ABSTRACT

(Under the direction of Daniel Kamykowski.)

Many laboratories have solely used the Wilson isolate to characterize the red tide dinoflagellate species *Karenia brevis*. Recent work has provided new isolates from different geographical locations. Prior to detailed biochemical investigations, laboratory studies were conducted on ten different isolates representing geographical locations around the coast of Florida. The investigation of the physiological parameters for the ten different isolates provided a foundation for the proper selection of a single isolate that would closely represent the species in the field. Each isolate was capable of a range of responses with primary dependence on cell counts within the cultures. Evidence suggests that either carbon limitation or bacterial negative feedback caused changes in the physiological parameters as cell counts increased. The Apalachicola isolate was selected for further biochemical investigations because it was representative of the group response.

A nutrient-replete intermediate-light mesocosm experiment investigated the taxis patterns, and the biochemical composition of vertically migrating *K. brevis* populations. This experiment confirmed the internal biochemical status of the cell controlled migratory behavior, growth and reproduction. Young cells fixed carbon at the surface while older cells limited vertical migration toward the middle depth in anticipation of cellular division. The cells were capable of performing complex lipid control during vertical migration. Quick turnover of these lipids indicated *K. brevis* was more than capable of taking advantage of changing physical conditions.
Finally, nutrient replete and deplete mesocosms investigated how *K. brevis* responded to field-simulated high light conditions. Results from high light and nitrogen-limited exposure suggested *K. brevis* may utilize the benthos by minimizing exposure to oxidative stress and as a nitrogen source supplied from benthic pore-water flow in permeable sediments. High light also caused *K. brevis* to increase toxin concentrations while decreasing its sterol lipid class concentrations. This response caused *K. brevis* to be more toxic at particular times of the day. Data from this experiment led to the conclusion that cell division yielded unequal daughter cells.
Biochemical Analysis of Diel Vertical Migration
in the Red Tide Dinoflagellate *Karenia brevis*

by

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Blake A. Schaeffer was born in Manhattan, New York and grew up in a small town located 10 minutes from the New Jersey shore called Toms River. Here, his family introduced him to life in the ocean and all its spectacular sights. From the beginning of grade school Blake knew he wanted to be a scientist and help out the world wherever he found his niche. Biochemistry became his area of focus upon entering college. During his senior year at Albright College a course on coral reef ecology at Bermuda’s Biological Station for Research brought Blake back to the ocean where he rediscovered his enthusiasm for marine sciences. An application to the Marine, Earth and Atmospheric Sciences program at North Carolina State University led him to be introduced to what he felt was his place in the world. In the future, Blake looks forward to using his expertise to contribute his small part of the pie to the world.
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CHAPTER 1

A comparison among ten different *Karenia brevis* isolates.

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INTRODUCTION

Typically, experiments in phytoplankton ecology assume a single isolate culture of a species is representative of all cells of that species in the field. This assumption is based on the premise that there is no genetic variability in the species. Therefore, ecological statements generated from an isolate culture must assume genetic variation is limited in the studied species. Even though there is general agreement that genetic variability exists within species (Brand et al. 1981; Greer and Amsler 2004), it is still necessary to perform laboratory experiments for a better understanding of field populations. For example, physiological research with laboratory isolates of marine phytoplankton species can provide useful information that can be representative of the physiology of field populations (Wood and Leatham 1992). Since experiments cannot examine all cells of a particular species, a representative must be identified. A single isolate culture may provide a representation of the species if multiple isolate cultures are examined to identify an isolate that is within the range of observations (Brand et al. 1981).

Steidinger et al (1998b) reported that blooms of *K. brevis* usually develop 18-75 km offshore of Florida’s West Coast and then moved shoreward. This initial transportation from offshore to nearshore presented dramatic changes in light exposure to which the cells must adapt to survive. In 1987, *K. brevis* blooms were reported as far north as North Carolina. This report showed *K. brevis* can sustain its population in changing light conditions from the Gulf of Mexico, to the Gulf Loop Current, through the Gulf Stream, and into the coast and estuaries of North Carolina (Tester et al. 1988; Warlen et al. 1998). During transportation, these blooms passed through oligotrophic and
eutrophic waters, and light conditions that changed with latitude. The blooms also crossed dramatic temporal oceanic and atmospheric conditions. *K. brevis* was also found to survive in waters ranging in salinity of 22 to 46 (Aldrich and Wilson 1960) and temperatures of 13 to 30 °C (McKay 2004). Toxin production varied among isolates of *K. brevis* from 0.42 pg cell\(^{-1}\) to 42 pg cell\(^{-1}\) as did growth rates ranging from 0.2 to 0.94 divisions day\(^{-1}\) in identical culture conditions (Loret et al. 2002). Genetic variability had also been identified between different isolated cultures of *K. brevis* (Campbell, pers. comm.). Clearly, *K. brevis* is an organism that is well-adapted to surviving and proliferating in constantly changing regimes.

Though isolates of the same species can vary in physiological responses when grown under the same conditions (Gallagher, 1980; Wood and Leatham, 1992), laboratory characterization of the red tide dinoflagellate species *K. brevis* has focused primarily on Wilson isolated in 1953 (Wilson and Collier 1955). Laboratory experiments reported here investigated the photosynthetic ranges of 10 different *K. brevis* isolates. Geographically, Texas B3 and Texas B4 isolates represent the east coast of Texas. Piney Island, Mexico Beach, and Apalachicola isolates represent the northwest region of Florida. Charlotte Harbor, New Pass, and Manasota represent the west coast of Florida and the Jacksonville isolate represents the east coast of Florida. Experiments investigated whether or not all of these *K. brevis* isolates have the same photosynthetic ranges. If all isolates have the same capabilities, then any isolate, including Wilson, is representative of the *K. brevis* species. If not, can a few functional groups be identified?
METHODS

Culturing Conditions

*Culture.* Texas (B3 and B4), Mexico Beach (C5), Apalachicola (C6), Piney Island (C4), Charlotte Harbor (C2), and Jacksonville (C3) isolates were obtained from the Florida Marine Research Institute, St. Petersburg, Florida. Manasota (CCMP2229) and New Pass (CCMP2228) isolates were obtained from Mote Marine Laboratories, Tampa, Florida. The Wilson isolate (CCMP718) was obtained from the Bigelow Center for Culture of Marine Phytoplankton, McKown Point, Maine.

Non-axenic, or bacteria containing, cultures of the *K. brevis* were incubated in 250 ml Erlenmeyer flasks using modified L/2 culture medium (Guillard and Hargraves 1993), with Cu removed and soil extract added at a salinity of 35. Soil extract was obtained from autoclaving 100 ml of Hyponex potting soil with 1 L of deionized water. Flasks were grown in a 22°C incubator, at 100 μmol quanta m⁻² s⁻¹ PAR (photosynthetically active radiation), by cool white fluorescent bulbs with 12 hours of light and 12 hours of dark. Pre-experiment cultures were grown in 9 L containers under 100 μmol quanta m⁻² s⁻¹ within 12 hour light/dark cycles at 22°C and allowed to photoacclimate for one week. Cultures were thoroughly homogenized and all subsamples were drawn from the container before lights-on.

*Photosynthetron.* Experiments were conducted in a modified radial photosynthetron (RP) (Babin et al., 1994), which provided controlled conditions of light and temperature (Fig. 1.1A). The photosynthetron included 12 black boxes extending as spokes around a 250 watt metal halide lamp (OSRAM HQISE250DX). Each black box held ten 25ml or 50ml Falcon tissue culture flasks. A light gradient was formed by the
distance of each bottle within a box from the light source. Typically, light intensity decreased 15% between bottles. Light levels ranged from a maximum of \(~2,000 \text{ \(\mu\text{mol quanta m}^{-2}\text{s}^{-1}\)}\) to 55 \(\text{\(\mu\text{mol quanta m}^{-2}\text{s}^{-1}\)}\). A darkened bottle could be included per set of incubated bottles to complete the irradiance range. Each box was water jacketed to keep constant temperature and to remove infrared light. All twelve boxes had equal exposure to the same light source, but their range of the absolute light gradient in each was reduced with the use of neutral filters in front of the bottle. This photosynthetron design allowed for triplicate replication and sub-samples with an identical light source. The light spectrum of each black box was recorded with a Lab Spec Spectroradiometer VNIR 512 from 400 nm to 700 nm (Fig. 1.1B). The light attenuation of each bottle, within the two black boxes, was measured with a Biospherical Instruments, Inc. QSL-100. These two measurements assured the spectral quality and quantity was identical throughout the photosynthetron.

**Analytical Measurements**

*Cell Counts and Size.* Cell counts and size were determined using a Coulter Multisizer II Particle Analyzer (Beckman-Coulter Inc., Miami, FL) with threshold settings of 13.3 and 30 \(\mu\text{m}\) and a 200 \(\mu\text{m}\) orifice.

*Pulsed Amplitude Modulated Fluorometer.* A Walz Water-Pulsed Amplitude Modulated Chlorophyll Fluorometer (PAM-FL) (Walz, Effeltrich, Germany) was used to measure relative electron transport rate (ETR), yield (Fv Fm \(^{-1}\)) and chlorophyll \(a\) (Schreiber and Bilger 1993; Schreiber 1998). The PAM-FL was auto-zeroed with 0.45 \(\mu\text{m}\) filtered cell media from each culture the morning of the experiment (Cullen and Davis 2003). A 650 nm measuring light was used to determine the dark state Fo.
saturating pulse representative of the light intensity measured in the RP was used to measure Fm. The spectral quality of the PAM-FL saturating pulse and the RP light source did not match. The PAM-FL light emitting diode saturating pulse peaked at 660 nm while the RP light source peaked between 470 and 600 nm (Fig. 1.1B). Therefore, the light intensity used in the PAM-FL saturating pulse was relative to the RP light intensity as measured with a Biospherical Instruments, Inc. QSL-100. Furthermore, relative ETR was recorded because the absorption cross section was not determined (Hartig et al. 1998) and there was no normalization of spectral differences (Boyd et al. 1997). Samples were measured on the PAM-FL after a 30-minute dark acclimation dictated by experimental procedures as sub-samples were distributed for other analytical techniques. Samples were exposed to the saturating pulse once and then discarded.

*DCMU yield.* Herbicide photoinhibitor 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea (DCMU) was used for photosynthetic yield determinations on a Turner fluorometer at a final concentration of 1x10^-5 M. Each sample was dark adapted for 30 minutes. DCMU blocked the reduction of O2 by PS I. Therefore, DCMU measured the yield of PS II indicating its degradation. The ratio of Fv/Fm^-1 determined the PS II yield (Vincent et al. 1984).

*Extracted chlorophyll a.* Chlorophyll a was measured with a Turner fluorometer after passive extraction in 90% acetone for 24 hrs in a dark freezer at -20°C on a 47 mm Whatman GF/F filter. The Turner fluorometer was calibrated with chlorophyll a standard from *Anacystis nidulans* algae (Sigma Chemical Co. St. Louis, MO) against a Jasco V-530 scanning spectrophotometer (Jasco Corp. Tokyo, Japan). Fluorescence by phaeopigments was corrected by acidifying with 1.2M HCl (JGOFS 1994).
14C primary productivity. 14C primary productivity was determined with 5 μCi H14CO3 injections into acid washed 20 ml culture bottles. Culture bottles were incubated in the RP at a specified light level for 30 minutes. The sample was filtered onto an ashed GF/C filter, rinsed with artificial seawater and placed in a scintillation vial with 1 ml of filtered seawater (FSW). A 1N HCl aliquot was added to the scintillation vial and placed on a shaker for 6 hrs to purge remaining inorganic 14C. ICN Ecolume scintillation fluid was added for analysis on a Perkin Elmer TriCarb 2900 scintillation counter (Parsons et al., 1984).

Primary productivity is often measured by radioactive 14C uptake across a light gradient to yield photosynthesis-irradiance curves often described using α, Pmax, and β parameters (Platt 1964; Platt and Gallegos 1980; Parsons et al. 1984; Zimmerman et al. 1987). Similarly, these parameters were used here to determine the isolates’ ability to utilize light for primary production. Low light levels made photosynthesis dependent on irradiance and was described in the linear slope of α. Saturating light levels made photosynthesis dependent on internal biochemical processes, which defined the photosynthetic capacity of Pmax. High light levels made photosynthesis dependent on the scavenging mechanisms ability to control photooxidative damage and was described by β (Miller 1984).

The DCMU yield, extracted chlorophyll a and 14C primary productivity methods of measuring the photosynthetic capabilities were time consuming, volume consuming, and potentially hazardous to human health. The PAM-FL had the capability to represent all three standard laboratory techniques within minutes, without the use of large volumes of sample, and there was no need to handle hazardous chemicals. Once it was shown
how the PAM-FL results related to the three standard techniques for *K. brevis*, all measurements were continued on the PAM-FL. Experiments incorporating DCMU-FL, extracted chlorophyll *a*, $^{14}$C primary production and PAM-FL measurements were conducted only once on the Jacksonville, Manasota, Wilson, and Piney Island isolates. Only the PAM-FL was used to assess the photosynthetic ranges of the remaining isolates.

**Experimental Approaches**

*Experiment 1: Sequential Multiple Isolates.* The sequential-multiple-isolates experiment determined if isolates were related by their geographic origin relative to the coast of Florida. Each light curve was composed of an isolate distributed between two black boxes on the RP totaling 20 bottles with light ranging from 0 μmol quanta m$^{-2}$s$^{-1}$ to ~2,000 μmol quanta m$^{-2}$ s$^{-1}$. Triplicates were collected by repeating this distribution in 4 more black boxes. A total of 6 black boxes provided 3 light curves for a 4 hr incubation and another 6 black boxes provided light curves for the 8 hr incubation.

Each isolate was removed from its 9 L container on a different day prior to lights-on. The isolate was distributed into three aliquots. The first aliquot was used for measurements prior to lights-on including a 30 minute incubation for $^{14}$C primary production. Cell counts, DCMU yield, extracted chlorophyll *a* and PAM-FL measurements were also taken prior to lights-on the morning of the experiment. The second aliquot was used for the 4 hr incubation with only PAM-FL measurements to capture the culture prior to the onset of photoinhibition. The third aliquot was used for an 8 hr incubation which included PAM-FL, cell counts, DCMU yield, extracted chlorophyll *a* and a 30 minute $^{14}$C primary productivity incubation.
**Experiment 2: Simultaneous Multiple Isolates.** The simultaneous-multiple-isolates experiment eliminated any bias in culturing/handling techniques by inoculating and experimenting on all isolates at the same time. Each light curve was composed of an isolate distributed within one black box on the photosynthetron totaling 10 bottles with light ranging from 50 μmol quanta m⁻² s⁻¹ to ~2,000 μmol quanta m⁻² s⁻¹. The light in the box was manipulated with neutral filters between bottles to provide a distributed range of light. All 10 isolates were grown at the same time and distributed into the RP prior to lights-on the morning of the experiment. Measurements on the PAM-FL were recorded prior to lights-on (data not shown) and again after an 8 hr incubation. Each isolate was randomly selected for sampling. All 10 bottles representing the isolate were removed from the RP at the same time to reproduce similar sample timing and dark adaptations as experiment 1. In the mean time, the remaining isolates stayed in the incubator with the light on. Complete sampling of all 10 isolates took 1 hr. Sampling of the 10 isolates on the same day was repeated on three separate days. Cell counts for each isolate were recorded each day prior to the experiment for the determination of growth rate.

**Experiment 3: Apalachicola Cell Number Gradient:** Experiment 3A. The Apalachicola-cell-number-gradient experiment 3A determined if cell count influenced physiology. Each light curve was composed of an Apalachicola culture with a different cell count in one RP black box. The light in the box was manipulated with neutral filters between bottles to provide a distributed range of light between 50 and 2,000 μmol quanta m⁻² s⁻¹. Apalachicola was grown at 5, 10, 20, 40, 80 and 160 times a relative inoculation concentration for 1 week. The morning of the first dilution experiment each culture container was split in half. One half of the culture returned to grow for an additional
week while the other half was used for the first dilution experiment. Each culture in the experiment was then split into two aliquots. One aliquot was incubated at its relative cell concentration and the other aliquot was diluted in half with fresh 0.2 μm filtered sea water (FSW). The FSW contained no L/2 nutrient media. Both aliquots were incubated in the RP for 8 hrs. The experiment was repeated for a second time with the now 2 week old 5, 10, 20, 40, 80 and 160x cultures. Cell counts for each culture were recorded each day prior to the experiment for the determination of growth rate.

**Experiment 3B.** The Apalachicola-cell-number-gradient experiment 3B determined if cell count influenced physiology with the support of ¹⁴C primary productivity regardless of relative measurements on the PAM-FL. A second dilution experiment included ¹⁴C primary productivity curves conducted on the Apalachicola isolate grown at 8x and 32x a relative inoculation concentration. This experiment was to prove that the observed trends in the PAM-FL measurements were not due to an unknown bias with that approach. Both culture concentrations were incubated in the radial photosyntheticron for 8 hrs in triplicate using 2 black boxes for a light gradient.

**Experiment 3C.** The Apalachicola-cell-number-gradient experiment 3C determined how much dilutions with FSW influenced self-shading. This experiment used Apalachicola grown in one container for one week at a relative inoculation concentration. On May 28th, 2004, the culture was split in half. One half of the culture was grown for an additional 4 days. The other half of the culture was divided into 6 aliquots. The culture was incubated at its original concentration and diluted with FSW to 80, 60, 50, 40 and 20% of the original concentration. All aliquots were incubated in the RP for 8 hrs and measured with the PAM-FL. On June 1st, 2004 the remaining original culture was
divided in an identical manner, diluted and incubated for 8 hrs. Cell counts for the original culture were recorded each day prior to the experiment for the determination of growth rate.

Experiment 4: Apalachicola Media Dilutions: Experiment 4A. Apalachicola-media-dilutions experiment 4A determined how FSW, fresh media or filtered media from the same culture influenced physiology. Apalachicola isolate was grown for 1 week in one container. Prior to lights-on the culture was divided into 5 aliquots. The culture was incubated at its original concentration and diluted to 50% with FSW, GF/C filtered used media, fresh media and 0.2 μm filtered used media. The used media was from the same culture and filtered the morning of the experiment. The FSW contained no L/2 nutrients. The fresh media was FSW with L/2 nutrients. All aliquots were incubated in the RP for 8 hrs and measured with the PAM-FL.

Experiment 4B. Apalachicola media dilutions in experiment 4B determined how FSW, fresh media or filtered media from another culture with different cell counts influenced physiology. The second experiment included Apalachicola grown for 1 week in two separate containers. One container was inoculated with 2x the relative inoculation concentration as the other container (1x). Prior to lights-on, both containers were split in half. One half of each container was used to filter the used media for dilutions. The media was filtered through a GF/C filter or a 0.2 μm filter. The 2x and 1x cultures were each divided into 4 aliquots. Apalachicola was incubated in its original 2x and 1x cell concentrations. A 2x and 1x culture aliquot was diluted to 50% with FSW. The remaining two aliquots were also diluted to 50% but with the GF/C or 0.2 μm used filtered media of the opposite culture.
Experiment 4C. Apalachicola media dilutions in experiment 4C determined how FSW, fresh media or filtered media from another culture with different cell counts influenced physiology with the support of $^{14}$C primary productivity regardless of relative measurements on the PAM-FL. For the third experiment, $^{14}$C primary productivity curves were measured on the Apalachicola isolate grown at 5x and 1x the relative inoculation concentration. This experiment was to prove that the observed trends from the isolate media dilutions measured with the PAM-FL were not due to an unknown bias with this approach. The 5x culture was incubated at the original cell concentration, a 50% dilution with FSW and a 50% dilution with GF/C used media filtrate from the 1x culture. The 1x culture was incubated its original cell concentration and a 50% dilution with the GF/C used media filtrate from the 5x culture. Both culture concentrations were incubated in the radial photosynthetron for 8 hrs using 2 black boxes for a light curve.

Analysis

The data were plotted and analyzed using Microsoft Excel, Sigma Plot, Sigma Stat and SAS. Error bars are reported as 1 standard deviation.

RESULTS

Experiment 1: Sequential Multiple Isolates: Prior to lights-on, cell diameter (Fig. 1.2A) ranged between 17.36 +/- 0.06 μm (Mexico Beach) and 20.96 +/- 0.07 μm (Jacksonville), PAM-FL chlorophyll a (Fig. 1.2B) varied between 21.3 pg cell$^{-1}$ (Mexico Beach) and 58 pg cell$^{-1}$ (Piney Island), and extracted chlorophyll a (Fig. 1.2C) was between 44.8 pg cell$^{-1}$ (Piney Island) and 25.1 pg cell$^{-1}$ (Manasota). PAM-FL yield and DCMU yield were between 0.60 +/- 0.01 and 0.57 +/- 0.03, respectively, prior to lights-on (Fig. 1.2D, E).
The Jacksonville $^{14}$C primary productivity $\alpha$ measurement prior to lights-on (Appendix A) was 0.368, which was significantly different ($p<0.001$) from Piney Island, Manasota and Wilson isolates (Fig. 1.3A-D). Piney Island, Manasota and Wilson exhibited no significant difference ($p=0.15$) between their $\alpha$ values and an average of 0.174. $P_{\text{max}}$ was significantly different between all isolates ($p<0.001$) and ranged from 21.83 to 88.53.

PAM-FL ETR $\alpha$ measurements after 4 hr incubations (Appendix B) were significantly different between isolates ($p<0.001$), ranging from 0.15 +/- 0.001 to 0.295 +/- 0.045 with an average of 0.214 (Fig. 1.4A-J). $P_{\text{max}}$ was also significantly different between isolates ($p<0.001$) ranging from 236.76 to 86 with an average of 145.5. Only Apalachicola, Piney Island, Jacksonville and Wilson showed signs of photoinhibition after 4 hrs of incubation. The $\beta$ slope for these isolates ranged from 0.055 to 0.078. Yield decreased from 0 $\mu$mol quanta m$^{-2}$ s$^{-1}$ to 2000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ by an average of 80% for all isolates, ranging between a 61% decrease for New Pass to a 97% decrease for Apalachicola after 4 hrs of incubation.

PAM-FL relative ETR $\alpha$ measurements after 8 hr incubations (Appendix C) were significantly different between isolates ($p<0.001$), ranging from 0.148 +/- 0.001 to 0.176 +/- 0.03 with an average of 0.166 (Fig. 1.5A-J left). $P_{\text{max}}$ was also significantly different between isolates ($p<0.001$), ranging from 71.8 to 164.58 with an average of 107.9. All isolates showed signs of photoinhibition after 8 hrs of incubation. The $\beta$ slope ranged from 0.048 +/- 0.014 to 0.077 +/- 0.007 with an average of 0.064. Yield decreased from 0 $\mu$mol quanta m$^{-2}$ s$^{-1}$ to 2000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ by an average of 90% for all isolates.
ranging between a 82% decrease for Piney Island to a 100% decrease for Wilson after 8 hrs of incubation.

$^{14}$C primary productivity $\alpha$ measurements after 8 hr incubations were significantly different between isolates ($p<0.001$), ranging from 0.06 +/- 0.004 to 0.19 +/- 0.004 with an average of 0.118 (Fig. 1.5E, H, I, J right). $P_{\text{max}}$ was between 33.7 to 59.09 with an average of 41.10. All isolates showed signs of photoinhibition after 8 hrs of incubation. The $\beta$ slope ranged from 0.011 +/- 0.004 to 0.048 +/- 0.002 with an average of 0.028. All measured isolates had significantly different $\beta$ values ($p<0.001$). Yield decreased from 0 $\mu$mol quanta m$^{-2}$ s$^{-1}$ to 2000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ an average of 46% for all measured isolates ranging between a 27% decrease for Jacksonville to a 65% decrease for Wilson after 8 hrs of incubation.

PAM-FL chlorophyll $a$ (Fig. 1.6A-J left) and extracted chlorophyll $a$ (Fig. 1.6E, H, I, J) decreased from 0 $\mu$mol quanta m$^{-2}$ s$^{-1}$ to 2000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ by an average of 27% and 23%, respectively. Cell diameter (Fig. 1.6A-J left) increased an average of 10% after the 8 hr incubation for all isolates except Piney Island and Wilson, which decreased 12% and 5%, respectively.

Experiment 2: Simultaneous Multiple Isolates: All isolate cultures were in the exponential growth phase prior to experiments (Fig. 1.7A-J left) (Appendix D). Cell counts of the same isolate over three different experiments varied on average +/- 1555 cells ml$^{-1}$. Growth rates for all isolates averaged 0.25 divisions day$^{-1}$ +/- 0.07 (Fig. 1.7A-J right). Maximum growth rates occurred just after inoculation but typically evened out after the third day.
PAM-FL relative ETR $\alpha$, $P_{\text{max}}$ and $\beta$ values were assumed to carry an average standard deviations of +/-0.002, +/-6.2, and +/-0.006, respectively, as indicated from *Experiment 1: Sequential Multiple Isolates*. Samples without replication were assigned these standard deviations for statistical comparison purposes. All isolates exhibited a significant difference in their $\alpha$, $P_{\text{max}}$ and $\beta$ parameters for at least one out of the three experiments (Fig. 1.8A-J left).

PAM-FL percent change in yield was assumed to carry an average standard deviation of +/-2.65 as indicated from the previous *Experiment 1: Sequential Multiple Isolates*. Samples without replication were assigned this standard deviation for statistical comparison purposes. Texas B3, Texas B4, Mexico Beach, Charlotte Harbor, Manasota, Jacksonville and Wilson were significantly different (p<0.001) between two out of the three experiments (Fig. 1.8A-J right). Mexico Beach, Piney Island and New Pass were significantly different (p<0.001) between all three of the experiments.

*Experiment 3: Apalachicola Cell Number Gradient: Experiment 3A*. All isolate cultures were in the exponential growth phase prior to experiments (Fig. 1.9A-J left). Isolate cultures on average tripled in cell count relative to the inoculation concentration prior to the week 1 experiment (Appendix E). Cell counts were between 4 times and 17 times higher than the inoculation concentration prior to the week 2 experiment. Growth rates for all cell concentrations averaged 0.40 divisions day$^{-1}$ +/- 0.08 (Fig. 1.9A-J right). Cultures 80x and 160x the relative inoculation (or 80xRI or 160xRI) had noticeably lower growth rates of 0.34 and 0.30 divisions day$^{-1}$ respectively. Prior to the week 2 experiment the culture grown at 40xRI the relative inoculation began to slow to 0.38
divisions day$^{-1}$. Cultures 5, 10 and 20xRI respectively, maintained growth rates of 0.42, 0.55, 0.41 divisions day$^{-1}$ throughout both experiments.

PAM-FL relative ETR $\alpha$, $P_{\text{max}}$, and $\beta$ values again were assumed to carry an average standard deviation of +/-0.002, +/-6.2, and +/-0.006, respectively, as indicated from Experiment 1: Sequential Multiple Isolates. Samples without replication were assigned these standard deviations for statistical comparison purposes.

Comparison between the week 1 and week 2 experiments indicated both $\alpha$ and $\beta$ increased for the 5 and 10xRI cultures but decreased for the 20, 40, 80 and 160xRI cultures (Fig. 1.10A-F left). $P_{\text{max}}$ decreased for all cultures between the week 1 and week 2 experiments ($p<0.001$). Comparison between the parameter differences of the original cultures and the diluted cultures for the week 1 experiment were complex. However, the week 2 experiment indicated the diluted culture $\alpha$, $P_{\text{max}}$ and $\beta$ were greater than the original culture. This trend increased with increased cell count.

The change in yield from 0 $\mu$mol quanta m$^{-2}$ s$^{-1}$ to 2000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ for the Apalachicola 5, 10, 20, 40, 80 and 160xRI cultures (Fig. 1.10A-F right) decreased an average of 80.7% in the week 1 experiment and 95.4% in the week 2 experiment. The 5, 10, 20, 40, 80 and 160xRI cultures that were diluted in half decreased an average of 67.9% in the week 1 experiment and 93% in the week 2 experiment.

Experiment 3B. Apalachicola culture grown at 8xRI (Appendix F) had an $\alpha$ of 0.426, $P_{\text{max}}$ of 109.74 and $\beta$ of 0.087 (Fig. 1.11). Culture grown at 32xRI had an $\alpha$ of 0.27, which was 36% lower ($p=0.046$) than the 8xRI culture and a $P_{\text{max}}$ of 72.02, which was 34% lower ($p=0.041$) than the 8xRI culture. The $\beta$ value for the 32xRI culture was not significantly different from the 8xRI culture ($p=0.062$).
Experiment 3C. Apalachicola cell counts (Fig. 1.12 left) increased approximately 1.75 times prior to the experiment on May 28th and more than 4 times prior to the experiment on June 1st. The culture had an average growth rate of 0.30 divisions day\(^{-1}\) from the time of the inoculation through both experiments (Fig 1.12 right). In the May 28th experiment (Fig. 1.13A left), \(\alpha\) typically increased in the diluted culture when compared to the original 100% culture. Alpha increased the maximum of 12.6% (p<0.001) in 80% diluted culture and the minimum of 6% (p<0.001) in the 20% diluted culture. All diluted culture \(\alpha\) values were significantly different from each other (p<0.001). \(P_{\text{max}}\) significantly increased in all diluted cultures when compared to the original 100% culture. The 80% diluted culture increased the minimum of 19% (p<0.001) and the 20% diluted culture increased the maximum of 48% (p<0.001). All diluted culture \(P_{\text{max}}\) values were significantly different (p<0.001). There was no significant difference between the \(\beta\) values of all cultures in the May 28th experiment.

In the June 1st experiment (Fig. 1.13B left), all \(\alpha\) values were significantly different when compared to the original 100% culture. Alpha values did not follow the same stepwise changes in relation to dilution amount as seen in the May 28th experiment. \(P_{\text{max}}\) increased in all diluted cultures when compared to the original 100% culture. The 80% diluted culture increased the minimum of 15% (p<0.001) and the 20% diluted culture increased \(P_{\text{max}}\) the maximum of 66% (p<0.001).

PAM-FL yield of the May 28th experiment (Fig. 1.13A right) decreased an average of 88.72%, with the 100% original culture decreasing by 92% and the 20% diluted culture decreasing by 77%. PAM-FL yield of the June 1st experiment (Fig. 1.13B right) decreased an average of 89%, with the 100% original culture decreasing by 100%.
and the 20% diluted culture decreasing by 77%. All culture yields were significantly
different from each other (p<0.001) for both experiments.

Experiment 4: Apalachicola Media Dilutions: Experiment 4A. The original
100% culture of Apalachicola had an $\alpha$ of 0.182, a $P_{\text{max}}$ of 97.99 and $\beta$ of 0.080 (Fig.
1.14 left) (Appendix H). A 50% dilution of the culture with FSW significantly increased
$\alpha$ by 6% (p<0.001) and $P_{\text{max}}$ by 29% (p=0.002). Dilutions with GF/C filtered media,
fresh media or 0.2 $\mu$m filtered media had no significant impact on $\alpha$. However, a dilution
with fresh media increased $P_{\text{max}}$ by 22% (p=0.012) and a dilution with 0.2 $\mu$m filtered
media increased $P_{\text{max}}$ by 23% (p=0.008). PAM-FL yield change (Fig. 1.14 right) from 0
$\mu$mol quanta m$^{-2}$ s$^{-1}$ to 2000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ declined 97% for the original 100%
culture. A 50% dilution with FSW significantly reduced the decrease to only 84%
(p=0.001). No other dilutions with GF/C filtered media, fresh media or 0.2 $\mu$m filtered
media caused significant changes in the decline of PAM-FL yield.

Experiment 4B. There was a significant increase in Apalachicola 2x $\alpha$ (p=0.036)
and in $\beta$ (p=0.021) with the FSW dilution. All other dilutions of the 2x culture had no
significant change in $\alpha$. $P_{\text{max}}$ significantly increased by 22% with FSW dilution
(p=0.022), by 15% with a GF/C filtered media dilution (p=0.049) and by 16% with a 0.2
$\mu$m filtered media dilution (p=0.044) from the 1x culture. A 50% dilution with FSW in
the 2x culture significantly reduced the PAM-FL yield decrease from 96% to 88%
(p=0.021).

Dilution of the 1x culture (Fig. 1.15B left) with FSW significantly decreased $\alpha$ by
6% (p<0.001) and increased $P_{\text{max}}$ by 18% (p=0.025). All other dilutions of the 1x culture
had no significant change in $P_{\text{max}}$ or $\beta$. The change in PAM-FL yield from 0 $\mu$mol quanta
m$^{-2}$ s$^{-1}$ to 2000 μmol quanta m$^{-2}$ s$^{-1}$ was a decrease of 99% for the 1x culture. A 50% dilution with FSW (p=0.016) or GF/C filtered media (p=0.019) in the 1x culture significantly reduced the PAM-FL yield decrease from 99% to 90.5% (p=0.021).

Experiment 4C. Finally, Apalachicola culture grown at 1x (Fig. 1.16 left) and 5x of the original inoculation culture (Fig. 1.16 right) were significantly different in $\alpha$ (p<0.001), $P_{\text{max}}$ (p=0.046) and $\beta$ (p=0.003) values. For the 1x culture $\alpha$ was 0.374, $P_{\text{max}}$ was 129.36 and $\beta$ was 0.102. The 1x culture $\alpha$ significantly increased by 41% (p<0.001), $P_{\text{max}}$ increased by 56% (p<0.001) and $\beta$ increased by 53% (p<0.001) with dilutions using 5x filtered media. For the 5x culture, $\alpha$ was 0.352, $P_{\text{max}}$ was 114.93 and $\beta$ was 0.075. The 5x culture $\alpha$ decreased by 60% (p<0.001) with a FSW dilution and increased by 29% (p<0.001) with a 1x filtered media dilution. The 5x culture $P_{\text{max}}$ decreased by 31% (p=0.001) with a FSW dilution and increased by 10% with a 1x filtered media dilution. The 5x culture $\beta$ decreased by 23% (p=0.028) with a FSW dilution and increased by 35.5% with 1x filtered media dilution.

DISCUSSION

Techniques

PAM-FL yield slightly overestimated DCMU yield but was in good agreement with a slope of 0.67 (r=0.70, p<0.001) (Fig. 1.17). PAM-FL chlorophyll $a$ also slightly overestimated extracted chlorophyll $a$ values but was in good agreement with a slope of 0.52 (r=0.76, p<0.001) (Fig. 1.18). Yield and chlorophyll $a$ measurements were expected to be similar between the two techniques since the fluorescence for both measurements originated primarily from the PSII reaction centers related to chlorophyll $a$ (Barranguet and Jacco 2000). Slight differences may have resulted from the PAM-FL’s direct
physical measure of fluorescence when compared to the chemical extraction of chlorophyll with acetone or inhibition of electron transport between PSII and PSI with DCMU (Johnsen et al. 1997).

There was no correlation for $\alpha$, $P_{\text{max}}$ or $\beta$ when the PAM-FL relative ETR and the $^{14}$C primary productivity techniques were compared. A lack of correlation was expected since corrections for absorption cross section of the cells and spectral normalization were not completed (Hartig et al. 1998). The absorption cross section of a cell estimated the light harvesting capacity of PSII and its efficiency to transfer light energy to chemical energy. Variability in the capacity of PSII was influenced by the xanthophylls cycle, cyclic electron flow around PSII and degradation of PSII at high light intensities (Kolber and Falkowski 1993). These corrective measures were not conducted because the intent of this experiment was acquiring a relative scale of $K. \text{brevis}$ physiological state for use in future experiments focused on biochemical measurements. Nevertheless, PAM-FL relative ETR provided a complimentary measurement for $^{14}$C primary productivity in this situation. The $^{14}$C primary production technique provided information on carbon incorporation into the cell while PAM-FL relative ETR estimated the electron flow from PSII (Hofstraat et al. 1994). There was always a good linear agreement between the two techniques up to the $E_k$ point in $^{14}$C primary productivity. All correlations were fit to a two parameter curve (Fig. 1.19A-D) (Jassby and Platt 1978). The linear regression up to $E_k$ estimated electron transport efficiency for carbon incorporation (Barranguet and Jacco 2000). Each isolate was significantly different ($p<0.001$) in its efficiency coefficient with Piney Island at 0.925 +/-0.09, Manasota at 0.645 +/-0.16, Jacksonville at 1.84 +/-0.01 and Wilson at 1.02 +/-0.11. After the $E_k$ point in $^{14}$C primary productivity, the linear
relationship disappeared. A change in the relationship could be due to a decrease in PSII from photoinhibition or a decrease in electron transport efficiency (Barranguet and Jacco 2000) from electron sinks such as the Mehler reaction (Hartig et al. 1998). The PAM-FL relative ETR was corrected (Fig. 1.20) using the efficiency coefficients from each isolate and improved the correlation to 0.89 (r=0.97, p<0.001) between the two techniques up to E_k.

**Isolate by geographic origin**

The **sequential multiple isolates experiment** indicated isolates were not physiologically distinguished by their origin relative to the coast of Florida. Previous studies have shown phytoplankton species from the same locations were related to each other when compared to the same species from different geographic regions (Hayhome et al. 1987; Rynearson and Armbrust 2004). For example, work on the species *Gymnodinium catenatum* (Bolch et al. 1999) and *Cryptethodinium cohnii* (Beam and Himes 1982) indicated geographic origin played a role in genetic variation. However, work with species *Peridinium* showed isolates from the same geographic region had genetic variability (Hayhome et al. 1987). The only two *K. brevis* isolates actually from the same local were Texas B3 and Texas B4. The other isolates were from different locales even though they represent isolates from the same coastal regions relative to Florida. All isolates, including Texas B3 and B4, had a variable range of photosynthetic parameters regardless of origin. Therefore, future *K. brevis* experimental isolate selection does not have to be dependent on geographic location at least as far as photo-response is concerned.
Isolate by cell count

The simultaneous multiple isolates experiment indicated that all *K. brevis* isolates exhibited similar variation. This experiment, however identified a possible trend with cell count and diameter. The *Apalachicola cell number gradient experiment A* determined cell count influenced physiology. The higher the cell count, the lower $\alpha$, and when the same culture was diluted in half with FSW, $\alpha$ increased an average of 6%. $P_{\text{max}}$ was directly influenced by cell count with values decreasing with increasing cell count. The observation that cell count influenced physiology was further supported through *Apalachicola cell number gradient experiment B* using $^{14}$C primary productivity. The higher the cell count, the lower the $\alpha$, and $P_{\text{max}}$.

**Physiological parameters**

A detailed investigation into the PAM-FL relative ETR parameters and cell measurements provided some significant correlations. Higher culture growth rates were associated with higher $\alpha$ values with a slope of 0.2 ($r=0.72, p<0.001$) (Fig. 1.21). The relationship between growth rate and photosynthetic activity was expected. A higher $\alpha$ means a culture under limited light had a more efficient capacity to transport electrons and to covert light energy into photosynthetic end products for cell division leading to faster growth rates (Falkowksi et al. 1985; Langdon 1988). Each dilution with FSW increased $\alpha$, which indicated the culture again had the capacity to increase the growth rate.

Chlorophyll $a$ generally increased with larger cell diameters. Prior to lights-on, chlorophyll $a$ increased with larger cell diameter with a slope of 4.6 ($r=0.69, p=0.027$) (Fig. 1.22A). After 4 hrs of incubation chlorophyll $a$ still increased with larger cell
diameters with a slope of 4.3 (r=0.68, p<0.001) (Fig. 1.22B). After 8 hrs of incubation and the onset of photoinhibition chlorophyll $a$ lost its linear relationship with cell diameters with a slope of 2.55 (r=0.32, p=0.17) (Fig. 1.22C). Cells with larger diameters after 8 hrs had less chlorophyll $a$ and higher expressions of $\beta$ photoinhibition with a slope of 0.02 (r=0.80, p<0.001) (Fig. 1.23). Although no biochemical measurements were recorded in this experiment, the increase in cell diameter possibly resulted from the production of storage products. Typically, the demand for required compounds related to cell growth will be less than the supply of energy from excess light, so the cell synthesizes non-nitrogen storage products such as carbohydrates and lipids (Morris 1981).

There was a negative relationship between cultures with higher cell counts and the $P_{\text{max}}$ values with a slope of -0.004 (r=0.54) (Fig. 1.24). The relationship with $E_k$ also decreased with increased cell counts with a slope of -0.02 (r=0.54) (Fig. 1.25). The explanation for a decrease in $P_{\text{max}}$ was not obvious. Initially, $P_{\text{max}}$ was expected to increase with an increase in cell counts due to self-shading from the unique set-up of the RP (Agusti 1991; Babin et al. 1994; Schaeffer 2006a) The Apalachicola cell number gradient experiment $3C$ was conducted to determine the impact of self-shading. Cultures grown at higher cell counts and then diluted in half should experience less self-shading and a reduction in $P_{\text{max}}$ due to the increased light exposure. The experiment indicated the exact opposite, an increase in dilution lead to an increase in $P_{\text{max}}$ and $\alpha$. The results from this dilution experiment meant something in the FSW dilution had to have influenced the physiological parameters.
Apalachicola media dilutions experiments 4A and 4B were run to determine how FSW, fresh media or filtered media influenced physiology. Dilutions with the filtered media from the same culture caused no change in $\alpha$ whether from the GF/C or 0.2 $\mu$m filters. Filtered media from the same culture did increase $P_{\text{max}}$ if it was from a 0.2 $\mu$m filter, which removed bacteria or fresh media. $P_{\text{max}}$ was not different in the diluted culture with GF/C filtered media, which contained bacteria. Dilutions of a culture with filtered media from a culture one half the cell count typically increased $P_{\text{max}}$ and did not influence $\alpha$. When a culture was diluted with filtered media from a culture twice the cell count, both $\alpha$ and $P_{\text{max}}$ did not change. The $^{14}$C primary productivity supported the same trends found from dilutions of higher cell count cultures with the filtered media of lower cell count cultures. A culture diluted with filtered media from a culture one half the cell count always increased $\alpha$ and $P_{\text{max}}$. These trends suggested some type of feedback mechanism or limiting factor in the filtered media. The trends were further supported when the average value for PAM-FL relative ETR (Fig. 1.26A) and $^{14}$C primary productivity (Fig. 1.26B) from 1000 to 1600 $\mu$mol quanta m$^{-2}$ s$^{-1}$ decreased with increased cell count for both the 4 hr and 8 hr experiments. The average value for PAM-FL yield (Fig. 1.27A) and DCMU yield (Fig. 1.27B) between the same light ranges also decreased with increased cell count for both the 4 hr and 8 hr experiments.

Two possible explanations for the reduction of $P_{\text{max}}$ and yield with increased cell count are carbon limitation or bacterial/biochemical biofeedback. The Redfield ratio dictated phytoplankton required ratios of carbon and nitrogen 106 to 16 (Redfield et al. 1963). Cultures were incubated in L/2 media, which contained $4.41 \times 10^{-4}$ mol L$^{-1}$ nitrogen or a total of $31 \times 10^7$ pg of inorganic nitrogen. Cultures contained $13.22 \times 10^8$
pg of carbon based on alkalinity measurements (Parsons et al. 1984). The Redfield ratio indicated that if cells in culture consumed all the inorganic nitrogen a carbon deficit of $7.32 \times 10^8$ pg would occur. Calculations based on nitrogen uptake experiments (Steidinger et al. 1998b; Bronk et al. 2004; Sinclair 2005), however, showed most cultures did not consume all the inorganic nitrogen. Some cultures with cell counts above 10,000 cell ml$^{-1}$ or cultures that were allowed to grow more than one week could consume all the inorganic nitrogen. Calculations based on cell counts with 12 hrs of photosynthesis (respiration not included) over the one week of acclimation and the 8 hr incubation showed most cultures did not exceed carbon limits. Most calculated carbon consumption quotas were between 5 and 30%. However, some cultures did reach calculated carbon consumption quotas of 50 to 100%. Cultures with carbon consumption quotas over 50% typically had increased $\beta$ and suppressed $P_{\text{max}}$ values. Therefore, a carbon threshold may have been limiting for *K. brevis* in these cases. Although this line of thinking goes against all accepted principles of marine phytoplankton ecology, we propose the possibility that *K. brevis* could experience some carbon limitation in the field.

Carbon for *K. brevis* populations came from available CO$_2$ in seawater. The diffusion of CO$_2$ from the atmosphere into seawater and conversion of HCO$_3^-$ to available CO$_2$ for the population was slow. Cellular carbon fixation with ribulose-1,5-biphosphate carboxylase (Rubisco) had a half saturation concentration of approximately 20 $\mu$M but seawater had CO$_2$ concentrations of only 12$\mu$M (Kirk 1983). The slow input and conversion of available CO$_2$ coupled with the low concentrations relative to Rubisco saturation could cause carbon limitation in *K. brevis* populations. However, *K. brevis*
may utilize carbon concentrating mechanisms such as carbonic anhydrase to catalyze the conversion of HCO$_3^-$ to CO$_2$ and to transport available CO$_2$ into the cell (Falkowski and Raven 1997).

A *K. brevis* population exposed to an unlimited supply of nitrogen from the sediment-water interface may remove enough CO$_2$ from the water where cells aggregate to reach a similar carbon limitation as indicated by the laboratory experiments. Under limited CO$_2$ conditions, cells may increase their carbon concentration mechanisms. Work with the fresh water dinoflagellate *Peridinium gatunense* indicated decreasing CO$_2$ supply changed cellular carbon uptake mechanisms such as increasing a carbon concentration mechanism (Berman-Frank et al. 1995; Berman-Frank and Erez 1996). This response, increased energy spent on carbon concentration mechanisms and decreased growth rates in *P. gatunense* (Berman-Frank et al. 1998). The *K. brevis* response to limited carbon may be an increase in carbon concentration mechanisms, but the increase may not be efficient enough or fast enough and therefore $P_{\text{max}}$ decreased.

The water column could be divided into three sections including the surface limited by CO$_2$ supply but with high light; middle depth limited by CO$_2$ supply and intermediate light, and bottom limited by low light (Talling 1979; Kirk 1983). Estimates of respiration from the population would not be enough to remove cells from their carbon-limited states. First, respiratory rates decline after several hours as cells utilize their carbon storages to produce nitrogen-containing compounds. Second, respiration rates account for approximately 10% of the photosynthetic rate (Falkowksi and Raven 1997). Theoretically, if a population started with a 100% relative carbon supply and only consumed 5% over one day for the initial population size, respiration would leave 95.5%
the following morning. Overnight, that population experienced an average 0.3 divisions and increased the cell count 30%. That same population, 30% larger, would consume 6.5% of the carbon supply over day 2 and respiration would leave 89.65% the following morning. The net change in available carbon, with respiration, in the water column would be negative for a growing *K. brevis* population even if the respiration rate was a larger percentage. A young cell located at the bottom of a water column may vertically migrate toward the surface (Schaeffer 2006b) where light was not limiting and there was greater probability of increased carbon from turbulence, wind mixing, shear with a surface fresh water plume, or shear from frontal boundaries (Mann and Lazier 1996).

A better understanding of the possible role carbon limitation plays in *K. brevis* populations can be explained with the use of $\delta^{13}C/^{12}C$ ratios in future experiments. Typically, marine phytoplankton uptake of inorganic carbon $\delta^{13}C$ was between -19 to -24‰, but a carbon-limited environment would have produced increased $\delta^{13}C$ values. Phytoplankton preferentially utilizes $^{12}C$ but carbon limitation would have caused an increase of $^{13}C$ utilized (Peterson and Brian 1987).

The second explanation involved a bacterial/biochemical feedback mechanism. Cultures diluted with GF/C filtered media did not increase $\alpha$ or $P_{max}$. Media from a GF/C filter would have contained bacteria or related biochemicals already in the non-axenic cultures. Cultures diluted with 0.2 μm filtered media did increase $\alpha$ and $P_{max}$. Media from a 0.2 μm filter would not have bacteria but would contain viruses and biochemicals. Since dilutions with 0.2 μm did increase the photosynthetic parameters similarly to dilutions with FSW or fresh media, it was concluded that viruses or biochemicals alone were not directly responsible for these changes.
A single algal cell may have as few as 5 (Vaque et al. 1989) or as many as 100 (Doucette 1995) bacterial cells associated within its phycosphere or area around the algal cell to which bacteria are attracted (Bell and Mitchell 1972). Bacteria associated with phytoplankton are dependent on the secretion of organic carbon for growth (Vaque et al. 1989). Field studies have identified greater bacterial productivity inside blooms of *K. brevis* when compared to bacterial productivity outside the bloom (Heil et al. 2004). The relationship between primary production and bacterial abundance is positive with greater primary production leading to higher bacterial populations. Bacteria metabolism could provide negative feedback which would lead to inhibitory responses in the phytoplankton cells (Cole 1982). The influence of bacteria on harmful algal cells has been found to be mostly inhibitory (Doucette et al. 1998). Particular strains of bacteria were reported to have the capacity to kill *K. brevis* (Doucette et al. 1999). Doucette et al. (1999) proposed a 4-stage model. The model started with the bacteria and *K. brevis* in low abundance and then progressed to an increase in *K. brevis* and bacterial abundance where the bacteria eventually provided negative feedback destroying *K. brevis* cells. Although we did not necessarily have the same bacteria with a capacity to kill *K. brevis*, it was possible that a similar negative feedback mechanism did occur. Competition between *K. brevis* cells and bacteria for survival may be one explanation for differences in toxicity and growth rate reports in lab cultures (Loret et al. 2002). Cultures may have been grown under identical conditions but cell counts are usually never identical (this study). Cultures could be under slightly different phases of the Doucette et al. (1999) proposed 4-stage model. The balance between inhibitory processes select the survival of either the phytoplankton cells or the bacterial cells (Cole 1982).
Many laboratories have solely used the Wilson isolate to represent the *K. brevis* species (Shanley 1985; Kamykowski et al. 1998; Evens et al. 2001). Photosynthetic parameters presented here ranged around previous experiments conducted both in the field and in the laboratory (Shanley 1985; Bendis et al. 2004). Our previous report on preliminary isolate studies indicated *K. brevis* could be separated into two functional groups: light-sensitive and light-capable. The Wilson isolate was believed to only partially represent *K. brevis* in the light-sensitive group (Schaeffer et al. 2004). This report now revises that statement to indicate all isolates are representative of *K. brevis* at least in the tested parameters. However, caution must be taken in laboratory culture preparations prior to any experiments. Until there is better understanding of the carbon-limitation or bacterial/biochemical feedback, we recommend *K. brevis* cultures in modified L/2 media be grown for no more than two weeks at cell counts below 10,000 cells ml\(^{-1}\). Counts above 10,000 cell ml\(^{-1}\) typically passed calculated carbon consumption rates of 50\% or greater and showed the greatest decreases in \(P_{\text{max}}\) and increases in photoinhibition.
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Figure 1.1. (A) The radial photosynthetron box and bottle design allowed for 12 black boxes. Each box held 10 bottles. Two boxes could be used for creating a light curve where the T bottle had the highest light intensity. Light intensity decreased 15% between each bottle towards the A bottle. Each box was also used independently to create a light curve from T to K or J to A decreasing light intensity with neutral filters. (B) Radial photosynthetron lamp spectra.
Figure 1.2. Isolate (A) cell diameter, (B) PAM-FL chlorophyll $a$, (C) extracted chlorophyll $a$, (D) PAM-FL yield and (E) DCMU yield prior to lights-on.
Figure 1.3. Isolate $^{14}$C primary productivity for (A) Piney Island, (B) Manasota, (C) Jacksonville and (D) Wilson prior to lights-on.
Figure 1.4. PAM-FL relative ETR (■) and yield (○) after 4 hrs incubation for (A) Texas B3, (B) Texas B4, (C) Mexico Beach, (D) Apalachicola, (E) Pinney Island, (F) New Pass, (G) Charlotte Harbor, (H) Manasota, (I) Jacksonville and (J) Wilson.
Figure 1.5. PAM-FL relative ETR (●) and yield (○) (left) compared to 8 hr $^{14}$C primary productivity (■) and DCMU yield (○) (right) for (A) Texas B3, (B) Texas B4, (C) Mexico Beach, (D) Apalachicola, (E) Piney Island, (F) New Pass, (G) Charlotte Harbor, (H) Manasota, (I) Jacksonville and (J) Wilson.
Figure 1.6. PAM-FL chlorophyll $a$ (●) and cell diameter (○) (left) compared to 8 hr extracted chlorophyll $a$ (right) for (A) Texas B3, (B) Texas B4, (C) Mexico Beach, (D) Apalachicola, (E) Piney Island, (F) New Pass, (G) Charlotte Harbor, (H) Manasota, (I) Jacksonville and (J) Wilson.
Figure 1.7. Cell count (left) and growth rate (right) from 3 separate multiple isolate experiments 1(○), 2(●), 3(△) for (A) Texas B3, (B) Texas B4, (C) Mexico Beach, (D) Apalachicola, (E) Piney Island, (F) New Pass, (G) Charlotte Harbor, (H) Manasota, (I) Jacksonville and (J) Wilson.
Figure 1.8. Relative ETR (left) and PAM-FL yield (right) from 3 separate multiple isolate experiments 1(○), 2(●), 3(△) for (A) Texas B3, (B) Texas B4, (C) Mexico Beach, (D) Apalachicola, (E) Piney Island, (F) New Pass, (G) Charlotte Harbor, (H) Manasota, (I) Jacksonville and (J) Wilson.
Figure 1.9. Cell count (left) and growth rate (right) for Apalachicola isolate at (A) 5, (B) 10, (C) 20, (D) 40, (E) 80 and (F) 160x.
Figure 1.10. Apalachicola isolate at (A) 5x and 5/2, (B) 10x and 10/2, (C) 20x and 20/2, (D) 40x and 40/2, (E) 80x and 80/2 and (F) 160x and 160/2 for PAM-FL relative ETR (left) and PAM-FL yield (right) after 1 week culture growth (○, △) and the same culture an additional week later (■, ▲). Full culture was represented with circles and diluted culture represented with triangles.
Figure 1.11. Apalachicola $^{14}$C primary productivity at 8x and 32x the relative inoculation concentration.
Figure 1.12. Apalachicola (A) cell count and (B) growth rate for 100% culture.
Figure 1.13. Apalachicola PAM-FL relative ETR (left) and PAM-FL yield (right) for (A) May 28th and (B) June 1 of the same culture diluted to 80, 60, 50, 40 and 20%.
Figure 1.14. Apalachicola PAM-FL relative ETR (left) and PAM-FL yield (right) for the same culture diluted to 50% using FSW, GF/C filtered used media, fresh media and 0.2 μm filtered used media.
Figure 1.15. Apalachicola PAM-FL relative ETR (left) and PAM-FL yield (right) for (A) 1x the inoculation concentration and (B) 2x the inoculation concentration with each culture diluted to 50% using FSW, GF/C filtered used media and 0.2 μm filtered used media.
Figure 1.16. Apalachicola $^{14}$C primary productivity for 1x (left) and 2x (right) the original culture diluted to 50% using FSW and media from the opposite culture.
Figure 1.17. Linear regression of DCMU yield measured on a Turner-FL and PAM-FL yield.
Figure 1.18. Linear regression of extracted chlorophyll $a$ and PAM-FL chlorophyll $a$. 
Figure 1.19. $^{14}$C Primary production compared to relative ETR prior to lights-on (left) and after 8 hrs of incubation (right) for (A) Piney Island, (B) Manasota, (C) Jacksonville and (D) Wilson.
Figure 1.20. Relationship between $^{14}$C primary productivity up to $E_k$ and corrected ETR.
Figure 1.21. Linear regression of growth rate and $\alpha$. 
Figure 1.22. Linear regression of diameter and chlorophyll a for (A) prior to lights-on, (B) after 4 hrs of incubation and (C) after 8 hrs of incubation.
Figure 1.23. Linear regression of diameter and $\beta$. 
Figure 1.24. Linear regression of cell count and $P_{\text{max}}$. 
Figure 1.25. Linear regression of cell count and $E_k$. 
Figure 1.26. Average value between 1000 μmol quanta m⁻² s⁻¹ and 1600 μmol quanta m⁻² s⁻¹ for (A) PAM-FL relative ETR and (B) ¹⁴C primary productivity versus cell count at 4 hrs (○) and 8 hrs (■).
Figure 1.27. Average value between 1000 μmol quanta m⁻² s⁻¹ and 1600 μmol quanta m⁻² s⁻¹ for (A) PAM-FL Yield and (B) DCMU yield versus cell count at 4 hrs (○) and 8 hrs (●).
Appendix A. Experiment 1- Sequential Multiple Isolates: Isolates individually incubated during separate experiments prior to lights-on.

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### Appendix B. Experiment 1- Sequential Multiple Isolates: Isolates individually incubated during separate experiments after 4 hrs.

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Appendix C. Experiment 1- Sequential Multiple Isolates: Isolates individually incubated during separate experiments after 8 hrs.

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Appendix D. Experiment 2-Simultaneous Multiple Isolates: All 10 isolates incubated for 8 hrs. at the same time over three separate experiments.

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Appendix E. Experiment 3-Apalachicola Cell Number Gradient: Apalachicola isolate was grown at 5, 10, 20, 40, 80, and 160x the relative inoculation concentration. Half was used for the experiment and the other half was allowed to grow for an additional week. The experimental culture was incubated for 8 hrs at the original concentration and diluted in half.

<table>
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<th>Growth rate</th>
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Appendix F. Experiment 3- Apalachicola Cell Number Gradient: Apalachicola isolate was grown at 8x and 32x the relative inoculation concentration. The experimental culture was incubated for 8 hrs.

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Appendix G. Experiment 3- Apalachicola Cell Number Gradient: Apalachicola isolate was grown for one week. May 28th the culture was split in half. Half the culture was used for the experiment and the other half was allowed to grow until the second experiment on June 1. On the day of both experiments the original culture was incubated along with an 80, 60, 50, 40 and 20% dilution using FSW.

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<th>Diameter</th>
<th>Growth rate</th>
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**Appendix H.** Experiment 4-Apalachicola Media Dilutions: Apalachicola isolate was grown for one week. The day of the experiment the original culture was incubated for 8 hrs with 50% dilutions using FSW, GF/C filtered media, fresh media and 0.2μm filtered media.

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<th>Diameter SD</th>
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Appendix I. Experiment 4- Apalachicola Media Dilutions: Apalachicola isolate was grown for one week at 2x and 1x the original starting culture. The day of the experiment both the 2x and 1x culture was incubated for 8 hrs at the original concentration and diluted to 50% using FSW, GF/C filtered media, and 0.2μm filtered media from the opposite culture.

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<th>PAM-FL</th>
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<th>Growth rate</th>
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Appendix J. Experiment 4- Apalachicola Media Dilutions: Apalachicola isolate was grown for one week at 1x and 5x the relative inoculation concentration. The day of the experiment both the 1x and 5x culture was incubated for 8 hrs at the original concentration and diluted to 50% using FSW, GF/C filtered media, and 0.2μm filtered media from the opposite culture.

<table>
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<th>Count SD</th>
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</table>
CHAPTER 2

Biochemical analysis of *Karenia brevis* under intermediate light and nutrient replete mesocosm conditions.

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INTRODUCTION

*Karenia brevis* is a harmful algal bloom (HAB) dinoflagellate typically located in the Gulf of Mexico. Blooms of *K. brevis*, or red tides, usually develop 18-75 km offshore of Florida’s West Coast or within an epicenter between Tampa Bay and Charlotte Harbor (Steidinger et al. 1998b). Blooms most typically occur between the months of July and October. These microalgal cells produce neurotoxins and hemolytic substances. Eventually the blooms move shoreward, to the west coast of Florida, causing fish kills (Steidinger et al. 1998a). Though the development of these blooms is determined by a number of physical, chemical, and biological environmental factors, laboratory studies suggest that the internal biochemical status of the cell controls growth, reproduction, and possibly migratory behavior (Kamykowski et al. 1998). The internal biochemical status of the cell is directly influenced by the cell’s ability to utilize ambient light and nutrients for growth and reproduction. Understanding mechanisms that allow *K. brevis* to efficiently use light and nutrients are crucial in identifying the organism’s ability to proliferate.

Dinoflagellates typically aggregate at the surface during the day and disperse or migrate as bands throughout the water column at night in a process termed diel vertical migration (DVM) (Eppley and Harrison 1975; Heaney and Eppley 1981). The DVM cycle is related to the taxis abilities of the organism. Phototaxis allows for carbon fixation at the surface during the day (Cullen et al. 1985) and geotaxis (Kamykowski et al. 1999)/chemotaxis (Burkholder and Springer 1999) allows for nitrogen uptake during the night. Two alternate, but nonexclusive, DVM forcing patterns originate from mesocosm observations (Kamykowski et al. 1998). The first case hypothesized a passive
mechanical effect when photosynthate accumulates during the day and is used during the night. These cyclic changes in the mass distribution, relative to the cell’s center of mass cause a change in swimming direction. The second case hypothesized an optimization scheme based on threshold limits through utilization or formation of photosynthate and/or dissolved nitrogen pools to actively control taxis of the organism in the local environment (Kamykowski et al. 1998; Liu et al. 2001).

Kamykowski et al. (1998) investigated laboratory measurements of the taxis patterns, and the biochemical composition of a vertically migrating quantized K. brevis (Wilson) population (Fig. 2.1a). A quantized population is defined when all cells divide together, as a population, every few days. Laboratory experiments included bulk analyses of DNA, RNA, carbohydrate, protein, chlorophyll, and lipid concentrations within the vertically migrating population. The most notable results were the significant decrease of lipid in the surface population and the increase of lipid in the mid-mesocosm population during the day (Fig. 2.1B). Chlorophyll concentrations also followed a similar pattern as shown in Figure 2.1C.

Conclusions from these observations led to the hypothesis of unequal daughter cell division within a quantized population or equal daughter cell division within a quantized division. Initially, unequal daughter cell division was considered a more realistic theory, due to the required rapid internal biochemical turn-around in the lipid component. The unanswered debate, between unequal and equal daughter cell division within a population, spurred the investigation into the biochemical mechanisms responsible for the results reported by Kamykowski et al. (1998).
This project investigated the biochemical changes of *K. brevis* during DVM in more detail. DVM patterns are impacted by the utilization or formation of photosynthate and/or dissolved nitrogen pools. Positive phototaxis attempts to satisfy carbon requirements of the cell, while positive geotaxis/chemotaxis attempts to satisfy the nitrogen requirements of the cell. A cell’s carbon and nitrogen requirements are not always met throughout its diel cycle. If a cell does not fulfill these requirements, ascent or descent continues to position the cells at optimal depths throughout the vertical water column.

Monogalactosyldiacylglycerols (MGDG) are predominant lipids in the photosynthetic membranes such as the thylakoid and the inner envelope membrane of chloroplasts. Studies with cyanobacteria have showed MGDG and phosphatidylglycerol (PG) are integrated with the pigment-protein complexes of photosynthesis in the thylakoids of chloroplasts. Therefore, MGDG, its precursors such as fatty acids and monoacylglycerol (MG), and triacylglycerol storage pools play an important role supporting photosynthesis (Jordan et al. 2001). MGDG is susceptible to lipid peroxidation from ROS because of its polyunsaturated fatty acid side-chains (Halliwell and Gutteridge 1999). For *K. brevis* to maintain a viable photosystem it must consume the fatty acids, MG and triacylglycerol pools to produce sufficient MGDG. This project indicated *K. brevis* successfully maintained a functional photosynthetic state by utilizing fatty acids, MG and triacylglycerol pools to produce sufficient MGDG at intermediate and low light.
METHODS

Culture

Non-axenic, or bacteria containing, cultures of the *K. brevis* Apalachicola isolate (APA-c6), provided by Florida Marine Research Institute, were incubated in 250 ml Erlenmeyer flasks using modified L/2 culture medium (Guillard and Hargraves 1993), with Cu removed and soil extract added at a salinity of 35. Soil extract was obtained from autoclaving 100 ml of Hyponex potting soil with 1 l of deionized water. Flasks were grown in a 22 °C incubator, at 100 µmol quanta m\(^{-2}\)s\(^{-1}\) PAR (photosynthetically active radiation), by cool white fluorescent bulbs with 12 hours of light and 12 hours of dark. Larger volumes were grown in 9 l carboys of modified L/2 medium, in a temperature controlled room at 22 °C, using soft white fluorescent bulbs to provide 100 µmol quanta m\(^{-2}\)s\(^{-1}\) for 12 hours of light and 12 hours of dark.

Mesocosm

Mesocosm experiments were used to investigate how subpopulations respond to light during DVM. The mesocosm had nutrient replete conditions to isolate the response to light as the only variable. Mesocosm experiments were conducted in a translucent fiberglass column (155 cm height x 44 cm diameter, 225 L volume) housed in a 22 °C temperature-controlled room. The mesocosm was washed with a weak soap solution and rinsed thoroughly with tap water prior to the addition of culture media. A 6 cm water bath was circulated between the mesocosm and a 500 W tungsten-halogen lamp suspended above to provide an infrared filter. The circulated water was kept clean with a Beckett bio-filter (BF350A20). A 12 hr light:12 hr dark cycle allowed for diel vertical migration (standard conditions). The light intensity at the water surface was 250 µmol
quanta m$^{-2}$ s$^{-1}$, and 90 µmol quanta m$^{-2}$ s$^{-1}$ PAR at 90 cm depth. The mesocosm was covered with a fiberglass top and supported with rubber stripping around the edges. Two 2 cm holes in the top allowed gas exchange and suppressed introduction of contaminants. A third covered opening was available to allow a weighted sample tube (Tygon 0.32 cm inner diameter) to enter the mesocosm. All collection tubes were autoclaved prior to use on the mesocosm. When tubing was handled for profiling, latex gloves were used to minimize introduction of contaminants. A detailed light profile of the mesocosm was measured with a sterilized Li-COR light meter prior to the addition of culture. Dissolved oxygen and temperature measurements were profiled with a sterilized Yellow Springs Instrument (YSI) handheld meter prior to and after the addition of culture. Nitrate was measured with a Lachat QuikChem 8000 Continuum Series Autoanalyzer using methods designed by Lachat Instruments for water samples. Mesocosm experiments were carried out after a two-week culture acclimation and growth period. Samples were removed from the mesocosm by siphoning to prevent physical damage to the cells from a surface depth (0-5 cm) and middle depth (90 cm), at 0600 (prior to lights on), 1200, and 1800 (prior to lights off). Preliminary high resolution profiling every 10 cm indicated aggregations primarily formed at the 0-5 cm and 100 cm depths. The experiment was conducted from 26-28 March 2003. Volume removed from the mesocosm, at the surface and middle depths, was calculated based on cell concentrations at those depths. This volume was replaced after each sampling period with the equivalent 22 °C nutrient rich media along the bottom of the mesocosm using autoclaved Tygon tubing.
Photosynthetron

A “snap-shot” of mesocosm surface and middle depth subpopulations were incubated in a radial photosynthetron (RP) (Babin et al. 1994) which allowed for controlled conditions of light and temperature during experiments. This photosynthetron had 12 black boxes that allowed 10 culture flasks (50 ml) to fit inside each box (120 samples) (Fig. 2.2). A light gradient was formed by the distance of each bottle to the light source, which allowed samples to be exposed to multiple light levels. Self-absorption in the RP was more like the mesocosm than any other type of photosynthetron. This experiment helped isolate physiological state from the effect of DVM. All twelve boxes had equal exposure to the same light source, and their range of the absolute light gradient was reduced with the use of neutral filters between culture flasks. Maximum light levels were 2000 μmol quanta m$^{-2}$ s$^{-1}$ and minimum light levels were 50 μmol quanta m$^{-2}$ s$^{-1}$ PAR. The RP had light levels beyond the light attenuation of the mesocosm to fully resolve photo-responses. Each box was water-jacketed to keep constant temperature and to remove infrared light. Cells at surface and middle depths from the mesocosm were placed into the radial photosynthetron for an 8 hr incubation just prior to mesocosm lights-on. Samples were drawn from the photosynthetron at 2 pm. This sample time was 2 hrs after the withdrawal of the 12 pm mesocosm sample allowing time for processing mesocosm samples. Samples were pooled for filtration by combining bottles from Apalachicola P-E curves determined from previous isolate experiments (Schaeffer et al. 2004; Schaeffer 2005; Schaeffer 2006c). Low light or $\alpha$ was 50-92 μmol quanta m$^{-2}$ s$^{-1}$, intermediate light or $P_{\text{max}}$ was 298-963 μmol quanta m$^{-2}$ s$^{-1}$ and high light or $\beta$ was 1198-1660 μmol quanta m$^{-2}$ s$^{-1}$.
Biochemistry and Physiology

All biochemical measurements were normalized to a per cell basis. Cell counts were determined using a Coulter Multisizer II Particle Analyzer (Beckman-Coulter Inc., Miami, FL) with threshold settings of 13.3 and 30 µm and a 200 µm orifice. Triplicate samples were collected in 20 ml scintillation vials for cell count and cell diameter determinations.

A Walz Water Pulsed Amplitude Modulated Chlorophyll Fluorometer (PAM-FL) was auto-zeroed with 0.45 µm filtered cell media (Cullen and Davis 2003) to measure photosynthetic yield (Fv/Fm-1) in triplicate.

Carbon and nitrogen samples were filtered on pre-combusted GF/F filters (0.7 µm) and stored in annealed glass scintillation vials at -20 °C freezer until analysis. All ashed filters and annealed glass were wrapped in aluminum foil and baked for 6 hrs at 510 °C. The day prior to analysis, all filters were freeze dried for 6 hrs in a Labconco Freeze dry system (-50 °C, 37 x 10⁻³ mbar). Filters were weighed and then combusted in a Sediments Analyzer Flash 1112 EA Series with an acetonilide (C₈H₉NO) standard from Costech Analytical Technologies, Inc.

Cells were filtered for pigment identification on 25 cm GF/F filters, folded in half, purged with N₂ gas, placed in a cryo-vial and stored in liquid nitrogen until analysis. Pigments were analyzed with reverse-phase Shimadzu LC-10AT high performance liquid chromatography (HPLC) (Wright et al. 1991) at Mote Marine Laboratories (Gary Kirkpatrick).

Lipid samples were collected on pre-combusted 55 cm GF/F filters, folded in half, purged with N₂ gas, placed in a cryo-vial and stored in liquid nitrogen until analysis.
Lipids were extracted with a methylene chloride:methanol:water (1.25:2.5:0.8) solvent mixture (Bligh and Dyer 1959). The methylene chloride phase was collected. The aqueous phase was rinsed twice more with methylene chloride. All methylene chloride collections were pooled and evaporated for 1 hr under a light flow of N\textsubscript{2} gas in a homemade evaporator. The evaporator was constructed from aluminum and covered with Teflon paint from Cotronics Corporation (Brooklyn, NY). This evaporator held 25 slots for vials and was water jacketed to keep a constant temperature of 35 °C. A lid contained holes centered over the vial slots to provide a steady stream of N\textsubscript{2} gas over the samples. Lipids were re-dissolved in 30 μl of methylene chloride and analyzed on the same day as the extraction with the MK-V Chromarod-Iatroscan (Iatron, Japan) thin layer chromatography/flame ionization detection (TLC/FID) system. Air flow rate was 2000 ml min\textsuperscript{-1} and the hydrogen flow rate was 160 ml min\textsuperscript{-1}. Samples were scanned at 25 seconds per scan. Lipid classes were identified with a 5 step separation technique on the Chromarods (Striby et al. 1999). Chromarods were SIII reusable quartz rods covered by silica gel particles (Parrish et al. 1992; Liu et al. 1998). Rods were acid cleaned with 33% HNO\textsubscript{3} after each sample, rinsed with deionized water and dried with 100% acetone. Samples were spotted (10-15 μl) by hand with a 1 μl syringe. Standards were purchased from Sigma Chemical Co. (St. Louis, MO) and all organic solvents were purchased from Fisher Scientific (Atlanta, GA). Data acquisition and processing was performed with Peak Simple v 2.97 software from SRI Instruments (Torrance, CA).

**Analysis**

The data were plotted and analyzed using Microsoft Excel, Sigma Plot, Sigma Stat and SAS. Error bars are reported as 1 standard deviation.
RESULTS

Mesocosm

Surface NO$_3$ decreased 28% at the end of day 1 and 32% at the end of day 2. On day 3, surface NO$_3$ exhibited no net change throughout the day. The concentration of NO$_3$ averaged 28 µM at the middle depth for all three days (Fig. 2.3). Surface cell count maximums occurred at 6, 36 and 66 hrs which represented each of the three sampling times; morning, mid-day and late afternoon progressing through the daylight period over the three days. Maximums were between 84% and 98% larger than counts taken at the surface within 6 hrs. Middle depth cell counts decreased each of the three days from the morning sample to the late afternoon (Fig. 2.4A). Surface diameters decreased 1% on day 1 and day 2 but decreased 4% on day 3 (Fig. 2.4B). Middle depth diameters increased 5% from 20 µm to 21 µm throughout each of the three days. Surface and middle depths had identical within-day-patterns that differed between days through the three days of the experiment for both internal cellular carbon (Fig. 2.4C) and internal cellular nitrogen (Fig. 2.4D). Total lipid content in surface and middle depths generally increased throughout the day for all three days. After mid-day on day 3 there was a 50% decline in surface and middle depth total lipid (Fig. 2.4E). Surface dissolve oxygen was consistently higher than middle depth dissolved oxygen for all three days, but both surface and middle depth increased from morning until later afternoon (Fig. 2.4F). Yield decreased significantly (p<0.001) at the surface each day, while middle depth showed no significant change each day (Fig. 2.4G).

Triacylglycerol, fatty acid, MG and MGDG lipid classes all showed large variability at surface and middle depth throughout all three days of the experiment (Fig.
2.5A, 2.5B, 2.5C, 2.5D). Day 1 and day 2 triacylglycerol ended higher for surface (p=0.021) and middle depth (p=0.013). Day 3 showed no significant net change in surface triacylglycerol (p=0.05) or middle depth triacylglycerol (p=0.106). There was no significant change from the beginning to the end of all three days for surface fatty acid (p=0.18) and middle depth fatty acid (p=0.209). Surface MG increased significantly on day 1 (p=0.006) and day 2 (p=0.039) while middle depth MG did not significantly change throughout all three days (p=0.917). MGDG concentration was higher for surface at the end of day 1 (p=0.014) and day 2 (p<0.001). MGDG was also higher for middle depth at the end of day 1 (p=0.009) and day 2 (p=0.024). Day 3 MGDG did not significantly change in surface (p=0.067) and middle depth (p=0.12).

Chlorophyll a and gyroxanthin exhibited identical patterns throughout all three days of the experiment (Fig. 2.6 A, 2.6B) as did diatoxanthin and diadinoxanthin (Fig. 2.6C, 2.6D). All four reported pigments showed large variability at surface and middle depth throughout the experiment. All four pigments at the surface increased from 6 to 12 hrs with no change at 18 hrs. Hours 30 to 42 exhibited no significant change in concentration for all surface pigments. Surface chlorophyll a, gyroxanthin, diatoxanthin and diadinoxanthin increased from 54 to 60 hrs, but there was a significant decrease at 66 hrs. Over the first two days of the experiment, middle depth chlorophyll a (p<0.001), gyroxanthin (p<0.001), diatoxanthin (p=0.003) and diadinoxanthin (p<0.001) increased significantly during each day.

**Radial photosynthetron**

Surface (250 μmol quanta m\(^{-2}\) s\(^{-1}\) in mesocosm) and middle depth (90 μmol quanta m\(^{-2}\) s\(^{-1}\) in mesocosm) cell counts sampled before lights-on were consistent
throughout the three pooled RP light levels (data not shown). Surface (p=0.024) and middle depth (p=0.022) diameters increased significantly from low to intermediate light and decreased at high light (Fig. 2.7A). There was no significant change in surface yield at all light levels but middle depth yield significantly decreased at high light (p<0.001) (Fig. 2.7B). Triacylglycerol concentration did not change from low to intermediate light, but significantly increased at high light for surface (p<0.001) and middle depth (p<0.001) (Fig. 2.8A). Surface (p=0.169) and middle depth (p=0.283) fatty acid did not significantly change at all three light levels (Fig. 2.8B). MG had no significant change for surface and middle depth (Fig 2.8C). MGDG middle depth had no significant change, but surface MGDG significantly increased (p<0.001) at intermediate light (Fig. 2.8D). Surface chlorophyll a (Fig. 2.9A) and gyroaxanthin (Fig. 2.9B) increased about 43% from low to intermediate light and about 35% from intermediate to high light. There was no significant change in middle depth chlorophyll a (Fig. 2.9A) or gyroaxanthin (Fig. 2.9B). Surface diatoxanthin decreased from low to high light, and middle depth diatoxanthin did not significantly change (Fig. 2.9C). Diadinoxanthin increased at surface 229% from low to high light, and middle depth did not significantly change from low to high light (Fig. 2.9D).

**DISCUSSION**

**General**

Gyroaxanthin was used as an additional verification that samples were pure *K. brevis*. This pigment had only been reported for two additional toxic dinoflagellates *Gyrodinium aureolum* and *Gymnodinium galatheanum* (Millie et al 1995). The linear relationship of gyroaxanthin to chlorophyll a was unique to *K. brevis* (r=0.99) (Fig. 2.10).
In the field this ratio is used as a biomarker to identify *K. brevis* from other populations. The constant ratio between gyroxanthin and chlorophyll *a* was observed in this experiment and can be used in future field sampling.

The epoxidation state, defined as the ratio of [diatoxanthin] / [diadinoxanthin + diatoxanthin], was low and did not exceed 0.2 throughout the mesocosm experiment (Fig. 2.11). Low expoxidation state indicated light at the surface and middle depth was not stressful. Biochemical constituent changes were most likely not a result of photooxidation or photoinhibition but instead from the photosynthetic responses during diel vertical migration.

**Surface aggregation**

In order to gain insight into the character of the cells that accumulate at the air-sea interface, the following discussion focuses on the surface mesocosm aggregations that occurred at 0600 on day 1, 1200 on day 2 and 1800 on day 3. Biochemical constituents of these three surface aggregations were the lowest when compared to populations at all other times irrespective of depth. Cell diameter was smallest during the 1800 aggregation of day 3 (Fig 2.12A). Internal cellular carbon (Fig 2.12B) and nitrogen (Fig 2.12C) were lowest during the 0600 aggregation and significantly increased with decreased cell count for the 1800 (p=0.001) and 1200 (p<0.001) aggregations. The biochemical patterns suggest that the 6 hr delay in surface aggregation over the 3 day period was not random. The lowest observed internal carbon and nitrogen at 0600 on day 1 suggested a morning aggregation which resulted from cells that newly divided over night and were ready for photosynthetic production at first light. The 1800 aggregation had intermediate internal carbon and nitrogen relative to the other two aggregates. An 1800 aggregation suggested
cells low in carbon that would be decreased further during the night but would be replenished at first light and could become a 0600 aggregation on day 4. The 1200 aggregation had the highest internal cellular carbon and nitrogen relative to the other two aggregates. A 1200 aggregation suggested cells had ample amounts of carbon prior to the end of day 1 (Fig. 2.4E). On day 2, at 0600, internal cellular carbon was still elevated relative to 0600 on day 1. Cells did not have to aggregate to the surface until carbon decreased by 1200 of day 2. Diel vertical migration allowed cells with decreased biochemical constituents to aggregate at the surface where higher light was available for carbon fixation.

Total lipid (Fig. 2.12D) of the 0600 aggregation was 76.85 pg cell\(^{-1}\) and significantly decreased (p<0.001) in the 1800 aggregation. Total lipid significantly increased (p<0.001) in the 1200 aggregation. Yield followed a trend similar to total lipid (Fig. 2.12E). Photosynthetic efficiency (Fv Fm\(^{-1}\)) was greatest after night recovery in the 0600 aggregation. Most of the absorbed light energy was dedicated toward carbon fixation and required biochemical constituents such as lipids. At 1200 the photosynthetic efficiency was decreased due to 6 hrs of light exposure, therefore decreasing the amount of absorbed light energy dedicated toward production of lipids and increasing thermal dissipation. Finally, 1800 had the longest exposure of 12 hrs light and the greatest decrease in photosynthetic efficiency. Lowered photosynthetic efficiency at the end of the day meant that new cells with low total lipid would have to wait overnight for Fv Fm\(^{-1}\) recovery. The following morning cells would have higher Fv Fm\(^{-1}\) and produce similar results as the 0600 day 1 surface aggregation.
Trend lines were fit to the cell count and biochemical constituent relationships for further analysis. Internal cellular carbon decreased with increased cell count (Fig. 2.13A); however, no clear relationship existed between cell diameter and internal cellular carbon or internal cellular nitrogen (data not shown). The relationship between surface internal cellular carbon and cell count had a linear fit with a slope of -0.0008 (r=0.88, p=0.0017). The relationship between middle depth internal cellular carbon and cell count fit to the inverse first order equation $y=y_0 + a/x$ (r=0.79, p=0.01). Internal cellular nitrogen also decreased with increased cell count (Fig. 2.13B). The relationship between surface internal cellular nitrogen and cell count had a linear fit with a slope of -0.0002 (r=0.75, p=0.019). Middle depth internal cellular nitrogen and cell count also had a linear fit with a slope of -0.0032 (r=0.89, p=0.001). Surface total lipid decreased with cell count which fit to the inverse first order equation $y=y_0 + a/x$ (r=0.85, p=0.003) as did middle total lipid but could not be significantly fit to a curve (Fig. 2.13C). All three cell aggregations were located at the right end of these fits and contained the lowest biochemical constituents per cell. Carbon and nitrogen clearly acted as motivators for the cell. Young cells resulting from a recent division had less carbon and nitrogen whether the division was equal or unequal. The reduction of carbon stimulated cells to swim toward higher light at the surface. Older cells had more carbon and could remain at depth where light intensity was not as intense. Intermediate aged cells had filled their carbon partly but were not as full as older cells. One could think of the aggregates in relation to cell age while sliding back and forth on the fits to carbon and nitrogen versus cell count. The youngest cells aggregated at the surface during the three maximums at 0600 1200 and 1800 on the right side of the plots. These cells created the highest cell counts at the
surface but had the lowest carbon and nitrogen pools. Older cells remained at middle depth with fuller carbon pools. After carbon and nitrogen pools are full the cell could restart the cycle with cellular division. These trends indicated that diel vertical migration was a result of an optimization scheme based on threshold limits through utilization or formation of photosynthate and/or dissolved nitrogen pools to actively control taxis of the organism in the local environment. Also, lipid pools did not influence *K. brevis* cell buoyancy in their migration patterns. Bouyancy control with lipids is a widely accepted form of migration (Lewis 1970; Sargent and Falk-Peterson 1988; Campbell and Dower 2003). It would be expected that lipids were positively buoyant and would cause the cell to arrive at the surface from buoyancy changes. All cells at the surface were low in total lipid.

**Lipids**

MGDG and its association with the pigment-protein complex was indicated by its linear relationship with chlorophyll \( a \) (\( r=0.65, \) slope=0.71, \( p=0.002 \)) (Fig. 2.14A). The intermediate MG was slightly positively related to chlorophyll \( a \) (\( r=0.64, \) slope=0.006, \( p=0.004 \)) (Fig. 2.14B). MG and its end product MGDG were also slightly positively related (\( r=0.66, \) slope=0.007, \( p=0.004 \)) (Fig. 2.14C). Fatty acid also held a slightly positive relationship with MGDG (\( r=0.45, \) slope=0.001, \( p=0.046 \)) (Fig. 2.14D). These relationships for MG to chlorophyll \( a \), MG to MGDG, and fatty acid to MGDG were not meant to indicate the precursor MG or fatty acid was solely utilized for the MGDG final product. Instead the positive relationship was used as an indicator that there was enough precursor fatty acid or MG to provide for biosynthesis into the end products MGDG without cell stress. If the cell was exposed to a stressful light environment, this
relationship would turn negative with decreasing concentrations of the precursor fatty acid and MG to provide for biosynthesis into an end product such as MGDG (Schaeffer 2006b). Under low (middle) and intermediate (surface) light, \textit{K. brevis} exhibited no biochemical stress. Biochemical compositions are the result of supply of energy from photosynthesis (Smith et al. 1989). The supply of energy for photosynthesis was sufficient but not inhibiting so the cell had not been stretched beyond a maximum which resulted in the positive lipid relationships.

When lipid class concentration was averaged at surface and middle depth over the entire course of the experiment there was great variability with average surface S.D. +/- 21.1 and average middle S.D. +/- 14.1. However, triacylglycerol and MGDG were consistently the two largest classes of the lipids measured on a percent basis. Surface triacylglycerol was 22% and MGDG was 41% while middle triacylglycerol was 32% and middle depth MGDG was 36%. Fatty acid and MG changed little from surface and middle depth with 1.6% and 0.5% respectively. Phospholipids such as PG, PE and PC also changed little from surface and middle depth 9%, 8% and 1.5% respectively. Finally, hydrocarbons represented 17% at surface and 13% at middle depth. The greatest changes in concentration occurred in the two largest classes, triacylglycerol and MGDG (Fig. 2.15A, 2.15B). All other classes remained relatively unchanged in percentage concentration between surface and middle depth. Surface triacylglycerol was 36 pg cell$^{-1}$ and surface MGDG was 48 pg cell$^{-1}$. Middle depth nearly doubled the MGDG to 84 pg cell$^{-1}$ with a small increase in triacylglycerol to 42 pg cell$^{-1}$.

The thylakoid membranes of algae are the main intracellular membrane and primarily composed of MGDG and some PG lipids. PG is the main phosphoglyceride
and the only phospholipid in the thylakoid (Harwood 1998). However, PG is also located in the endoplasmic reticulum, plasma membrane and mitochondria (Dormann 2005). PE and PC are located in the endoplasmic reticulum, plasma membrane and mitochondria and Golgi. However, PE and PC do not occur in chloroplasts and thylakoid membranes (Harwood 1998; Dormann 2005). MGDG of algae typically account for 40-55% while PG is 10-20% of total lipid. For example, total cell lipid for the green alga *Chlamydomonas reinhardtii* contained 42% MGDG, 5% PE and 4% PG (Mendida-Morgenthaler et al. 1985). Lipid percentages from this experiment on *K. brevis* are within the expected percentages for algae cells. Middle MGDG increased because of reduced light at depth while surface MGDG decreased when exposed to the higher light. MGDG form the structure of photosynthetic cellular membranes and increase when exposed to low light (Khotimchenko and Yakovleva 2005). Lower triacylglycerol percentage and concentration at surface depth indicated cells may have been at the surface for CO₂ fixation to increase triacylglycerol pools. Middle depth cells had increased triacylglycerol percentage and concentration because the carbon pool was previously filled at the surface. Lack of significant percentage change in PE, PC and PG at different light intensities was expected since these phospholipids are primarily in membranes of organelles that do not respond to light such as the endoplasmic reticulum, plasma membrane and mitochondria.

Changes in concentration of MGDG and triacylglycerol suggested these two lipid classes were the predominant classes impacting the total lipid results measured in Kamykowski et al (1998). Although the present experiment was not quantized, similar trends resulted from both experiments. Surface had reduced total lipid and chlorophyll *a*
while middle had increased total lipid and chlorophyll \(a\). The linear relationship between MGDG with chlorophyll \(a\) presented here, MGDGs predominant association with the protein-pigment complex and MGDGs crucial role in photosynthetic light reactions lead to the conclusion that the total lipid changes presented by Kamykowski et al (1998) were largely a result of decreased MGDG at the surface and increased MGDG at middle.

**Physiology vs Behavior/Physiology**

The rate that cells were migrating and time that cells remained at a particular depth were not known in the mesocosm. When we placed the 0600 aggregate on day 1 into the RP, we were able to control physical conditions and remove the behavior of the cells. Surface triacylglycerol had a positive net change while middle depth triacylglycerol had a negative net change in both the mesocosm and RP (Fig. 2.16A). Fatty acid had a negative net change in the mesocosm and a positive net change in the RP for both surface and middle depth (Fig 2.16B). MG showed a positive net change for surface and middle depth mesocosm and RP samples (Fig. 2.16C). Surface MGDG had a negative net change in both the mesocosm and RP while middle depth MGDG had a positive net change in the mesocosm and a negative net change in the RP (Fig. 2.16D). Surface chlorophyll \(a\) had a positive net change in both the mesocosm and RP, while middle depth had a positive net change in the mesocosm but a negative net change in the RP (Fig. 2.16E). EPS had a negative net change in the mesocosm and a positive net change in the RP for surface while middle depth EPS was negative for both the mesocosm and RP (Fig. 2.16F).

Almost all of the biochemical constituents in the RP showed similar results to the mesocosm’s 1800 sample time. Therefore RP samples were accelerated 4 hrs when
behavior was removed. This accelerated trend in the RP held well for surface triacylglycerol, fatty acid, and MG. MGDG followed the accelerated trend only after closer review. Even though MGDG in the RP sample was less than the initial 0600 mesocosm, it was still greater than the 1200 mesocosm sample. In the mesocosm, surface MGDG increased from 1200 to 1800. The surface MGDG signal may have been suppressed in the RP because of the higher light bracket (250-500 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \)) than that of the surface mesocosm (250 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \)). This suppression of MGDG signal was supported by the 2-fold higher EPS value in the radial photosynthesetron when compared to the mesocosm. Middle depth MGDG did not significantly change from 1200 to 1800 (p=0.139) in the mesocosm. The RP sample may have begun to indicate a decrease in MGDG which was seen on day 2 at 0600. RP surface chlorophyll \( a \) increased as much as the 1200 mesocosm sample. After 1200 the mesocosm showed no significant change (p=0.474) at 1800. Middle chlorophyll \( a \) decreased in the RP instead of increased as expected from the 1800 mesocosm sample. However, on day 2 at 0600 chlorophyll \( a \) was significantly less (p=0.039) than the 1800 sample on day 1. Overall, cells took longer to acquire biochemical constituents with behavior in the mesocosm. It appears that competition for optimal positioning and self-shading reduced efficiency.

**Conclusions**

This mesocosm experiment confirmed the internal biochemical status of the cell controlled migratory behavior, growth and reproduction. The data were inconclusive as to whether cell division yielded unequal and equal daughter cells. Scatter around the trend lines of internal cellular carbon and internal cellular nitrogen plotted against cell
count was large enough to indicate cells could divide unequally. Data points located on the trend lines showed cells may also have divided equally. The light level for this mesocosm allowed young cells with decreased biochemical constituents to migrate toward the surface. At the surface photosynthetic carbon fixation increased the internal cellular carbon and total lipid pools followed by a decent to middle depth. No light stress was observed due to *K. brevis*’ capacity to produce ample precursors such as free fatty acids and MG with increased end products such as MGDG. The cell was capable of performing a complex lipid balancing act. At the surface *K. brevis* reduced its MGDG constituent upon high light exposure while photosynthetic processes increased carbon pools and lipid precursors such as free fatty acids and MG. MGDG accounted for up to 40% of the total lipid pool in the cell while precursors along with triacylglycerol make up approximately 25% of the cells total lipid. In all, the cell is able to manipulate more than 50% of its lipids, while maintaining other lipid classes such as hydrocarbons and phospholipids during DVM. Quick turnover of these lipids within 6 hrs indicated *K. brevis* was more than capable of taking advantage of a changing physical condition such as light. The only indicated limiting factor in this experiment was competition in the behavioral aspect of the cell. When large aggregates form with young cells photosynthetically fixing carbon, there appeared to be competition for space, which was compounded by self-shading. Colored dissolved organic matter may also provide a shading mechanism at certain wavelengths for surface *K. brevis* cells in the field (Walsh et al. 2003). Higher light levels, as typically seen in the field from upwards of 2000 μmol quanta m⁻² s⁻¹, may remove some of the competition in space and time but it adds the stress through oxidation and photoinhibition.
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Figure 2.1. Time course of (A) cell count, (B) total lipid and (C) chlorophyll $a$ from the Kamykowski et al. 1998 experiment for surface (○) and middle (●) mesocosm samples. The y-axis was scaled for comparison to this experiment.
Figure 2.2. The radial photosynthetic box and bottle design allowed for 12 black boxes. Each box held 10 bottles. The T bottle has the highest light intensity while light intensity decreases 15% between each bottle towards the A bottle.
Figure 2.3. Time course of nitrate for surface (○) and middle (●) mesocosm depths.
Figure 2.4. Time course of (A) cell count, (B) cell diameter, (C) cell carbon, (D) cell nitrogen, (E) total lipid, (F) dissolved oxygen and (G) photosynthetic yield for surface (○) and middle (■) mesocosm samples. Error bars are ±1 standard deviation.
Figure 2.5. Time course of (A) triacylglycerol, (B) fatty acid, (C) MG and (D) MGDG for surface (○) and middle (●) mesocosm samples. Error bars are +1 standard deviation.
Figure 2.6. Time course of (A) chlorophyll $a$, (B) gyroxanthin, (C) diatoxanthin and (D) diadinoxanthin for surface (○) and middle (●) mesocosm samples. Error bars are +1 standard deviation.
Figure 2.7. Radial photosynthetron light gradient of (A) cell diameter and (B) photosynthetic yield for surface (○) and middle (●) mesocosm samples incubated for 8 hrs. Error bars are +1 standard deviation.
Figure 2.8. Radial photosynthetic light gradient of (A) triacylglycerol, (B) fatty acid, (C) MG and (D) MGDG for surface (○) and middle (●) mesocosm samples incubated for 8 hrs. Error bars are +1 standard deviation.
Figure 2.9. Radial photosynthetron light gradient of (A) chlorophyll $a$, (B) gyroxanthin, (C) diatoxanthin and (D) diadinoxanthin for surface (○) and middle (■) mesocosm samples incubated for 8 hrs. Error bars are +1 standard deviation.
Figure 2.10. The ratio of gyroxanthin to chlorophyll $a$ remained consistent for all samples at surface (○) and middle (■) which provided an additional indicator the sample was pure *K. brevis*. 
Figure 2.11. Time course of the epoxidation state (EPS = [diatoxanthin] / [diatoxanthin + diadinoxanthin] of the xanthophyll cycle for surface (○) and middle (●) samples.
Figure 2.12. Cell specific comparison of (A) cell diameter, (B) cell carbon, (C) cell nitrogen, (D) total lipid and (E) photosynthetic yield for surface (○) and middle (■) mesocosm samples. Error bars are +1 standard deviation.
Figure 2.13. Cell specific comparison of (A) cell carbon, (B) cell nitrogen and (C) total lipid for surface (○) and middle (●) mesocosm samples.
Figure 2.14. Linear regressions of (A) MGDG to chlorophyll $a$, (B) MG to chlorophyll $a$ and (C) MG to MGDG (D) fatty acid to MGDG for surface (○) and middle (●) mesocosm samples.
Figure 2.15. An average of measured lipid classes over all times for surface and middle. Pie fractions are percentage of the total lipid classes measured. The largest changes between surface and middle occurred in MGDG.
Figure 2.16. Comparison of (A) triacylglycerol, (B) fatty acid, (C) MG, (D) MGDG, (E) chlorophyll $a$ and (F) EPS between 0600 and 1200 sample periods in the mesocosm and radial photosynthetron for surface and middle. Net change in mesocosm indicated as $+/-$ Mesocosm and net change in radial photosynthetron $+/-$RP.
CHAPTER 3

Biochemical comparison of *Karenia brevis* under high light and nitrate replete/reduced mesocosm conditions.

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INTRODUCTION

*Karenia brevis* is a harmful algal bloom (HAB) dinoflagellate typically located in the Gulf of Mexico. Blooms of *K. brevis*, or red tides, usually develop 18-75 km offshore of Florida’s West Coast or from an epicenter between Tampa Bay and Charlotte Harbor (Steidinger et al. 1998b). Blooms most typically occur between the months of July and October. These microalgal cells produce neurotoxins and hemolytic substances. Eventually the blooms move shoreward, to the west coast of Florida, causing fish kills (Steidinger et al. 1998a). Though the development of these blooms is determined by a number of physical, chemical, and biological environmental factors, laboratory studies demonstrated that the internal biochemical status of the cell controls growth, reproduction, and migratory behavior (Kamykowski et al. 1998; Schaeffer 2006). The internal biochemical status of the cell is directly influenced by the cell’s ability to utilize ambient light and nutrients for growth and reproduction. Understanding mechanisms that allow *K. brevis* to efficiently use light and nutrients are crucial in identifying the organism’s ability to use local waters to proliferate.

Diel vertical migration (DVM) patterns are impacted by the utilization or formation of photosynthate and/or dissolved nitrogen pools. Positive phototaxis attempts to satisfy carbon requirements (Cullen et al. 1985) of the cell while positive geotaxis (Kamykowski et al. 1999)/chemotaxis (Burkholder and Springer 1999) attempts to satisfy the nitrogen requirements of the cell. Cell carbon and nitrogen requirements are not always met during the first iteration of ascent for carbon production and descent for nutrients. Ascent or descent continues, without completing these requirements, to position the cells at optimal depths throughout the vertical water column. Lack of
fulfilling these requirements during the cell diel cycle leads to phased cell division at
night. Cells must have sufficient reserves to achieve a permissive state before responding
to a circadian dark/light cue and moving into the cell division cycle (Van Dolah and
Leighfield 1999).

Phototaxis and exposure to increasing light intensity comes at a cost to *K. brevis.*
When exposed to excess energy, photooxidation occurs and eventually leads to
photoinhibition (Krause 1994). Photooxidation is the process that induces lipid
peroxidation and the bleaching of light absorbing pigments. Photoinhibition is caused by
absorption of excess light energy that inhibits photosynthetic activity by inactivating the
photosystems. The amount of inhibition is determined by internal factors such as
photoacclimation, defense mechanisms, and external factors such as environmental stress
(Foyer and Harbinson 1994).

Excessive light can cause the thylakoid electron transport system to move into full
production, increasing the thylakoid membrane pH (Asada 1994; Evens et al. 2001). An
increase in pH activates the carotenoid de-epoxidase that allows xanthophylls to remove
the excess energy as heat. Several scavenging mechanisms exist to protect the
photosystems from degradation by singlet oxygen, superoxide radicals, and hydrogen
peroxide collectively termed reactive oxygen species (ROS). Some of the energy is
transferred to chlorophyll creating an excited triplet state. Carotenoids (CAR) play a
large role in protecting chlorophylls from ROS due to their antioxidant characteristics
either through direct quenching of the singlet oxygen (Equation 1) or through quenching
the triplet chlorophyll (Equation 2) (Montenegro et al. 2002).

\[ ^1O_2 + CAR \rightarrow O_2 + ^3CAR \] or \[ ^1O_2 + CAR \rightarrow CARO_2 + \text{heat} \] 

Equation 1
\( ^3\text{Chl} + \text{CAR} \rightarrow ^3\text{CAR} + \text{Chl} \rightarrow \text{CAR} + \text{heat} \)  

Equation 2

The carotenoid triplet excited state can then return to the ground state by removing the excess energy through heat (Montenegro et al. 2002). Carotenoids with nine or more conjugated double bonds (ex. diatoxanthin) have a low enough energy in the triplet-excited state to become an efficient quencher. Carotenoids out-compete molecular oxygen for the additional electrons from the excited triplet chlorophylls. The energy of the triplet-excited carotenoid is also lower than that of the singlet oxygen so the excited triplet carotenoid will not react with molecular oxygen and cause further damage (Fraser et al. 2001).

Monogalactosyldiacylglycerols (MGDG) are predominant lipids in the photosynthetic membranes such as the thylakoid and the inner envelope membrane of chloroplasts (Harwood 1998). MGDG is susceptible to lipid peroxidation from ROS because of its polyunsaturated fatty acid side-chains (Halliwell and Gutteridge 1999). For \textit{K. brevis} cells to maintain a functional photosynthetic state it must consume the fatty acids, MG and the triacylglycerol pools that they produce to maintain sufficient MGDG.

The ability for the cell to repair its photosystems and lipid membranes should ideally equal, or surpass, the rate at which its photosystems and lipid membranes are degraded. Photoprotection is important to the organism’s survival since energy production must continue during times of stress for growth and reproduction (Niyogi 1999). Carotenoids may be responsible for maintaining \textit{K. brevis’} ability to survive photooxidation and photoinhibition. If the capacity of the carotenoids is exceeded then the lipids in the membranes may be at risk of photooxidation.
Previous work showed *K. brevis* maintained a functional photosynthetic state under intermediate and low light (Schaeffer 2006). It is still unclear if colored dissolved organic matter (CDOM) provides shade protection to *K. brevis* in the field (Walsh et al. 2003). If CDOM does provide protection, the effect would not occur at the surface where cells aggregate, but a few meters below the surface. However, high light conditions such as those seen in field surface populations may limit this protective effect as Evens et al. (2001) indicated with experiments over 3 days of different light intensity (partly sunny, mostly cloudy and sunny days). As light intensity increased, so did the xanthophylls epoxidation state, or ratio of monoepoxide diadinoxanthin to diatoxanthin. They also showed an increase in bulk lipid concentration when sun-light is minimal (i.e. middle mesocosm) compared to a decrease the other days of higher light (i.e. surface mesocosm). Measurement of PSII also shows degradation at high light, but degradation is less severe and recovery is possible during the cloudy day. Although Evens et al (2001) carried out similar measurements, they specifically were investigating the xanthophyll cycle of *K. brevis* and did not have the same intentions as this project. Evens et al (2001) did not include behavior, lipid class, free amino acid, cellular carbon, cellular nitrogen and toxins. This project expands upon previous work (Schaeffer 2006) in intermediate and low light conditions to show how *K. brevis* responded to field-simulated, high-light-nitrate*-replete* and high-light-nitrate*-reduced* conditions. From now on, the italicized terms *replete* and *reduced* indicate the particular nutrient conditioned mesocosm to distinguish from the normal use of these words.
METHODS

Culture

Non-axenic, or bacteria containing, cultures of the *K. brevis* Apalachicola isolate (APA-c6), provided by Florida Marine Research Institute, were incubated in 250 ml Erlenmeyer flasks using modified L/2 culture medium (Guillard and Hargraves 1993), with Cu removed at salinity of 35. Mesocosm medium had soil extract and Tris buffer removed. This reduced version of the L/2 culture medium allowed the only nitrogen source to be NO₃. Culture flasks were grown in a 22°C incubator, at 100 μmol quanta m⁻²s⁻¹ PAR (photosynthetically active radiation), by cool white fluorescent bulbs with 12 hours of light and 12 hours of dark. Larger culture volumes were grown in 9 l carboys of modified L/2 medium, in a temperature controlled room at 22°C, using soft white fluorescent bulbs to provide 100 μmol quanta m⁻²s⁻¹ for 12 hours of light and 12 hours of dark.

Mesocosm

Mesocosm experiments were used to investigate how subpopulations respond to light and nitrate limitation during DVM. One mesocosm had nutrient *replete* conditions and the other mesocosm had nitrate *reduced* conditions with replete phosphate and iron. Mesocosm experiments were conducted in a translucent fiberglass mesocosm housed in a temperature-controlled room set at 22 °C as described in Schaeffer (2006). Six cm water baths were circulated between the mesocosms and a 1,500 W tungsten-halogen lamp suspended above to provide an infrared filter. The light intensity was 960 μmol quanta m⁻² s⁻¹ at water surface and 100 μmol quanta m⁻² s⁻¹ PAR at 90 cm depth. Mesocosm experiments were carried out after a two-week culture acclimation and growth period.
Samples were removed from the mesocosms by siphoning to prevent physical damage to the cells from the surface depths (0-5 cm) and middle depths (100 cm), at 0600 (lights on), 1200, and 1800 (prior to lights off). The experiment was conducted from 27-29 October 2003. Volume removed from the mesocosms was calculated based on cell concentrations at the surface and middle depths. This volume was replaced after each sampling period with the equivalent nutrient rich media or nitrate reduced media along the bottom of the mesocosms using autoclaved Tygon tubing. Dissolved oxygen and temperature measurements were profiled with a sterilized Yellow Springs Instrument (YSI) handheld meter prior to and after the addition of culture.

**Biochemistry and Physiology**

All biochemical measurements were normalized to a per cell basis. Cell counts were determined using a Coulter Multisizer II Particle Analyzer (Beckman-Coulter Inc., Miami, FL) with threshold settings of 13.3 and 30 µm with a 200 µm orifice. Triplicate samples were collected in 20 ml scintillation vials for counts and diameter determinations.

A Walz Water Pulsed Amplitude Modulated Chlorophyll Fluorometer (PAM-FL) was auto-zeroed with 0.70 µm filtered cell media (Cullen and Davis 2003) to measure photosynthetic yield (Fv/Fm) in triplicate.

Carbon and nitrogen samples were filtered on ashed GF/F filters and stored in annealed glass scintillation vials at -20 °C freezer until analysis. All ashed filters and annealed glass were wrapped in aluminum foil and baked for 6 hrs at 510 °C. The day prior to analysis, all filters were freeze dried for 12 hrs in a Labconco Freeze dry system (-50 °C, 37 x 10^{-3} mbar). Filters were weighed and then combusted in a Sediments
Analyzer Flash 1112 EA Series with an acetonilide (C₈H₉NO) standard from Costech Analytical Technologies, Inc.

Cells were filtered for pigment identification on 25 cm GF/F filters, folded in half, purged with N₂ gas, placed in a cryo-vial and stored in liquid nitrogen until analysis. Pigments were analyzed with reverse-phase Shimadzu LC-10AT high performance liquid chromatography (HPLC) (Wright et al. 1991) at Mote Marine Laboratories (Gary Kirkpatrick).

Lipid samples were collected on pre-combusted 55 cm GF/F filters, folded in half, purged with N₂ gas, placed in a cryo-vial and stored in liquid nitrogen until analysis. Lipids were extracted, evaporated and analyzed as described in Schaeffer (2005). Lipid classes were identified with a 5 step separation technique on the Chromarods (Striby et al. 1999). Only lipid data for the first day was presented due to loss of temperature control in the lab during analysis for part of day 2 and all of day 3 samples.

Fifty ml of cells were filtered on pre-combusted Whatman GF/F glass fiber filters and rinsed with artificial seawater (ASW). Boiling, distilled, deionized water was passed through the same filter and collected in an acid washed nutrient bottle. Internal nitrate was measured with a Lachat QuikChem 8000 Continuum Series Autoanalyzer using methods designed by Lachat Instruments for water samples (Thoresen et al. 1982). Free amino acids (FAA) were measured with an adapted technique from (Clayton et al. 1988) based on (Lowry et al. 1951). Cells for free amino acid analysis were collected in 50ml Nalgene centrifuge vials, centrifuged at 3200 rpm for 10 min and frozen until later analysis. The frozen pellet was osmotically shocked with 0.5 ml of 0.2 μm filtered distilled water. Samples were then diluted with 0.5 ml of deionized water which 0.1 ml
of sodium deoxycholate solution and 0.1 ml of trichloroacetic acid, from a Sigma Diagnostics protein assay kit (Procedure No. P 5656), were added to induce precipitation. After centrifugation, the 50 µl of the supernatant was saved and 1.75 ml of deionized water, 1.20 ml of 0.2 M borate buffer (pH 9.0) and 0.5 ml of 0.5 mM fluorescamine reagent was added and mixed. Fluorescence at 390 nm excitation and 475 nm emission was measured after 30 sec. FAA concentration was determined with a DL-glutamic acid standard.

Cells were collected on pre-combusted GF/F filters for particulate phase toxin analysis. Filtrate was collected in 10% HCl washed glass vials for dissolved toxins. Brevetoxins Btx-1, Btx-2, Btx-3 and Btx-5 were analyzed by Dr. Damian Shea at North Carolina State University with electrospray ionization tandem mass spectrometry (Hua and Cole 2000).

Analysis

The data were plotted and analyzed using Microsoft Excel, Sigma Plot, Sigma Stat and SAS. Error bars are reported as 1 standard deviation.

RESULTS

Replete mesocosm

The concentration of NO₃ was approximately 58 µM and the concentration of PO₄ was approximately 2 µM in the nutrient replete mesocosm (Fig. 3.1). Replete surface cell counts reached a maximum at 12, 36 and 60 hrs or mid-day for each three days of the experiment (Fig. 3.2A). Maximums were between 60% and 91% larger then counts taken at surface prior to lights on and late afternoon. Middle cell counts decreased 50% each of the three days from the morning sample to the late afternoon. Surface diameters
remained lower than middle diameters throughout the experiment (Fig. 3.2B) while middle cell diameters increased from 6 hrs to 18 hrs. Surface diameter decreased by the end of day one at 18 hrs and showed no significant net change on day 2 (p=0.267) and day 3 (p=0.128). Internal cellular carbon (Fig. 3.2C) and internal cellular nitrogen (Fig 3.2D) had identical patterns for both surface and middle depths throughout all three days of the experiment. Day 2 surface and middle diverged from 30 hrs to 42 hrs. Surface carbon (p=0.001) and surface nitrogen (p<0.001) significantly decrease, while middle carbon (p<0.001) and middle nitrogen (p=0.005) significantly increase. Surface total lipid increased from 6 hrs to 12 hrs but finished the day 52% lower (Fig 3.2E). Middle total lipid increased 45% from 6hrs to 18 hrs. Dissolved oxygen in the replete mesocosm increased 20% on day 1, 8% on day 2 and 30% on day 3 for surface and 26% each day for middle (Fig. 3.2F). Middle dissolved oxygen was consistently higher than surface all three days. Each morning the dissolved oxygen concentration returned to its approximate beginning concentration from the previous day for both surface and middle. Surface yield decreased for all three days of the experiment (Fig 3.2G): 32%, 42%, and 48%. Middle yield declined 15% on day 1, exhibited no change on day 2, and decreased 40% on day 3. Each morning the yield for surface and middle began close to the ending yield from the previous day showing little recovery overnight.

Total lipid was placed at the top of Figure 3.3 for reference. Replete surface (p=0.08) and middle (p=0.239) triacylglycerol concentrations did not significantly change from 6 hrs to 18 hrs (Fig. 3.3A). Fatty acid surface and middle concentrations inverted at 18 hrs. Surface fatty acid decreased 51% and middle fatty acids increased 158% (Fig. 3.3B). MG concentrations did not significantly change for surface and middle from 6
hrs to 18 hrs but middle concentration was almost double surface (Fig 3.3C). MGDG surface did not significantly change at surface (p=0.922) or middle (p=0.668) from 6 hrs to 12 hrs. However, at 18 hrs the concentrations inverted and surface decreased 65% (p=0.042) while middle increased 81% (p=0.334) (Fig. 3.3D).

Chlorophyll \(a\) and gyroxanthin had identical patterns throughout all three days of the experiment (Fig. 3.4A, 3.5B) as did diatoxanthin and diadinoxanthin (Fig. 3.4C, 3.4D). All four reported pigments showed large variability at surface and middle throughout the experiment. All four pigments at the surface increased from 6 to 12 hrs and significantly decreased at 18 hrs for chlorophyll \(a\) (p<0.001), gyroxanthin (p<0.001), diatoxanthin (p<0.001) and diadinoxanthin (p<0.001). Hours 30 to 42 had no significant change in concentration for surface chlorophyll \(a\) (p<0.684), gyroxanthin (p<0.580), diatoxanthin (p<0.320) and diadinoxanthin (p<0.436). Surface chlorophyll \(a\), gyroxanthin, diatoxanthin and diadinoxanthin increased from 54 to 60 hrs but there was a significant decrease at 66 hrs. Over 3 12 hr periods middle chlorophyll \(a\) (p<0.001), gyroxanthin (p<0.001), diatoxanthin (p<0.001) and diadinoxanthin (p=0.001) increased significantly.

Day 2 replete mesocosm brevetoxins 1, 2, 3 and 5 increased from 30 hrs to 42 hrs for surface and middle (Fig. 3.5A, 3.5B, 3.5C and 3.5D). Surface began the day with higher concentrations of brevetoxins 1, 2 and 3. However, middle brevetoxin 2 and 3 increased to the same concentration as surface while brevetoxin 1 increased throughout the day but remained less than surface. Brevetoxin 5 surface and middle had identical concentrations throughout the day.
Replete surface free amino acid (FAA) ended day 1 without change, day 2 with a 68% increase and day 3 with a 46% decrease. Replete middle FAA increased throughout all three days and where higher than surface FAA (Fig 3.6A). Surface internal nitrate ended day 1 without change, day 2 with a significant increase (p<0.001) at 42 hrs and a significant decrease (p=0.023) at 60 hrs with no change at 66 hrs (p=0.85). Middle internal nitrate also increased day 1 (p<0.001) and no significant change day 2 (p=0.085) or day 3 (p=0.838) (Fig. 3.6B).

**Reduced mesocosm**

The concentration of NO\textsubscript{3} was approximately 2.20 μM and the concentration of PO\textsubscript{4} was approximately 2.0 μM in the reduced mesocosm (Fig. 3.7). Surface cell count maximums occurred at 12 hrs and 60 hrs both at mid-day (Fig. 3.8A). Maximums were 67% and 70% larger than counts taken 6 hrs previously. Middle cell counts decreased 60%, 73% and 85% each of the three days respectively from the morning sample to the late afternoon. Surface and middle diameters increased throughout day 1 as did middle on day 2. Unexpectedly, surface diameter decreased 11% on day 2. Day 3 showed no significant change at surface (p=0.14) and middle (p=0.188). Internal cellular carbon and nitrogen followed similar patterns between surface and middle except during day 3 surface and middle diverge (Fig. 3.8C and 3.8D). Total lipid increased more than 2-fold in surface and more than 10-fold in middle during day 1 (Fig. 3.8E). Surface dissolved oxygen was continuously lower than middle for all three days (Fig. 3.8F). Yield lacked recovery at middle but was higher than surface all three days. Surface declined the greatest throughout the day but recovered 65% by the next morning (Fig 3.8G). Compared to the replete mesocosm, the reduced surface and middle had the greatest
decline in yield with an average decrease of 80% at surface and a decrease of 20%, 30% and 53% over the three respective days for middle.

Total lipid concentration was placed at the top of Figure 3.9 for reference. Surface triacylglycerol did not change significantly ($p=0.249$) from 6 hrs to 18 hrs (Fig. 3.9A). Middle triacylglycerol significantly ($p=0.003$) increased 10 fold from 6 hrs to 18 hrs. Surface fatty acid increased 60% at 12 hrs ($p=0.023$) with a decrease ($p=0.009$) at 18 hrs (Fig. 3.9B). Middle fatty acid increased 23% at 12 hrs ($p=0.02$) with a decrease ($p=0.048$) at 18 hrs. MG followed the identical trend of fatty acid with no significant difference between surface and middle (Fig. 3.9C). Surface and middle MGDG had no significant difference but significantly increased three fold ($p=0.037$) from 12 hrs to 18 hrs (Fig. 3.9D).

Chlorophyll $a$ and gyroxanthin had identical patterns throughout all three days of the experiment (Fig. 3.10 A and 3.9B) as did diatoxanthin and diadinoxanthin (Fig. 3.10C and 3.9D). All four reported pigments showed large variability at surface and middle throughout the experiment. Day 1 ended with no significant net change in any of the four pigments at surface or middle. Day 2 all four pigments increased throughout the day and finished at the same concentration as Day 1. Day 3 there was no significant change among all four pigments in middle with a significant increase among all four pigments in surface at 66 hrs.

Brevetoxins 1, 3 and 5 showed no significant net change in concentration throughout day 2 for both surface and middle (Fig. 3.11A, 3.11C and 3.11D). Brevetoxin 2 increased 60% for surface and middle throughout day 2 from 30 hrs to 42 hrs (Fig. 3.11B). Reduced mesocosm brevetoxin 2, 3 and 5 were not significantly different
between surface and middle. Surface brevetoxin 1 was greater than middle brevetoxin 1 throughout the day.

Surface and middle FAA ended day 1 and day 2 with no significant net change (Fig. 3.12A). The end of day 3 surface and middle FAA increased 52% and 75% respectively at 66 hrs. Except for day 2 when surface more than doubled (p=0.001) (Fig. 3.12B), surface and middle internal nitrate had no other significant net change all three days of the experiment.

DISCUSSION

Initial examination of the results for both the replete and reduced mesocosms looked random. Cell behavior in the mesocosms contributed to day-to-day constituent variation. Cell division was phased in both mesocosms. Therefore to explain the results, let’s assume that a conglomeration of young, old and cells with ages in between were behaving based on their biochemical constituent requirements. Cell division rate was essentially 0.0 day\(^{-1}\) on day 1 and day 2 in the reduced mesocosm corresponding to a lower supply of nitrogen. The replete mesocosm cell division rate was averaged approximately 0.25 day\(^{-1}\). The oldest cell in the replete mesocosm was between 3-4 days where the oldest cell in the reduced mesocosm was greater than 3 days. Surface aggregations only occurred on day 1 and day 3 in the reduced mesocosm, while surface aggregations formed all three days in the replete mesocosm (Fig 3.13A). The following conditions were evidence of slower cell division in the reduced mesocosm. Reduced mesocosm cells had larger diameters at surface (Fig. 3.13B) and middle depths (Fig. 3.14B). Internal cellular carbon and internal cellular nitrogen were generally greater in the reduced mesocosm for surface (Fig. 3.13C and D) and middle depths (Fig. 3.14C and
D). Total lipid cell$^{-1}$ also increased to a greater extent in the *reduced* mesocosm at surface (Fig. 3.13E) and middle depths (Fig. 3.14E). Increased cell diameter and the trends in the above biochemical constituents indicated *reduced* mesocosm cells were storing reserves for division until nitrogen became more readily available. *Replete* mesocosm cells could utilize their biochemical reserves for division because there was no nitrogen limitation. Slower phased cell division may have resulted in the *reduced* mesocosm because the available nitrogen was less than that required. This explained why no surface aggregate was observed on day 2. Almost none of the population divided over night between day 1 and day 2.

*Reduced* mesocosm cells had increased concentrations of the triacylglycerol storage lipid by the end of day 1 at surface (Fig. 3.15A) and particularly at middle depth (Fig. 3.16A) relative to the *replete* mesocosm. Increased triacylglycerol storage lipid in the *reduced* mesocosm continued to indicate cells were storing biochemical constituents for cell division in anticipation of adequate nitrogen. Increased triacylglycerol has been associated with halted cellular division and cells entering stationary phase of the growth cycle (Hodgson et al. 1991; Dunstan et al. 1993; Fidalgo et al. 1998). Precursors such as fatty acids and MG followed similar trends for both mesocosms at surface (Fig. 3.15B and C) and middle depths (Fig. 3.16B and C). Surface fatty acids and MG exhibited no net change by the end of the day for both mesocosms. These similarities at surface were associated with light stress. Middle depth fatty acids and MG had a net increase by the end of the day indicating reduced light stress.

Surface pigments showed opposite trends on day 1 and 3 at mid-day (Fig. 3.17A-D) while middle pigments had similar patterns (Fig. 3.18A-D). Light was the primary
influence on pigment differences between the *replete* and *reduced* mesocosms. High light at the surface stimulated *reduced* mesocosm cells to decrease their pigments mid-day. An order of magnitude less cells in the *reduced* mesocosm meant they were exposed to more light at the surface because self-shading had less of an impact. *Replete* cell counts were greater and therefore self-shading was pronounced at the surface. *Replete* mesocosm cells increased pigments because there was less relative light at the surface when compared to the *reduced* mesocosm. Middle depth pigments for both the *replete* and *reduced* mesocosms exhibited similar trends because light decreased through attenuation and surface cell shading. The *reduced* mesocosm cells had less chlorophyll *a* (Fig 3.18A) and gyroxanthin (Fig. 3.18B) throughout most of the 3 days relative to the *replete* mesocosm. Although light decreased at middle depth for both mesocosms through attenuation, the surface population played a significant role in shading. Lower cell counts at the *reduced* surface meant more light was reaching the middle depth relative to the *replete* mesocosm.

Phytoplankton use nitrogen in numerous constituents such as amino acids, protein, RNA and pigments. These constituents decrease as nitrogen becomes limiting. When nitrogen was available, cells manufactured required constituents for division such as free amino acids (Dortch et al. 1984). Evidence of lower nitrogen resources were supported by low free amino acids pools and generally low internal nitrate pools in *reduced* mesocosm cells when compared to *replete* mesocosm cells (Fig. 3.19A and B, 3.20A and B). The *reduced* mesocosm cells only had 5 to 10 pmol cell\(^{-1}\) less internal nitrate than *replete* mesocosm cells at middle depth. However, day 2, *reduced* surface cells had more internal nitrate than *replete* surface cells, which indicated the *reduced*
mesocosm was not depleted in nitrogen. Cells could continue to store nitrogen in the form of internal nitrate until enough required nitrogen was available for cell division. The division rate in the reduced mesocosm was considerably lower. Lack of nitrogen for the production of amino acid building blocks lowered cell division. The cells apparently decide not to use their internal pools, though adequate, to produce amino acids based on the lack of sufficient external nitrate. Higher internal cellular carbon and internal cellular nitrogen pools also indicated cells in this reduced mesocosm were storing resources until nitrogen became available for future cell division. Reduced mesocosm nitrogen did not limit uptake, but it did limit growth (Morel 1987).

Greater similarities occurred at middle depths than at the surface for both the replete and reduced cellular biochemical constituents because older cells swam toward the lower depth in the mesocosms. Older cells fulfilled their biochemical requirements and were prepared for cell division. Younger cells were at the surface with a variety of different biochemical requirements from previous cell division. The reduced mesocosm cells lacked the readily available nitrogen supply that existed in the replete mesocosm. Younger cells in the reduced mesocosm compensated for the lower nitrogen supply by building reserves until a threshold of nitrogen was passed.

Gyroxanthin was used as an additional verification that samples were pure K. brevis. This pigment had only been reported for two additional toxic dinoflagellates Gyrodinium aureolum and Gymnodinium galatheanum (Millie et al 1995). The linear relationship of gyroxanthin to chlorophyll a was unique to K. brevis for the replete mesocosm (r=0.72, slope=0.028) (Fig. 3.21A) and the reduced mesocosm (r=0.85,
slope=0.034) (Fig. 3.21B). The gyroxanthin – chlorophyll $a$ ratio was not altered by nitrogen concentration or high light intensity.

The epoxidation state (EPS), defined as the ratio of $[\text{diatoxanthin}] / [\text{diadinoxanthin} + \text{diatoxanthin}]$, increased to levels above 0.2 for the replete mesocosm (Fig. 3.22A) and the reduced mesocosm (Fig. 3.22B). The replete mesocosm surface EPS increased an average 80% over the three days of the experiment. At the beginning of each day, replete EPS was fully recovered to below 0.1. Replete middle EPS also increased an average of 50% over the three days. Complete recovery at replete middle also occurred each morning. Reduced surface EPS increased 84%, 53% and 30% respectively over the three days. Reduced surface EPS reached a maximum of approximately 0.65 each day; however, there was minimal reduced EPS recovery each morning. Reduced surface recovered to the level of reduced middle EPS from the previous day’s 1800 sample. Reduced middle EPS increased 60%, 19% and 25%, respectively, over the three days. Reduced middle EPS continued to increase each day with a lack of recovery over night. In the field, EPS increased as sunlight increased throughout the morning. EPS peaked with maximal sunlight mid-day and recovered in the evening when sunlight decreased (Evens et al. 2001). Figure 3.23 provides a theoretical representation of relative EPS during relative sunlight exposure on a cloudless day from sunrise at 0600 to sunset at 1800. Laboratory mesocosms did not have changing light gradients throughout the day. A maximal light intensity was reached in this laboratory experiment within 15 minutes after the lights turned on and remained at a maximum until the lights turned off at 1800. Although these experimental conditions did
not exactly simulate field light conditions they provided information about the cell’s capacity to handle high light and to recover.

Nitrate reduction played a crucial role in the lack of EPS recovery. The influential role of nitrate on EPS is better understood when photosynthetic yield also is considered. Yield was inversely proportional to EPS ($r=0.75$, slope=0.6, $p<0.001$) (Fig. 3.24). Cells under high light such as surface moved along the slope to the right toward a high EPS and low yield. Cells under decreased light such as middle moved along the slope toward the left with higher yields and lower EPS. Excess energy was greater in surface under high light as indicated by a lower Fv/Fm$^{-1}$. Increased EPS led to increased thermal dissipation of excitation energy under high light to lower the efficiency with which excitation energy was transferred to PSII reaction centers. The reduced mesocosm lacked EPS recovery to reduce photosynthetic efficiency because nitrogen was less available to produce required nitrogen containing constituents. Once nitrogen did become available, the cell’s EPS would recover and efficiency would return increasing carbon fixation and incorporation of nitrogen requiring constituents.

Internal cellular carbon and nitrogen decreased with increased cell count for the replete mesocosm (Fig. 3.25A and B; note x-axis change) and the reduced mesocosm (Fig. 3.26A and B; note x-axis change). The relationship between internal cellular carbon and cell count is described with an inverse first order $y = y_o + a/x$. All three replete mesocosm maximum cell counts are located toward the right end of these curves and contain the lowest internal carbon and nitrogen per cell. There is a clear separation between the replete surface and middle internal cellular carbon along the fit ($r=0.79$, $p<0.0001$). This separation also occurs with the replete surface and middle internal
cellular nitrogen fit \( r=0.82, \ p<0.0001 \). Reduced surface and middle cells show intermixed internal cellular carbon along the fit \( r=0.79, \ p=0.002 \). Internal cellular nitrogen show the same intermixed trend for the reduced mesocosm along the fit \( r=0.70, \ p=0.0023 \). Young replete cells migrate toward the surface to acquire carbon through photosynthesis. Once carbon thresholds are passed they returned to middle. This replete high light mesocosm followed a similar migratory behavior as described for a previous replete intermediate light experiment (Schaeffer 2006). In the reduced mesocosm, no young cells were motivated by carbon limitation to migrate toward the surface. This intermixing between surface and middle provided further evidence that cells were older and not dividing frequently due to lack of required biochemical constituents from nitrogen reduction. In a nitrogen reduced mesocosm, the cell had no added benefit with increased light since it would be required to reduce photosynthetic efficiency and combat possible oxidative stress.

A *K. brevis* bloom advected along the West Florida shelf from off-shore to on-shore may encounter conditions similar to the reduced mesocosm first and then conditions similar to the replete mesocosm (Fig. 3.27). Dark circles represented older cells that had fulfilled their biochemical requirements and primarily used geotaxis during vertical migration. Open circles were young cells with unequal partial fulfillment of their biochemical requirements from unequal daughter cell division. Young cells primarily used phototaxis during vertical migration. Equal daughter cell division was not a realistic assumption because data from this experiment and Schaeffer (2006) indicated a population that achieved quantized divisions (all cells divided together) (Kamykowski et al. 1998) with equal daughter cells would have all of the newly divided young cells
vertically migrate to the surface. In the Kamykowski et al (1998) experiment, the *K. brevis* population was quantized but only part of the population vertically migrated toward the surface while a proportion of the population remained at middle depth. If daughter cell division occurred unequally, newly divided young cells with less biochemical requirements would use phototaxis toward the surface and young cells with a larger fill of biochemical requirements could have remained at middle depth. Therefore, we assume *K. brevis* has unequal daughter cell division.

Off-shore young cells may not vertically migrate completely to the surface where light intensity is the greatest. These cells may remain sub-surface because of a lower threshold due to decreased photosynthetic efficiency to remove excess energy. Exposure to lower nitrogen concentrations lowers the need for photosynthetic production because essential building blocks containing nitrogen can not be synthesized. Lack of nitrogen containing building blocks leads *K. brevis* to divide at a lower rate (one division indicated by red and black dashed arrows) or slow the division rate and store reserves until nitrogen becomes available (solid black arrow). Once *K. brevis* is exposed to nitrogen replete conditions essential building blocks containing nitrogen can be synthesized and cell division can resume (red and black dashed arrows above the replete section). The threshold level increases toward higher light so young cells can migrate toward the surface. The cells fill of biochemical requirements influences migration rate toward the surface. Those cells with less biochemical requirements are highly motivated to migrate to the surface (red dashed arrows) while cells with more biochemical requirements are less motivated to migrate to the surface (red dashed arrows). Throughout the vertical
migration *K. brevis* is also capable of adjusting its lipid classes and pigments to sustain itself under changing light conditions.

When *replete* mesocosm (Fig. 3.28A and B) and *reduced* mesocosm (Fig. 3.29A and B) lipid class concentrations were averaged at surface and middle over day 1 of the experiment, the *replete* variability for surface was S.D. +/- 16.2 and middle was S.D. +/- 12.26. The *reduced* variability for surface was S.D. +/- 10.05 and middle was S.D. +/- 24.26. Triacylglycerol and MGDG were the two largest classes of the lipids measured in both mesocosms. *Replete* surface triacylglycerol was 9.6% and MGDG was 77% while middle triacylglycerol was 22% and middle MGDG was 61%. *Reduced* surface triacylglycerol was 17% and MGDG was 50% while middle triacylglycerol was 47% and middle MGDG was 38%. *Replete* fatty acid and MG changed little from surface and middle with 1% and 0.2%, respectively. *Reduced* fatty acid and MG also changed little from surface and middle with 2% and 0.5% respectively. *Replete* phospholipids such as PG, PE and PC changed from surface to middle 3.3-7.2%, 9-1.3% and 0.9-1.3% respectively. *Reduced* phospholipids changed from surface to middle 11-4.6%, 9-3% and 3-2.3%. Finally, *replete* mesocosm hydrocarbons represented 2.8% at surface and 4.4% at middle. *Reduced* mesocosm hydrocarbons were 7% at surface and 2.5% at middle. The greatest changes in concentration occurred in the two largest classes, triacylglycerol and MGDG for both mesocosms. *Replete* surface triacylglycerol was 22 pg cell\(^{-1}\) and surface MGDG was 190 pg cell\(^{-1}\). *Replete* middle more than doubled the triacylglycerol concentration to 46.2 pg cell\(^{-1}\) when compared to the surface. *Reduced* surface triacylglycerol was 26 pg cell\(^{-1}\) and surface MGDG was 80 pg cell\(^{-1}\). *Reduced* middle nearly quadrupled the concentration of triacylglycerol at 99 pg cell\(^{-1}\) and increased its
MGDG to 106 pg cell$^{-1}$. The replete surface MGDG was a much larger fraction than expected. Surface MGDG concentration decreased more than half, while middle MGDG concentration doubled by the end of day 1. Like numerous other species of algae, lower light increased the structural lipid MGDG (Klyachko-Gurvich et al. 1999; Khotimchenko and Yakovleva 2005). Decreased amounts of triacylglycerol at the surface agreed with the low internal cellular carbon. This suggested newly divided cells migrated toward the surface for photosynthesis of carbon products such as triacylglycerol. Reduced surface and middle triacylglycerol were nearly double that of replete surface and middle as a percentage of total lipid. Triacylglycerols are synthesized when energy input exceeds the cell’s capability to use that energy for production of constituents that require nitrogen. Production of triacylglycerol also requires utilization of ATP and NADPH which help reduce damage to the photosystems. Triacylglycerols can be used as a form of energy and carbon for basic metabolic processes until nitrogen became available. It may also indicate cessation of cell growth during nitrogen limitation (Roessler 1990). These results suggested middle populations of $K. brevis$ were capable of increasing triacylglycerol for storage which could be used for metabolism at a later time when a nitrogen source became available (Kamykowski et al. 1998; Kamykowski et al. 1999).

MGDG and its association with the pigment-protein complex was indicated by its linear relationship with chlorophyll $a$ ($r=0.52$, slope=1.1, $p=0.002$) (Fig. 3.30A). However no significant relationship was found between the intermediate MG and chlorophyll $a$ ($r=0.3$, slope=-0.003, $p=0.375$) (Fig. 3.30B), between MG and the end product MGDG ($r=0.46$, slope=-0.002, $p=0.155$) (Fig. 3.30C) and between fatty acid and MGDG ($r=0.1$, slope=0.002, $p=0.046$) (Fig. 3.30D). The relationships for MG to
chlorophyll \(\alpha\), MG to MGDG and fatty acid to MGDG were not meant to indicate the precursor MG or fatty acid was only used for the MGDG final product. Instead, the relationship was used as an indicator whether enough precursor fatty acid or MG was available for biosynthesis into end products such as MGDG. The lack of a positive relationship between precursors and end products indicated cells were light stressed in the replete and reduced mesocosms, unlike exposure to low and intermediate light (Schaeffer 2006). At low and intermediate light cells were capable of increasing precursor concentration along with increasing end product concentration. \(K.\ brevis\) exhibited biochemical stress under this high light experiment. Cells were not capable of increasing precursor concentration while increasing end product concentrations. As precursor concentration increased end product concentrations decreased. This indicated end product constituents such as MGDG were broken down to supply basic precursors such as fatty acids and MG for other essential functions. This laboratory experiment pushed cultures toward 1000 \(\mu\text{mol quanta m}^{-2}\ \text{s}^{-1}\) or saturated light which was unique for laboratory studies (Wolfe-Simon et al. 2005). The unique high light conditions presented in this experiment was critical in understanding how field populations responded since most marine carbon fixation occurs in high light (Wolfe-Simon et al. 2005). Supply of energy from photosynthesis and the capability of the cell to deal with this energy determines the cellular biochemical composition (Smith et al. 1989). The supply of energy for photosynthesis in this experiment was high and the capability of the cell to deal with higher light was stretched to the point where \(K.\ brevis\) was required to degrade end products to generate essential precursors to continue vital processes.
Total lipid and individual lipid classes had similar responses to saturating light. Lipid concentration increased to a maximum toward an optimal EPS between 0.3 and 0.6. Lipid concentration decreased after the optimal EPS was surpassed. Total lipid (Fig. 3.31A) \((r=0.64)\) high EPS was 0.73 and low EPS was -0.0013. Triacylglycerol (Fig. 3.31B) \((r=0.66)\) high EPS was 0.69 and low EPS was 0.07. Fatty acid (Fig. 3.31C) \((r=0.51)\) high EPS was 0.80 and low EPS was -0.09. MG (Fig. 3.31D) \((r=0.77)\) high EPS was 0.95 and low EPS was 0.07. MGDG (Fig. 3.31E) \((r=0.79)\) high EPS was 0.72 and low EPS was 0.03. Sterol (Fig. 3.31F) \((r=0.52)\) high EPS was 0.79 and low EPS was 0.08. A negative EPS was not possible so negative values for total lipid, triacylglycerol and fatty acid should be considered zero. Saturating light is difficult to achieve in laboratory cultures. Issues such as voltage supply and heat production limited the total light intensity during experiments. The ability to stress cells and have higher EPS is rare. This is indicated by a lack of points with an EPS of 0.5 or higher. Continued sampling will provide more points and a better fit. Lipid end products, such as the primary lipid storage product triacylglycerol and structural lipid MGDG, had an upper EPS range of 0.7. Whereas precursor lipid classes such as fatty acid and MG had broader ranges of EPS. Precursors stretched the upper range beyond 0.7 that was the upper range for triacylglycerol and MGDG. This indicates that precursors during saturating light conditions are from the degradation of existing cell structure (Wolfe-Simon et al. 2005).

Theoretical lipid concentrations are plotted against time of day from the results in Figure 3.23 (Fig. 3.32). At 0600, sunrise low light exposure cells respond with low EPS and low lipid concentration (1). As mid-morning light intensity increases, cells exhibit an intermediate EPS while the capacity to fix carbon and produce lipids increases (2). At
1200 light intensity is the highest as is EPS. At maximum EPS, cells are light saturated which degrades existing cell structure and decreases lipid concentrations (3). Mid-afternoon light intensity decreases and cell EPS decreases. Cells are able to recover lipid concentrations but not necessarily to mid-morning levels after the saturating light conditions are removed (4). Finally, just before sunset light intensity is minimal and cells respond with low EPS and low lipid concentrations (5).

Toxins increased with exposure to light and increased EPS (Fig. 3.33). *Replete* mesocosm toxins were always greater in concentration than *reduced* mesocosm toxins. Toxin production fluctuates with culture growth stage (Baden and Tomas 1987; Sundstroem et al. 1989). Lower toxin production in the *reduced* mesocosm resulted from cells remaining in stationary phase until nitrogen became available for cell division. Meanwhile the *replete* mesocosm was closer to exponential phase with increased toxins remaining elevated while lipid classes such as sterol decreased with increased EPS. This pattern of high EPS and low sterol concentration may be a unique survival strategy for *K. brevis*. Two sterols (24R)-4a-methyl-5a-ergosta-8(14),22-dienol and (24R)-4a-methyl-27-nor-5a-ergosta-8(14),22-dienol are unique to *K. brevis* (Leblond and Chapman 2002; Giner et al. 2003). One hypothesized function was protection from invertebrate predators. Invertebrates lack the capability to convert these sterols to a nutritional source (Giner et al. 2003). Another suggested function was these sterols made *K. brevis* immune to the membrane disrupting effects of their brevetoxins (Place and Deeds 2003). Iatroscan measurements were not capable of identifying the two unique sterols, however, they should be included in the sterol class measurement. When the cells were exposed to high light at the surface sterol concentrations decreased while toxins increased. *K. brevis*
may take advantage of its toxicity by reducing sterol concentrations and releasing toxins into the surrounding environment limiting competition.

**Conclusions**

Trends presented from this experiment have significant impacts in understanding the ecology of *K. brevis* because light levels were closer to field observations. Young cells vertically migrated toward the surface for carbon fixation (Schaeffer 2006) after division even though this meant *reduced* photosynthetic efficiency. This trend seemed counterproductive since *K. brevis* decreased lipid concentrations and photosynthetic efficiency. However, this may have been dinoflagellate survival of the fittest. Competition for space and time at the surface may have induced cells to expose themselves to saturating light. Exposure to the high light may have initially resulted in the reduction of biochemical constituents, but the average time a cell spent in this location may be insignificant compared to the reward of obtaining an optimal position for carbon fixation later.

Lower nitrate forced *K. brevis* to limit EPS recovery and lower photosynthetic efficiency until nitrogen became available for required N-containing constituents. This limited *K. brevis* in its use of the surface for higher light and carbon fixation. *Reduced* photosynthetic efficiency combined with lack of required constituents for replication decreased the overall rate of cell division. These trends suggested at least one reason why *K. brevis* migrates along the benthos. When nitrogen was limiting near the surface where high light occurs, lower light allowed *K. brevis* to maintain decreased EPS levels and minimize exposure to oxidative stress. Additionally, *K. brevis* can utilize nitrogen sources supplied from benthic pore-water flow in permeable sediments (Huettel and
Webster 2001) for N-containing constituents to support growth and reproduction (Sinclair 2005). The reduced mesocosm may better represent field populations because nitrogen sources are typically undetectable in the field (Lester et al. 2000).

High light caused *K. brevis* to increase EPS and toxin concentrations while decreasing its lipid concentrations, specifically the sterol class. These trends indicated that *K. brevis* was relatively benign from sunrise to mid-morning. From mid-morning until mid-afternoon *K. brevis* had the potential to be highly toxic. As sterol concentrations recovered *K. brevis* may lose some of its potency. The physiological response of *K. brevis* to changing light in the field could have dramatic consequences for people at the beach. Peak time for beach attendance occurred between 1100 and 1500 at Sarasota beaches according to Sarasota County lifeguard supervisor Scott Montgomery (B. Kirkpatrick pers. comm.). Experimental persons involved in brevetoxin aerosolized studies (Backer et al. 2005; Fleming et al. 2005) had suggested the greatest symptoms occurred between 0900 and 1500 (B. Kirkpatrick pers. comm.). These times coincide with the estimated time *K. brevis* would be highly toxic by producing the highest toxin concentrations and reducing sterol concentration. Increased toxin concentrations with the enhanced transport through lower sterol concentrations, combined with breaking waves, onshore winds and peak beach attendance set the stage for human respiratory problems.
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Figure 3.1. Time course of (A) phosphate and (B) nitrate for surface (○) and middle (■) replete mesocosm depths.
Figure 3.2. Time course of (A) cell count, (B) cell diameter, (C) cell carbon, (D) cell nitrogen, (E) total lipid, (F) dissolved oxygen and (G) photosynthetic yield for surface (○) and middle (●) replete mesocosm samples. Error bars are +1 standard deviation.
Figure 3.3. Time course of (A) triacylglycerol, (B) fatty acid, (C) MG and (D) MGDG for surface (○) and middle (●) *replete* mesocosm samples. Error bars are +1 standard deviation.
Figure 3.4. Time course of (A) chlorophyll $a$, (B) gyroxanthin, (C) diatoxanthin and (D) diadinoxanthin for surface (○) and middle (●) replete mesocosm samples. Error bars are $\pm 1$ standard deviation.
Figure 3.5. Time course of (A) toxin 1, (B) toxin 2, (C) toxin 3 and (D) toxin 5 for day 2 surface (○) and middle (■) *repelete* mesocosm samples. Error bars are ±1 standard deviation.
Figure 3.6. Time course of (A) free amino acids and (B) internal nitrate for surface (○) and middle (●) *replete* mesocosm samples. Error bars are +1 standard deviation.
Figure 3.7. Time course of (A) phosphate and (B) nitrate for surface (○) and middle (■) reduced mesocosm depths.
Figure 3.8. Time course of (A) cell count, (B) cell diameter, (C) cell carbon, (D) cell nitrogen, (E) total lipid, (F) dissolved oxygen and (G) photosynthetic yield for surface (○) and middle (■) reduced mesocosm samples. Error bars are +1 standard deviation.
Figure 3.9. Time course of (A) triacylglycerol, (B) fatty acid, (C) MG and (D) MGDG for surface (○) and middle (●) reduced mesocosm samples. Error bars are +1 standard deviation.
Figure 3.10. Time course of (A) chlorophyll $a$, (B) gyroxanthin, (C) diatoxanthin and (D) diadinoxanthin for surface (○) and middle (●) reduced mesocosm samples. Error bars are +1 standard deviation.
Figure 3.11. Time course of (A) toxin 1, (B) toxin 2, (C) toxin 3 and (D) toxin 5 for day 2 surface (○) and middle (●) reduced mesocosm samples. Error bars are +1 standard deviation.
Figure 3.12. Time course of (A) free amino acids and (B) internal nitrate for surface (○) and middle (●) reduced mesocosm samples. Error bars are +1 standard deviation.
Figure 3.13. Time course of (A) cell count, (B) cell diameter, (C) cell carbon, (D) cell nitrogen, (E) total lipid, (F) dissolved oxygen and (G) photosynthetic yield for replete surface (△) and reduced surface (○) mesocosm samples. Error bars are +1 standard deviation.
Figure 3.14. Time course of (A) cell count, (B) cell diameter, (C) cell carbon, (D) cell nitrogen, (E) total lipid, (F) dissolved oxygen and (G) photosynthetic yield for replete middle (△) and reduced middle (●) mesocosm samples. Error bars are +1 standard deviation.
Figure 3.15. Time course of (A) triacylglycerol, (B) fatty acid, (C) MG and (D) MGDG for replete surface (△) and reduced surface (○) mesocosm samples. Error bars are +1 standard deviation.
Figure 3.16. Time course of (A) triacylglycerol, (B) fatty acid, (C) MG and (D) MGDG for replete middle (△) and reduced middle (●) mesocosm samples. Error bars are +1 standard deviation.
Figure 3.17. Time course of (A) chlorophyll $a$, (B) gyroxanthin, (C) diatoxanthin and (D) diadinoxanthin for replete surface ($\triangle$) and reduced surface ($\circ$) mesocosm samples. Error bars are +1 standard deviation.
Figure 3.18. Time course of (A) chlorophyll $a$, (B) gyroxanthin, (C) diatoxanthin and (D) diadinoxanthin for *replete* middle ($\triangle$) and *reduced* middle ($\bullet$) mesocosm samples. Error bars are $\pm 1$ standard deviation.
Figure 3.19. Time course of (A) free amino acids and (B) internal nitrate for *replete* surface (△) and *reduced* surface (○) mesocosm samples. Error bars are +1 standard deviation.
Figure 3.20. Time course of (A) free amino acid and (B) internal nitrate for replete middle (△) and reduced middle (●) mesocosm samples. Error bars are +1 standard deviation.
Figure 3.21. The ratio of gyroxanthin to chlorophyll *a* remained consistent for all samples at surface (○) and middle (●) in the (A) *replete* mesocosm and (B) *reduced* mesocosm which provided an additional indicator the sample was pure *K. brevis*. 
Figure 3.22. Time course of the epoxidation state (EPS = [diatoxanthin] / [diatoxanthin + diadinoxanthin]) of the xanthophyll cycle for surface (○) and middle (●) (A) replete mesocosm and (B) reduced mesocosms.
Figure 3.23. Theoretical response of EPS (●) to relative sunlight (○) on a cloudless day.
Figure 3.24. Linear regressions of Fv/Fm to EPS for *replete* surface (○), *replete* middle (●), *reduced* surface (△) and *reduced* middle (△) mesocosm samples.
Figure 3.25. A comparison of cell carbon to cell count. *Replete* mesocosm (A) internal cellular carbon and (B) internal cellular nitrogen. Surface samples are represented with open circles (○) while middle are represented with closed circles (●).
**Figure 3.26.** A comparison of cell carbon to cell count. *Reduced mesocosm* (A) internal cellular carbon and (B) internal cellular nitrogen. Surface samples are represented with open circles (○) while middle are represented with closed circles (■).
Figure 3.27. Relative schematic of cell division over *reduced* and *replete* conditions as expected in the field. Red dashed arrows indicated highly motivated cells and faster vertical migration. Black dashed arrows indicated less motivated cells and slower vertical migration. Solid black arrow indicated no cell division.
Figure 3.28. An average of measured lipid classes over all times for *replete* surface and middle. Pie fractions are percentage of the total lipid classes measured. The largest changes between surface and middle occur in triacylglycerol.
Figure 3.29. An average of measured lipid classes over all times for reduced surface and middle. Pie fractions are percentage of the total lipid classes measured. The largest changes between surface and middle occur in triacylglycerol.
Figure 3.30. Linear regressions of (A) MGDG to chlorophyll $a$, (B) MG to chlorophyll $a$, (C) MG to MGDG and fatty acid to MGDG for replete (○) and reduced (●) mesocosm samples.
Figure 3.31. Total lipid (A), triacylglycerol (B), fatty acid (C), MG (D), MGDG (E) and Sterol (F) versus EPS for both *replete* and *reduced* mesocosms.
Figure 3.32. Theoretical response of surface lipid (●) to relative sunlight (○) on a cloudless day.
Figure 3.33. Toxin 1 (A), toxin 2 (B), toxin 3 (C) and toxin 5 (D) versus EPS for both replete (○) and reduced (△) mesocosms.