Spring 5-2013

Identification of an Unknown Intracellular Organism in *Karenia brevis*

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Identification of an Unknown Intracellular Organism in *Karenia brevis*

by

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A Thesis

Submitted to the Honors College of
The University of Southern Mississippi
In Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2013

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Abstract

The dinoflagellate *K. brevis* is a mixotrophic marine alga that is of ecological importance in coastal waters especially along the Gulf of Mexico. They are one of a few species of algae responsible for the production of "harmful algae blooms" or HABs in which they release neurotoxins called brevetoxins which negatively impact fishing industries, local wildlife, tourism, and coastal health. Because of the danger these algae pose their lifecycle and characteristics merit intensive study.

During a previous experiment in Dr. McLean's lab involving salinity stressing cultured *K. brevis*, the stressed cultures exhibited an unusual data pattern when having their ribosomal RNA profile bioanalyzed. The rRNA pattern for these stressed cultures showed an additional set of rRNA bands that were absent in all healthy non-shocked cultures, one smaller than the *K. brevis* small ribosomal subunit and one larger than the *K. brevis* large ribosomal subunit. Cultures of *K. brevis* that were bioanalyzed before shocking and after shocking clearly showed the anomalous rRNA bands appearing directly after stressing.

Looking at the stressed cells under a light microscope revealed that the stressed cells had their membranes pinching off in small bubbles with what looked like small motile agents moving about within. Because intracellular organisms are known to inhabit other dinoflagellates we believe that the second set of rRNA signals and movement within stressed cells could be explained by an intracellular parasite or mutualistic symbiote that remains dormant until its host begins to die.

In order to test if an intracellular organism was actually the source of the abnormal rRNA data a variety of tests were applied to stressed *K. brevis* cultures. Lugol staining, DAPI staining,

PCR amplification, microscopy and cloning were all used in an attempt to find any indication of a candidate organism. Despite all initial observations and data being consistent with an intracellular organism these later tests effectively disproved the initial hypothesis. Because the new data makes the intracellular organism explanation unlikely the source of the anomalous rRNA bands during stressing remains unknown.

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Introduction

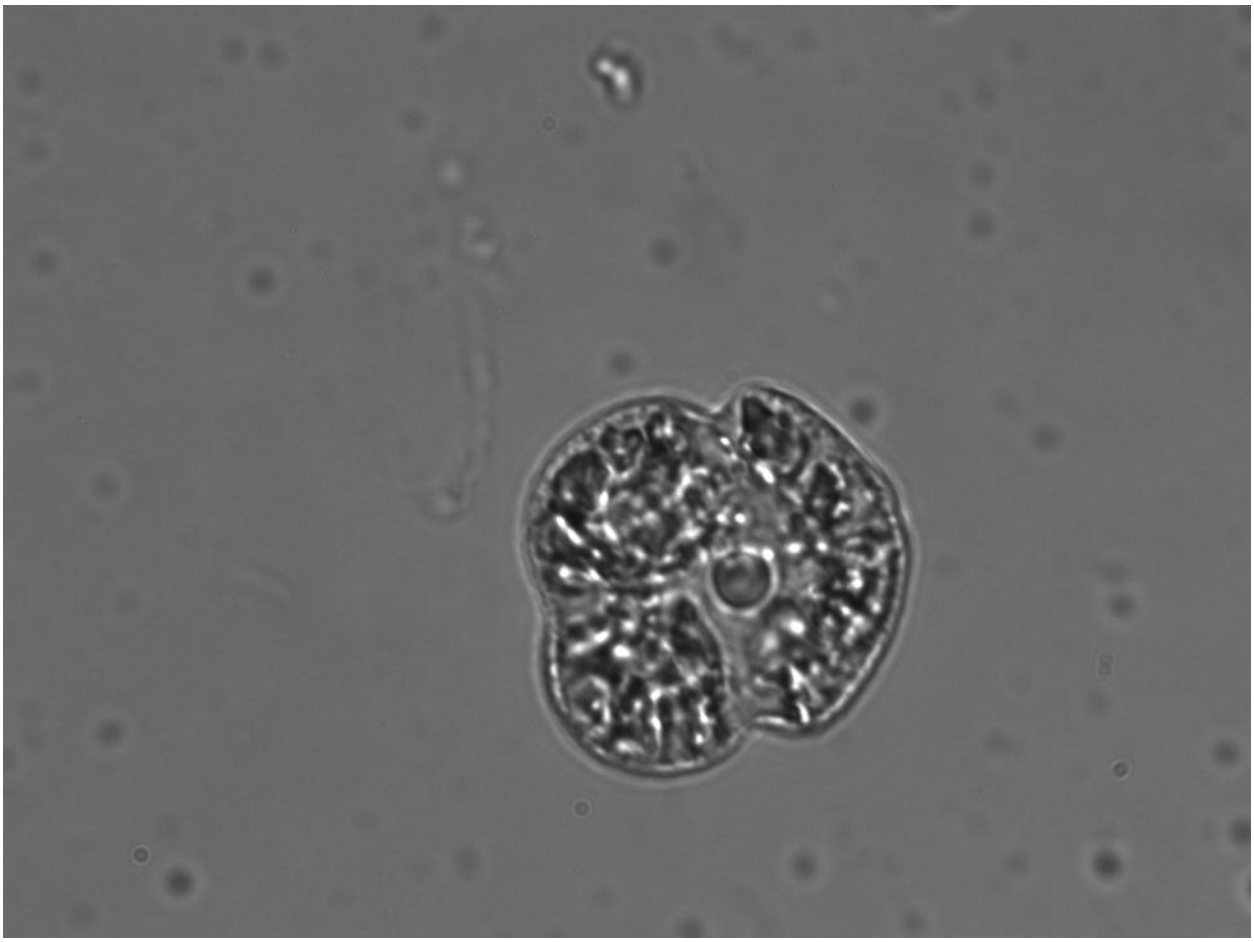

Fig. 1 A *K. brevis* cell viewed under a light microscope at 100x magnification

Dinoflagellates, which live in marine or freshwater environments, represent a large group of flagellated protists. The characteristics of a dinoflagellate vary dramatically from species to species in terms of ecology, lifecycle and effects on other living things. Because of this diversity there is much to discover about the biology of the individual species and their role in the ecosystem.

A few species of dinoflagellates have been noted as being of special commercial, industrial, and ecological interest since the 1700s as they produce harmful algae blooms (HABs)

which release neurotoxin into the surrounding environment (Contreras, Magna, and Villareal 2003). Harmful algae blooms in marine coastal environments are known more commonly as "red tides" because of the discoloration of surrounding water associated with them. *Karenia brevis* is one of the dinoflagellate species responsible for red tides especially around the coastal waters of Texas, coastal Spain, and Florida (Steidinger and Tester 1997). It is particularly important to study *Karenia* as their red tide blooms have been gradually increasing in duration and frequency since the earliest recorded incidences, and it directly impacts sea life, birds, and humans (Kirkpatrick et al 2004). Effects of the blooms range from mass mortality of marine organisms, seafood poisoning, human respiratory problems from brevetoxins kicked into the air by the surf, and devastation of entire ocean bottom communities (Martin and Martin 1976). Terrestrial ecosystems can be affected by the brevetoxins influx as well due to birds consuming tainted fish and dying inland ("Botanica Marina" 2013). Brevetoxins build up to the high concentration in filter feeders such as bivalves posing a risk to any humans or animals that consume them (Michael Echevarria et. all 2013).

During a previous experiment in Dr. McLean's lab, in which *Karenia brevis* was salinity stressed by diluting the culture over the period of a week, two unique sets of ribosomal RNA (rRNA) bands were detected on a bioanalyzer that were not present when cells were grown under normal salinity conditions. After trying multiple cultures under different types of stresses we found that after chemically fixing the culture we could see what would seem to be the source of this extra set of rRNA: a group of small motile objects that lysed their way out of the dying *Karenia* cells. Initial observations on the membrane section pinching off the cells revealed that

they move in a matter consistent with the presence of flagella.

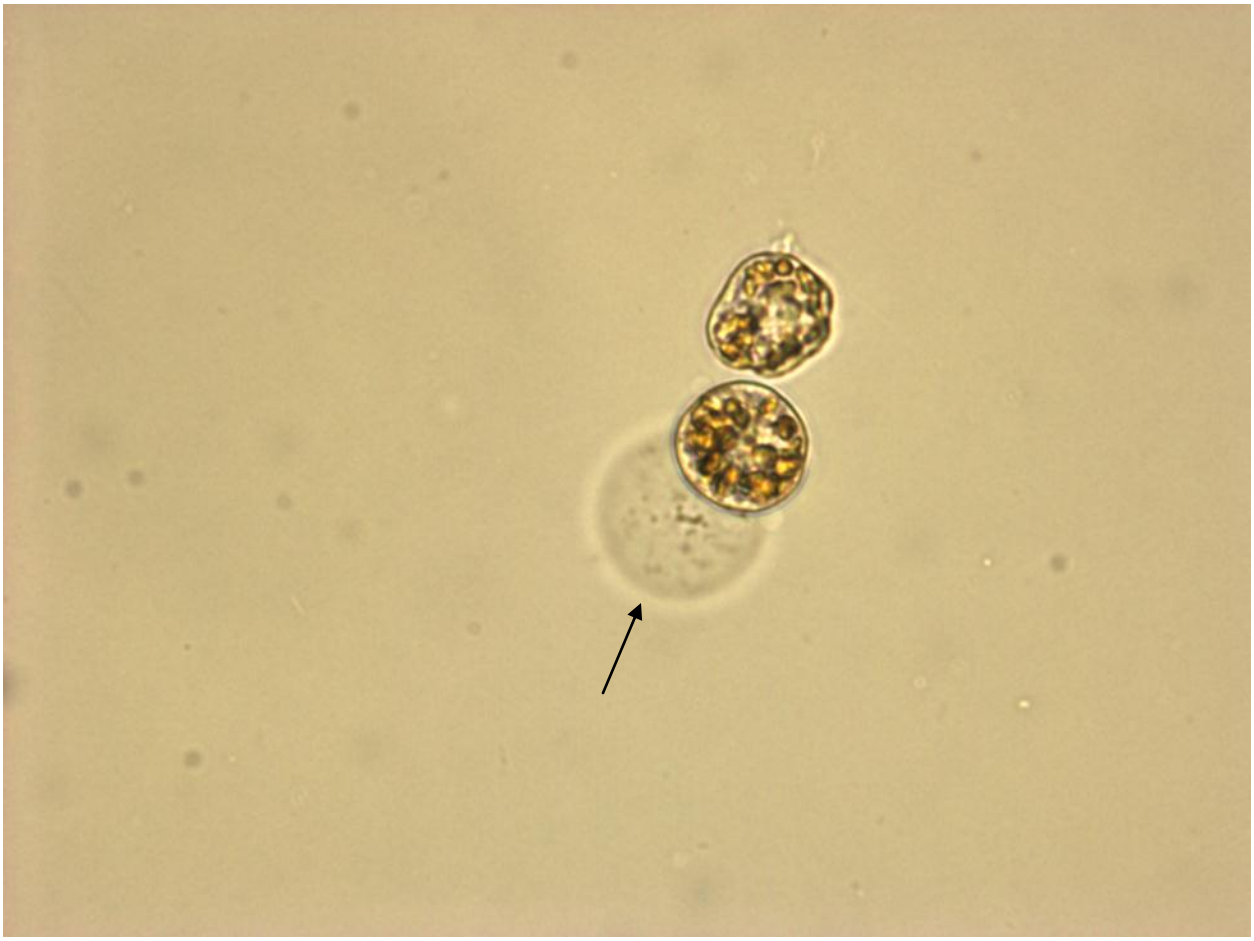

Fig 2. Salinity-stressed *K. brevis* under a light microscope at 100x magnification. The lower cell shows distortion in shape and membrane blebbing shown by arrow, small black objects within membrane bleb were highly motile under microscope.

Parvilucifera sinerae, which infects species of several different genera of dinoflagellates including *Karenia brevis*, looks like a possible candidate for the origin of this rRNA (Garces and Hoppenrath 2010). It is possible that this second rRNA set of bands could be associated with such a parasite or symbiote but the production of a distinct rRNA signal while the host is salinity stressed is not a recorded behavior in any of the known *K. brevis* associated intracellular organisms. *Parvilucifera sinerae* was originally isolated in another species of dinoflagellates

Alexandrium minutum from the coasts of Spain and identified as a different species separate from *Parvilucifera infectans* (Figueroa, Garcés, Massana and Camp 2008).

The overall goal of these experiments is to isolate and identify any organism inside the *Karenia brevis* cells and determine if they are indeed the source of this rRNA behavior. Since this unusual rRNA activity is tied to the environmental conditions of *K. brevis*, it may be a factor in determining the length and severity of red tide. The first goal of our research is proving or disproving the presence of an intracellular organism within *K. brevis*.

Throughout these experiments there were a number of observations that allow for some preliminary predictions. First, because of this previously undocumented behavior of these unknown intracellular organisms, they are most likely a novel parasite or a new strain of an older, better understood microbe. Also the fact that this intracellular organism has biological mechanisms to escape its host suggests that it is a facultative parasite or that it is part of a longer life cycle which includes parasitism. And finally and perhaps most importantly, because these microbes respond to stress caused by dilution of media, there may be a link to the cessation or induction of harmful algae blooms.

I hypothesize that this organism is a novel strain of an intracellular parasitic organism that is related to one of other species of parasites that are found in *Karenia brevis*. Also, the unique behavior this organism has of releasing rRNA upon the stressing of its host is part of a larger series of steps in its lifecycle. These predictions will be tested by comparing the nucleotide sequence of the rRNA from the unknown organism to the National Center for Biotechnology database.

Methods

Bioanalyzer

Bioanalyzers (Agilent Technologies) are the chip-based electrophoresis machines that were used to obtain the rRNA measurements revealing the two set of ribosomal RNA units found in stressed *K. brevis*. RNA extracted from stressed cells was denatured for 2 minutes on ice and loaded into one of the twelve sample wells on the RNA bioanalyzer nanochip. The prepared chip was loaded into the bioanalyzer and the automated software ran a series of capillary electrophoresis assays separating out RNA by size and purity. Data was collected in the form of figures 4 and 5 in the results section.

Salinity stressing *K. brevis*

Salinity was reduced in normal *K. brevis* cultures from Maine and Mississippi seawater by dilution with freshwater media over the course of a week. The cells are kept in an incubator with enough light and CO2 to support themselves through photosynthesis. The salinity dilution was performed by removing 20% of the culture each day and replacing it with distilled saline free water slowing reducing the overall salinity until it becomes a stressful environment for the algae. The salinity concentration of the culture at the start was 3.5 % similar to seawater the saline concentration after seven days of dilution was 0.7 % much too low for sea-living *K. brevis*. Salinity stressed cultures produce the rRNA with unusual patterns as seen in the bioanalyzer data.

Cold-shocking *K. brevis*

The bioanalyzer data reveals that the second set of rRNA signals appear after the *K. brevis* culture has been salinity stressed. Additional procedures for stressing the cultures were performed in order to ensure that other stressing methods produced the same result. A healthy one liter culture of *K. brevis* in fresh media was placed in a 17°C waterbath and allowed to sit for five days. Cold-shocking *K. brevis* for long periods of time places the cells near death but does not kill all of them. The intended result was to induce the stressed state in *K. brevis* in which the second set of rRNA signals appear so the potential intracellular organism could be more easily isolated though subsequent techniques.

Centrifugation isolation techniques of cold-shocked *K. brevis*

After stressing by cold-shocking aliquots of the shocked culture were pelleted down in 2 ml centrifugation tubes by centrifugation (Rotanta 460 centrifuge by Hettich) at various RCF values. This was done to find the exact RCF value, time and centrifuge settings that results in the shocked *K. brevis* becoming totally pelleted down but the potential intracellular organism remaining suspended. Six different aliquots of cold-shocked *K. brevis* were performed before the optimum centrifugation settings were found. Ramping up/down refers to the acceleration settings on the centrifuge in a 1-9 scale all settings were set to maximum for this assay. The supernatant above the pellet was observed under a light microscope at 100x to search for organisms remaining suspended.

Attempt	RCF	Time in minutes	Ramping up/down	Result(in supernatant)

1	350	4:00	9/9	*K. brevis* still visible.
2	700	4:00	9/9	*K. brevis* still visible.
3	1050	4:00	9/9	*K. brevis* still visible.
4	1400	4:00	9/9	*K. brevis* still visible.
5	1750	4:00	9/9	One *K. brevis* cell spotted under microscope.
6	1900	5:00	9/9	*K. brevis* absent, movement of possible intracellular organism spotted.

Table 1. Centrifugation Parameters Tested to Find Conditions for Clearing *K. brevis* Cells

Antibiotic control

Four cultures (kanamycin-treated, carbenicillin-treated, penicillin G-treated and an untreated control) were used to determine if killing bacteria outside *K. brevis* would have a noticeable effect on the cultures rRNA signatures. Each culture was 400 mls in volume and stored in a half liter bottle. Aliquots of each antibiotic treated culture and the control was cold-shocked for 5 days at 17 °C A second set of aliquots from each antibiotic treated and control cultures were instead placed in the incubator at 37 °C to provide a comparison for the effect of shocked versus unshocked antibiotic treated cultures. The concentration of each experimental culture was as follows: kanamycin 50 ug/ml, carbenicillin 100 ug/ml, penicillin G 10 units/ml from a 1596 units/ml stock.

Flagella stain

Lugol's iodine can be used as a flagella stain in cell cultures. It is prepared as a solution of 4% elemental iodide and 6% potassium iodide in distilled water. The solution should have the iodine stirred in the night before use and filtered through a 0.45 um syringe filter for best use. The slides used for microscope viewing of stained cells have to be cleaned in a 70% ethanol solution overnight and heated over a Bunsen burner for 3-4 seconds before use to ensure maximum clarity. 10 um of 5-day cold-shocked sample was added to a slide with 10 um of Lugol's stain and let air dry for 5 minutes. Once dried the samples were observed under a light microscope at 100X magnification and I searched for anything with a visible flagella other than *K. brevis*.

PCR amplification

The possible intracellular organism was registering bands suggesting a large subunit made with 28S rRNA and a small subunit with a 16S rRNA. Such a pattern is distinguishable from *K. brevis* by the fact that *K. brevis* has a 24S rRNA large subunit and a 18S rRNA small subunit. Seven sets of primers for generic eukaryotic rRNA genes were used in the hopes of amplifying and identifying a sequence that did not belong to *K. brevis*. All primers were ordered from the company Integrated DNA Technologies. Amplicon size is the exact number of base pairs between the forward and reverse primers the estimated size is where the amplified bands would appear on an agarose gel based on its resolution power. The purified nanodrop reading was used to ensure the amplification of each primer set produced enough usable copies.

Primer Set	Amplicon Size (bp)	Estimated Size (bp)	Purified Nanodrop Reading (ng/ul)
F63mod & R635	572	750	9.0
F635sq & R1630	995	950	3.9
F635sq & R1411	776	650	4.1
F635sq & R2077	1442	1250	10.9
F1580 & R2077	491	500	10.7
F63mod & R1630	1567	1500	9.7

F63mod & R1411	1348	1300	6.5

Table 2. Amplicons Produced by Combinations of 18S Primers

The PCR solution was set up with the following materials and concentrations for each set of primers. The solution was amplified using a standard PCR program on an Eppendorf thermocycler.

PCR solution:

10X PCR buffer ordered from Sigma-Aldrich: 2.5ul

100 μM dNTP solution from New England BioLabs: 0.2 ul

Taq polymerase (isolated and quantitated in lab): 0.25 ul

10 μM Forward primer: 0.5 ul

10 μM Reverse Primer: 0.5 ul

Deionized H2O: 19.05 ul

Sample DNA: 2 ul

Ligation of amplified gene products to pUC19 plasmid vector

All PCR amplification products were run on an agarose gel to separate the amplified bands from any leftover fragments of culture DNA. The bands corresponding to the PCR products were then cut out and purified with a NucleoTrap PCR band purification kit. The isolated nucleotide insert was added to the pUC19 vector ordered from Clontech in a 6:1 ratio of insert to vector in nanograms along with 1.0 ul 10X T4 ligase buffer and 0.5 ul T4 ligase. (The T4 reagents were ordered from Promega, Inc.) The mixture was vortexed for 10 seconds and let sit for 30 minutes at 22.5°C. The ligase was then denatured at 67°C for 10 minutes. The resulting vector with insert was stored at -20°C until being used in the subsequent protocols.

Transformation into *E. coli*

For each set of ligated plasmids 5 ul of 10ng/ul pUC19 plasmid vector with the PCR amplified DNA were added to 50 ul of *E. coli* culture that were grown overnight then gently stirred for 30 seconds and incubated on ice for 5 minutes. EcoR1 and Sac1 restriction enzymes were used to open the plasmid and ligase was used to ligate the insert to the pUC19 vector. All enzymes were ordered from Promega. The vector contained a gene for resistance to kanamycin so for each set of *E. coli* with vector and insert agarose plates with kanamycin were prepared and 10 ul of transformed *E. coli* were spread across the plate with a sterilized rod and allowed to grow in an incubator at 37 °C for one day. To ensure that the cultures had taken up the plasmid with the vector and not just the vector itself ten cultures from each agarose plate were grown in broth at 37 °C in an incubator for one day. Two of the healthiest looking sets of these transformed *E. coli* had their plasmids extracted, cut into a linear strand with a restriction enzyme and run on an electrophoresis gel to determine which *E. coli* cultures had a taken

plasmids with the insert based on size of the linearized plasmid. Plasmids were extracted following protocol from a Promega plasmid purification kit.

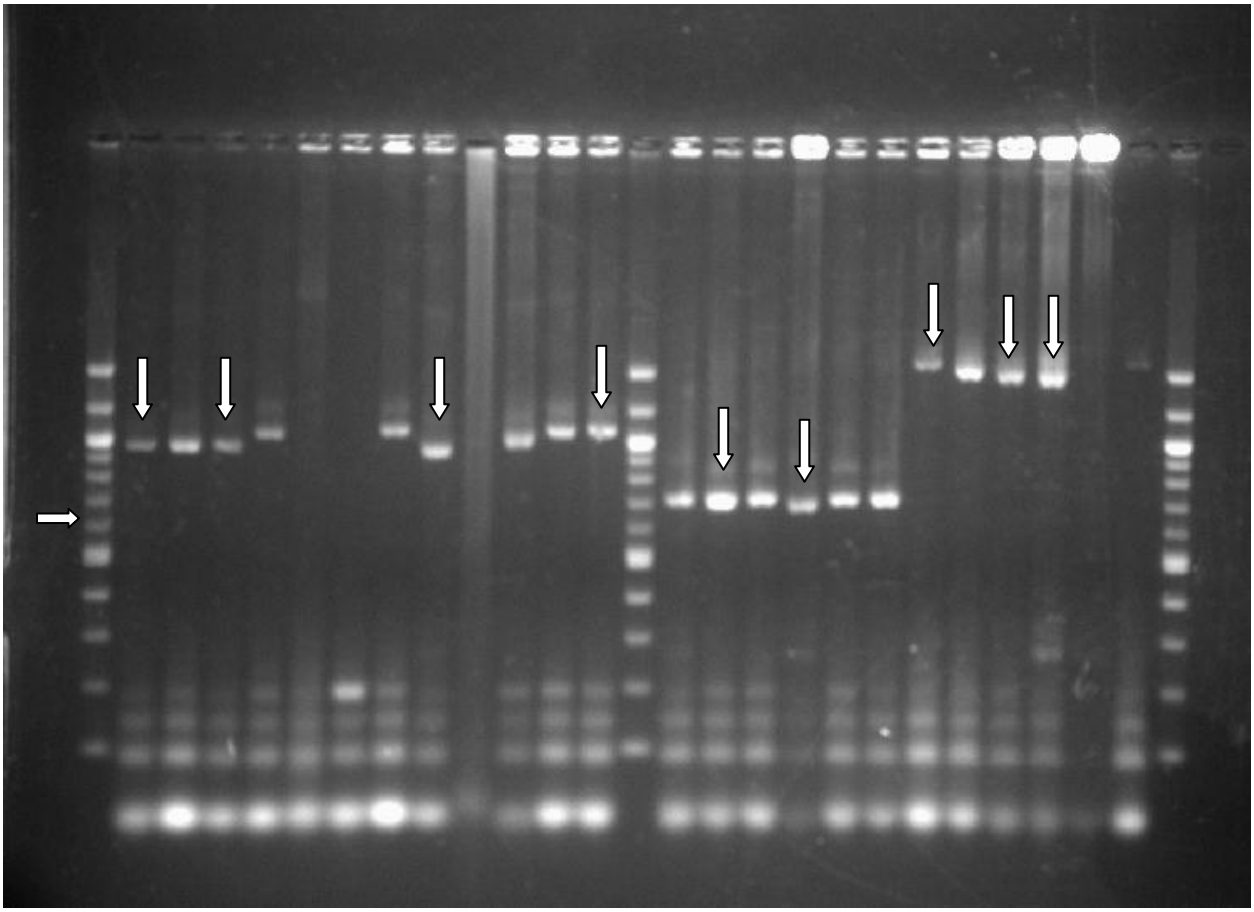

Fig 3. Electrophoresis gel of plasmid inserts extracted from transformed *E. coli* from left to right the gel was loaded: 10kb ladder, samples A5, A7, B4, B6, B7, C7, C4, C5, D7, D8, D9, 10kb ladder, samples E8, E5, E6, F7, F8, F9, G3, G10, H5, H7, and 10kb ladder. The bar at the left indicate the approximate position of the empty pUC19 vector. The arrows denote samples that were isolated from the gel for sequencing.

pUC19 has 2686 base pairs so all bands that corresponds to a point on the ladder larger than roughly 2.7 kb include an insert and were extracted via plasmid extraction kit and prepared for sequencing and BLAST. A5, B4, C4, D9, E5, F7, G3, H5, and H7 were selected for

sequencing and BLAST alignment they are marked on figure 3 by arrows. The horizontal arrow shows the size of the pUC19 plasmid without an insert any band higher is assumed to have taken up an insert.

BLAST

The cloned and extracted DNA from the PCR products had to be sequenced and BLAST compared against NCBI's database to make sense of the data. BLAST stands for Basic Local Alignment Search Tool it is a software program available on the NCBI's website that compares gene sequences against several genome libraries and finds similarities in sequences. The plasmids were extracted from the *E. coli* and mailed to another lab to be sequenced. Sequences received from the nucleotide sequencing service were input into the NCBI database and any non *K. brevis* results were examined for any similarity to the known characteristics of the potential intracellular organism.

Douncing

Douncing is the physical breaking of cells in a mortar and pestle like test tube with a glass rod. In order to better visualize the potential intracellular organism as a separate entity from *K. brevis* cells a douncing method was used for breaking open *K. brevis* cells and isolating nuclei in a sucrose gradient by centrifugation.

K. brevis cells from a healthy culture were pelleted down at 1500 RCF for 5 minutes and the supernatant was poured off the pellet. The pellet without supernatant was resuspended in 500ul of CE solution composed of 10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% NP40, 1mM DTT and 1 mM PMSF . The resulting suspension was incubated on ice for 10 minutes.

After incubating the cells were broken open by douncing 25 strokes within a 10 ml douncing mortar and pestle. Two sucrose buffers at different concentrations were used to separate dounced products; sucrose buffer 1 at 10% sucrose and sucrose buffer 2 at 20% sucrose. The lysed cells were moved to a new 1 ml centrifuge tube and 500 ul of sucrose buffer 1 was added to them. The resulting mixture was spun down at 600 RCF for 10 minutes in a 4 °C cold centrifuge. The supernatant was removed from the centrifuged tube isolating a pellet. Another 500 ul of sucrose buffer 1 was added to the microcentrifuge tube and the tube was spun down again at 600 RCF for 10 minutes in a 4 °C cold centrifuge. The resulting supernatant was removed and the pellet was resuspended in 500 ul of a 1:1 ratio of sucrose buffer 1 and sucrose buffer 2. A new 1 ml microcentrifuge tube was prepared and 500 ul sucrose buffer 2 was added to it. Then 500 ul of the cell pellet suspension was slowly added to the new tube. The new mixture was spun at 16,000 RCF for 30 minutes in a 4 °C cold centrifuge. The resulting gradient had the nuclei of the *K. brevis* culture and the unknown organism isolated at the bottom of the sucrose gradient in the microcentrifuge tube.

DAPI Stain and confocal microscope viewing

K. brevis nuclei extracted by douncing, whole cells from normal cultures and 5 day cold-shocked whole cells were stained with DAPI and viewed under a confocal microscope picking up DAPI florescence as well as autoflorescence of the cells. DAPI was used at 20 ug per ml of culture from a 500 ug/ml stock and added immediately before viewing of the cells for best result. 10 ul of DAPI+culture mixture was used per viewing and the coverslip of each slide was held in place with dried acrylic nail polish to stabilize the viewing. The DNA of any cells inside or outside the *K. brevis* cells should be visible by this method.

Results

Bioanalyzer results

Results from the bioanalyzer samples both from stressed cultures of *K. brevis* and normal *K. brevis* cultures were collected and compiled in figures 4 and 5. The size of the bands is measured on the y-axis by nucleotides (nt).

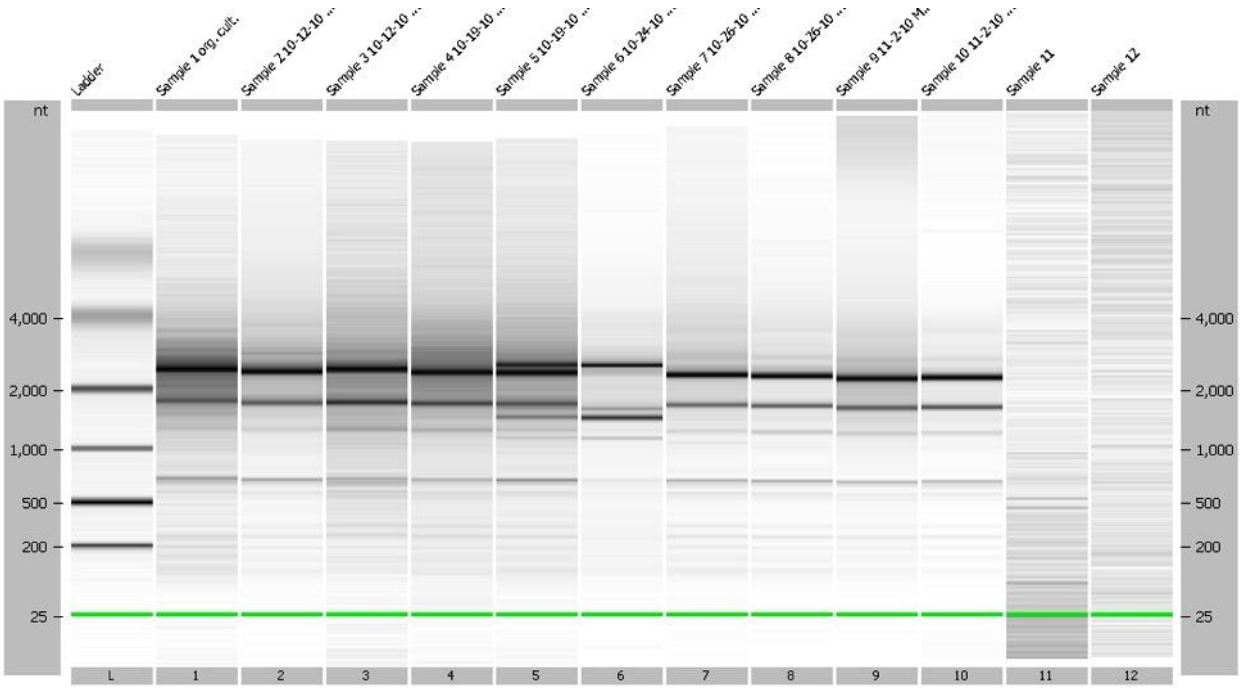

Fig 4. *K. brevis* cultures during multiple stages of shock Lane L: ladder of ribonucleic acid fragments of known length, lane 1: original culture, lane 2: Maine seawater 32% salinity, lane 3: Mississippi seawater 25% salinity, lane 4: Maine seawater 32% salinity, lane 5: Mississippi seawater 21% salinity, lane 6: Mississippi seawater 19% salinity, lane 7: Maine seawater 32%, lane 8: original culture, lane 9: 400 ml MS + 100 ml ME culture, lane 10: 400 ml ME + 100 ml MS culture

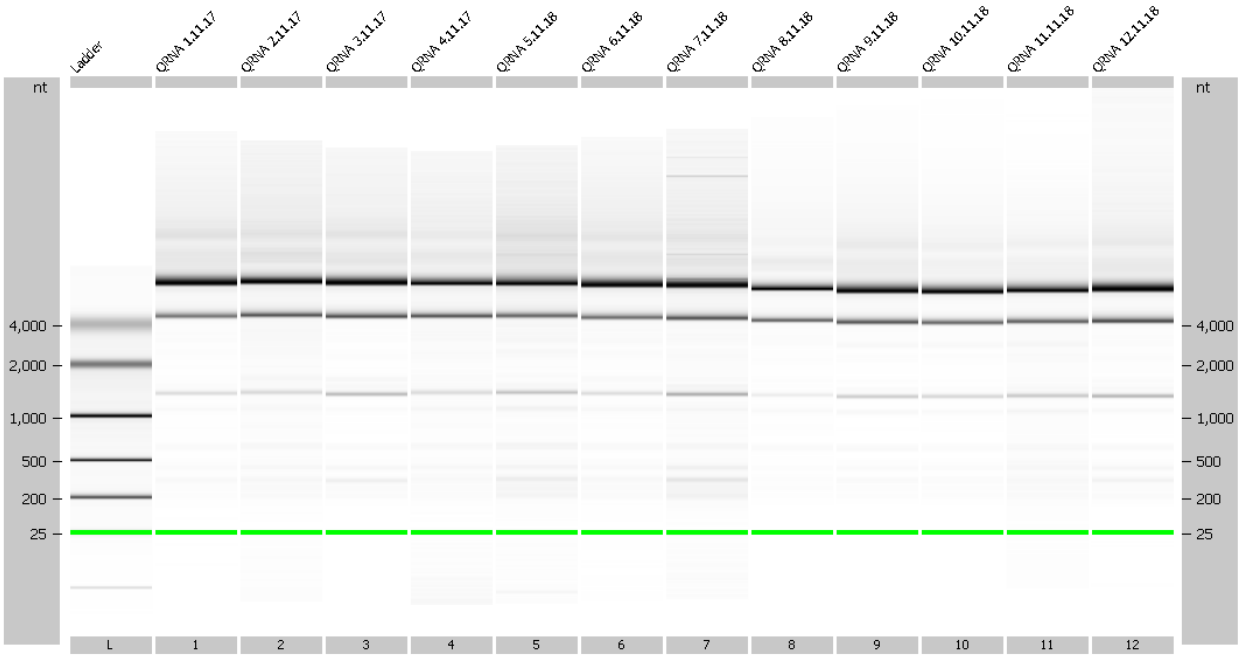

Fig 5. *K. brevis* normal total RNA banding patterns

As salinity was decreased through dilution of their original saltwater media two separate patterns emerged (See Figure 4). These results are not consistent with RNA degradation because the original *K. brevis* bands remain, but they are joined by a new set of slightly larger and slightly smaller bands. The larger band could not have been produced by degradation of *K. brevis* rRNA. Culture of *K. brevis* not exposed to stress never develop these additional rRNA bands even if a healthy culture contains several dead cells (See Figure 5). This observation means that the death of the cells alone does not cause this phenomenon only slowly crashing cultures exhibit this effect.

Cold-Shocked *K. brevis*

K. brevis cells cold shocked for 5 days at 17 °C were used in nuclei extractions as seen in in the DAPI stain extracted nucleus results and imaged under a light microscope at 100X magnification.

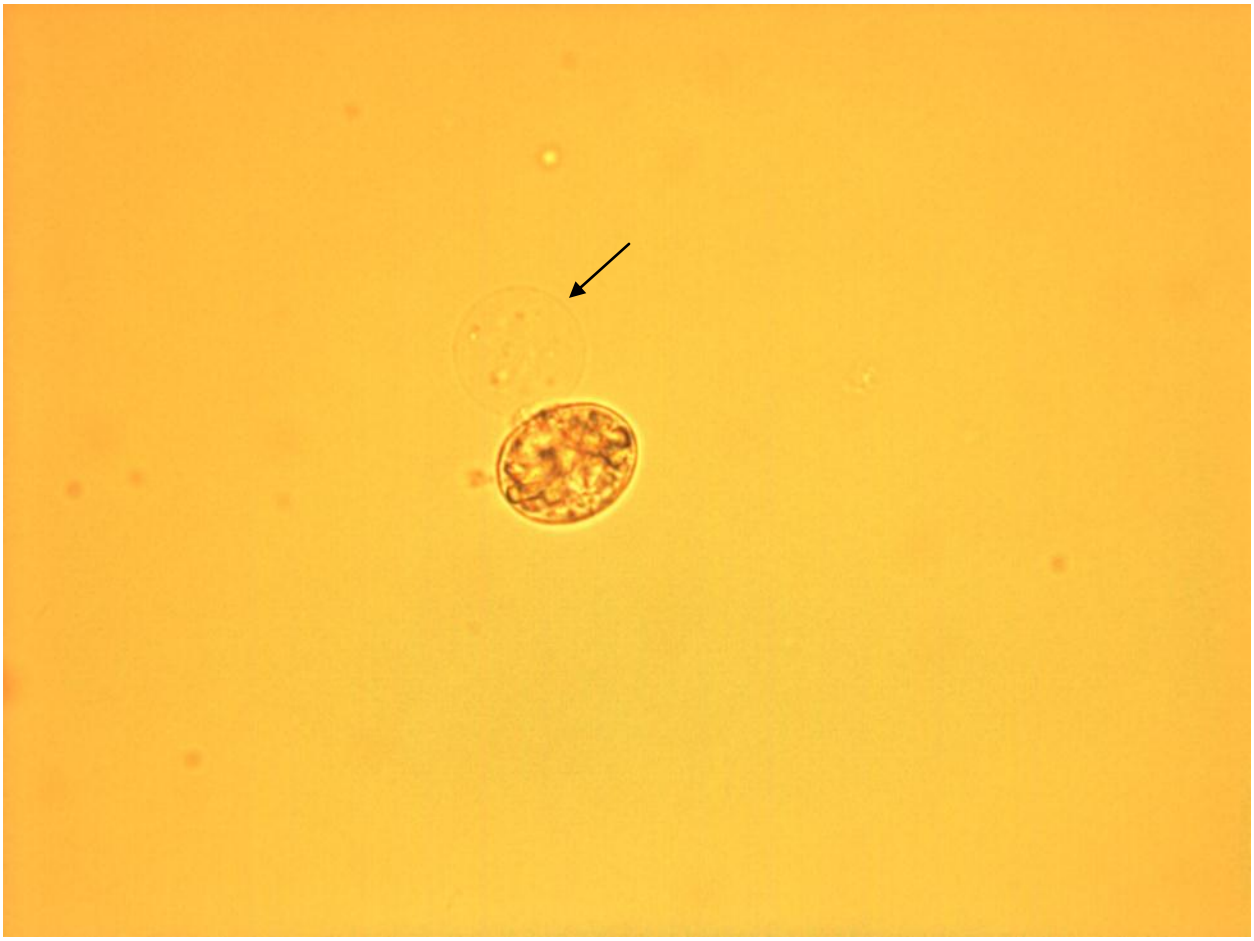

Fig 6. Cold-shocked *K. brevis* cells under 100X light microscope magnification

Cold-shocked cultures show the same pattern of membrane blebbing and small motile objects moving within marked by arrow compare Figure 2 and Figure 6.

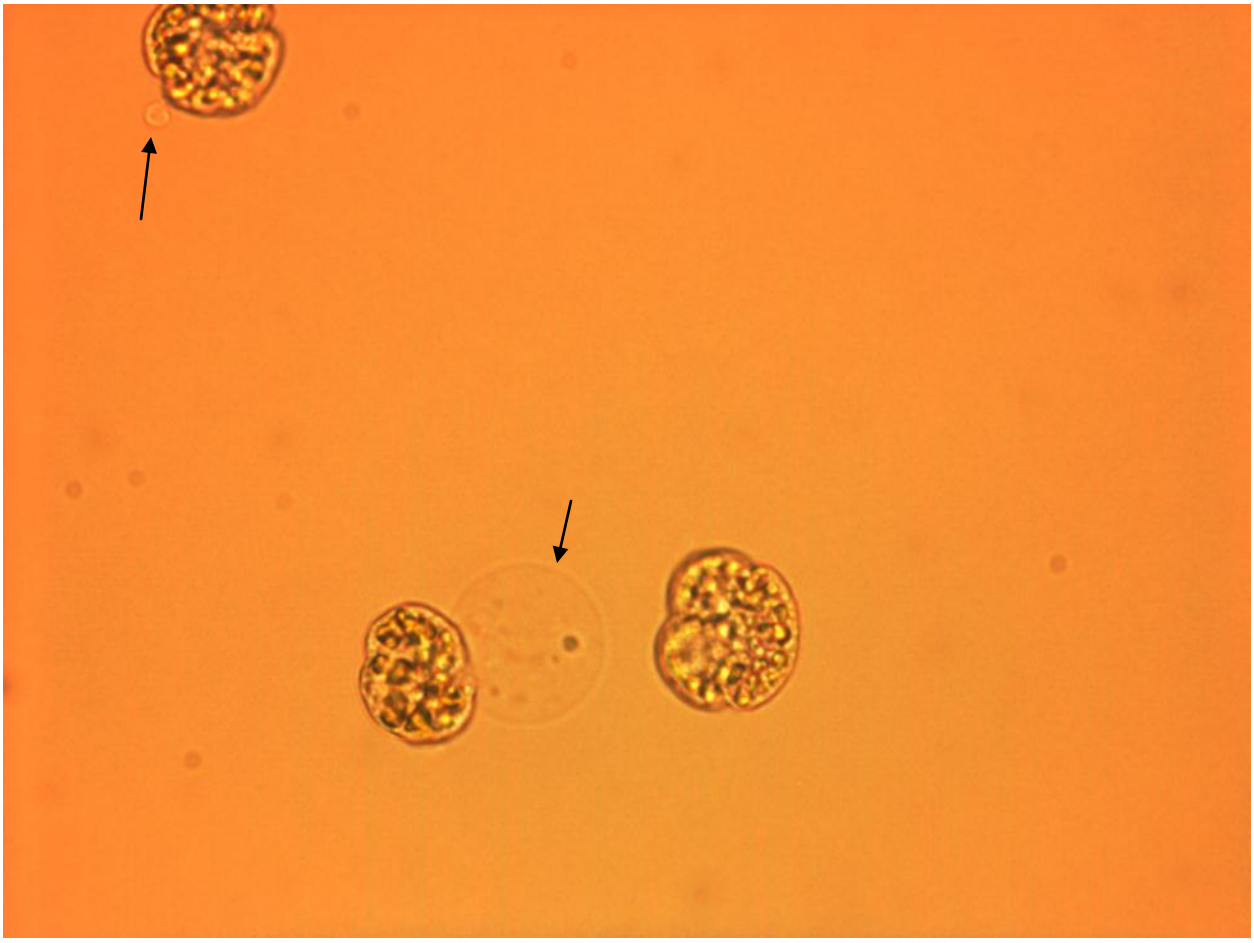

Fig 7. Cold-shocked K. brevis cells under 100X light microscope magnification

Effects of cold-shocking vary from cell to cell as seen in Figure 7. The lower left cell shows a large notable membrane bleb with motile elements while the lower right cell looks normal and healthy. Upper left cell is developing a small growing membrane bleb with no

movement within it at the time of the viewing. Blebbing is marked by arrows.

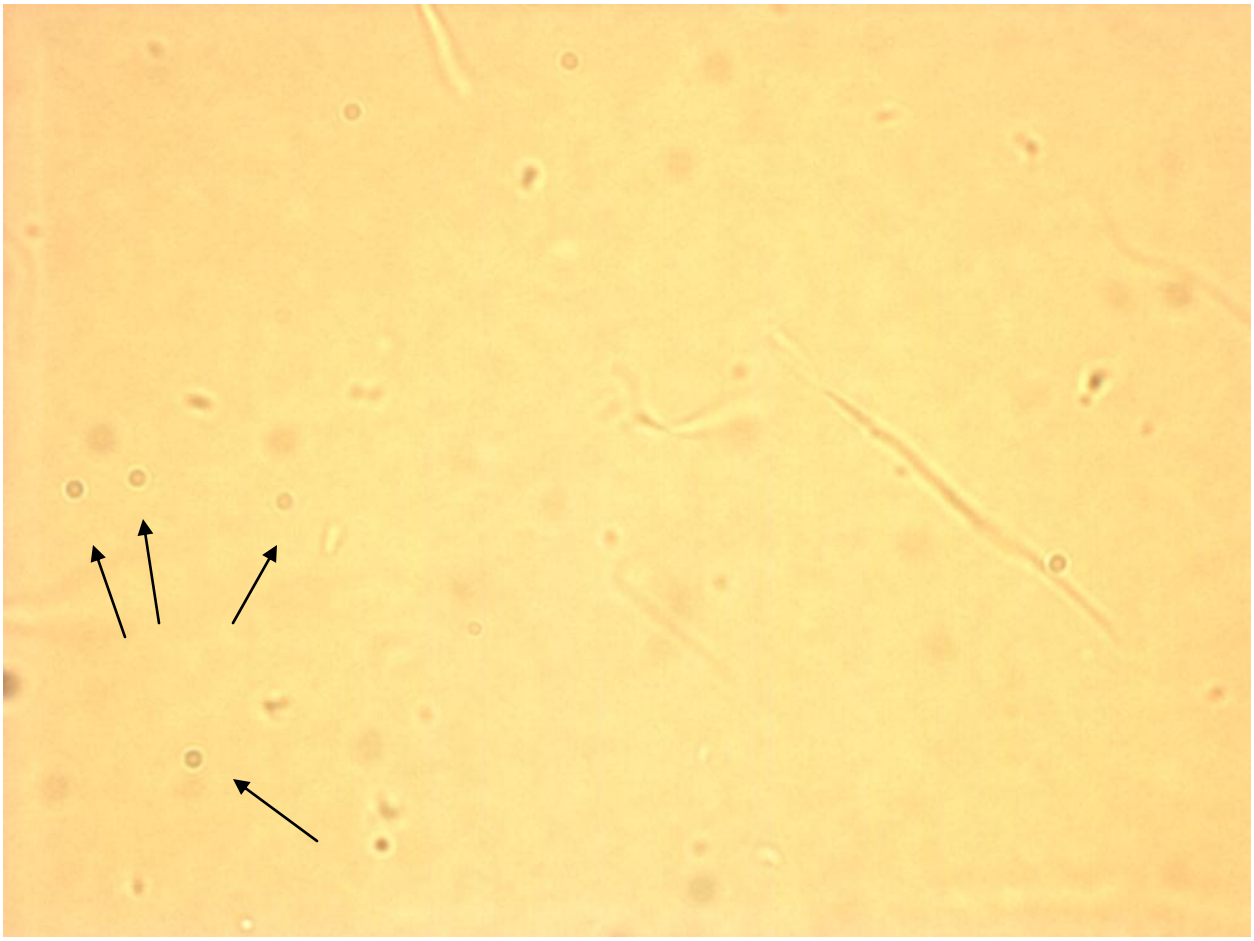

Fig 8. Supernatant taken from cold-shocked *K. brevis* cells using centrifugation techniques from Table 1 under a light microscope at 100X magnification.

Small round motile objects are highly prevalent in the media when cold-shocked cells are spun down seen in Fig 8. The objects are marked by arrows and are about a tenth the size of a *K. brevis* and highly motile on the slide. A small cluster of the objects can be seen of the left side of the image in Figure 8. They highly resemble the motile elements seen in the membrane blebs of Figure 6 and 7.

Antibiotic-Treated *K. brevis*

Antibiotic-treated *K. brevis* were bioanalyzed using the same methods used on salinity-stressed *K. brevis* cells that produce the results seen in Figure 4. However, the bioanalyzer data from the antibiotic treated cultures were rendered unreadable by data noise or contamination. Time constraints prevented repeating the week long preparation and data extraction but further research should test if failed results are a result of the antibiotic treatment itself or a machine error.

Lugol's Stain

Under observation of a light microscope Lugol's iodine did not show a positive sign of flagella other than that of *K. brevis*. The stain was functional on *K. brevis* flagella but only slightly with small clumps of pigmentation sticking to the flagella rather than making the entire structure visibly stained. Several staining attempts with several variations on preparation, *K. brevis* cultures used and concentration of Lugol's stain but all produced the same negative result.

PCR and BLAST

Results of BLAST alignment for each selected pUC19 plasmid clone and insert based on closest genetic matches. All matches were for the 18S eukaryotic ribosomal subunit amplified by the primers described in Table 2.

A5: *K. brevis*

B4: *K. brevis*

C4: *Gymnodinium aureloum, Amphidinium semilunatum, Prorocentrum donghaiense, Karenia mikimotoi, takayama acrotrocha*

D9: *Gymnodinium aureloum, Amphidinium semilunatum, Prorocentrum donghaiense, Karenia mikimotoi, Takayama acrotrocha*

E5: *Gymnodinium aurelous, Prorocentrum micons, Prorocentrum donghaiense, Akashino sanguinea, Cochlodium polykrikoide*

F7: *Gymnodinium aurelous, Prorocentrum micons, Prorocentrum donghaiense, Akashino sanguinea, Cochlodium polykrikoide*

G3: *Komikimotoi, G. aureolum, K. umbrella, T. aerotrocha, Karlodinium antarcticum*

H5: *Komikimotoi, G. aureolum, K. umbrella, T. aerotrocha, Karlodinium antarcticum*

H7: *K. brevis*

The BLAST results we obtained other than *K. brevis* are all dinoflagellates that are not present in our culture. The reason they were returned from the database search is because the gene sequences obtained from our primer sets are similar in most dinoflagellates. None of the organisms that match our sequences are small enough to be an intracellular organism in *K. brevis*.

DAPI Stain Extracted Nucleus

DAPI stains DNA by intercalating between the turns of the helical molecule the stain itself fluoresces blue under a confocal microscope. Many organelles within *K. brevis* naturally

fluoresce green due to their contact with chlorophyll. These wavelengths were used to check for living things other than *K. brevis* in samples with extracted nuclei and stressed whole *K. brevis* cells. The expected size of the hypothetical unknown organism is in the average prokaryotic range of between 1-3 micrometers the nuclei of *K. brevis* is about 20 micro meters in diameter for comparison.

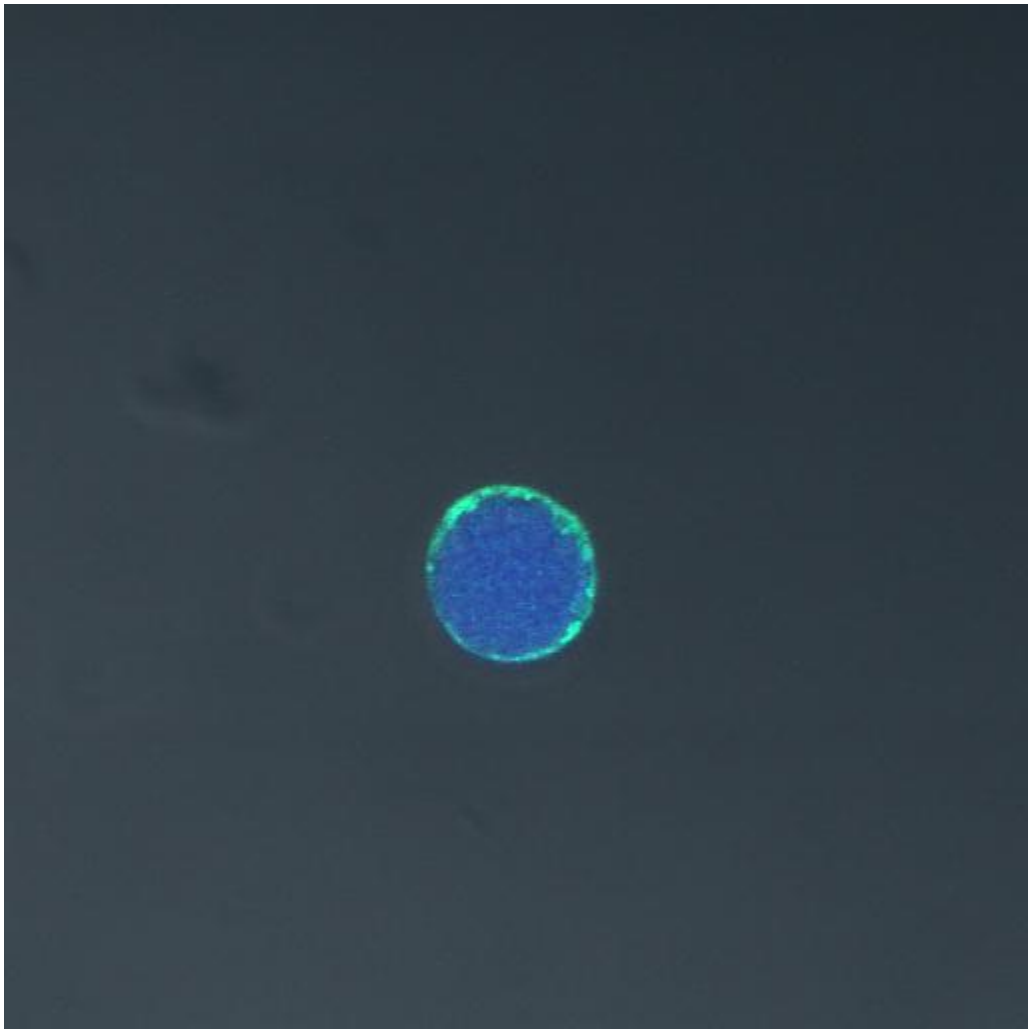

Fig 9. Douncing-extracted nuclei with blue DAPI fluorescence and green autofluorescence

Fully intact *K. brevis* nuclei under confocal microscopy contain small autofluorescent coat but no sign of other organisms (Figure 9). Some non-fluorescent objects moving around the nucleus could be seen but nothing consistent with an intracellular organism.

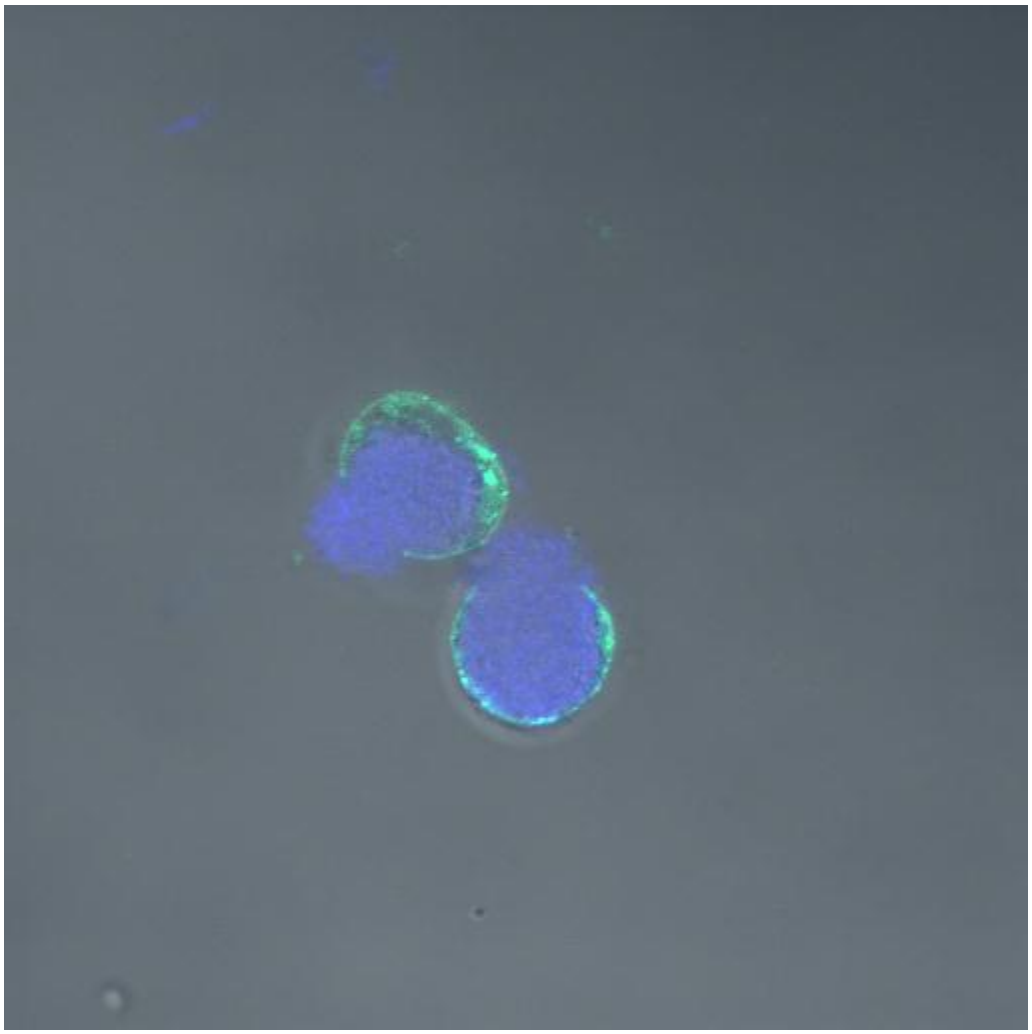

Fig 10. Douncing-extracted broken nuclei with blue DAPI fluorescence and green autofluorescence

Douncing broke some of the nuclei apart resulting in these crescent structures with chromosomal DNA spilling through the broken envelope (Figure 10).

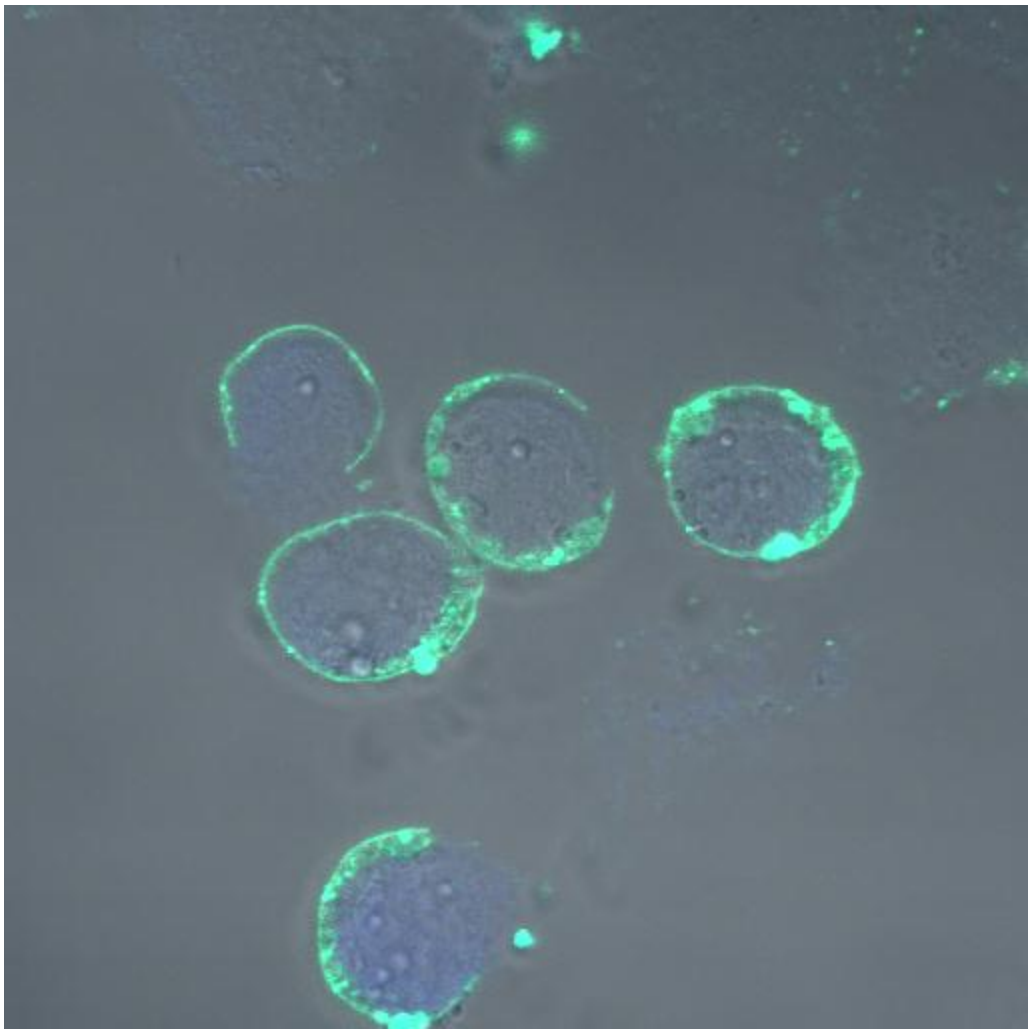

Fig 11. Douncing-extracted nuclei with blue DAPI fluorescence and green autofluorescence

More nuclei some intact and some broken are seen in this image (Figure 11). The cells have large dots of autofluorescence on the outsides of the nucleus which are likely segments of photoactive pigments that got stuck to the nuclear envelope somehow during douncing.

DAPI Stain on Whole Cells

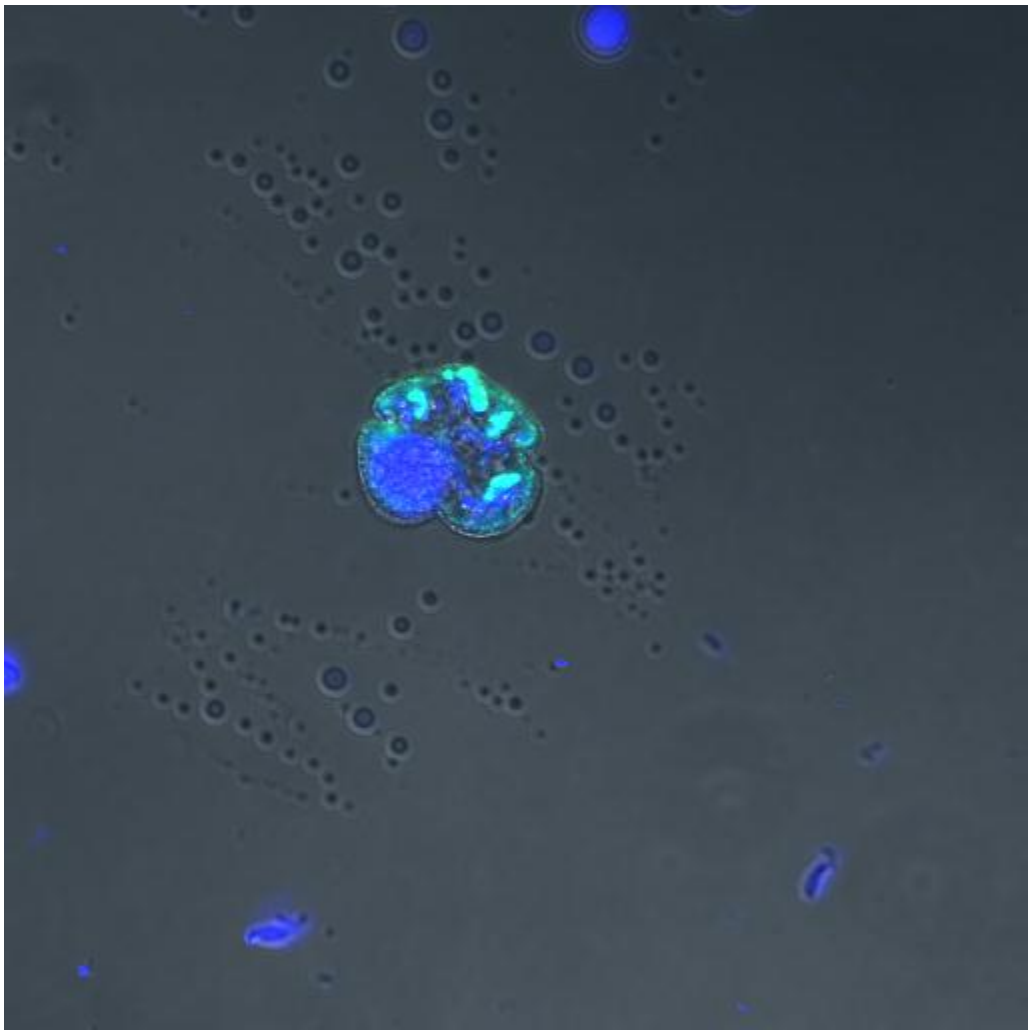

Fig 12. Whole *K. brevis* cell from a non cold-shocked culture blue DAPI fluorescence and green autofluorescence

In Figure 12 the cell is intact and shows DAPI activity outside of nucleus but within cell for unknown reason. Other healthy cells have DAPI fluorescence restricted to nucleus.

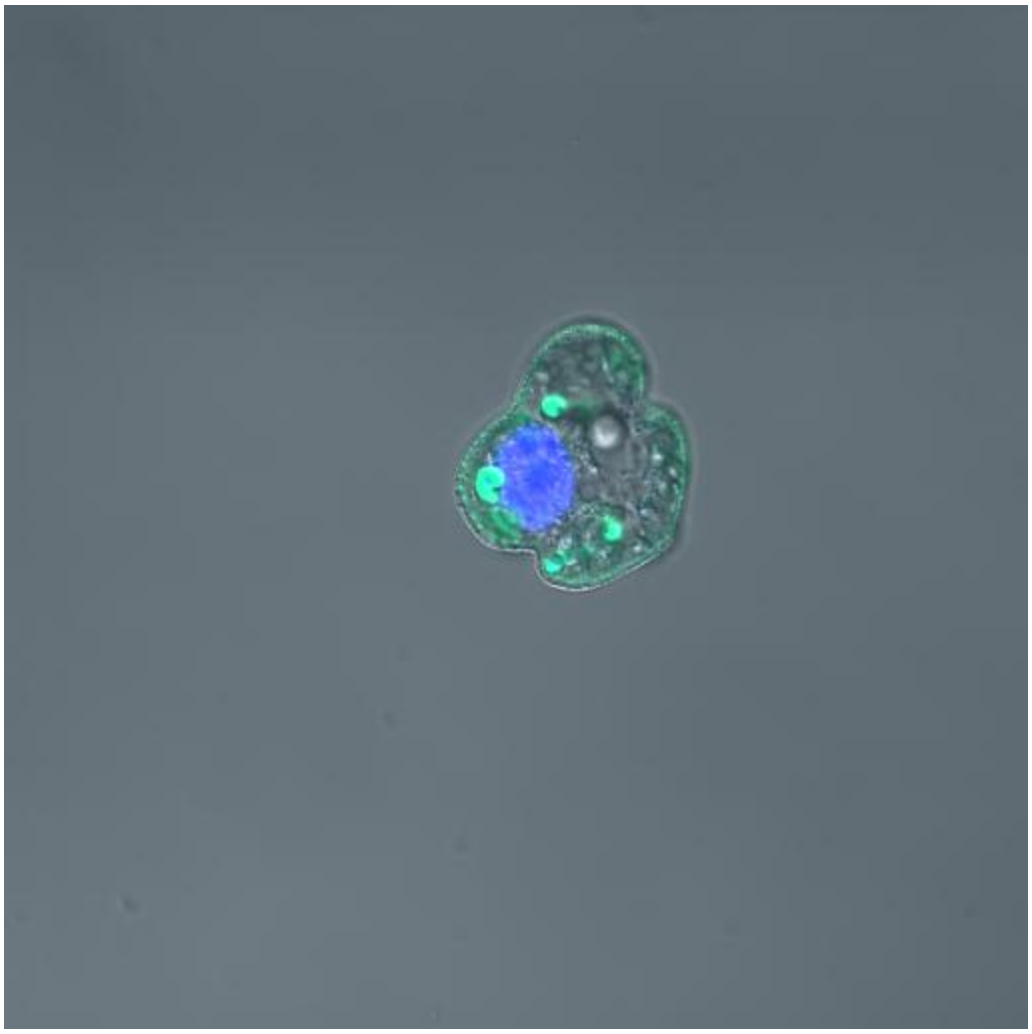

Fig 13. Whole *K. brevis* cell from a 5-day 17 °C shocked culture blue DAPI fluorescence and green autofluorescence

Not all shocked *K. brevis* cells would deform or lyse. The cell in Figure 13, for example, seemed perfectly intact after shocking. No sign of unusual intracellular activity.

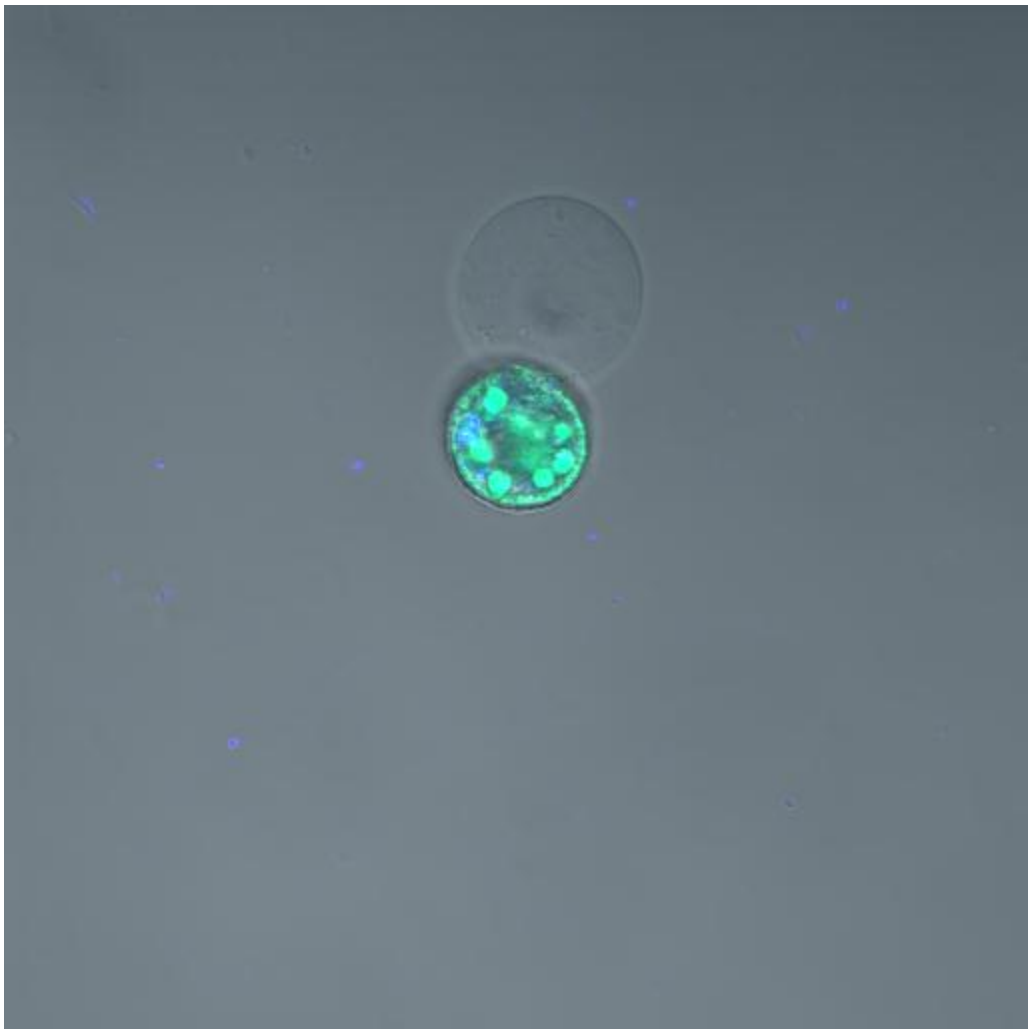

Fig 14. Whole *K. brevis* cell from a 5-day 17 °C shocked culture blue DAPI fluorescence and green autofluorescence

I captured an image of a cell undergoing membrane blebbing (Figure 14). The cell shows characteristic deformation and membrane-blebing but there is no fluorescence within the pinched off section of membrane. Small dots of DAPI fluorescence can be seen around the cell which is likely debris from less intact cells and not organisms associated with *K. brevis* due to the lack of interaction with the extended membrane.

Results discussion

Although initial results were all consistent with the presence of an intracellular organism as the source of the anomalous rRNA activity the final results of our experiments provide strong evidence against the presence of an intracellular organism as the explanation for unusual rRNA activity in *K. brevis*. Flagella staining did not show any conclusive evidence of flagellated organisms outside *K. brevis* meaning the movement seen within the membrane blebs of shocked *K. brevis* cells is likely just Brownian motion of cell debris.

Multiple rounds of DAPI staining and viewing with a confocal failed to show any sign of an intracellular organism living within *K. brevis*. There was also an absence of fluorescent activity within pinched off segments of *K. brevis* membranes which was where the objects originally believed to be intracellular organisms were first spotted further reinforcing that the movement under the light microscope was simply due to debris.

NCBI database BLAST analysis also showed nothing except *K. brevis* DNA and alignments with other species of dinoflagellates that were likely just conserved sequences among the Dinoflagellata phylum all ultimately from *K. brevis*. None of the organisms that were matches on the NCBI BLAST program had ribosomal subunits the size implied by the bioanalyzer data and although some have parasitic or symbiotic behavior they were all too large to be live inside *K. brevis* without being easily detected. Eukaryotic primers instead of prokaryotic primers were used because in all three antibiotic treated cultures the motile elements were still seen when treated and most prokaryotes are vulnerable to at least one of the antibiotics we used making a prokaryotic source unlikely.

The ultimate source of the unusual rRNA behavior remains unexplained but due to the importance of studying harmful algal bloom associated organisms other hypotheses should be explored. Because the second set of rRNA bands is probably not from another organism the next most likely explanations is that the second set of rRNA is due to alternate splicing or the expression of completely different genes. Other cultures of *K. brevis* from multiple locations should be analyzed to see if it is only our *K. brevis* cultures that exhibit this phenomenon or if it is a trait common to all *K. brevis*. Determining if close relatives of *K. brevis* that belong to different families of Dinoflagellata also exhibit anything similar would also help in establishing a pattern and making sense of the origin and function of this behavior.

Future research should focus on transcriptional and translation activity during culture stressing. Total cDNA of stressed cultures could be collected and examined for possible alternate splicing or expression of normal *K. brevis* ribosomal proteins that could result in a change in size. Other molecular techniques such as attempting to isolate all ribosomal proteins from stressed cells and then separating those proteins out by size and getting a proteins structure of the unusually sized ones by crystallography may be the most direct approach for future work. Because of its large size of the *K. brevis* genome it has not been fully sequenced so molecular techniques should be used more extensively than genome searching.

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