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EFFECT OF UV-A IRRADIANCE ON LIPID ACCUMULATION IN

NANNOCHLOROPSIS OCULATA

A Thesis
presented in partial fulfillment of requirements
for the degree of Masters of Biology
in the Department of Biology
The University of Mississippi

By

RATHY SRINIVAS

2011

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ABSTRACT

The marine microalgae *Nannochloropsis* spp. are of both ecological and economic importance as they contribute to the base of the food chain of marine systems, and can accumulate a considerable amount of lipids. Their lipids can be grouped into two categories, storage lipids, which are mainly triglycerides (TGs), and structural lipids, which are composed of polyunsaturated fatty acids (PUFAs). PUFAs are essential nutrients for both aquatic animals and humans, while TGs can be transesterified to produce biodiesel. Lipid formation in microalgae depends on several environmental factors and *Nannochloropsis* spp. have been shown to accumulate excess storage lipids under stressful conditions. Changes in either TGs or PUFAs in response to environmental factors is therefore of economic and ecological concern.

The objectives of this project were (1) to determine the effect of UV-A (320 – 400 nm), a potential environmental stress factor, on total lipid accumulation in *Nannochloropsis oculata*, (2) to examine if there is reciprocity in the relationship of exposure duration to exposure irradiance on the stress response; and (3) to study the interactive effect of UV-A and nutrient concentration on lipid accumulation in *N. oculata*. The first and second objectives were addressed using a series of 7-day bioassay experiments, in which *N. oculata* cells were exposed to three UV-A irradiances (I), 6, 12, and 24 W m⁻². For each irradiance there were three different durations of exposure (T) such that they resulted in three Doses (I x T); 24, 48 and 72 J m⁻². If the same doses have a similar effect, irrespective of UV-A irradiance and duration of exposure, reciprocity holds. Lipid

analysis was done using the lipid soluble fluorescent dye Nile Red. UV-A treatments significantly increased the chlorophyll-specific lipid concentration of *N. oculata* cells, and I was unable to reject that reciprocity holds. The third objective was addressed by a pair of factorial bioassay experiments. There was a synergistic effect of UV-A exposure and nutrient depletion on lipid content of *N. oculata* cells.

DEDICATION

I dedicate this thesis to my husband, Hari. Without his support and encouragement it would not have been possible to attend graduate school.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Clifford Ochs, for his excellent guidance, caring, patience, and helping me to develop my background in ecology. He was always accessible, actively involved in the work of all his students, and clearly always has their best interest in mind. Had he not agreed to mentor me, my graduate school dream would have remained such.

I would like to thank my committee members; their expertise has made my research successful. Dr. Mossing was always willing to help and give his best suggestions. He trusted and encouraged me from day one; my research would not have been possible without his support. Dr. Marjorie Holland kept me motivated during tough times with her moral support. I appreciate her kind concern and consideration regarding my academic requirements. Dr. Jason Hoeksema was the last and crucial addition to my committee; I thank him for his excellent support and guidance with statistical analysis.

Apart from my committee members I am very grateful to Dr. Randy Wadkins and Dr. Gail Stratton for allowing me to use their labs like one of their own students. I thank Rani Menon and Leah Wang for discussing several research related issues with me and for always being ready to help me.

Last but not the least I would like to thank Dr. Stephen D'Surney for giving me the initial opportunity to experience graduate school at Olemiss.

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INTRODUCTION

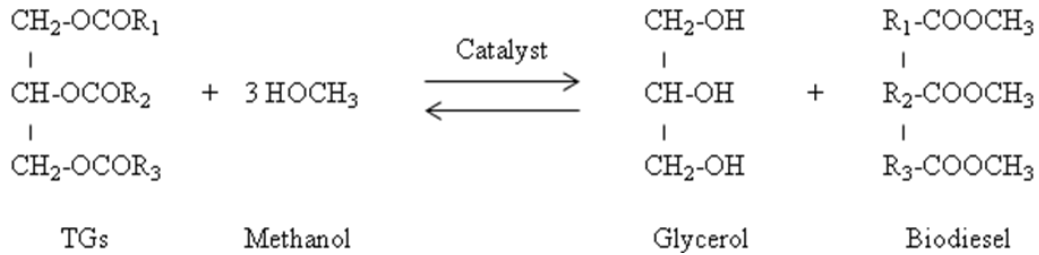
For organisms living in the photic zone of the open ocean or clear lakes, ultraviolet radiation is a daily fact of life. Although most of the shorter wavelengths of ultraviolet radiation (UV-B: 280-320 nm) are blocked by the ozone layer, longer wavelengths of radiation (UV-A: 320-400 nm) can penetrate for tens of meters. UV-A irradiation is not photosynthetically active, but can be a potent force regulating biological productivity in aquatic systems, capable of impairing nutrient assimilation, photosynthesis, and photorepair of molecular damage (Arts et al. 2000, Zhou et al. 2009). Thus, all living organisms must tolerate, counteract, or avoid being exposed to it (Larkum & Wood 1993). Microalgae, being unicellular, can undergo direct or indirect damage when exposed to UV radiation, with the effect being species-specific (Kim & Watanabe 1994; Zacher et al. 2007). The effect of UV-A on an organism depends on UV-A irradiance (I ; $W\ m^{-2}$) and duration of UV-A exposure (T), resulting in a corresponding dose ($I \times T$; $J\ m^{-2}$). Low UV-A irradiance over a long period of time may or may not have the same effect as high UV-A irradiance for a shorter period of time (with dose constant). Therefore dose, irradiance, and duration of exposure are important factors to be considered when studying the effects of UV-A irradiance on organisms (Grad et al. 2001). If the effects of the same doses are similar, regardless of irradiance and exposure duration, there is “reciprocity”. Reciprocity fails if the effects of exposure at similar doses depend on I or T (Grad et al. 2001).

Nannochloropsis spp. are marine microalgae which were first described by D. J. Hibberd (Hibberd 1981). They are mixotrophic, non-flagellated algae that belong to the class Eustigmatophyceae, which includes unicellular and small (< 5µm) coccoid organisms that lack chlorophyll b (Hoek 1995). *Nannochloropsis* spp. are of ecological and potential economic importance since they are abundant and contribute to the base of the food chain in marine habitats, and can accumulate significant amounts of eicosapentaenoic acid (EPA), a component of structural lipids (Chaturvedi & Fujita 2006). EPA is an essential source of nutrition for marine organisms and humans, and it has potential pharmaceutical applications in preventing various human diseases (Wand & Chai 1994).

The quantity and proportion of different types of lipids within *Nannochloropsis* spp. cells is determined by the growth conditions. Microalgal lipids are basically grouped into two classes; storage and structural lipids. Structural lipids contribute to membrane integrity, and storage lipids can be drawn on as energy sources. Storage lipids are mainly saturated fatty acids such as triglycerides (TGs), while structural fatty acids are polyunsaturated fatty acids (PUFAs). Nitrogen deficiency (Xu et al. 2004, Suen et al. 1987), saturating light conditions (Sukenic & Carmeli 1989), and an increase in temperature from 20-25 °C (Converti et al. 2009) resulted in excess accumulation of mainly storage lipids. Under low light conditions excess structural lipids enriched with PUFAs are produced, which can enhance photosynthetic efficiency (Sukenic & Carmeli 1989).

TGs can be transesterified (Equation 1) to biodiesel with glycerol as a byproduct (Li 2008). Due to their rapid reproduction, and accumulation of lipids, *Nannochloropsis* spp. have recently been regarded as potential sources for biofuels (Gouveia & Oliveira 2009). Recently, it was shown that continuous illumination of *Nannochloropsis* spp. with very high UV-A doses

increases the ratio of storage lipids (TGs) to structural lipids (PUFA) (Forjan et al. 2010). UV-A is a cause for oxidative stress in *Nannochloropsis* cells which increases the saturated:unsaturated fatty acid ratio.



Equation 1: Transesterification of TG to biodiesel with glycerol as byproduct.

The objectives of this project were (1) to determine the effect of UV-A (320 – 400 nm), a potential stress factor, on total lipid accumulation in *Nannochloropsis oculata*; (2) to examine if there is reciprocity in the relationship of exposure duration to exposure irradiance on the stress response; and (3) to study the interactive effect of UV-A and nutrient concentrations on lipid accumulation in *N. oculata*. This was accomplished by a series of laboratory experiments using *N. oculata* cultures exposed to artificial UV-A at different irradiances, and for different durations, and under several nutrient (N and P) regimes. Irradiances employed in this experiment are within the UV-A irradiance range in the marine environment (Koronakis et al. 2002).

METHODOLOGY

Culture Conditions

The culture of *N. oculata* used in these experiments was obtained from Carolina Biological (# 153220). For all experiments, 20 ml of *N. oculata* cultures were incubated in Whirlpak bags (UV-transparent) at approximately 25°C. All growth media were made with sea water (35 gm sea salt mixed with 1 L pure water). Nutrient concentrations of the sea water medium were 0.06 mg L⁻¹ of PO₄, 0.14 mg L⁻¹ of NH₃ and 0.28 mg L⁻¹ of NO₃. For experiments a semi-continuous culture technique was used in which 50% of the sample volume was removed daily for lipid and growth analysis, and replaced with fresh sea water media. The culture bags were clipped to a stand on a shaker table and exposed to different UV-A irradiances. UV-A irradiance was manipulated by using neutral density filters (NDFs) that block all UV-A wavelengths equally. All experiments were run for seven days with samples taken every day or two days and included a control treatment that was not exposed to UV-A irradiance.

Light Sources

The source of UV irradiance was a Psoralite series 2400 light system fitted with F24T12BL/HO UV-A fluorescent lamps situated behind an acrylic shield. The spectrum of UV irradiance produced by this system was determined by a spectroradiometer (Analytical Spectral

Device; FH HH 325-1075) (Fig. 1). The maximum integrated UV-A irradiance to which organisms were exposed was 24 W m^{-2} (320-400 nm). During experiments, the cultures also received photosynthetically active radiation (PAR) provided by a bank of fluorescent lights (tube no. RB15T8) on a 12 hour L:D cycle. PAR used in this experiment was between $55\text{-}60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

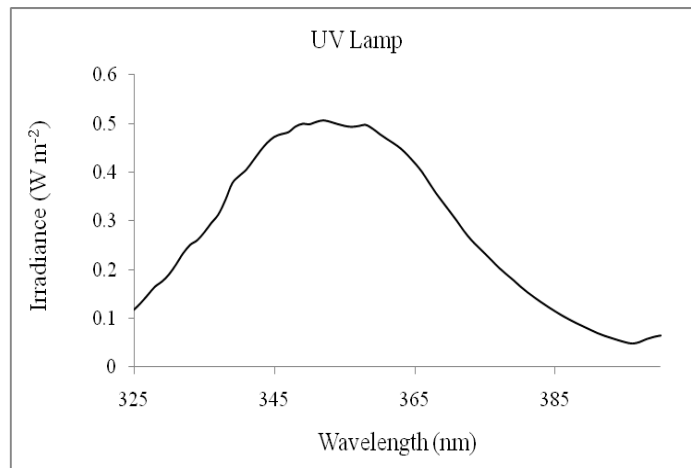


Fig. 1: Maximum UV-A irradiance received by cultures.

Experimental Design

Five experiments were performed. Following is a description of each experiment:

Experiment 1: The purpose of Experiment 1 was to evaluate the effect of UV-A dose & UV-A duration on cell lipid content. NDFs were used to generate three different irradiances (Fig. 2), low (L), medium (M), and high irradiance (H). Durations of UV-A exposures were manipulated such that at each irradiance there were three different dose treatments; L1, L2, L3; M1, M2, M3; H1, H2, H3. Dose 1 was identical across all three irradiances (L1, M1, H1), as was dose 2 (L2,

M2, H2) and dose 3 (L3, M3, H3). Controls were exposed to PAR only. The incubation medium consisted of 0.071 mg L⁻¹ of PO₄, 0.16 mg L⁻¹ of NH₃ and 0.3 mg L⁻¹ of NO₃. Due to space limitations, Experiment 1 was performed as a randomized block experiment. There were four blocks in which each block contained one replicate per irradiance-duration combination. The block experiments were performed on different days.

Experiment 2: Experiment 2 was not a randomized block experiment and was designed to check for consistency of results obtained from Experiment 1. It included 3 independent replicates per treatment, The UV-A treatments used in this experiment were the high doses L3, M3 and H3. Nutrient medium was as in Experiment 1.

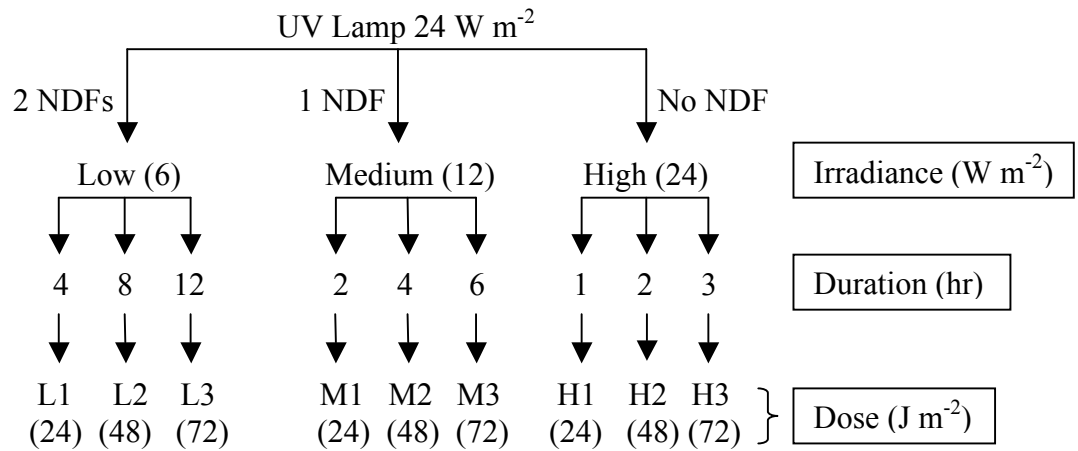


Fig. 2: Experiment 1, 2, & 3 UV-A treatment design. L1 = M1 = H1 = 24 J m⁻²; L2 = M2 = H2 = 48 J m⁻²; L3 = M3 = H3 = 72 J m⁻².

Experiment 3: The purpose of Experiment 3 was to evaluate the effect of UV-A irradiance alone on lipid content. *N. oculata* cultures were exposed to control (0 W m⁻²), low (6 W m⁻²), and high (24 W m⁻²) UV-A irradiances for 12 hrs per day with UV-A doses of 72 J m⁻² and 288 J m⁻²,

respectively. Incubation medium was as in Experiment 1 & 2. There were three replicates per treatment.

Experiment 4: The purpose and design of Experiment 4 were identical to Experiment 3, except that the UV-A exposure was for 6 hrs per day. This produced UV-A doses of 0 J m^{-2} , 36 J m^{-2} and 144 J m^{-2} for the low and high irradiances respectively.

Experiment 5: The purpose of Experiment 5 was to examine the main and interaction effects of nutrient concentrations and UV-A on cell lipid content. *N. oculata* cultures were exposed to irradiances of 0 W m^{-2} or 6 W m^{-2} for 12 hrs (dose 24 J m^{-2}) in low and high nutrient conditions. The low nutrient incubation medium contained approximately 0.062 mg L^{-1} of PO_4 , 0.15 mg L^{-1} of NH_3 and 0.2 mg L^{-1} of NO_3 . The high nutrient incubation medium was as in Experiment 1. There were three replicates per treatment.

Experiment 6: The purpose and design of Experiment 6 was identical to Experiment 5 except that low and sea water growth media were used to examine in finer detail the interaction effect of nutrients and UV-A on lipid production.

Measurement of response variables

a) Cell count: In preliminary experiments, cell abundance and chlorophyll fluorescence (explained below) was determined. Cells were counted using a hemocytometer. The relationship between cell number and chlorophyll fluorescence was similar regardless of the UV-A irradiance

or dose (Fig. 3). Therefore, in our experiments only chlorophyll fluorescence was used for biomass analysis.

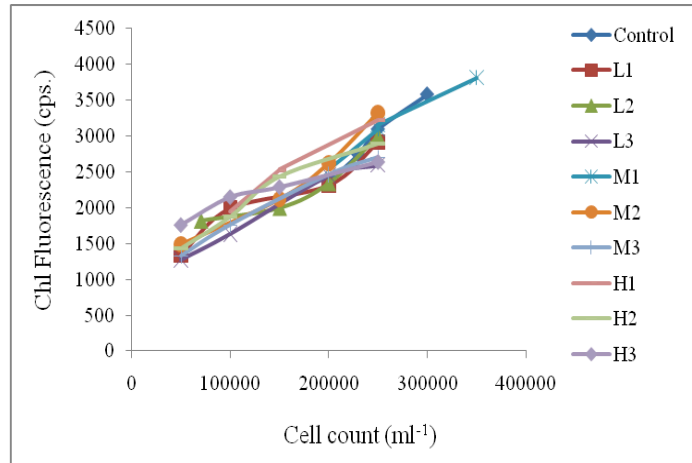


Fig. 3: Correlation between cell number and chlorophyll fluorescence for all UV-A treatments and control ($R^2 > 0.95$ in all cases).

b) Chlorophyll analysis: Algal biomass was estimated by chlorophyll fluorescence measured by spectrofluorometry (Photon Technology International; PTI). Excitation of chlorophyll fluorescence was at 425 nm and emission measured at 680 nm. Serially diluted *N. oculata* cells were used to derive a correlation between chlorophyll fluorescence and chlorophyll concentration, measured by spectrophotometry (Wetzel et al. 1991) (Fig. 4). Chlorophyll fluorescence was converted to chlorophyll concentration using a regression equation (Chl concentration = $0.05 \times \text{Chl fluorescence} - 11.5$).

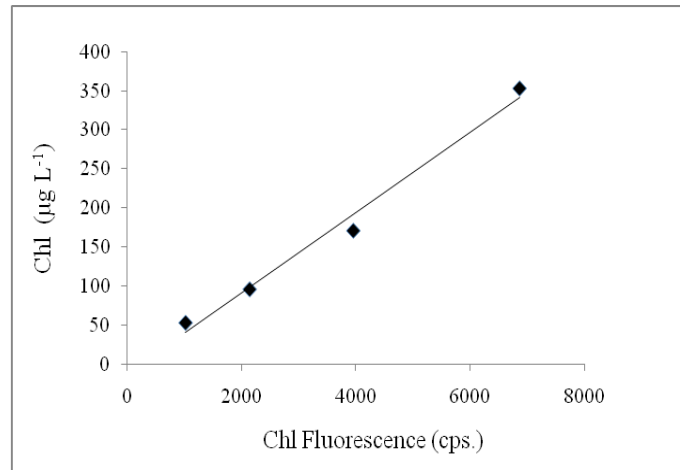


Fig. 4: Correlation between chlorophyll fluorescence and chlorophyll concentration of serially diluted *N. oculata* cells ($R^2 = 0.98$).

c) Lipid Analysis: Intracellular lipid analysis was by Nile red fluorescence. Nile Red, also known as Nile blue oxazone, is a lipid soluble fluorescent dye. It is a hydrophobic fluorescent probe as it fails to fluoresce in an aqueous environment (Fowler & Greenspan 1985), but does fluoresce when dissolved in lipids. It has been used successfully for the detection of lipids (Cooksey et al. 1987). I used Nile red with live cells of *N. oculata* for detection of intracellular lipid droplets by spectrofluorometry. For this measurement, 3 µl of 0.5 mg ml⁻¹ Nile Red in acetone were added to 3 ml of *N. oculata* culture in a cuvette, gently mixed, and placed in the dark for one minute, after which cellular lipid fluorescence was measured.

d) Excitation/emission wavelength selection for lipid fluorescence: Excitation/emission wavelengths of 560/640 nm were selected based on a preliminary study using lipid standards prepared according to Alonzo et al. (1999). Fluorescence of the 0.5 mg ml⁻¹ lipid standards was compared to fluorescence of *N. oculata* cultures after adding Nile red, as described above. Storage and structural lipid standards used were triolein (T) and phosphatidylcholine (PC)

respectively. Lipid standards, both separate (0.5 mg ml^{-1}) and combined (1 mg ml^{-1}), consistently had a peak at approximately 620 nm, and *N. oculata* cultures had a peak at around 640 nm (Fig. 5). Based on this test, in the experiments we measured lipid fluorescence of *N. oculata* cultures at 640 nm.

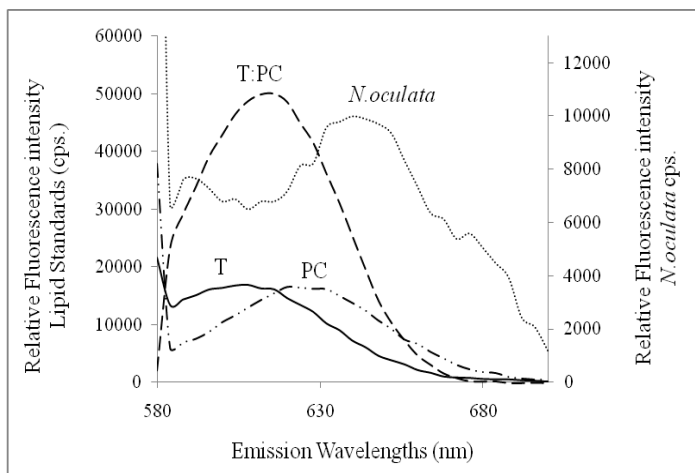


Fig. 5: Relative fluorescence intensity of *N. oculata* cells stained with Nile red and lipid standards separate (T & PC) and combined (T:PC; 1:1) stained with Nile red. Relative fluorescence intensity plotted here is the difference between stained samples and control (unstained cells for *N. oculata* and stained blank without lipid for lipid standards).

e) Gravimetric estimation of total lipids: Total lipid weight in *N. oculata* cells was determined gravimetrically and a correlation derived between lipid weight and lipid fluorescence. *N. oculata* cells were serially diluted to four different concentrations. A portion of each dilution was used for spectrofluorometric analysis of lipid using Nile red. The remaining portion of each dilution was sonicated for a minute and lipids extracted using the Bligh & Dyer method (Bligh & Dyer 1959). Dry weights of extracted lipids were plotted against the respective dilution's fluorescence in whole cells (Fig. 6). All experimental lipid fluorescence data were converted to lipid dry

weight based on a regression (Lipid concentration = $8E-08 * \text{Lipid fluorescence} + 0.0009$).

Chlorophyll-specific lipid weight is expressed as lipid:chlorophyll ratio (lipid:chl).

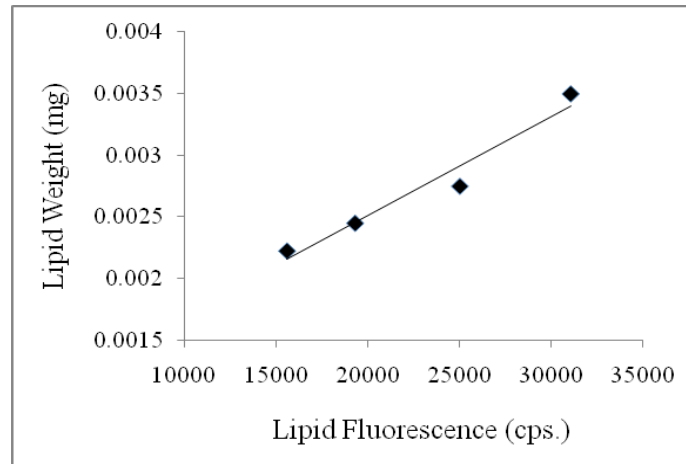


Fig. 6: Correlation between lipid fluorescence and lipid weight of serially diluted *N. oculata* cells ($R^2 = 0.95$).

Statistical Analysis

Results of experiments were analyzed using repeated-measures ANOVA, where the within-subject factor was day of sampling and the between-subject factors were UV-A dose, durations of exposure, UV-A irradiance and nutrients. The following comparisons were made:

a) Experiment 1: The response variable was $\ln(\text{treatment/control})$ where treatment and control refer to the lipid:chl ratio at different levels of UV-A. To evaluate if reciprocity held, treatments that received a similar dose, but different irradiances and exposure times, were compared to each other using post-hoc comparisons of means (Tukey's HSD). For example, L1, M1 and H1 were compared with each other, as was L2, M2, H2, and L3, M3, and H3. As this experiment was performed using a randomized block design, we also included block as a factor in the ANOVA.

b) For Experiments 2, 3, 4, 5, and 6 did not include blocks and the response variable lipid:chl ratio ($\mu\text{g } \mu\text{g}^{-1}$) was analyzed by ANOVA with three replicates per treatment. Experiments 5 and 6 were analyzed using Factorial ANOVA.

RESULTS

I. Effect of UV-A treatments on lipid accumulation in N. oculata

There was a significant effect of UV-A on lipid:chl ratio in *N. oculata* after 7 days of UV-A treatment. Lipid:chl ratios in all UV-A treatments except L1 and M1 were significantly higher from the control ($P < 0.0001$). The chlorophyll contents of all the UV-A treatments except M1 were lower than control ($P < 0.0001$). Cultures exposed to UV-A treatments had slower growth rates when compared to the control, as the increase in chlorophyll concentration per volume for all the UV-A treatments over time (except for M1) was significantly lower than the control (Table 1).

Table 1: Effect of UV-A treatments on chlorophyll concentration and lipid:chl ratio in *N. oculata* cells on day 7 (mean \pm SE, n = 4).

Treatments	Lipid:Chl ($\mu\text{g } \mu\text{g}^{-1}$)	Chl ($\mu\text{g L}^{-1}$)
Control	0.009 ± 0.001	332 ± 11
L1	0.012 ± 0.001	243 ± 16
L2	0.017 ± 0.003	188 ± 22
L3	0.021 ± 0.005	165 ± 23
M1	0.013 ± 0.003	291 ± 44
M2	0.016 ± 0.003	227 ± 26
M3	0.021 ± 0.004	185 ± 20
H1	0.016 ± 0.003	236 ± 18
H2	0.017 ± 0.004	229 ± 24
H3	0.016 ± 0.003	230 ± 34

a. Effect of dose

In Experiment 1, we were unable to falsify that reciprocity holds, that is, equal UV-A doses in this experiment, irrespective of UV-A irradiance and duration of exposure, had similar effects on the lipid:chl ratio and chlorophyll concentration in *N. oculata*. However there was a significant difference between different doses ($P = 0.0006$) in the lipid:chl ratio. The high UV-A dose of 72 J m^{-2} (L3, M3, H3) had a significantly higher lipid:chl ratio than the low dose of 24 J m^{-2} (L1, M1, H1). The medium dose of 48 J m^{-2} (L2, M2, H2) had an intermediate mean value and was not different from high and low UV-A doses (Fig. 7). The trend in chlorophyll concentrations with UV-A level was the opposite of that for lipid:chl ratio. The low UV-A dose treatments had the highest mean chlorophyll concentration, and the high dose had the least while the medium dose treatments were intermediate, and did not significantly differ from either low or high dose (Fig. 7). There was an increase in lipid:chl ratio from day 2 to day 7 in all treatments (Fig. 8), with trend towards a synergistic effect of treatment and time ($P = 0.05$).

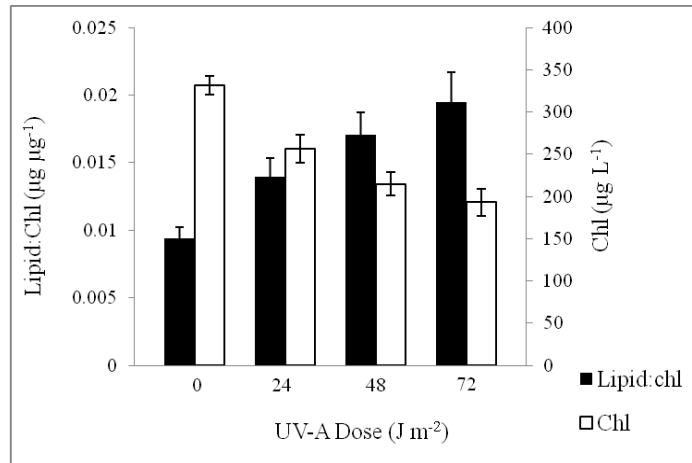


Fig. 7: Effect of UV-A doses on lipid:chl ratio (black bars) and chlorophyll concentration (white bars) on day 7 in Experiment 2 (mean \pm SE, n = 3). For UV-A dose details refer to Fig. 2.

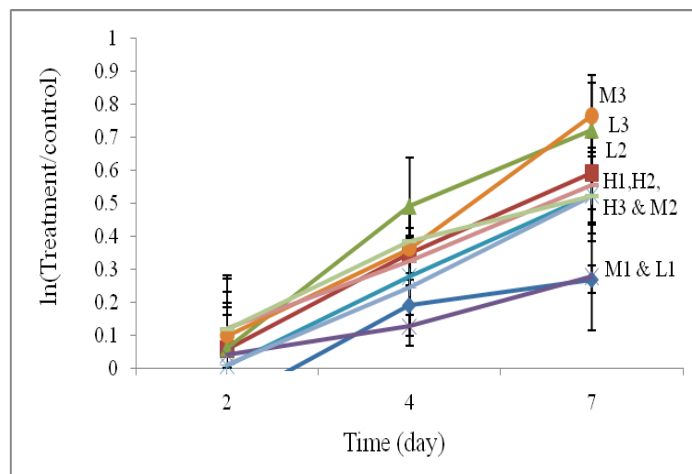


Fig. 8: Effect of time on lipid:chl ratio for UV-A treatments with respect to the control in Experiment 2. Data are expressed as $\ln(\text{treatment/control})$; (mean \pm SE, n = 4). For UV-A treatment details refer to Fig. 2.

In order to check for consistency in the effect of similar doses on lipid:chl ratio and chlorophyll concentration in *N.oculata*, Experiment 2 was set up with the treatments L3, M3, H3 and control, with three replicates per treatment. The results were in agreement with Experiment 1 (Fig. 9). With UV-A, algal growth rates were reduced as indicated by chlorophyll concentration

on day 7 ($P = 0.01$), and we were unable to falsify that reciprocity holds. There was an overall increase in lipid:chl ratio with day ($P < 0.0001$) for all three UV-A treatments and the control (Fig. 10). The data suggest a synergistic effect of UV-A and time on the lipid:chl ratio ($P = 0.02$).

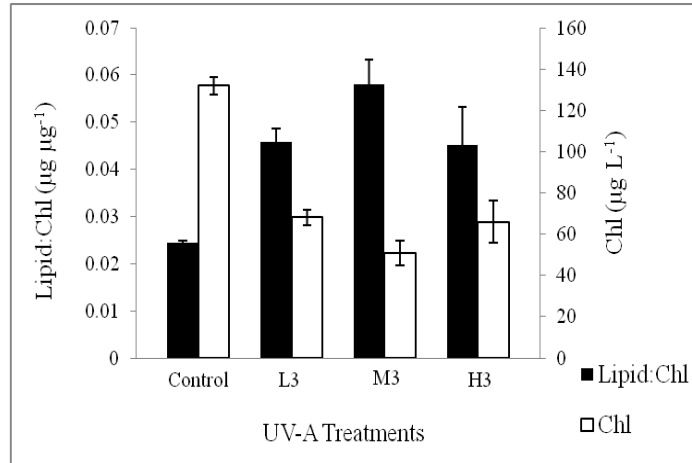


Fig. 9: Effect of dose 72 J m^{-2} on lipid:chl ratio (black bars) ratio and chlorophyll concentration (white bars) on day 7 in Experiment 2 (mean \pm SE, $n = 3$). For UV-A treatment details refer to Fig. 2.

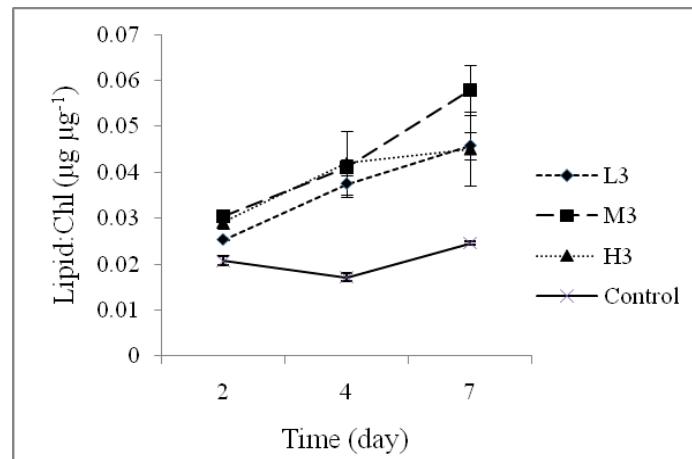


Fig. 10: Effect of time and dose 72 J m^{-2} on lipid:chl ratio in Experiment 2 (mean \pm SE, $n = 3$). For UV-A treatment details refer to Fig. 2.

b. Effect of duration

Data from Experiment 1 were used to analyze the effect of duration of exposure at a constant irradiance. Treatments L1, L2 and L3, representing different durations of UV-A exposure at the low irradiance level, were compared with each other. Similarly, M1, M2, M3, and H1, H2, H3 were compared (Fig. 11). Of L1, L2 and L3, with UV-A exposure of 4, 8 and 12 hrs respectively, L3 was significantly higher in lipid:chl ratio from L1 ($P = 0.001$). Treatment L2 was intermediate and not statistically different from L1 and L3. Among M1, M2 and M3, with 2, 4, and 8 hrs of UV-A exposures, respectively, M3 was statistically higher from M1 and M2. M2 was intermediate and not statistically different from M1 and M3 ($P = 0.0003$). There was no significant difference between the high UV-A treatments H1, H2, and H3, having 1, 2 and 3 hrs of UV-A exposure, respectively.

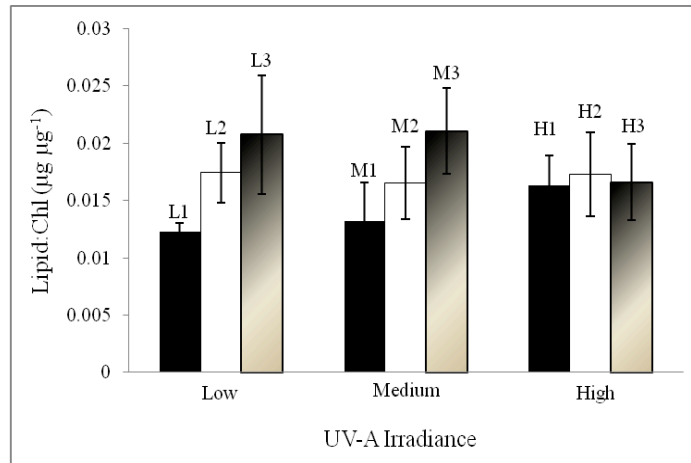


Fig. 11: Effect of UV-A duration on lipid:chl ratio on day 7 in Experiment 1 (mean \pm SE, n = 4). For UV-A treatment details refer to Fig. 2.

c. Effect of irradiance

Experiment 3 was designed to determine the effect of irradiance alone. At day 7 all three UV-A treatments were significantly different from each other ($P < 0.0001$) in lipid:chl ratio (Fig. 12). The high UV-A treatment had the greatest lipid:chl ratio, followed by the low UV-A treatment and control. At day 7, chlorophyll content was negatively impacted by UV-A ($P < 0.0001$), with the control having the highest chlorophyll followed by low UV-A and high UV-A treatments, indicating that the reason for differences in lipid:chl ratio can be attributed to differences in the amount of lipid produced relative to cell growth rates in different treatments (Fig. 12).

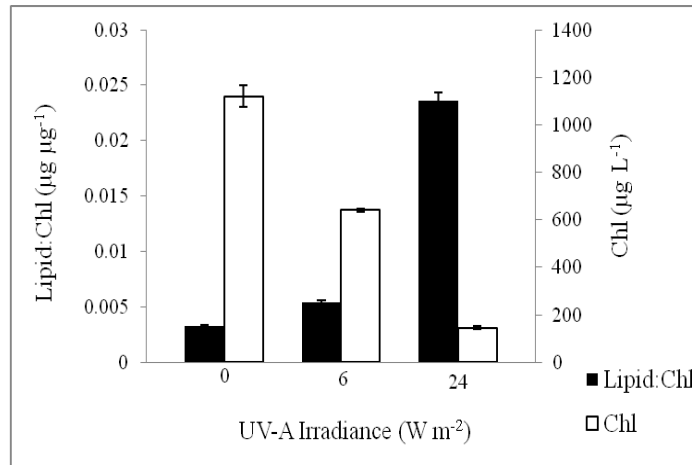


Fig. 12: Effect of low (6 W m⁻²) & high (24 W m⁻²) UV-A irradiance for 12 hrs on lipid:chl ratio (black bars) and chlorophyll concentration (white bars) on day 7 in Experiment 3 (mean ± SE, n = 3).

Experiment 4 was also designed to determine the effect of irradiance, but with 6 hrs of UV-irradiance, rather than 12 hrs as in Experiment 3. As in Experiment 3, all the three treatments were significantly different from each other ($P < 0.0001$) for lipid:chl ratio. High UV-A had the greatest lipid:chl ratio followed by low UV-A irradiance and the control had the least lipid:chl ratio (Fig. 13). Again, changes in the lipid:chl ratio were a consequence of reduced growth rate of cells, relative to lipid production, with increasing UV-A exposure (Fig. 13).

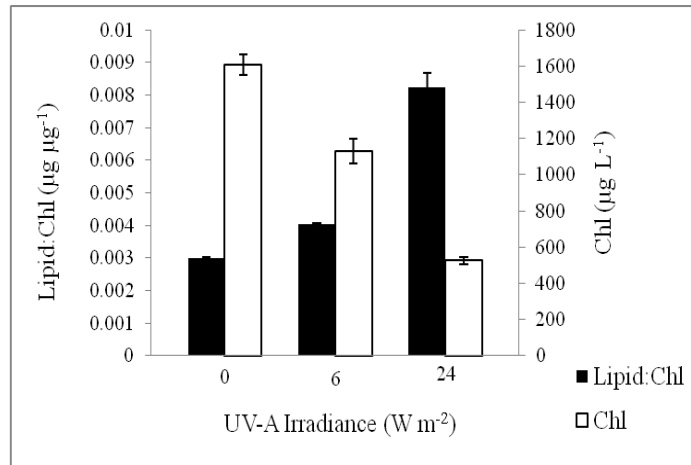


Fig. 13: Effect of low (6 W m⁻²) & high (24 W m⁻²) UV-A irradiance for 6 hrs on lipid:chl ratio (black bars) and chlorophyll concentration (white bars) on day 7 in Experiment 4 (mean ± SE, n = 3).

Although Experiments 3 and 4 were designed to test the effect of irradiance on lipid:chl ratio in *N. oculata* cells, the same data can be used to examine the effects of UV-A dose and duration. Ignoring irradiance, and just focusing on duration, it is clear that as duration of UV-A exposure increases, lipid:chl ratio also increases (Table 2). Therefore lipid:chl ratio increases with UV-A irradiance at a single duration of exposure with duration of exposure at a single irradiance, and hence with UV-A dose.

Table 2: Comparison between effect of different UV-A irradiances and durations of exposure on lipid:chl ratio and chlorophyll content in *N. oculata* on day 7 in Experiments 3 & 4.

Treatment	Lipid:Chl (µg µg ⁻¹) 6 hrs	Lipid:Chl (µg µg ⁻¹) 12 hrs
Control	0.00299 ± 0.00002	0.0031 ± 0.0002
Low UV-A (6 W m ⁻²)	0.00404 ± 0.00004	0.0054 ± 0.0001
High UV-A (24 W m ⁻²)	0.00824 ± 0.00043	0.0235 ± 0.0007

*II. Effect of nutrients and UV irradiance on lipid accumulation and growth of *N. oculata**

From Experiments 1-4, it is evident that an increase in UV-A dose, by increasing either irradiance or duration of exposure, increases lipid:chl ratio in *N. oculata* cells. Experiment 5 was designed to test the combined effect of nutrients and UV-A on lipid accumulation. There were significant main effects of both nutrients and UV-A ($P < 0.0001$) on lipid:chl ratio in *N. oculata*. By day 7, the UV-A control treatment in low nutrients had significantly higher lipid:chl ratio when compared to the control grown in high nutrient media (Fig. 14). Similarly, the UV-A treatment in low nutrient media had greater lipid:chl ratio when compared to UV-A treatment in high nutrient media. There was a synergistic interaction between nutrients and UV-A ($P = 0.003$). There was a 108% and 140% increase over controls in lipid:chl ratio due to UV-A exposure in low and high nutrient media cultures respectively. The increase in lipid:chl ratio due to reduced nutrients in the control treatments was 40% with and without UV-A exposure. UV-A and nutrients decreased chlorophyll content significantly ($P = 0.001$) (Fig. 14). The data strongly suggest a trend towards synergistic interactive effect of UV-A and nutrients on chlorophyll content in *N. oculata* after 7 days of treatment ($P = 0.08$).

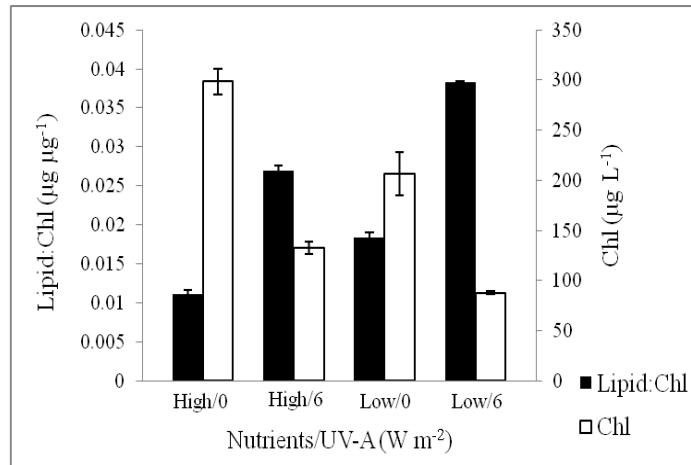


Fig. 14: Effect of high and low nutrient media along UV-A irradiance (6 W m^{-2} for 12 hrs) on lipid:chl ratio (black bars) and on chlorophyll concentration (white bars) on day 7 in Experiment 5 (mean \pm SE, $n = 3$).

Results of Experiment 6 using two different nutrient concentrations were similar to Experiment 5. Cultures in low nutrient media were compared to cultures grown in sea water (SW) media after 7 days of 6 W m^{-2} UV-A irradiance for 12 hrs each day. There was an overall significant effect of nutrients ($P = 0.001$) and UV-A ($P < 0.0001$) on lipid:chl ratio in *N. oculata* cultures. There was no effect of nutrients on lipid:chl ratio in the control treatments; this may be due to the small difference in the two media compositions (Fig. 15). However, UV-A treatments were significantly different from each other; UV-A treated cells in sea water media had higher lipid:chl ratio when compared to low nutrient media cells. There was a strong indication of synergistic effect of UV-A and nutrients on lipid:chl ratio in *N. oculata* ($P = 0.07$). There was a 180% and 144% increase over controls in lipid:chl ratio due to UV-A exposure in SW and low

nutrient media cultures respectively. Increase in lipid:chl ratio due to reduced nutrients along with UV-A exposure was 45%. Nutrients and UV-A had an overall significant effect on the chlorophyll content ($P < 0.0001$). The control treatments had higher chlorophyll content than the UV-A treatments (Fig. 15). The effect of UV-A on chlorophyll concentration was additive, not interactive.

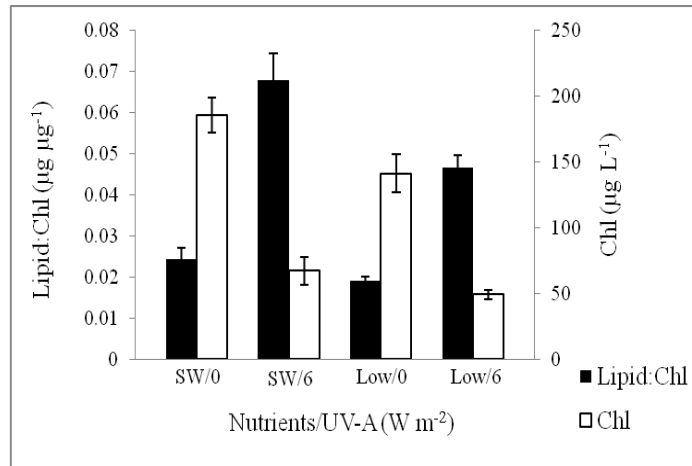


Fig. 15: Effect of low nutrient and sea water (SW) media along with UV-A irradiance (6 W m^{-2} for 12 hrs) on lipid:chl ratio (black bars) and chlorophyll concentration (white bars) on day 7 in Experiment 6 (mean \pm SE, $n = 3$).

There was an overall increase in lipid:chl ratio over time ($P < 0.0001$) with a significant interaction between day and treatments ($P < 0.0001$). UV-A treatment in SW and low nutrient media showed significant increase in lipid:chl ratio from day 2 to day 7. Control treatments for both the media types showed no consistent change in lipid:chl ratio over time (Fig. 16).

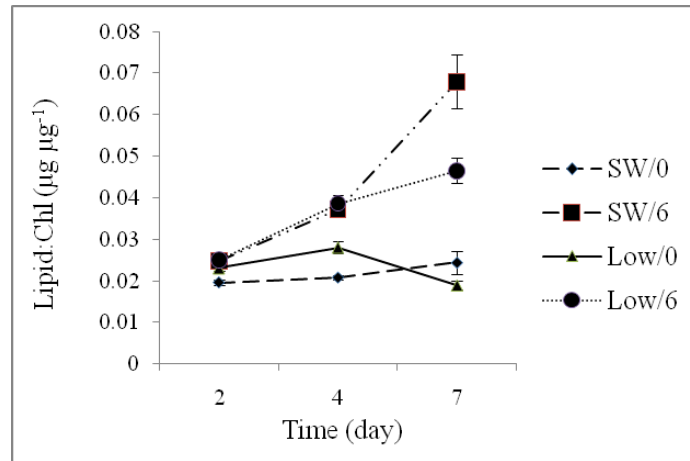


Fig. 16: Effect of time, nutrients and UV-A irradiance (6 W m^{-2} for 12 hrs) on lipid:chl ratio in Experiment 6 (mean \pm SE, $n = 3$). Experimental conditions as indicated in Fig. 20.

DISCUSSION

Effect of UV-A irradiation on N. oculata lipid ratio

We made two predictions for the first objective which was to study the effect of UV-A on lipid accumulation in *N. oculata*; first that UV-A will increase lipid:chl ratio, and second that lipid:chl ratio will be directly proportional to UV-A treatments employed in this experiment. The results corroborated our predictions. UV-A exposed cultures had a higher lipid:chl ratio after 7 days than the control. Further, an increase in UV-A irradiance, or duration of exposure, and hence UV-A dose generally resulted in greater lipid:chl ratio in *N. oculata* cells. For the second objective to examine reciprocity, irrespective of UV-A irradiance and duration of exposure, identical UV-A doses showed uniform lipid:chl ratio after 7 days. Thus, for the UV-A dose range utilized ($24 - 72 \text{ J m}^{-2}$) the concept of reciprocity cannot be rejected, which may or may not be true for doses outside the range used in these experiments.

As a follow up to the first objective, I attempted to determine whether the increase in lipid accumulation after UV-A exposure was due to an increase in structural lipid, storage lipid or both. Unfortunately, I was unable to confidently differentiate between storage lipids and structural lipids using Nile red, because of an overlap in the fluorescence peak region (Fig. 5). Storage and structural lipid standards did not yield two distinct fluorescent peaks when combined together and treated with Nile red. However, other studies indicate that in response to certain kinds of environmental stressors, there can be an increase in the ratio of storage to structural

lipids. For example, Forjan et al. (2010) showed using high performance liquid chromatography (HPLC) that UV-A increases the saturated: unsaturated fatty acids ratio, and hence an increase in TGs (storage lipids) in *N.gaditana*. UV-A can increase the saturated: unsaturated fatty acid ratio by accelerating the formation of intracellular free radicals and strong oxidants like peroxides (Halliwell & Gutteridge 1989) that cause reduction in long-chain PUFAs. Fatty acids with relatively few or zero double bonds, like most of saturated fatty acids and few unsaturated fatty acids, are resistant to oxidative attack, and their accumulation seemed to be largely unaffected by UV-A (Hessen et al. 1997).

The UV-A irradiances used in this experiment are within the range encountered by algae in the environment (Holzinger et al. 2006). UV-A penetration into aquatic ecosystems depends mainly on aerosols in the atmosphere and particulate organic carbon (POC) in the water column (Kazadzis et al. 2009; Hader et al. 2007). Aerosols control UV-A irradiance through various processes like scattering and absorption of the solar and Earth's radiation, or by changing atmospheric thermodynamics and cloud formation (Charlson et al. 1992; Rosenfeld & Lensky 1998). In Greece a 2% per decade increase in UV-A has been predicted due to changes in aerosol properties (Kazadziz et al. 2009). POC along with chromophoric dissolved organic matter strongly attenuates UVR in the marine environment (Tedetti et al. 2006).

UV-A penetration in the water column could affect the cell-specific mass of lipids, or the fatty acid ratio of *N.oculata* in the environment with potentially cascading effects on the foodweb. *Nannochloropsis* spp. are a good source of nutrition for marine organisms because of accumulation of EPA, a component of PUFAs. PUFAs are vital for maintaining somatic and population growth, and survival and reproductive success in invertebrates (Guschina & Harwood 2009), and a decrease in PUFAs as result of UV-A stress may also alter grazing preferences by

consumers. There is evidence that UV-A alone could determine the composition and biomass of the marine microbial community by altering primary production and predator grazing preferences by consumers (Ochs & Eddy 1998). Various UV-A mediated qualitative changes in phytoplankton have been reported that affect interactions among organisms in aquatic food-webs. These include changes in stoichiometry, cell biochemistry, and the fatty acid profile of phytoplankton, which are major determinants for food quality for aquatic herbivores (Hessen et al. 1997).

Microalgae are considered one of the potential sources of biodiesel because of their ability to accumulate a considerable amount of TGs (Li 2008). They can produce up to 250 times the amount of oil per acre as soybeans. Their growth rate has been estimated to be up to 50 times more than that of switch grass (*Panicum virgatum*), which is the fastest growing terrestrial plant (Li 2008). Biodiesel derived from oil crops is an alternative to petroleum fuels, but recent studies have showed that nitrogen fertilizers used to produce these crops contribute to global warming by N₂O emission (Chang & Yang 2003). High-efficiency CO₂ mitigation can be coupled with biofuel production as microalgae are tolerant of high CO₂ concentration. Due to their inexpensive nutritional requirements and their ability to accumulation TGs, *N. oculata* has potential to be an ecologically safe source of biodiesel.

Effect of UV-A on N. oculata biomass

We predicted that chlorophyll content, which was used as a measure of biomass, would decrease per unit biomass with an increase in UV-A dose. Results confirmed these predictions, and also that identical doses, regardless of irradiance or exposure duration, had a similar effect on chlorophyll content, and thus we were unable to falsify that reciprocity holds. We used chlorophyll content as a measure for cell biomass and growth rate because UV-A treated cells were larger in volume than the control treatment exposed to only PAR and therefore expressing data as lipid:cell would be inappropriate. In preliminary experiments, measurements of both chlorophyll fluorescence and cell count were made to ensure that the difference in cell number between control and UV-A treatments was also reflected in the chlorophyll fluorescence (Fig. 3). The UV-A treatments to which *N. oculata* cells were exposed in this experiment reduced their growth rate, but did not alter their chlorophyll content per cell. However, as UV-A treated cells were larger than control cells, probably due to elevated lipid storage, UV-A did result in a decrease in chlorophyll per cell volume.

UV-A radiation has been reported to inhibit the maximum effective quantum yield of photosystem II in microalgae (Forster & Schubert 1997; Hermann et al. 1997; Nilawati et al. 1997). UV radiation targets photosynthesis by damaging the D1 protein of photosystem II or the enzyme Rubisco causing loss of photosynthetic pigments as well as reduced expression of genes involved in photosynthesis (Holzinger et al. 2006). Decreased growth rates and increased cell

volume may be due to decoupling between cell growth and cell division in UV-A stressed *N. oculata* cells in our experiment.

Effect of nutrients on lipid accumulation in N. oculata

For the third objective we studied the combined effect of nutrients and UV-A on lipid accumulation in *N. oculata*. The effect of nutrients on lipid:chl ratio was evident from the analysis of control treatments grown in high and low nutrient media without UV-A exposure. Cultures in low nutrient media had significantly greater lipid:chl ratio compared to cultures grown in high nutrient media. The quantity and quality of lipids within *Nannochloropsis* cells vary as a result of changes in growth conditions (temperature and light intensity) or nutrient concentration. For example, *Nannochloropsis sp.* QII was shown to synthesize more than one-half of its cell dry weight in neutral or storage lipids when grown under nitrogen-deficient conditions (Suen et al. 1987). It has been reported that, nitrogen deficiency resulted in synthesis of nitrogen-poor compounds, such as lipids and carbohydrates as storage compounds or as extracellular compounds, instead of structural lipids (Xu et al. 2004). Production of PUFAs, including EPA, and their incorporation into TGs is different between species and also different during phases of growth within a species. For example, in *N. oculata* 68% of total EPA was accumulated as TGs at the end of the stationary phase in comparison to only 8% during the exponential growth phase (Sheeham 1998). It was concluded that in *N. oculata*, pathways responsible for partitioning of EPA into TGs are induced during transition from exponential to stationary phase. In other words, as the medium gets nutrient depleted and cells reach stationary phase, EPA decreases as it is increasingly incorporated into TGs. One possible explanation for an

increase in TGs under nutrient deficiency could have to do with the enzyme acetyl-coenzyme A carboxylase, an important enzyme in fatty acid synthesis. Acetyl-coenzyme A carboxylase depends on ATP and requires large amount of ATP for the process of fatty acid chain elongation and unsaturation (Hardwood 1988; Thompson et al. 1990). Reduced nitrogen in the medium will result in decrease in ATP and chain elongation and an eventual decrease in PUFAs.

UV-A exposure in both high and low nutrient media caused an increase in lipid:chl ratio when compared to the control treatments. Further there was a interactive effect of UV-A and nutrients on lipid:chl ratio. Lipid peroxidation is one of the likely explanations for an increase in the saturated: unsaturated fatty acid ratio in microalgae in response to UV-A. The other possibility that has been proposed is that UV radiation reduces nutrient uptake (Goes et al. 1994), which decreases ATP production thereby inhibiting long chain fatty acid formation; This mechanism would explain the interactive effect of UV-A and nutrients on lipid:chl ratio in *N. oculata* cells.

It is evident from this research project that UV-A exposure increases lipid:chl ratio in *N. oculata*. Decrease in essential PUFAs as a result of UV-A irradiance and reduced nutrients could be altering the nutritional quality and grazing patterns in the aquatic foodweb. On the other hand increase in TGs due to UV-A exposure and reduced nutrients could be made use of for mass cultivation of *N. oculata* for the production of biodiesel. Since UV-A exposure decreases the growth rate, subjecting cultures to UV-A irradiance and nutrient depletion after the cultures have attained a significant biomass can be an alternative approach to increase lipid accumulation per biomass. Therefore change in either PUFAs or TGs due to UV-A exposure in *N. oculata* will be of economic and ecological importance.

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