## UV-PHYSIOLOGY OF A DIATOM (*PSEUDO-NITZSCHIA DELICATISSIMA*) ISOLATED FROM THE SARGASSO SEA

by

#### ERIC S. PORTERFIELD

(Under the Direction of Brian Binder)

#### ABSTRACT

Ultraviolet light can be a major source of stress to phytoplankton, especially in the optically clear waters of the oligotrophic gyres. Diatoms are important primary producers in these gyres, yet the responses of diatoms that occur here to UV-radiation have not been characterized. In this study, *Pseudo-nitzschia delicatissima* was isolated from the Sargasso Sea and its response to UV-A and full spectrum UV was monitored over the course of a two week experiment. The diatom showed significant growth rate inhibition and decreased chlorophyll fluorescence in response to UV-A, indicating a loss in photosynthetic reaction center efficiency. The UV-A cultures also showed an increase in optical density indicative of UV-screening pigments. The full spectrum treatment evoked significantly less response than the UV-A treatment. This indicates that UV-A is more damaging than UV-B for this diatom. Overall, the observed responses are consistent with UV-resistance in *Pseudo-nitzschia delicatissima*. INDEX WORDS: Ultraviolet, Sargasso Sea, diatom, growth rate

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## FROM THE SARGASSO SEA

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B.S. College of Charleston, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

## MASTER OF SCIENCE

ATHENS, GEORGIA

2006

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#### ACKNOWLEDGEMENTS

I would like to thank my major professor, Brian Binder, and my committee members, Mary Ann Moran and Bill Miller for their guidance in the production of this work. I would also like to thank Chris Burbage, Brad Blythe, Cedric Fichot, Erin Biers, Kristina Spooner and Loren Jablonowski for material and moral support. Finally, I would like to thank the National Science Foundation for funding this research (Grant No. OCE-0241740 to Brian Binder).

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#### Chapter 1

#### Introduction

Diatoms are important members of the open ocean phytoplankton community. Cell densities in the Sargasso Sea commonly range from 1-3 ml<sup>-1</sup>, but occasionally reach 100 ml<sup>-1</sup> (Riley 1957). Although in comparison to nano- and pico-phytoplankton these are low cell concentrations, diatoms can nevertheless contribute between 15-48% of annual primary production in the Sargasso Sea because of their relatively large size (Brzezinski and Nelson 1995, Nelson and Brzezinski 1997). Furthermore, diatoms may disproportionately contribute to the vertical flux of particulate organic carbon (POC) due to their rapid growth rates, large size, silica frustules (i.e. rapid sinking), and preferential grazing by metazoans (Goldman 1993). Finally, diatoms may influence the nitrogen cycle by regulating their buoyancy to "mine" nitrogen from deep water (Villareal and Lipschultz 1995, Villareal et al. 1999).

The oligotrophic gyres are large ecosystems that contribute significantly to global biogeochemical cycles (Steinberg et al. 2001, Benitez-Nelson et al. 2001, Bates 2001). Because of relatively low primary productivity and distance from terrigenous inputs these gyres are optically clear. This clarity makes the penetration and biological effects of ultraviolet radiation particularly important in these regions (Kirk 1994a). It is surprising, therefore, that while the effects of ultraviolet radiation on diatoms have been studied in the Antarctic, coastal waters, and fresh water, these effects have been largely ignored in the oligotrophic gyres. These gyres should, in fact, be an excellent setting for studying the effects of ultraviolet radiation on primary producers, given their seasonal stratification and optical clarity.

Ultraviolet light is comprised of wavelengths from 200 nm to 400 nm. The UV-C range (200-280 nm) is completely absorbed by the upper atmosphere and is not relevant when considering biological processes on the surface of the earth (Kirk 1994). However, UV-C does generate ozone, which absorbs UV-B radiation (Madronich 1994). Ultraviolet-B radiation (280 nm-315 nm) has important biological effects, primarily related to DNA damage (Karentz et al. 1991, Buma et al. 1995, Buma et al. 1997). Ultraviolet-B radiation can also damage other cellular components through oxidation, either directly or indirectly through the formation of Reactive Oxygen Species (ROS) (Day and Neale 2002). It is UV-B radiation that is most strongly absorbed by ozone, and therefore it is the biological effects of UV-B that have received the greatest attention to date (Day and Neale 2002, Flint et al. 2003). Ultraviolet-A does not damage DNA and is not a major source of oxidative stress (Rijstenbil 2002). However, UV-A is the primary source of UV damage to the photosynthetic components of algae and higher plants, primarily by damaging the light harvesting complex and photosystem proteins (Helbling et al. 1992, Villafane et al. 1995, Neale, Fritz et al. 2001, Neale, Litchman et al. 2001, Zudaire and Roy 2001). Absorption of UV-A radiation by photosynthetic pigment increases the rate of photodegradation of these pigments as well as reaction center proteins (Kirk 1994, Hazzard et al. 1997, Xiong 2001).

Diatoms obviously rely on their pigments for photosynthetic light capture, but pigments also play a role in adapting to ultraviolet stress. Mycosporine-like amino acids (MAA) are well known for their role in shielding cells from UV damage, and are inducible in diatoms (Zudaire and Roy 2001). Carotenoids also play an important role in UV adaptation, primarily by reducing ROS and by quenching excess energy absorbed by chlorophyll (Young and Frank 1996, Goss et al. 1999, Zudaire and Roy 2001). There is a great deal of species-specific variability in the effects of ultraviolet radiation on pigment content, but generally chlorophyll content is reduced and carotenoids are increased under UV-stress (Dohler 1984, Xiong et al. 1999).

Beyond inhibiting photosynthesis, ultraviolet radiation has a range of effects on diatom biochemistry. The nitrogen metabolism of Antarctic and coastal diatoms under UV stress has been thoroughly studied. These diatoms show a decrease in overall nitrogen uptake and a preference for ammonium, along with decreased amino acid production and protein synthesis (, Dohler 1984, Dohler 1985, Dohler and Stolter 1986, Dohler 1992, Dohler 1997, Dohler 1997a). Several studies have shown an effect on lipid content as well. Most diatoms experience a reduction in overall lipid content, especially short-chain poly-unsaturated fatty acids (PUFA) (Skerratt et al. 1998, Hessen et al. 1997). However, this is not always the case, as UV irradiation has also been shown to increase PUFA in some species (Dohler and Biermann 1988). Such species-specific responses to ultraviolet irradiation have been demonstrated repeatedly (Xiong et al. 1999, Dohler 1984, Hessen et al. 1997, Cockell and Rothschild 1999, Kustenko 1990, Molis and Wahl 2004, Forster and Schubert 2001).

Ultraviolet radiation has also been shown to have an effect on diatom morphology and cell cycle. Several studies report an increase in size in irradiated diatoms due to an uncoupling of photosynthesis and mitosis (Higley et al. 2001, Dohler 1984). Ultraviolet light has also been shown to induce sexual reproduction in diatoms, which has been attributed to UV-induced oxidative stress (Rijstenbil et al. 1994, Rijstenbil 2001). In general, the light environment (PAR and UV) is known to affect the timing of sexual reproduction in the diatom life cycle (Chepurnov et al. 2004). Sexual reproduction has in turn been positively correlated with increased sinking rates (hence increased POC flux) (Crawford 1995).

The research presented in this paper addresses the UV-mediated growth rate and physiological responses of an open-ocean diatom. A *Pseudo-nitzschia sp.* was isolated from the Sargasso Sea and incubated under UV radiation treatments on a light/dark cycle for two weeks. Population growth rate, cell size, pigment content, oxidative stress, and superoxide dismutase activity were monitored daily. The responses of these variables to PAR-only, PAR+UV-A, and PAR+UV-A+UV-B were compared. While the studies cited previously were primarily performed using short-term (~4 hour) UV treatments, this experiment will look at the response of this diatom to long-term (16 days) UV irradiation. Short-term incubations have been very illuminating as to the importance of cellular repair mechanisms in mitigating UV damage. This study seeks to determine the ramifications of cellular acclimation on more ecologically relevant time scales.

#### Chapter 2

#### **Materials and Methods**

*The organism.* The diatom used in this study was isolated on 25-May-2005 from a depth of 10 m in the Sargasso Sea ( $35^{\circ}$  9.316'N  $66^{\circ}$  33.834'W), approximately 370 km northwest of Bermuda. Aliquots of niskin bottle-water samples were enriched with individual components of F/2 medium (trace metal solution, nitrogen, phosphorus, vitamins and silica) (Guillard and Ryther 1962, Guillard 1975) in 25 ml test tubes at various concentrations. These tubes were incubated at room temperature under a window on the R/V Oceanus before being transported in an open cooler back to the laboratory in Athens, GA, where they were kept in an incubator (25°C, 14:10 day:night cycle,  $4 \times 10^{15}$ quanta·cm<sup>2</sup>·s<sup>-1</sup> PAR).

These cultures were monitored by epifluorescence microscopy for diatom growth. All of the cultures were initially contaminated with picoeukaryotes and picocyanobacteria. The picoeukaryotes were removed during a 4 day dark treatment. The diatoms survived this treatment, possibly as a result of producing resting auxospores (Chepurnov et al. 2004). The picocyanobacteria were removed with 30  $\mu$ l/ml Provasoli's concentrated antibiotic solution (Sigma). Serial dilutions were performed to achieve unialgal cultures, although the cultures were not assumed to be clonal. In preparation for the experiment, a 2 L culture was grown in F/2 medium under 6×10<sup>15</sup> quanta·cm<sup>2</sup>·s<sup>-1</sup> PAR in constant light for 5 days prior to the beginning of the UV treatments.

*Experimental conditions.* All cultures were exposed to PAR (Sylvania Cool White fluorescent lamps) on a 14:10 light:dark cycle. The maximum scalar PAR irradiance was adjusted with neutral density filters to provide  $6 \times 10^{15}$  quanta·cm<sup>2</sup>·s<sup>-1</sup>. This

total PAR irradiance was chosen to achieve saturation in the growth versus irradiance curve (Fig. 1), while preventing the effects of PAR photoinhibition.

The UV radiation was provided by a bank of four ESU 7% Reptile Lamps mounted above the experimental cultures (Energy Savers Unlimited). These were turned on one at a time in series, starting one hour after the beginning of the light period and adding one lamp every hour. The UV lamps were then turned off one at a time starting 5 hours before the end of the light period. In this way the cultures were exposed to maximum irradiance for 6 hours, with a 1 hour period of PAR-only at the beginning and end of the day.

The experimental UV irradiance was set to match the natural irradiance at 350 nm  $(33 \text{ mW} \cdot \text{m}^{-2} \cdot \text{nm}^{-1})$  calculated for  $45^{\circ}$ N in May (Norwegian Institute for Air Research <u>http://zardoz.nilu.no/~olaeng/fastrt/fastrt.html</u>). Even though the experimental irradiance spectrum has a different shape than the American Society for Testing and Materials



Figure 1 Growth vs. irradiance data. The maximum PAR irradiance chosen for the experiment was  $0.6 \times 10^{16}$  quanta·cm<sup>2</sup>·s<sup>-1</sup> to remove the effects of PAR photoinhibition. Error bars represent one standard deviation. The curve is the fitted hyperbolic tangent function.

standard solar spectrum, the UV:PAR integrated irradiance ratio was approximately the same (Fig. 2).



Figure 2 Irradiance spectra of the cellulose acetate shielded lamps used in the experiment (red) and the American Society for Testing and Materials (ASTM) Terrestrial Reference Spectra for Photovoltaic Performance Evaluation (blue).

The experimental cultures were all shielded with a 7 mil cellulose acetate shield (changed daily) to screen out any UV-C produced by the reptile lamps. The PAR-only control was grown in 500 ml borosilicate glass flasks in a polycarbonate box to screen out all UV. The UV treated cultures were grown in quartz flasks, with or without Mylar D screening, for the UV-A or UV-A+UV-B ("full spectrum") treatments, respectively. The PAR irradiance was 5% lower in the PAR-only control treatment, due to the polycarbonate shielding.

The initial culture volume was 500 ml for the PAR-only control treatments and for two replicates of each of the UV treatments. One additional replicate of each of the UV treatments was established with 2000 ml initial volume, with inocculum volume adjusted to ensure the same initial cell concentration in all treatments. The larger flasks were used to ensure that enough cells were available for the daily assays given the anticipated potential for severe growth rate inhibition in the UV treatments. All of the flasks were diluted daily to a constant optical density with fresh F/2 medium in an attempt to keep the diatoms growing in exponential phase and limit self-shading. Daily dilution ranged from no dilution to 14-fold, in accordance with the cell density and volume of the culture at the time of sampling. Cultures were maintained in this way for 16 days (24-Mar-06 to 9-Apr-06). After this period, the UV lamps were turned off, and the cultures were monitored for a further five days (10-Apr-06 to 14-Apr-06) under the same dilution regime.

*Growth rate.* Samples (1 ml) were taken daily from each flask before the beginning of the light period, before and after dilution. The former were stained with dihydrofluorescein (see below), all were fixed with paraformaldehyde (1% final concentration), and frozen in liquid nitrogen. After at least 4 hours in liquid nitrogen they were counted on a modified EPICS 753 flow cytometer (Coulter Corporation) (Binder et al. 1996). Diatoms were distinguished using forward angle light scatter (FALS, related to cell size) and red (chlorophyll) fluorescence (680 nm band pass filter, 40 nm bandwidth), and cell concentration calculated from the known sample infusion rate. Samples were run on the flow cytometer until at least 1,000 diatoms were enumerated. Using the diluted cell count from the previous day as density at time zero (N<sub>0</sub>), and the undiluted cell count as N<sub>t</sub>, the exponential growth rate was determined by  $\mu(t) = \ln(N_t/N_0)$ .

*Dihydrofluorescein.* Dihydrofluorescein becomes fluorescent when oxidized within cells, and was used as an indicator of ROS production. During the daily sampling, 1 ml from each replicate was stained with 40  $\mu$ M (final concentration) dihydrofluorescein (DHF, Sigma) for 40 minutes at room temperature in the dark. The DHF stocks were 20 mM in DMSO, as per Hempel et al. (1999). Green fluorescence was measured using the flow cytometer (580 nm band pass filter, 50 nm bandwidth) and normalized to the fluorescence of polystyrene beads (1.0  $\mu$ m diameter, Polysciences, Inc., Warrington, PA, USA). Green autofluorescence was measured on unstained, post-dilution samples and subtracted from the DHF stained samples.

*Cell size*. Diatoms were sized using a Coulter Multisizer II with a 100  $\mu$ m aperture. Various electrolytes were tried, with NaCl (40.5 ppt) plus MgSO<sub>4</sub> (13.8 ppt) providing the lowest background noise. For each replicate, 1 ml of culture was diluted with 19 ml of electrolyte to minimize noise. Two ml of each of these diluted samples were counted. However, these measures did not eliminate small particle noise (mostly silica precipitates). As a result, the mode of the size distribution was used as a measure of the central tendency of cell volume in these samples.

*In vivo autofluorescence*. At each time point, 4 ml were taken from each flask and placed in a 13 ml borosilicate tube. The fluorescence was immediately measured in a Turner 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA) equipped with a chlorophyll-a specific filter set (Welschmeyer 1994).

*Optical density.* Throughout this paper the term optical density will be used instead of absorbance to minimize the confusion of absorbance and absorption (Kirk 1994). Optical density of each daily sample was measured at 350 nm (UV screening

pigments), 440 nm (chlorophyll) and 750 nm (cell density) in a 1 cm quartz cuvette using a Shimadzu 1600 UVA dual-beam spectrophotometer (Shimadzu Corp., Kyoto, Japan). Package effect was assumed to be constant because *Pseudo-nitzschia delicatissima* possesses a single large, two-lobed chloroplast. To deal with scattering, preliminary experiments were performed to test the effectiveness of moving the cuvette closer to the collector in the spectrophotometer, as well as the efficacy of turning the cuvette so that the light from the spectrophotometer would pass through the frosted sides (Xiong et al. 1995). Both of these methods indicated that scattering was approximately constant as a proportion of total optical density, so scattering was assumed to be constant in this experiment. However, bacterial counts were not performed for these preliminary experiments, so it is impossible to determine the effects of bacteria on scattering in the data reported here.

Absorbance spectra of extracted pigments were measured on the last day of the irradiation period. One hundred ml from each flask was filtered onto a Whatman 25 mm GF/F, and stored in liquid nitrogen. Frozen samples were thawed, ground with a tissue grinder in cold methanol and the ground filters were then centrifuged at 7,000 ×g for 10 minutes at  $0^{\circ}$ C. The absorbance spectrum of the supernatant was then measured in a 1 cm quartz cuvette, and the optical density of the peaks recorded (Zudaire and Roy 2001).

*Bacteria.* Bacteria could not be completely excluded from the stock cultures before the experiment, but replicate samples for cell counts allowed them to be enumerated over the course of the experiment. For each replicate sample, 1 ml was stained with Hoechst 33341 (0.5  $\mu$ g/ml final concentration). Blue fluorescence (408 nm long pass and 475 nm short pass filters) from the stained DNA was the trigger for cell counts using the EPICS 753 flow cytometer with UV laser excitation (Binder 2000). As with diatoms, at least 1,000 bacteria were counted for each sample.

Superoxide dismutase. Superoxide dismutase activity was determined by the cytochrome *c* method (Flohé and Ötting 1984). Briefly, this assay is based on competition for superoxide radicals between cytochrome *c* reduction on one hand and the superoxide dismutase reaction on the other. Superoxide radicals are generated by a mixture of xanthine oxidase and xanthine. Reduction of cytochrome *c* in the reaction mixture is monitored by the increase in absorbance at 550 nm; the concentration of SOD is proportional to the decrease in the rate of this reduction, relative to the blank. Final concentrations of the reaction mixture were 10 µg/ml xanthine oxidase (Sigma), 0.1 mM xanthine (Sigma), and 10 µM cytochrome *c* (VWR International), all in the extraction buffer (50 mM K<sub>2</sub>HPO<sub>4</sub> + 0.1 mM EDTA, Sigma).

For each replicate at each sampling, 100 ml of culture were filtered onto a 25 mm Whatman GF/F. These were immediately frozen in liquid nitrogen until just before being assayed. Filters were thawed, ground in extraction buffer (7 ml), and centrifuged at 7,000 ×g for 15 minutes at 4°C. Four ml of the supernatant was transferred to a 1 cm cuvette, into which the cytochrome and superoxide generators were then added. The absorbance at 550 nm was recorded initially and after two minutes. Extraction buffer blanks (+generator+cytochrome) were analyzed along with the samples. The ratio between the rate of change in absorbance from the SOD sample and the blank has a linear relationship to SOD concentration.

*Statistics.* The variables presented here were analyzed using nonparametric statistics. Given the small number of replicates for each treatment (3), it was impossible

to directly confirm that the data were normally distributed. Therefore, the daily data for individual treatments and variables were pooled to produced a larger number of pseudoreplicates (63 pseudo-replicates). These were then compared to a normal distribution with the same mean and standard deviation via the Kolmogorov-Smirnov statistic, and in all cases found to be significantly non-normal ( $p \le 0.05$ ). As this could not be ameliorated by transformation, all statistics used here are non-parametric. Daily treatments were compared using the Wilcoxon U test. Data were compared across the entire irradiation period using a Kruskal-Wallis test (a non-parametric analog for twoway ANOVA).

#### Chapter 3

#### Results

*Growth rate.* Cell density for each replicate was measured daily and the data were used to calculate growth rate using the exponential model  $N_t = N_0 e^{\mu^* t}$  (where  $N_t$  and  $N_0$  are cell density at times t and 0, and  $\mu^*$  is the exponential growth rate). This produced data that showed a great deal of day-to-day variability (Fig. 3).



Figure 3 Unprocessed diatom population growth rate medians as calculated using daily cell counts and the exponential growth model. Black circles = PAR-only control Blue squares = UV-A treatment Red triangles = Full spectrum treatment

The relationship between observed growth rate and cell concentration suggests that these cultures were not always in the exponential growth phase; i.e. that growth rate was not independent of population size (Fig. 4). To correct for the effect of cell density variations on growth rate, these data were instead fitted to the logistic model  $\mu^* = \mu(1-$ N/K) where  $\mu^*$  is the observed exponential growth rate,  $\mu$  is the maximum intrinsic growth rate, N is cell density and K is the carrying capacity (Jensen 1975). Carrying capacity was estimated to be  $7.5 \times 10^4$  diatoms/ml from the plot of observed growth rates for the PAR-only control versus the cell density at the beginning of each measurement period (Fig. 4). This approximate method was used when both linear and exponential regressions yielded unreasonable growth rates for the logistic model. The experimental design did not justify the development of a more complicated growth model. The calculated intrinsic growth rate ( $\mu$ ) was then used in all statistical calculations.



Figure 4 Observed exponential growth rate of PAR-only control treatment. Abscissa is the diatom density at the beginning of each measurement period. Ordinate is the observed exponential growth rate. The x-intercept was estimated at 75,000 diatoms/ml for use as the carrying capacity (K) in the logistic growth equation.

Ultraviolet-A radiation resulted in significantly decreased growth rates relative to PAR controls (Kruskal-Wallis  $p = 2 \times 10^{-5}$ , Fig. 5A). The logistic model did produce a few unreasonable growth rates (e.g. >> 5 d<sup>-1</sup>), which were considered outliers but still

included in the statistics. Median growth rates during the irradiation period were 1.27 d<sup>-1</sup> (range 4.09 d<sup>-1</sup>) and 2.01 d<sup>-1</sup> (range 3.47 d<sup>-1</sup> without outliers), for the UV-A and PAR treatments respectively. The inhibition of growth rate can be expressed as the difference between the treatment growth rate and the PAR-only control, normalized to the control ([mean PAR-UVA]/mean PAR). This appeared to be time-dependent: if the irradiation period is separated into two segments (days 0 - 9, and days 10 - 16), growth rate inhibition for the first period ("induction phase") showed a significant positive correlation (p < 0.005) with time, while the second period did not show a significant trend (0.1 > p > 0.05) (Fig. 6).

In the case of the full spectrum treatment, the intrinsic growth rate (median 1.43  $d^{-1}$  range 6.37  $d^{-1}$  without outliers) was also significantly (though not dramatically) lower than the PAR-only control over the course of the irradiation period (Kruskal-Wallis p =  $3 \times 10^{-4}$ ) and significantly higher than the PAR-only control after the irradiation period (Kruskal-Wallis p = 0.007, Fig. 5B). Growth rate inhibition in the full spectrum treatment appeared to show an induction period (though of a shorter duration), without a significant trend afterwards (Fig. 6). The growth rates for the two UV-treatments were not significantly different from each other (Fig. 5C).

Figure 5. Estimated intrinsic growth rate. Median values for PAR (black circles), UV-A (blue squares) and full spectrum (red triangles) treatments. Error bars represent the range of the data (n=3). The vertical dotted line represents the end of the irradiation period. UV-A and full spectrum treatments were significantly different from the PAR control, but not each other.



17

Day



Figure 6 Percent growth rate inhibition, relative to PAR-only control (see text). Blue squares = UV-A Red triangles = Full spectrum. Lines show least squares linear fits over time period indicated.

*Cell size.* Cell volume in the UV-A treatment was not significantly different from the PAR-only control over the course of the irradiation treatment (Kruskal-Wallis p = 0.052, Fig. 7A). The median cell volume in the UV-A treatment was 57  $\mu$ m<sup>3</sup> with a range of 39  $\mu$ m<sup>3</sup>, and a single outlier of 254  $\mu$ m<sup>3</sup>. In contrast, the full spectrum treatment resulted in significantly larger cells (median volume = 65  $\mu$ m<sup>3</sup>, range = 25  $\mu$ m<sup>3</sup>) during the irradiation period, as compared to the PAR (median volume = 60  $\mu$ m<sup>3</sup>, range = 33  $\mu$ m<sup>3</sup>) and UV-A treatments (Kruskal-Wallis p = 5×10<sup>-5</sup>). Cell volumes in the full

spectrum treatment were always larger than or equal to the PAR and UV-A treatments (Fig. 7B, C). Volume did not correlate to growth rate inhibition (Kendall Rank Correlation p > 0.05) (not shown).

*In vivo autofluorescence*. In vivo fluorescence was normalized to optical density at 440 nm as an estimate of fluorescence yield per chlorophyll. Note that no OD440 nm measurements are available for the first four days of the irradiation. The normalized fluorescence was significantly lower in the UV-A treatment than in both the PAR-only control (Kruskal-Wallis  $p = 9 \times 10^{-8}$ , Fig. 8A) and the full spectrum treatment (Kruskal-Wallis  $p = 10^{-5}$ , Fig. 9C). The full spectrum treatment in turn resulted in lower normalized fluorescence than did the PAR-only control (Kruskal-Wallis  $p = 5 \times 10^{-4}$ , Fig. 8B).

*Optical density.* The ratio of optical density at 350 nm and 440 nm is taken as an approximation for the ratio of UV-protective pigments to chlorophyll *a* (Fig. 9) (Xiong 1995). This ratio was significantly higher in the UV-A treatment relative to the PAR-only control and full spectrum treatment (Kruskal-Wallis  $p = 6 \times 10^{-4}$  and 0.003, respectively) (Fig. 9A, C). The latter two were not significantly different (Kruskal-Wallis p = 0.36) (Fig. 9B).

Figure 7 Median cell volumes, as measured by Coulter Multisizer. Error bars represent the range of the data. The vertical dotted line represents the end of the UV-treatment. Blue squares = UV-A Red triangles = Full spectrum Black circles = PAR



Apparent cross section per diatom ( $\mu$ m<sup>2</sup>/diatom) was estimated as absorbance coefficient at 440 nm (1 cm path length) normalized to cell density (diatoms/cm<sup>3</sup>). Considered over the entire irradiation period, cultures in the UV-A treatment had a significantly higher cross section than in the PAR-only control (Kruskal-Wallis p=  $3 \times 10^{-6}$ , Fig. 10A) and the full spectrum treatment (Kruskal-Wallis p=0.003, Fig. 10C). Optical cross section in the full spectrum treatment was significantly larger than in the PAR-only control treatment (Kruskal-Wallis p=0.007, Fig. 10B). The median optical cross sections for the UV-A, full spectrum, and PAR treatments were 53  $\mu$ m<sup>2</sup>/cell (range 125  $\mu$ m<sup>2</sup>/cell), 38  $\mu$ m<sup>2</sup>/diatom (range 51  $\mu$ m<sup>2</sup>/cell) and 29  $\mu$ m<sup>2</sup>/cell (range 132  $\mu$ m<sup>2</sup>/cell), respectively.

Extracted pigments were obtained from each flask on the last day of the irradiance treatment, and optical density of these extracts were measured at selected wavelengths (337 nm, 444 nm, 615 nm and 666 nm, Fig. 11). The PAR-only control cultures exhibited the greatest optical densities at all wavelengths, while the UV-A cultures had the lowest, although the differences between PAR and UV-A were not statistically significant. The full spectrum treatment produced specific optical densities intermediate between the PAR-only and UV-A treatments at all of the selected wavelengths. The OD337 nm/OD444 nm ratio was not significantly different between any two treatments (not shown).

Figure 8 Median in vivo autofluorescence, normalized to optical density at 440 nm. Error bars represent the range of the data. The vertical dotted line indicates the end of the irradiation period. Blue squares = UV-A Red triangles = Full spectrum Black circles = PAR



Figure 9 Median optical density at 350 nm, normalized to OD440 nm. Error bars represent the range of the data. The vertical dotted line indicates the end of the irradiation period. Blue squares = UV-A Red triangles = Full spectrum Black circles = PAR



Figure 10 Median optical cross section at 440 nm ( $\mu$ m<sup>2</sup>), per diatom. Error bars represent the range of the data. The vertical dotted line indicates the end of the irradiation period. Blue squares = UV-A Red triangles = Full spectrum Black circles = PAR



Figure 11 Median optical density of methanol-extracted pigment normalized to diatom concentration. Error bars represent the range of the data. All data are from the last day of the UV irradiation. White = PAR Blue = UV-A Red = Full spectrum



*Bacterial load.* Hoechst-stained bacteria were counted via flow cytometry and normalized to the diatom count. Bacteria per diatom in the UV-A treatment was higher than the PAR-only control (Kruskal-Wallis p=0.001, Fig. 13A) and the full spectrum treatment (Kruskal-Wallis p= 0.004, Fig. 13C). The full spectrum treatment had more bacteria per diatom than the PAR-only control, though not dramatically so (Kruskal-Wallis p=0.01, Fig. 13B). All three treatments showed a large drop in bacteria per diatom during the first day of irradiation. This was significant for the UV-A treatment, but the loss of a replicate sample from both the full spectrum and PAR treatments prevents the determination of significance in these cases (a limitation of the Wilcoxon U statistic). Both UV treatments appeared to show an induction phase (10 days for UV-A, 4 days for full spectrum) and a recovery phase (all subsequent days). Bacteria per diatom significantly correlated to time over these periods (Kendall-Rank correlation p<0.01). The bacterial load appears strikingly similar to the optical cross section (440 nm) for the UV-A treatment. However, the bacterial load did not correlate significantly to optical density at either 440 nm and 750 nm in that treatment, indicating that bacteria are not the decisive component of the optical density measurements. This becomes apparent when bacterial loads are paired with their accompanying optical density (Fig. 12). However, the instrument used in this experiment did not allow the quantification of scatterance of either bacteria or diatoms.



Figure 12. Optical density versus bacterial load for the UV-A treatment. Blue squares = OD440 nm, Red squares = OD750 nm

*Superoxide dismutase*. Relative superoxide dismutase (SOD) concentration did not correlate as strongly to diatom number (or volume) as it did to bacteria number (Kendall Rank Correlation, table 1). When superoxide dismutase concentration was normalized to bacteria concentration, the UV-A treatment produced significantly less SOD than both the PAR-only control and the full spectrum treatments, while the full spectrum and PAR-only treatments were not significantly different (Fig. 14). When superoxide dismutase concentration was normalized to diatom density, both UV treatments produced significantly more SOD than the PAR, although the two treatments were not significantly different from each other (Fig. 15).

*Reactive oxygen species.* Dihydrofluorescein fluorescence was assayed twice on both the first and last days of the irradiation: in the morning prior to the onset of UV exposure, and after several hours of UV irradiation (Fig. 16). On the first day, all treatments (including the PAR control) exhibited increased DHF fluorescence after 9 hours of irradiation (statistically significant for UV-A and PAR). Before the irradiation, the UV-A flasks showed significantly lower DHF fluorescence than the PAR-only control and full spectrum flasks, whereas later in the day the full spectrum flasks showed significantly lower DHF fluorescence than and PAR control. No differences in DHF fluorescence were observed among or between treatments on day 16. Over the course of the irradiation dihydrofluorescein fluorescence (morning, before UV irradiation) was not significantly different between any two treatments (Kruskal-Wallis p  $\geq 0.2$ , Fig. 16). Fluorescence dropped dramatically in all three treatments in the post-irradiation period, though it did spike up again in the PAR control on day 2 of that period.

Figure 13 Bacteria per diatom. Markers represent median values, with error bars representing range. Blue squares = UV-A treatment Red triangles = Full spectrum treatment Black circles = PAR control



Table 1 SOD correlations. Kendall Rank Correlation p-values between the variable on the left and the total relative superoxide dismutase concentration. Diatom volume was calculated as volume/diatom × diatoms/filter. Total biovolume was diatom volume + estimated bacterial volume (assuming an average diameter of  $0.5 \mu m^2$ ).

	UV-A	Full spectrum	PAR-only
bacterial / ml	p = 0.009	p = 0.02	p = 0.009
diatoms / ml	p = 0.14	p = 0.68	p = 0.51
diatom volume*	p << 0.01	p >> 0.1	p >> 0.1
total biovolume	p = 0.006	p = 0.30	p = 0.94

\*The smaller number of data for diatom volume prevented the use of the Student's t approximation that allowed a more accurate p-value determination for the other variables. Figure 14 Relative superoxide dismutase concentration normalized to bacteria. Symbols represent median, error bars represent range. Blue squares = UV-A treatment Red triangles = Full spectrum treatment Black circles = PAR control



Figure 15 Relative superoxide dismutase concentration normalized to diatoms. Symbols represent replicate median, error bars represent range. Blue squares = UV-A treatment Red triangles = Full spectrum treatment Black circles = PAR control





Figure 16 Median DHF fluorescence before and after irradiation on days 0 and 16. Error bars represent the range of the data. White = PAR Blue = UV-A Red = Full spectrum Figure 17. Median dihydrofluorescein fluorescence, assayed every morning before UV exposure. Error bars represent the range of the data. The vertical dotted line indicates the end of the irradiation period. Blue squares = UV-A Red triangles = Full spectrum Black circles = PAR



#### **Chapter 4**

#### Discussion

*Growth rate.* The growth rate for all treatments showed a high degree of variability from day to day (Fig. 3). This appears to have resulted from variations in cell concentration, to which observed growth rate was strongly (and inversely) related (Fig. 4). The basis of this relationship is not known. The variations may reflect increased light or nutrient limitation at high cell densities. In order to compare growth rates among treatments with varying cell concentrations, intrinsic growth rates were calculated using a logistic model, with the carrying capacity estimated from the PAR-only control (Fig. 4). This procedure did create outliers when the model failed, for instance when cell density was larger than the estimated carrying capacity, but the observed exponential growth rate was non-zero (e.g. PAR-only control, day 4). Nevertheless, this is a reasonable method of dealing with density-induced growth rate variability, and the non-parametric statistics used are less sensitive to outliers than parametric statistics (Sokal and Rohlf 1995).

The median growth rate under UV-A irradiation  $(1.67 d^{-1})$  was 37% lower than in the PAR-only control (2.01 d<sup>-1</sup>). After the end of the irradiation period, the growth rate of the UV-A treated cultures increased to become higher than the PAR-only control (2.6 d<sup>-1</sup> vs. 2.0 d<sup>-1</sup>). This is evidence that repair processes are important in the survival of UV stress in this diatom. Previous studies have focused on short-term effects of UV on parameters such as carbon assimilation and pigments (e.g. Hazzard et al. 1997, Xiong 2001, Sinha and Hader 2002). These studies have indicated the importance of repair to damaged cellular components when considering the overall effects of ultraviolet radiation on phytoplankton populations (Neale, Fritz et al. 2001). Growth rate inhibition in the UV-A treatment appeared to increase over the first nine days of irradiation, indicating dose dependence in the growth rate response (Fig. 6). During the remainder of the irradiation period there was not a significant trend in growth inhibition (i.e. no significant slope), but the median value over this period was relatively low (35%). This suggests that active repair of damage contributed to the long-term acclimation of this diatom to UV-stress. These results are consistent with the findings of Zudaire and Roy (2001), who found an induction period of 10 days for the growth rate inhibition of the diatom *Thalassiosira weissflogii* irradiated with PAR + UV-A + UV-B.

The full spectrum treatment also resulted in a significant (though more modest) reduction in growth rate during the irradiation period (Fig. 5), and an apparent induction period for growth inhibition (Fig. 6). The fact that the full spectrum growth rate was not significantly different from the UV-A treatment suggests that the UV-B dose was too small to produce a measurable response in this diatom (Fig. 5C). Given that the irradiance spectrum used in the incubation was highly enriched in both UV-A and UV-B when compared to the standard solar spectrum (Fig. 2), this is evidence that UV-A radiation is more important than UV-B when considering the effects of UV on phytoplankton populations. Consistent with this conclusion, several studies have found that the UV-A wavelengths were responsible for most of the photosynthetic inhibition in diatoms and other phytoplankton under full spectrum treatments (Helbling et al. 2001, Cullen et al. 1992, Riegger and Robinson 1997). In a study of *T. weissflogii*, Zudaire and Roy (2001) found that "High UV-B" irradiance (0.3 W·m<sup>-2</sup>) did not significantly affect growth rate or pigment composition when compared to their "Low UV-B" treatment

 $(0.16 \text{ W}\cdot\text{m}^{-2})$ . The high resistance of this diatom to UV-B radiation is probably due to aggressive turnover of the photosynthetic units (Xiong 2001).

*Cell size.* Cells growing under the full spectrum treatment (Fig. 7) were significantly larger than cells in the UV-A or PAR-only treatments. This is consistent with previous studies (Karentz et al. 1991, Rijstenbil 2001, Rijstenbil 2003). The explanation given in the literature for increased cell size under full spectrum irradiation is that UV-B stress inhibits mitosis (by damaging DNA), but not cell growth (Buma et al. 1997, Higley et al. 2001). Under these circumstances, the cells would then continue to grow in size without dividing. If this were true in the present study, we might expect cell size to be correlated with growth rate inhibition. But the full spectrum population growth rates were not significantly different than those for UV-A (which showed strong inhibition but no change in cell volume), and a Kendall Rank Correlation shows no significant correlation between cell volume and growth rate inhibition within the full spectrum treatment. A speculative explanation for this phenomenon is that the increase in cell volume is a defensive adaptation: in the process of increasing cell size, the diatom is increasing the path length that a photon must pass through before reaching delicate cellular components (see Karentz et al. 1991).

*In vivo fluorescence*. The ratio of in vivo chlorophyll fluorescence to optical density at 440 nm (a proxy for chlorophyll concentration) reflects the relative efficiency of photosynthetic reaction centers (Kirk 1994). This ratio was significantly lower in the UV-A treatment relative to the PAR-only control (Fig. 8A), indicating a loss in reaction center efficiency in this treatment (Clendennen et al. 1996).

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The full spectrum treatment (Fig. 8B) also produced a significant decrease in normalized fluorescence, but not significantly more than the UV-A treatment (Fig. 8C). Again, this points to the importance of the UV-A wavelengths in damaging the photosynthetic apparatus (Cullen et al. 1992, Riegger and Robinson 1997, Helbling et al. 2001). In fact, the UV-A ratio of in vivo chlorophyll fluorescence to OD440 was significantly lower than the full spectrum treatment, suggesting that the UV-B wavelengths induced additional protective and/or repair mechanisms that ameliorated the harmful effects of the UV-A wavelengths. This effect has been observed in the cyanobacterium Synechocystis when accompanied with low-PAR flux (Sicora et al. 2003). There has been a great deal of research focusing on the D1 protein in Photosystem II, and cyanobacteria have been shown to activate enhanced turnover mechanisms in response to UV-B irradiation (Vass et al. 2000). Turnover of this protein has been singled out as a marker of UV-B resistant algae (Xiong et al. 1999, Xiong 2001). The fact that normalized fluorescence rapidly increased to match the PAR-only control after UV irradiation ceased (Fig. 8) illustrated the important role of active repair of the photosynthetic reaction centers is an important part of the adaptive response to UV stress. The repair activity under UV stress is an acceleration of the repair processes occurring during PAR-only growth (Schofield et al. 1995, Hazzard et al. 1997, Marwood et al. 2000, Grzymski et al. 2001).

*Optical density.* The apparent optical cross section  $(\mu m^2/diatom)$  of exposed cells at 440 nm is used here as an estimate of cellular chlorophyll content. The UV-A treatment (Fig. 10A) showed a significantly higher cross section throughout the UV treatment. This is an atypical response for phytoplankton (Dohler 1984, Dohler 1997a,

Xiong et al. 1999, Lohmann et al. 1998), although increases in chlorophyll have occasionally been reported for diatoms (Nilawati et al. 1997) and higher plants (Mirecki and Teramura 1984). This variability is not surprising given the wide range of responses found between different diatom species (Buma et al. 1996). The mechanism responsible for increased chlorophyll production under UV irradiation has not been studied. The redox state of the electron transport chain (ETC) linking Photosystems I and II has been shown to regulate the synthesis of reaction centers (Kovacs et al. 2000), and UV radiation has been shown to cause a shift towards oxidation in the ETC (Nilawati et al. 1997). In this way, UV radiation could increase chlorophyll concentrations by disrupting the ETC and consequently up-regulating photosynthetic reaction center synthesis. This hypothesis has not been tested experimentally.

Optical density at 350 nm is taken here as a proxy for the presence of UVprotective pigments (e.g. mycosporine-like amino acids) (Xiong et al. 1995, Xiong et al. 1997, Cockell and Knowland 1999, Montecino et al. 2001), and was normalized to OD440 to account for changes in cell density and/or photosynthetic pigment concentration. The observed increase in the OD350:OD440 ratio in the UV-A treatment suggests the production of protective pigments; however no such increase was observed in the full spectrum treatment (Fig. 9). This is not surprising since the full spectrum treatment showed significantly better acclimation than the UV-A treatment for other optical properties (autofluorescence and OD440). This data indicates that the diatoms under the full spectrum treatment acclimated to UV-stress without needing photoprotective pigments. It should be noted, however, that the actual UV absorption peak for this diatom was at 337 nm, which is close to the peak UV output for the experimental UV source, but lower than the assayed wavelength of 350 nm. Therefore, the optical cross section of the relevant UV-protective pigments may not have been properly measured. As a result, any conclusions drawn from these absorbance data should be viewed with caution.

Optical density of methanol-extracted pigments showed a consistent pattern of PAR-only > Full spectrum > UV-A, for cell-normalized OD at all wavelengths tested (Fig. 11). This is in direct contradiction to the *in vivo* data, which showed the greatest optical cross section in the UV-A treatment and the lowest in the PAR-only control at 440 nm. This is likely a result of changes in package effect associated with UV-mediated changes in the number and structure of chloroplasts in the cells (Buma et al. 1996). Package effect refers to the effect on absorbance of concentrating pigments (chlorophyll and accessory pigments) into chloroplasts inside cells. This reduces the optical density of the pigments when compared to the same concentration of pigments distributed uniformly in medium (Kirk 1994). The observed difference in optical density between in vivo and extracted samples could also be a result of dissolved pigments (chlorophyll and MAA's) in the *in vivo* samples. Diatoms are known to release photosynthetic pigments along with high-molecular weight photosynthate when stressed (Bianchi et al. 1995, Carrillo et al. 2002). Released pigments would contribute to whole water (*in vivo*) absorbance, but not filtered sample absorbance. In the event that exuded chlorophyll is a significant factor in the observed *in vivo* optical cross sections, the data would still indicate an increase in the production of chlorophyll because all of the cultures were diluted daily. In order to maintain high optical density in these diluted cultures, the diatoms would have to produce (and exude) chlorophyll rapidly.

*Bacteria/diatom.* Bacterial standing stock (normalized to diatom concentration) was generally highest in the UV-A treatment (Fig. 13). Excretion of reduced carbon by phytoplankton and bacterial production fueled by this excretion have been shown to increase under UV-A radiation (Carrillo et al. 2002). On the other hand, UV-B radiation can significantly reduce bacteria growth rate (Aas et al. 1996, Joux et al. 1999). Thus under UV-A, increased DOM availability via enhanced diatom exudation likely resulted in the observed increases in bacterial standing stock, whereas under full spectrum UV this stimulation of bacteria may have been offset by UV-B-mediated growth inhibition. The fact that changes in bacterial concentration mirrored the changes in diatom growth rate inhibition in the UV-A treatment suggests that DOC exudation may have been correlated with UV-mediated growth rate inhibition (compare Figs. 6 and 12). Further experimentation that includes the quantitation of extracellular DOC and bacterial production is necessary before robust conclusions about the relationship between UV exposure and bacterial dynamics can be drawn.

*Superoxide dismutase*. The SOD assay proved problematic. The protocol from the literature (Rijstenbil et al. 1994) called for filtering samples onto polycarbonate filters, which would size-fractionate the sample and select only diatoms for the assay. However, these filters to not permit grinding of the sample, which could result in incomplete diatom extraction. For this reason GF/F glass fiber filters were used. These filters allowed all of the samples to be ground uniformly and extracted completely. Unfortunately they are also likely to retain bacteria as well as diatoms, and thus may have introduced bacterial contamination into the assay. Consistent with this possibility, bacterial density correlated significantly with SOD activity, suggesting that bacteria may be contributing significantly to the measured SOD activity (Table 1). Previous studies have shown that UV-A can increase SOD in bacteria (Farr and Kogoma 1991, Hoerter et al. 2005). However, SOD normalized to bacteria (Fig. 14) showed significant decreases under both irradiation treatments when compared to the PAR-only control, in contrast to expectations (Rijstenbil 2001). On the other hand, when SOD was normalized to diatoms (Fig. 15) it showed a significant increase in activity under both irradiation treatments compared to the PAR-only control. Thus it appears that this diatom had increased SOD activity in response to UV irradiation. However, because we are unable to distinguish between bacteria- and diatom-associated activity in this experiment, this conclusion must be considered tentative. Previous studies have shown an increase in diatom SOD activity under UV stress (e.g. Rijstenbil 2001).

*Reactive oxygen species*. Reactive oxygen species, as reflected in the DHF assay, did not respond to UV treatments in a consistent way. On the first day of irradiation, cells showed an increase in DHF oxidation in all treatments, including the PAR-only control (Fig. 17). Surprisingly, the PAR-only control showed the most fluorescence after irradiation, and the full spectrum treatment showed the least. Over the remainder of the experiment, no significant differences were observed among the three treatments. This is in direct contradiction to a previous study using a similar stain (Rijstenbil 2002), in which ROS-reactive stain fluorescence was significantly increased by UV-B irradiation in the diatom *T. pseudonana*. The present results may reflect the presence of some other factor that is oxidizing DHF in this diatom. The dramatic drop in fluorescence of all treatments after the end of the irradiation period suggests that total PAR dose is involved. Reactive

oxygen species (superoxide, hydroxyl radicals, and hydrogen peroxide) are produced under PAR as a byproduct of photosynthesis (Knox and Dodge 1985, Zolla and Rinalducci 2002, Ohnishi et al. 2005). For example, Photosystem II produces singlet oxygen when chlorophyll donates an electron to oxygen, and PSI produces superoxide when accepting electrons during acyclic photophosphorylation (Nishiyama et al. 2006). Superoxide radicals are converted to hydrogen peroxide by superoxide dismutase (Flohé and Ötting 1984), and hydroxyl radicals through metal-catalyzed reactions (Ryter and Tyrrell 1998).

This assay depended on secondary oxidation of dihydrofluorescein, since the residence times of reactive oxygen species are several orders of magnitude smaller than the time scale of the assay procedure (milliseconds vs. minutes). As a result, the assay may prove more reliable if cells are treated with dihydrofluorescein while they are being exposed to UV-irradiation. Large culture volume and long-term irradiation treatment made this difficult in the present study. In order to perform this assay properly, sample aliquots would be incubated in smaller vessels with stain inside the experimental incubator at each daily sampling.

#### Chapter 5

#### Conclusions

Because the Sargasso Sea allows penetration of UV radiation through a significant portion of the euphotic zone (Smith and Baker 1981, Kirk 1994, Piazena et al. 2002), phytoplankton living in this ecosystem might be expected to be resistant to UV damage (Xiong et al. 1997). The results of this study show that *Pseudo-nitzschia delicatissima* isolated from surface waters to be relatively UV-resistant, with growth rate inhibition of around 40%. This compares to other diatoms whose growth rates have been shown to be inhibited by UV by as much as 100% (Montecino et al. 2001, Rech et al. 2005). Other phytoplankton communities have been shown to have differential resistance to UV-mediated damage as a function of their historical UV exposure. This phenomenon is best illustrated in the Antarctic, where phytoplankton from shallow (i.e. high UV) sites were significantly more tolerant to UV stress than phytoplankton from deeper waters (Neale et al. 1998).

Although the diatom in this experiment showed a significant decrease in growth rate during the experiment, this decrease was not exacerbated by the increased cumulative dose of the long incubation. On the contrary, growth rate inhibition appeared to relax as the incubation progressed. The brief increase in growth rate, and decreases in bacteria load and optical cross section after the end of the irradiation period all show the importance of active repair in acclimation to UV radiation.

Optical density and in vivo autofluorescence provide estimates of the dynamics of the photosynthetic apparatus under UV-stress. However, they are sensitive to both scattering and package effect, which are difficult to quantify using a spectrophotometer and fluorometer. This study produced interesting results with these measurements, which need to be confirmed using more sensitive instruments such as HPLC and PAM fluorometry.

The greatest contribution of this experiment to the study of UV-responses in phytoplankton comes from the long irradiation period. Most studies involving phytoplankton and UV relied on short term exposures on the order of hours (e.g. Neale, Litchman et al. 2001). While this approach has been very useful in illuminating the components of UV acclimation (passive defense and active repair), they fail to extrapolate this acclimation process to ecological time scales. Diatom population dynamics in the Sargasso Sea are dominated by the Spring and Summer blooms (Deuser et al 1995). These blooms last approximately one to two months, during which time diatoms probably sexualize (Chepurnov et al. 2004). This study suggests that these bloom communities are able to acclimate to UV-stress, greatly reducing the applicability of earlier studies to natural phytoplankton assemblages.

Finding a diatom in the surface of the Sargasso Sea that is resistant to UV radiation is perhaps not particularly surprising given the unique optical qualities of this region (Kirk 1994). Although it is impossible to make broad generalizations about a large biome given an experiment on a single diatom, this study provides an important first step in engaging the UV-physiology of diatoms in the Sargasso Sea for further study.

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