression of the large and small rRNA subunit genes. Interestingly, we found that the pre-rRNA molecule was also significantly differentially expressed. This pre-rRNA transcript molecule is also a candidate for alternative splicing to produce different sized large and small rRNA subunits during stress. In all of the rRNA unigenes examined from the RNA-seq data, the stressed culture had significantly differentially expressed more transcripts of these genes. Included in this expression is any rRNA genes which have been alternatively spliced, or transcribed from a different loci. Perhaps this is observed because the *K. brevis* cell is desperately trying to make proteins and must first manufacture ribosomes. The mRNA transcription from chloroplast DNA genes was significantly differentially expressed lower in the stressed culture and differentially expressed lower in a mitochondrial DNA gene. The mRNA reduction in the stressed culture shows that the culture is not able to produce them at normal healthy levels due to the cold shock stress.

This study provides the first glimpse into rRNA expression levels in healthy and cold shock stressed cultures of *K brevis*, as well as novel formations of alternate large and small rRNA subunits in the presence of environmental stress including nutrient depletion, decreased salinity, and cold shock. The current state of understanding of dinoflagellate molecular biology is far from being completely described. It does not come as a surprise that they are capable of extraordinary, not previously described molecular feats. These

feats have enabled dinoflagellates to thrive through evolution. Next generation sequencing will continue to offer discoveries in dinoflagellate molecular biology. Understanding of *K. brevis* molecular capabilities will offer insights to what it will do in nature. *K. brevis* bloom maintenance is of particular interest because some blooms last up to 18 months one place. Meaning that the bloom endured drastic changes in environmental conditions from season to season. *K. brevis* bloom initiation, maintenance, and termination are in need of attention from researchers, because they are too poorly understood. The negative effects *K. brevis* has on its environment, economy, and human health will continue to push researchers to understand more biological functions it possesses.

This RNA-seq data was analyzed using bioinformatics tools available today. This data set will be uploaded to NCBI, and as more bioinformatics approaches are invented, the data can be re-evaluated. In this bioinformatics analysis the master transcriptome was assembled using only 25% of the raw paired-end reads. This was done in part because of price, and that we were interested in rRNA, which is very abundant in total RNA, giving good coverage of rRNA. A reasonable next step would be to not downsize the raw paired-ends reads, generating a more robust master transcriptome from de-novo assembly. The more robust a master transcriptome, the more reads will be mapped to unigenes. This will give a more broad understanding of cold shock stress adaptation of *K. brevis*. The data could also be mined for other interesting RNA species, increasing our knowledge of *K. brevis* biology.

## REFERENCES

- Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol*, 11(10), R106.
- Anders, S., Pyl, P. T., & Huber, W. (2014). HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics*, btu638.
- Brown, A. Dortch, Q. Van Dolah, F. Leighfield, T. Morrison, W. Thessen, A. Steidinger, K. Richardson, B. Moncreieff, C. Penncock, J. (2006) The Effect of Salinity On the Disturbution Growth And Toxicity Of *Karenia* spp. *Harmful Algae* 5 199-212.
- Coats, D. W., & Park, M. G. (2002). Parasitism of Photodynthetic Dinoflagellates by Three Strains of *Amoebophrya* (Dinophyta): Parasite Survival, Infectivity, Generation Time, and Host Specificity. *Journal of phycology*, 38(3), 520-528.
- Cheng, Yung Sung, Yue Zhou, Clinton M. Irvin, Richard H. Pierce, Jerome Naar, Lorraine C. Backer, Lora E. Fleming, Barbara Kirkpatrick, and Dan G. Baden, (2005), Characterization of Marine Aerosol for Assessment of Human Exposure to Brevetoxins. *Environmental Health Perspectives*, 113, 5
- Erard-Le Denn, E., Chrétiennot-Dinet, M. J., & Probert, I. (2000). First report of parasitism on the toxic dinoflagellate *Alexandrium minutum* Halim. *Estuarine, Coastal and Shelf Science*, 50(1), 109-113.
- Espina, S. M. D., Alverca, E., Cuadrado, A., & Franca, S. (2005). Organization of the genome and gene expression in a nuclear environment lacking histones and nucleosomes: the amazing dinoflagellates. *European journal of cell biology*, 84(2), 137-149.

- Franklin, D. J., & Berges, J. A. (2004). Mortality in cultures of the dinoflagellate *Amphidinium carterae* during culture senescence and darkness. *Proceedings of the Royal Society of London B: Biological Sciences*, 271(1553), 2099-2107.,
- Garcés, E., Fernandez, M., Penna, A., Van Lenning, K., Gutierrez, A., Camp, J., & Zapata, M. (2006). Characterization of NM Mediterranean Karlodinium Spp. (Dinophyceae) Strains Using Morphological, Molecular, Chemical, and Physiological Methodologies.1. *Journal of phycology*, 42(5), 1096-1112.
- Guillard and Hargraves, (1993), Methods to Make L1 Media, an Enriched Seawater Medium.
- Kelley, D. R., Schatz, M. C., & Salzberg, S. L. (2010). Quake: quality-aware detection and correction of sequencing errors. *Genome Biol*, 11(11), R116.
- Kirkpatrick, B., Fleming, L.E., Squicciarini, D., Backer, L.C., Clark, R., Abrahama, W., Benson, J., Cheng, Y.S., Johnson, D., Pierce, R., Julia, Z., Bossart, G.D. Baden, D.G. (2004) Literature review of Florida red tide: implications for human health effects. *Harmful Algae* 3. 99-115
- Kuvaridina, o.n., Leander, S. S., Aleshin, v.s., Myl'nikov, a.p., Keeling, p.j. & Simdyanov, t.g. (2002). The phylogeny of colpodellids (Alveolata) using small subunit rRNA gene sequences suggests they are the free-living sister group to apicomplexans. *Journal of Eukaryotic Microbiology*, 49(6), 498-504.
- Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods*, 9(4), 357-359.
- Li, W., & Godzik, A. (2006). Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*, 22(13), 1658-1659.

- Lidie, K. B., & Van Dolah, F. M. (2007). Spliced leader RNA-mediated trans-splicing in a dinoflagellate, *Karenia brevis*. *Journal of Eukaryotic Microbiology*, 54(5), 427-435.
- MacLean, H.L., 1979. Indo Pacific red tides. Toxic Dinoflagellate Blooms. In: Proceedings of the Second International Conference on Toxic Dinoflagellate Blooms, Key Biscayne, FL, 173-178
- Mastrangelo, A. M., Marone, D., Laidò, G., De Leonardis, A. M., & De Vita, P. (2012). Alternative splicing: enhancing ability to cope with stress via transcriptome plasticity. *Plant Science*, 185, 40-49.
- Monroe, Emily A., and Frances M. Van Dolah. (2008). The Toxic Dinoflagellate *Karenia brevis* Encodes Novel Type I-like Polyketide Synthases Containing Discrete Catalytic Domains, *Protist*, Vol. 159, 471—482,
- Morey, J. S., Monroe, E. A., Kinney, A. L., Beal, M., Johnson, J. G., Hitchcock, G. L., & Van Dolah, F. M. (2011). Transcriptomic response of the red tide dinoflagellate, *Karenia brevis*, to nitrogen and phosphorus depletion and addition. *BMC genomics*, 12(1), 346.
- Nakaminami, K., Matsui, A., Shinozaki, K., & Seki, M. (2012). RNA regulation in plant abiotic stress responses. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, 1819(2), 149-153.
- Pierce, R., Henry, M., Boggess, S., Rule, A., (1989). Marine Toxins in Bubble Generated Aerosol. In: Morohan, Implications of Bubble-Mediated Sea-Air Exchange. Connecticut Sea Grant Publications. 27-42

- Porter, K. R., & Kallman, F. (1953). The properties and effects of osmium tetroxide as a tissue fixative with special reference to its use for electron microscopy. *Experimental Cell Research*, 4(1), 127-141.
- Prince, E. Myers, T. Kubanek, J. (2008) Effects of Harmful Algal Blooms On Competitors: Allelopathic Mechanisms of The Red Tide Dinoflagellate *Karenia brevis*. *Limnology and Oceanography*. 53(2) pgs. 531-541
- Redalje, D. Lohrenz, S. Natter, M. Tuel, M. Kirkpatrick, G. Millie, D. Fahnenstiel, G. Van Dolah, F. 2008. The Growth Dynamics of *Karenia Brevis* With in Discrete Blooms Along The West Florida Shelf. *Continental Shelf Research*, 28. 22-44
- Roy, S., & Morse, D. (2012). A full suite of histone and histone modifying genes are transcribed in the dinoflagellate *Lingulodinium*. *PLoS One*, 7(4), e34340.
- Roy, S., & Morse, D. (2013). Transcription and Maturation of mRNA in Dinoflagellates. *Microorganisms*, 1(1), 71-99.
- Scholin, C. A., Anderson, D. M., & Sogin, M. L. (1993). Two Distinct Small-Subunit Ribosomal RNA Genes In The North American Toxic Dinoflagellate *Alexandrium Fundyense* (Dinophyceae) 1. *Journal of Phycology*, 29(2), 209-216.
- Sinclair, G. Kamykowski, D. Milligan, E. Schaeffer, B. 2006. Nitrate uptake by *Karenia brevis* 2. Behavior and uptake physiology in a nitrate depleted mesocosm with a bottom nutrient source. *Marine Ecology Progress Series*. Vol 328 125-131
- Smayda, T. J. (1997). Harmful algal blooms: their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnology and oceanography*, 42(5), 1137-1153

- Taylor, F. J. R., Hoppenrath, M., & Saldarriaga, J. F. (2008). Dinoflagellate diversity and distribution. *Biodiversity and Conservation*, 17(2), 407-418.
- Van Dolah, F.M., et al., The Florida red tide dinoflagellate *Karenia brevis*: New insights into cellular and molecular processes underlying bloom dynamics. *Harmful Algae* (2009), doi:10.1016/j.hal.2008.11.004
- Vargo, G. Heil, C. Fanning, K. Dixon, K. Neely, M. Lester, K. Ault, D. Murasko, S. Havens, J. Walsh, J. Bell, S. 2008. Nutrient Availability in support of *Karenia brevis* Bloom on the West Florida Shelf: What Keeps *Karenia* Blooming? *Continental Shelf Research*. 28 73-98
- Walsh, P. Smith, S. Fleming, L. Solo-Gabriele, H. Gerwick, W. 2008. Oceans and Human Health Risks and Remedies from the Seas. Copyright Elsevier. Burlington MA 01803 USA. Pgs 242-246
- Xie, Y., Wu, G., Tang, J., Luo, R., Patterson, J., Liu, S., & Wang, J. (2014). SOAPdenovo-Trans: de novo transcriptome assembly with short RNA-Seq reads. *Bioinformatics*, 30(12), 1660-1666.
- Yoon, H. S., Hackett, J. D., Van Dolah, F. M., Nosenko, T., Lidie, K. L., & Bhattacharya, D. (2005). Tertiary endosymbiosis driven genome evolution in dinoflagellate algae. *Molecular Biology and Evolution*, 22(5), 1299-1308.

