

CULTIVATING ALGAE TO GENERATE PUFAS, AND MACROMOLECULES  
USING STRUVITE AND STRESS FACTORS

by

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## DEDICATION

I would like to dedicate this to my family who's love and support help to carry me through all of the hard times. Especially my aunt and uncle Mari and Alvino Artelejo.

Thank you for everything love you both so much.

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## ABSTRACT

Agricultural and municipal wastewaters contain vital elements necessary for algal growth (i.e., N, P, Mg, K). One way to capture these elements, in an environmentally friendly way, is through struvite production ( $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ ), however, this process is costly to implement. High-value secondary commodities coupled to struvite production can enable implementation of this nutrient capture strategy for agricultural and municipal wastewater systems. In this study, struvite was tested as a nutrient source for cultivation of high-value algal biomass with the main focus on high-value lipids such as Eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). We compared the growth of microalgal strains *C. reinhardtii*, *N. oculata*, and *P. tricornutum* in two different growth media that include a standard media (Chu 13 and F2), and media supplemented with municipal struvite as the primary nitrogen and phosphorus source. A second experimental factor (temperature and nutrient stress) was used to evaluate stress effects on algal lipid production when struvite is used as the primary nutrient source for growth. Growth response, lipid content and quality, and nutrient sequestration were evaluated. Data indicate that all three species produce more or the same amount of biomass in struvite-amended treatments relative to controls, respectively (0.43 g/L struvite vs. 0.16 g/L in controls for *C. reinhardtii*, 2.76 g/L for struvite and 2.58 in controls for *P. tricornutum*, *N. oculata* control compared to struvite-amended treatments (0.15 g/L vs. 0.32 g/L, respectively). Lipid productivity and lipid profiles varied with respect to stress and media type as a function of algal strain. Overall, stress and media

had a limited effect on polyunsaturated fatty acids PUFA's production with linoleic acid being the only fatty acid to illustrate a significant response to nutrient stress in *N. oculata* (104.52 ug/mg compared to 5.84 ug/mg in struvite media). *C. reinhardtii* had a significant increase in linoleic acid in struvite amended media compared to standard media (13.2 ug/mg vs 7.96 ug/mg). *P. tricornutum* was the only strain out of the three to produce Eicosapentaenoic acid (EPA); however, while none of the treatments had a significant effect on EPA production, EPA was not produced in F2 under temperature stress. Based on the biomass composition of the algal we cultivated under our experimental conditions the highest market value of the biomass was a high-protein cattle feed. Although few lipids illustrate significant responses to our temperature and nutrient stresses, the directional trends in the responses observed across strains and lipids suggest future studies that limit single nutrients (i.e. N or P), rather than simultaneous limitation of N and P may result in more positive effects on yields of targeted lipids.

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## LIST OF ABBREVIATIONS

|       |                             |
|-------|-----------------------------|
| PUFAs | Polyunsaturated Fatty Acids |
| DHA   | Docosahexaenoic acid        |
| EPA   | Eicosapentaenoic acid       |
| DW    | Dry Weight                  |
| BSA   | Bovine Serum Albumin        |
| M+    | Municipal Plus              |

## INTRODUCTION

Microalgal biomass can be used for production of biofuels, plastics, and even food. The broad array of potential uses for algal biomass arises in part because of the broad species and strain diversity found in microalgae, their corresponding biochemical diversity, and the various macromolecules they can produce as a function of their growth (Chew et al., 2018). For example, algae can be a source of polyunsaturated fatty acids (PUFAs) which include Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) which can help reduce inflammation, protect against neuro-generative diseases, and maintain the membrane fluidity of the brain and retina (Ramesh Kumar et al., 2019). Microalgae are also adept at assimilating nutrients from a variety of water sources. As such, they can be used to clean wastewater produced from industries such as livestock farms, municipal resource recovery facilities, and industrial wastewater plants (Barbera et al., 2018). For example, one study found that algae was able to remove 80.3% of cadmium, and 66.3% of nickel from industrial wastewater (Khan et al., 2017). Algae can also be employed to treat nutrients from industrial and agricultural effluents. *Chlorella variabilis* was found to remove 78.17% of phosphate from textile effluent (Bhattacharya et al., 2017). Another study using *Chlorella vulgaris* removed 100% of ammonia nitrogen from dairy and meat processing wastewater (Lu et al., 2016). Combined these observations suggest that coupling algal cultivation systems to wastewater treatment can help minimize environmental pollution while potentially producing a secondary value added commodity (i.e. the algal biomass). This biomass can be used for different purposes

depending on the strain of algae. For example, *Chlorella* can be used as a food supplement as it contains all amino acids required by heterotrophs. Other strains are used for vitamins and different antioxidants (M. Bishop & M. Zubeck, 2012).

Although numerous studies have demonstrated that microalgae can be a renewable source for different commercial and food products, uncertainty remains in how to maximize the production of algae biomass, or algal bio-products, while minimizing inputs (Milano et al., 2016). Two key parameters of interest are the source and costs of nutrient and water inputs and the corresponding effects on biomass production rates and biomass quality (Slade & Bauen, 2013). Wastewater from dairies and by-products from agricultural and municipal wastewater treatment are being investigated as alternative sources of nutrients for microalgae cultivation with the goal of minimizing nutrient competition with traditional food crops such as soy and corn (Barbera et al., 2018). Wastewater from many different systems including agricultural systems are rich in the macronutrients required for algae cultivation (e.g. N, P, K) (Chew et al., 2018). For example, Idaho's dairy industry produces significant quantities of N, P, and K rich solid wastes. There are an estimated 425,000 dairy cows in the Magic Valley alone capable of annually producing as much solid waste as a city of 12 million people (Johnson, 2020). These solid wastes can be viewed as an opportunity to recover and reuse nutrients, including N and P (N.M. Moed et al., 2015) as well as carbon and energy via methane production (Milano et al., 2016). One means by which these nutrients can be captured is via precipitation to a solid mineral known as struvite (N.M Moed et al., 2015). Struvite is an N, P, K, and Mg containing mineral currently produced on commercial scales at some agricultural and municipal waste resource recovery facilities. For example, the city of



Boise has been producing struvite as part of a phosphorus recovery strategy since 2008. Struvite has also been shown to be able to supply the nutrients required for cultivation of algae (Davis et al., 2015).

Microalgae are often considered as a promising feedstock for biofuel production compared to more traditional land crops, due to their ability to produce up to 60% lipid content by dry weight (DW) compared to 5-7% from traditional land crops (As, 2019). Algae also tend to have higher growth rates, up to 200 times greater, than land plants. For example, high lipid-producing algae strains include *Chlorella vulgaris* (28-32% maximum lipid content per unit of dry weight), and *Nanochloris & Nanochloropsis* (20-68% maximum lipid content per unit of dry weight) (As, 2019), among others. *P. tircounutum*, *C. reinhardtii*, and *N. oculata* are also known for producing high concentrations of lipids when put under stressful conditions such as a temperature shock where there is a sudden decrease in temperature, or nutrient stress, typically induced by N or P-limitation (e.g. nitrogen (N) or phosphorus (P) limitation) (Sharma et al., 2012). Some of the same conditions that enhance lipid production noted in biofuel-based studies, can also induce production of high value lipids.

Past studies have shown that struvite can be used to replace major nutrients like nitrogen and phosphorus as well as grow a wide variety of algae strains, some of which include high PUFA producing strains (Davis et al., 2015). Therefore, struvite is seen as a potential cost-effective alternative to traditional nutrition sources for cultivation of microalgae and one that avoids nutrient competition for traditional food crops (Davis et al., 2015). For example, struvite can be used as the primary nutrient source for cultivation of *C. vulgaris* (Moed et al., 2015). When BBM medium was supplemented

with 721 mg/L of struvite, *C. vulgaris* produced 2.85 g/L lipids (Moed et al., 2015).

Cultures grown in crude struvite can also be as productive as those grown in traditional medium. *M. salina* cultivated using crude struvite for full phosphorus replacement along with a 1:1 N/P ratio replacement of nitrogen produced a higher cell density than using the standard L1 medium as measured by *chlorophyll a* absorbance (Liu et al., 2019). The increased productivity in a struvite-based medium vs. the standard medium could be from crude struvite providing trace metals needed for algal growth in addition to the required macronutrients (Davis et al., 2015).

Here we tested three different algal species known for producing PUFAs to determine if a municipal source of struvite can be used to grow these high lipids producing algal species and assess how struvite levels, and the elemental make-up of the struvite, affects the fatty acid composition of the microalgae (*i.e.* *C. reinhardtii*, *N. oculata*, *P. tricornutum*). We also applied stress to the cultures in the form of a 15°C temperature shock, and a nitrogen phosphorus nutrient deprivation with the goal of increasing PUFA production.

## MATERIALS AND METHODS

### Cultivation of Algae

Three algal strains were employed in these experiments *C. vulgaris* and *N. oculata* which were maintained in Chu 13 media, and *P. tricornutum* which was maintained in F/2 media. Algal strains were cultivated in 500 mL Erlenmeyer flasks (i.e. photobioreactors) filled with 250 mL of cultivation media and placed on a day:night cycle of 16:8 hr. Chu 13 media consisted of 2.0 mM KNO<sub>3</sub>, 0.41 mM MgSO<sub>4</sub>\*7H<sub>2</sub>O, 0.23 mM K<sub>2</sub>HPO<sub>4</sub>, 0.37 mM CaCl<sub>2</sub>\*7H<sub>2</sub>O, 0.027 mM FeNaEDTA, and Trace Elements (Furuhashi et al 2013). F/2 media consisted of 0.880 uM NaNO<sub>3</sub>, 0.036 mM NaH<sub>2</sub>PO<sub>4</sub>\*H<sub>2</sub>O, 0.106 mM Na<sub>2</sub>SiO<sub>3</sub>\*9H<sub>2</sub>O, trace metals, 0.1 nM vitamin B12 solution, 0.1 nM biotin vitamin solution, 2 nM thiamine vitamin solution, and 1 liter of non-pasteurized seawater (UTEX Culture Collection of Algae).

### Experimental Design

We determined if struvite can be used as the sole nutrient source for growth of these algal strains and for production of PUFAs, or if additional nutrient supplementation was required. Additionally, we determined the effect of supplemental nutrient amendments in struvite-based media on the lipid profile produced by each species. More specifically, all three algal strains were grown in 250 mL Erlenmeyer flasks with 125 ml of each treatment (n = 4). Control treatments consisted of the standard cultivation media. Experimental treatments for *C. vulgaris* and *N. oculata* consisted of crude struvite + Chu 13 backbone. The Chu 13 backbone consisted of all components needed to make Chu 13

with the exception of the N and P sources (i.e.  $\text{KNO}_3$ , and  $\text{K}_2\text{HPO}_4$ ). For *P. tricornutum* the standard media used is F/2. The experimental treatments consisted of crude struvite + F/2 backbone. The F/2 backbone included all nutrients except  $\text{NaNO}_3$ , and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ . The flasks mL were placed on a shaker until cultures reached stationary phase (i.e. approximately day 14).

### **Effects of Stress Conditions on Algal Lipid Yields**

#### Temperature Shock/Stress

Temperature shock of  $15^\circ\text{C}$  for 24 hours with constant shaking was applied to each treatment combination described above to determine temperature stress effects on lipid production as a function of algal strain and media type.

#### Nutrient Stress

Different levels of a nutrient stress were applied to each treatment combination described above by monitoring N content in the medium and modulating the duration of time cultures were held in stationary phase across all treatments using a factorial experimental design. Nutrient stresses were applied by collecting the biomass of each treatment combination via centrifugation (10 min at 5,000 RPM), discarding the cultivation media, washing the cells 2x in 40 ml of sterile 0.9% saline, and then resuspending the cells in the same media treatment in the absence of the nitrogen and phosphorus source for 4 days. Once cultures were reset they were placed back on the same day:night cycle of 16:8 hrs with constant shaking. This approach allowed us to assess both independent and interactive effects of media type and nitrogen/ phosphorus stress on lipid production.

### Nutrient Analysis

Initial nutrient content of each treatment and nutrient uptake rates were measured using Hach #8048 and Lovibond #535600 reagent kits. Nutrients measured included total dissolved phosphorus (TDP, Hach method 8190), nitrate (NO<sub>3</sub>-N, Hach method 8171), and ammonia (NH<sub>4</sub>-N, Hach method 10023). All samples were filtered using a 0.45 µm syringe tip filter prior to analysis. Absorbance was measured using an Aquamate spectrophotometer and nutrient content of each sample determined by comparison to a suite of known standard solutions for each analyte. To measure nutrient uptake by the algae, samples were taken at the start of the experiment, halfway through the experiment, and at the end. Nutrient uptake rates were calculated by taking the difference between time points divided by the time passed between sampling points:  $dx/dt=(X_t-X_o)/t$  and expressed as mg/L/d for each of the nutrients tested.

### Algal Growth Response

Biomass accumulation was determined by direct cell counts and absorbance. Direct cell counts were performed using a hemocytometer and absorbance was measured at 680 nm using a Thermo Helios Aquamate spectrophotometer (part no. 9423 AQA 2000E). Biomass measurements were taken every 48hrs to track algal growth. Biomass productivity was determined by endpoint measures of ash-free dry weight (AFDW). AFDW was measured on each culture to quantify total biomass produced and to enable analysis of the relative composition of the biomass via measures of total protein, total carbohydrates, and total lipid content.

### Carbohydrate Productivity

We employed a phenol-sulfuric acid method (Masuko et al., 2005) to determine the carbohydrate content of the algal biomass in each experimental treatment. Carbohydrate content was assessed at the end of the log phase of growth and at the end of each experiment on stationary phase biomass. Measurements were obtained using a Biotek Instruments Synergy Mx 96 well plate reader (part no. 7191000). To calculate carbohydrate productivity (mg/L/d) the difference in carbohydrate content between time points was measured and divided by the time between samples.

### Protein Productivity

Total protein content of the algal biomass was determined using the Lowry assay as described previously (Thermo Scientific modified Lowry kit). Briefly, algal biomass samples were centrifuged and lyophilized. 5 mg of the lyophilized biomass was treated with 24% trichloroacetic acid to extract and solubilize the protein in the sample and then the mg/L of protein determined by comparison to a Bovine Serum Albumin (BSA) standard using the Modified Lowry assay for total protein (ThermoFisher Scientific Part #232240).

### GC/MS Fatty Acid Composition

Lipid and fatty acid profile methods were adapted from Kukal et al. (2022). Briefly, to simultaneously extract lipids and convert them to fatty acid methyl ester derivatives (FAMES) the algal biomass was heat treated at 90°C for 90 min in a mixture of methanol, sulfuric acid, and chloroform, the latter of which contained a 1-naphthaleneacetic acid internal standard (Kukal et al., 2022). After heating, the samples were mixed with water and the organic fraction recovered. FAMES present in the organic

fraction were analyzed by GCMS on an ISQ-Trace1300 instrument (ThermoScientific) equipped with a ZB-5 (30 m x 0.25 mmØ, Phenomenex) capillary column and temperature program of 40 °C (1 min) to 250 °C (10 min) at the rate of 5 °C min<sup>-1</sup>. The detected FAMEs were identified by comparison to a C12 to C24 standard and palmitic acid-D31 (98%, Cambridge Isotope Laboratories). Detected FAMEs not present in the standard were identified by spectral matching to the 2017 NIST mass spectral library.

### Data Analyses

Statistical analyses were done using Bayesian generalized linear models. This was done on R using STAN. Packages used were Rstanarm using a normal distribution, and the normal default priories in the package as of 2022-04-08. In our analyses 0.90 confidence intervals that overlapped zero were considered to be significant (Goodrich et al. 2020). Linear models were designed to estimate the effect that media type, and stress had on biomass and macromolecule production.  $\beta_0$  was calculated by marginalizing across all possible combinations of treatments to calculate an intercept value representing the absence of all predictors. Models used 4 Markov chains that had ample coverage for  $\hat{R}$  values equal to 1.

## RESULTS

### **Media Effects on Growth ( $A_{680}$ ), Biomass Production (AFDW), and Growth Rates (k)**

We evaluated the growth ( $A_{680}$ ), biomass production (AFDW), and growth rates ( $k$ ) of *C. reinhardtii*, *N. oculata*, and *P. tricornutum* as a function of media type during a standard cultivation period of 12 days. Absorbance measures ( $A_{680}$ ) indicate that *C. reinhardtii* and *N. oculata* demonstrated more growth in the standard Chu 13 media relative to the municipal struvite-based media (M+). Whereas *P. tricornutum* grew equal amounts in the standard F2 media and the M+ treatment. In terms of biomass production, as measured by AFDW, there were no significant differences in biomass production between the standard media and the M+ treatments across all three species (Figure 3). However, all three organisms tended to yield higher biomass (i.e. mean AFDW levels) in the M+ treatment (Figure 3). Similarly, there was no statistically significant difference in growth rates between media types within each organism. *C. reinhardtii* in standard chu13 had a growth rate of 0.31 vs 0.29 (p value=0.38). *N. oculata* in chu13 had an average growth rate of 0.36 vs 0.32 in M+ media (p value=0.50). For *P. tricornutum* the average growth rate in standard F2 was 0.22 vs 0.27 in M+.

### **Stress Effects on Growth ( $A_{680}$ ) and Biomass Production (AFDW)**

Absorbance measures of biomass production indicate that there is an interaction between stress and algal species on yield (Figure 1). All statistical results from our linear models were compared to a marginalized mean. A marginalized mean is defined as a



method that requires summing over the possible values of one variable to determine the marginal contributions of another (Brooks-Bartlett, 2018). Temperature and nutrient stresses were applied at day 11 and 9 in each species treatment, respectively. Under both stress regimes each algal strain reached the stationary phase by day 12 of the experiment (Figure 1). The highest overall biomass yields were observed in the control (i.e. no stress treatment) cultures of *N. oculata* grown in chu 13 and in the M+ struvite supplemented media based on absorbance. However, based on AFDW yield estimates of the three strains, *P. tricornutum* had the highest overall yield in biomass production (Figure 3), and there was no significant difference between the M+ 2.76 g/L and F2 2.57 g/L treatments. In standard chu13, *C. reinhardtii* produced 0.16 g/L, and *N. oculata* produced 0.15 g/L of biomass. In the M+ media, the average biomass production for *C. reinhardtii* increased to 0.43 g/L and to 0.32 g/L for *N. oculata*.

Temperature and nutrient stress affected the median biomass produced in M+ media, relative to the standard media for each organism tested (Figure 4). However, the direction and magnitude of the effect was species dependent. *P. tricornutum* and *N. oculata* both produced more biomass in the M+ media under nutrient stress relative to expected yields. Whereas *C. reinhardtii* produced less biomass in the M+ media relative to control under nutrient stress. More specifically, *P. tricornutum* produced 3.52 g/L biomass when nutrient stress was applied, relative to 3.17 g/L produced in the F2 standard media under the same nutrient stress, an increase of approximately 0.35g/L (pd=0.997). Under nutrient stress *N. oculata* produced 0.055 g/L of biomass in M+ vs. 0.44 g/L in the control Chu treatment. Our model indicates that there is a 96% chance that the true effect of nutrient stress of *N. oculata* in M+ media is negative and decreases

biomass production by a coefficient of approximately -0.3g/L. Conversely, based on our data *P. tricornutum* under nutrient stress in M+ there is a 99.7% chance that the true effect is positive with biomass production being 3.52 g/L, an increase of approximately 0.7 g/L. While an increase in biomass production in the standard media under nutrient stress is observed for *P. tricornutum* (i.e. 3.16 g/L), based on the linear model it is not enough to be considered significant (supplementary figures 1). There was not a significant effect of the nutrient stress on *C. reinhardtii* biomass production. *C. reinhardtii* produced an average biomass of 0.15 g/L and 0.09 g/L in Chu and M+ under nutrient stress, respectively.

In terms of temperature stress on biomass production, none of the three strains tested produced a significantly different amount of biomass under the temperature stress relative to expected biomass yields. More specifically, *P. tricornutum* cultures subjected to temperature stress produced 2.84 g/L. *N. oculata* biomass yield under temperature stress in M+ was 0.475 g/L. In chu13 *N. oculata* produced 0.44 g/L during temperature stress. *C. reinhardtii* average biomass yield during temperature stress for chu and M+ media was 0.36 g/L, and 0.39 g/L, respectively.

### **Effects of Species, Media Type, and Stress on Algal Lipid Production (Fatty Acids and Poly-Unsaturated Fatty Acids (PUFAs)):**

Fatty acid production (types and amounts of fatty acids produced) varied between species, media, and stress treatments. The direction and magnitude of media and stress treatment effects also varied by organism. This indicates that individual algal strains respond differentially to our experimental treatments. We expressed concentrations of

each lipid produced as  $\mu\text{g}/\text{mg}$  of AFDW and tested for treatment effects using linear modeling and calculate estimations of treatment effects.

There were 8 unique fatty acids detected across all treatments, with two of those being PUFA's (i.e. linoleic acid and eicosapentaenoic acid). Production of linoleic acid (C18:2) was observed in all three strains, but C20:5 (Eicosapentaenoic acid) was only observed in *P. tricornutum* (Figure 5). *N. oculata* produced linoleic acid in non-stress conditions at 10.66  $\mu\text{g}/\text{mg}$  and 5.84  $\mu\text{g}/\text{mg}$  in Chu13 and M+ media treatments, respectively. Although these mean yield values trended differently, no significant difference was detected between media treatments based on our linear model (supplementary figure 10). Conversely, there was a significant yield difference in linoleic acid produced by *C. reinhardtii* in response to media type (7.96  $\mu\text{g}/\text{mg}$  and 16.37  $\mu\text{g}/\text{mg}$  in Chu13 vs. M+, respectively) (supplementary Figure 11). The median estimated effect of the no stress M+ media was a 8.9  $\mu\text{g}/\text{mg}$  increase in C18:2 production by *C. reinhardtii*. Of the three strains tested *P. Tricornutum* had the lowest overall yield in C18:2 production and no significant effect of media type on C18:2 production was observed (0.72  $\mu\text{g}/\text{mg}$  and 0.34  $\mu\text{g}/\text{mg}$  in F2 and M+ media, respectively).

When we applied the nutrient stress treatments, lipid yields were affected in a media and strain dependent fashion. *P. tricornutum* produced 0.38  $\mu\text{g}/\text{mg}$  of C18:2 in the M+ media when the nutrient stress was applied, relative to 0.69  $\mu\text{g}/\text{mg}$  produced in the F2 standard media under the same nutrient stress, however this difference was not significant based on our linear modeling. Under nutrient stress *N. oculata* produced 104.52  $\mu\text{g}/\text{mg}$  of C18:2 in M+ vs. 13.34  $\mu\text{g}/\text{mg}$  in the standard Chu 13 media, an increase of approximately 91.34  $\mu\text{g}/\text{mg}$  (supplementary figure 10). Lastly, *C. reinhardtii* under

nutrient stress produced 8.72 ug/mg vs 4.78 ug/mg of C18:2 in the Chu 13 and M+ media, respectively. Although an increase in C18:2 production by *C. reinhardtii* was observed in the standard media under nutrient stress, based on the linear model it was not enough to be considered significant (supplementary figure 11).

Temperature stress also affected lipid yields in a media and strain dependent fashion. However, in response to the temperature stress all three strains tested tended to produce more C18:2 in their respective standard media vs. the M+ media. More specifically, *P. tricornutum* cultures subjected to temperature stress produced 1.52 ug/mg in standard F2 media while in M+ an average C18:2 of 0.48 ug/mg was observed. *N. oculata*'s C18:2 yield under temperature stress in M+ was 4.39 ug/mg compared to 7.99 ug/mg in Chu13. The average C18:2 yield from *C. reinhardtii* during temperature stress was 5.38 ug/mg and 1.44 ug/mg in Chu13 and M+ media, respectively.

As stated earlier, eicosapentaenoic acid (C20:5), was only produced in the *P. tricornutum* treatments (Figure 5). The production of this lipid varied by media type and by stress treatment. In the standard F2 media, *P. tricornutum* produced 9.65 ug/mg vs. 10.24 ug/mg in M+. When applying nutrient stress, the average eicosapentaenoic acid production increased in the standard F2 media to 16 ug/mg and decreased in the M+ media to 6.50 ug/mg. When applying temperature stress only *P. tricornutum* in the M+ media produced eicosapentaenoic acid, at a level of 11.43 ug/mg. Linear modeling indicates that while the average C20:5 production decreased in the M+ media under nutrient stress by a factor of -1.70 ug/mg the effect was not significant. Similarly, the increase in C20:5 by a coefficient factor of 7.63 ug/mg in the standard media treatment under nutrient stress condition was not significant.

Palmitic acid production varied between species, media, and stress treatments. *P. tricornutum* was affected the most by the experimental treatments. In standard media, the average palmitic acid production in *P. tricornutum* was 25.77  $\mu\text{g}/\text{mg}$  compared to 8.30  $\mu\text{g}/\text{mg}$  in M+ in the no stress treatments. Based on our data there is a 97.6% probability that the effect of standard media with no stress is positive. Whereas, the M+ no stress treatment had the exact opposite effect with there being a 97.5% probability it had a negative effect on palmitic acid production. Under nutrient stress conditions, palmitic acid production decreased from 17.09  $\mu\text{g}/\text{mg}$  in F2 to 9.88  $\mu\text{g}/\text{mg}$  in M+. Under temperature stress palmitic acid production increased to 29.18  $\mu\text{g}/\text{mg}$  in the standard media cultures (i.e. F2 media). Bayesian modeling indicated that there is a 99.8% chance that the standard media with a temperature stress will have a positive effect on palmitic acid production by *P. tricornutum*. Conversely, no significant effect was seen in the M+ treatment under the temperature stress.

Out of all treatments and media types, nutrient stress in standard media was the only one to have a significant effect on palmitic acid production by *C. reinhardtii* (supplementary figure 15). *C. reinhardtii* had an average palmitic acid production of 23.51  $\mu\text{g}/\text{mg}$  in standard media and 7.24  $\mu\text{g}/\text{mg}$  in M+ media. Under the nutrient stress, mean palmitic acid production was 30.62  $\mu\text{g}/\text{mg}$  an increase by a factor of 5.07  $\mu\text{g}/\text{mg}$ . Conversely, media type and stress treatment had no significant effect on palmitic acid production by *N. oculata* (supplementary 14).

Stearic acid had a production level of 2.03  $\mu\text{g}/\text{mg}$  in Chu13 vs 0.86  $\mu\text{g}/\text{mg}$  in M+ for *C. reinhardtii* under no stress conditions. For nutrient and temperature stress conditions, the average stearic acid production was 2.65  $\mu\text{g}/\text{mg}$  and 1.86  $\mu\text{g}/\text{mg}$  in

Chu13, respectively. A decrease in production was observed in the M+ media to 1.59  $\mu\text{g}/\text{mg}$  and 1.44  $\mu\text{g}/\text{mg}$  for the same treatments, respectively. For *N. oculata* stearic acid was only produced in the M+ media and only at low levels (Figure 5). In the absence of stress 0.34  $\mu\text{g}/\text{mg}$  was produced and in the temperature stress, 0.31  $\mu\text{g}/\text{mg}$  was produced. When *N. oculata* is under nutrient stress only the standard media produced stearic acid (2.39  $\mu\text{g}/\text{mg}$ ). For *P. tricornutum* very low levels of stearic acid were produced in the M+ media (0.07  $\mu\text{g}/\text{mg}$ ). However, when nutrient stress conditions were applied, stearic acid levels increased to 0.12  $\mu\text{g}/\text{mg}$  in M+ and 0.22  $\mu\text{g}/\text{mg}$  in F2. Conversely, stearic acid levels were much higher when temperature stress was applied in the F2 media at 3.91  $\mu\text{g}/\text{mg}$ . While an increase in stearic acid production by a factor of 1.08  $\mu\text{g}/\text{mg}$  was observed, per our linear model when the temperature stress was applied to F2 media there was not a significant effect on stearic acid production ( $p=0.93$ ).

Oleic acid average production was 32.69  $\mu\text{g}/\text{mg}$  in *C. reinhardtii*, 38.81  $\mu\text{g}/\text{mg}$  in *N. oculata*, and 9.99  $\mu\text{g}/\text{mg}$  in *P. tricornutum* in standard media under no stress conditions. For M+ media *C. reinhardtii* produced 15.03  $\mu\text{g}/\text{mg}$ , *N. oculata* 65.24, and *P. tricornutum* produced 0.60  $\mu\text{g}/\text{mg}$ . Between the three strains, media only had a significantly positive effect on *N. oculata*. In the case of *N. oculata*, per our linear model the application of M+ media with no stress will have a positive effect on oleic acid production 95% of the time. Under the temperature stress treatment *C. reinhardtii* produced 35.58  $\mu\text{g}/\text{mg}$  in Chu13 and 31.45  $\mu\text{g}/\text{mg}$  in M+. *N. oculata* produced 32.73  $\mu\text{g}/\text{mg}$  of oleic acid in Chu13 and 63.48  $\mu\text{g}/\text{mg}$  in M+. The average oleic acid production response for *P. tricornutum* under temperature stress was 5.89  $\mu\text{g}/\text{mg}$  in F2 and 0.96  $\mu\text{g}/\text{mg}$  in M+. When *C. reinhardtii* was exposed to nutrient stress the average oleic acid

production was 38.96  $\mu\text{g}/\text{mg}$  in Chu and 31.96  $\mu\text{g}/\text{mg}$  in M+. For *N. oculata* 93.60  $\mu\text{g}/\text{mg}$  was produced in Chu13 and 1.15  $\mu\text{g}/\text{mg}$  in M+ under the nutrient stress. Indeed, nutrient stress applied to *N. oculata* had a strong effect in both media types. In the standard media, applying a nutrient stress will have a positive effect on oleic acid production in *N. oculata* 96% of the time. For the M+ treatment when that same stress is applied, oleic acid is reduced by a factor of 35  $\mu\text{g}/\text{mg}$  leading to a significant reduction (supplementary Figure 20). *P. tricornutum* produced 4.15  $\mu\text{g}/\text{mg}$  and 1.92  $\mu\text{g}/\text{mg}$  of oleic acid in F2 and M+, respectively. Based on our linear model, media nutrient stress did not have a significant effect on oleic acid production by *P. tricornutum*.

Myristic Acid was only produced by *P. tricornutum* in this experiment. The no stress conditions produced 6.83  $\mu\text{g}/\text{mg}$  in F2 and 4.60  $\mu\text{g}/\text{mg}$  in M+. Average production under nutrient stress conditions was 5.39  $\mu\text{g}/\text{mg}$  in F2 and 4.12  $\mu\text{g}/\text{mg}$  in M+. Temperature stress induced a response of 8.88  $\mu\text{g}/\text{mg}$  in F2 and 4.54  $\mu\text{g}/\text{mg}$  in M+. Per our linear model none of the treatment combinations had a significant effect on myristic acid (supplementary figure 23). Two additional lipids were only produced by *P. tricornutum* in F2 media under temperature stress conditions (i.e. behenic acid 8.36  $\mu\text{g}/\text{mg}$  and lignoceric acid 0.81  $\mu\text{g}/\text{mg}$ ).

#### **Effects of species, media type, and stress on carbohydrate and protein production:**

Carbohydrate and protein production varied by species, media type and stress treatment. The direction and magnitude of media and stress treatment effects also varied by organism. Indicating that the production of these two biomass components in the individual algal strains can respond differentially to our experimental treatments. We expressed carbohydrate and protein concentrations as mg of carbohydrate or protein per

mg of AFDW and tested for treatment effects using linear modeling and bayesian estimations of treatment effects.

When grown in standard media in the absence of a nutrient or temperature stress the two green algae strains, *C. reinhardtii*, and *N. oculata*, both produced an average of 0.10 mg of carbohydrate per mg of dry weight (mg/mg). In struvite amended media (M+) the average carbohydrate production increased for both strains. For *C. reinhardtii* the average carbohydrate production increased to 0.18 mg/mg; *N. oculata* average carbohydrate production increased to 0.29 mg/mg in M+ media (Figure3). Carbohydrate concentrations were analyzed by linear modeling to determine if the direction of effects were significant and what the magnitude of treatment effects were. Our linear modeling indicated that *C. reinhardtii* produced a 0.06 mg/mg lower carbohydrate concentration in standard media with no stress (temperature or nutrient) relative to the other treatments (pd=0.973) (supplementary figure 5). Similarly, *N. oculata* produced 0.08 mg/mg less carbohydrate in standard media in the absence of stressors relative to the average expected response (pd=0.990). Conversely, the M+ no stress treatment had a significant positive effect on carbohydrate production by *N. oculata*, increasing average production by 0.09 mg/mg (pd=0.995) (supplementary figure 6). *P. tricornutum* in standard media had an average carbohydrate content of 0.23 mg/mg, while in M+ media, the average carbohydrate content decreased to 0.05 mg/mg (figure 6). Based on our linear modeling *P. tricornutum* grown in standard media increased carbohydrate production by 0.06 mg/mg of (pd=0.973). Whereas, in M+ media the same strain had a significantly decreased carbohydrate production by 0.08 mg/mg of AFDW (pd=0.986) (supplementary figure 4).



Nutrient stress affected the carbohydrate produced in M+ media, relative to the standard media for each organism tested (Figure 7) and the effect of nutrient stress varied by organism and media type. In the M+ media under nutrient stress conditions, *C. reinhardtii* increased its average carbohydrate production to 0.29 mg of carbohydrate/mg of AFDW. For *N. oculata* under nutrient stress the average carbohydrate production slightly decreased to 0.25 mg/mg compared to the no stress conditions. When the nutrient stress was applied in the standard media *C. reinhardtii* produced 0.21 mg/mg of carbohydrates, an increase compared to the no stress treatment in the same media. For *N. oculata* an increase in carbohydrate production was observed with a mean carbohydrate yield 0.23 mg of carbohydrate/mg of AFDW. Based on our linear modeling, applying a nutrient stress while in M+ media results in a significant increase in carbohydrate production by both *C. reinhardtii* and *N. oculata* ( $p = 0.999$  and  $p = 0.967$ , respectively). For *P. tricornutum* in the M+ media, a nutrient stress resulted in an increase in the average carbohydrate content to 0.14 mg/mg compared to no stress (Figure 7). With that same nutrient stress applied to *P. tricornutum* in the F2 media the average carbohydrate content was 0.18 mg of carbohydrate/mg AFDW.

Temperature stress also affected the median carbohydrate produced in M+ media, relative to the control media for each organism tested (Figure 7) and the effect of temperature stress varied by organism and media type. In M+ media temperature stress decreased the average carbohydrate content of *C. reinhardtii* to 0.11 mg/mg. In response to the same treatment combination the carbohydrate concentration in *N. oculata* was not different from the no-stress control (Figure 7). Applying temperature stress to *P. tricornutum* led to a decrease in average carbohydrate production to 0.17 mg/mg in

standard media. Conversely, in M+ there was no change in average carbohydrate production by *P. tricornutum* compared to the no stress treatment 0.05 mg/mg.

Protein production varied by algal strain and media type. *P. tricornutum* had the highest protein production across all treatments and strains and produced a significantly higher protein content in the M+ media (pd=.995) (supplementary Figure 7).

Specifically, *P. tricornutum* produced 0.60 mg/mg of protein in the no-stress M+ treatment vs. 0.40 mg/mg in the standard F2 media (Figure 8). *N. oculata* had a lower protein concentration in the M+ media compared to the standard Chu media, 0.32 mg/mg in Chu vs. 0.26 mg/mg in the M+ treatment. *C. reinhardtii* produced an average protein content of 0.46 mg/mg in standard media and 0.48 mg/mg in M+ treatments, respectively. Unlike for the diatom, no significant differences were observed between the standard media and M+ for both green microalgae strains tested under no stress conditions.

Protein concentrations responded to the stress treatments as a function of stress type, algal strain and media type. For *C. reinhardtii* when the nutrient stress was applied to the standard media treatments, the average protein production (0.52 mg/mg) was higher than that observed in the non-stressed treatments. For the M+ treatment average protein content was lower than in the absence of the stress, 0.37 mg/mg. Linear modeling indicated that while applying a nutrient stress to *C. reinhardtii* in standard media protein production increased by 0.07 mg/mg. However, the stress treatments did not have a significant impact on protein content (Supplementary figure 8). When applying a nutrient stress to *N. oculata* in standard media, average protein content decreased to 0.22 mg/mg . However, in M+ media the average protein content increased to 0.56 mg/mg under the same stress conditions. Linear modeling indicated that when a nutrient stress is

applied, the average protein content for *N. oculata* in M+ increased by a coefficient of 0.19 mg/mg (pd=0.999). *C. reinhardtii* in the M+ treatment had a lower average protein content of 0.37 mg/mg under nutrient stress. Compared to the marginalized mean, where an increase in protein content was observed under the same nutrient stress (0.52 mg/mg). However, based on our linear modeling there was not a significant difference in protein yield between the two media types under nutrient stress. *P. tricornutum* in standard media saw a significant decrease in the protein content by a factor of 0.18 mg of protein/mg of AFDW when exposed to the nutrient stress (pd = 0.996). For *P. tricornutum* in M+ media under the same stress the average protein content was 0.38 mg of protein/mg of AFDW, a decrease in protein concentration relative to the no-stress condition in M+ media.

Under temperature stress and in standard media the average protein content by *N. oculata* was 0.30 mg of protein/mg of AFDW and 0.19 mg of protein/mg of AFDW in M+. For the M+ treatment the temperature stress had a significant effect, reducing protein content by 0.12 mg of protein/mg of AFDW (supplementary figure 9). Conversely, protein content of *C. reinhardtii* did not respond to the temperature stress in either media type. More specifically, the average protein content of *C. reinhardtii* in the temperature stress treatment was 0.40 mg of protein/mg of AFDW in the M+ media and 0.42 mg of protein/mg of AFDW in the standard media. *P. tricornutum* had a significant response to the temperature stress in both media types. In standard media the mean protein content was 0.20 mg of protein/mg of AFDW, a reduction in protein production by a coefficient of -0.17 mg of protein/mg of AFDW, relative to the no-stress control (pd=0.987). An opposite effect is seen with M+ where the protein content increased to

0.62 mg of protein/mg of AFDW, an increase of 0.20 mg of protein/mg of AFDW relative to the no-stress treatment (pd=0.996).

## DISCUSSION

There is a scarcity of studies exploring the utility of struvite as a source of nutrients for algal production, and to the best of our knowledge none that explore the combination of struvite as a source of nutrients and that simultaneously explored effects of temperature and nutrient stress on high-value lipid production. Therefore, here we compare our results to related work in the literature exploring the utility of wastewater for production of *C. reinhardtii*, *N. oculata*, and *P. tricornutum* and, when available, compare our findings to studies using similar organisms and struvite as a nutrient source. Whenever possible we compared our findings to studies where the starting nutrient concentrations and incubation conditions were similar to our work.

To the best of our knowledge, there are no other experiments using struvite to cultivate *C. reinhardtii*, and *N. oculata* in the literature. However, there are other works that employed wastewater as the primary source of nutrients for cultivation of these species. Biomass yields and first order productivity rates ( $k$ ) of *C. reinhardtii* that we observed in modified chu 13 were similar to those observed in other studies employing wastewater as a nutrient source. More specifically, in our study *C. reinhardtii* had a  $k$  value of 0.31 with a biomass density of 1.34 g/L. Similarly, Fields et al. measured *C. reinhardtii* productivity ( $k$ ) at 0.25 and biomass density of 1.76 g/L (Fields et al., 2018). In our study, *N. oculata* had a growth rate of 0.36 in Chu 13 and 0.324 in M+ and biomass yields were 0.975 g/L in standard Chu media and 0.715 g/L in M+ in the absence of a stress treatment. One study that looked at applying temperature stress to *N.*

*oculata* for 14 days at 15° C had a growth rate of 0.06 (Converti et al., 2009). This was far lower than what we achieved by only applying the stress for 24 hours. Another study looking at N starvation found dry weight to be approximately 0.55 g/L while in our experiment an average of 0.51 g/L in standard media was seen. They also had 13.1% DW of protein and 21 % DW of carbohydrates. In our experiment we had 10.3% carbohydrates and 9.64% protein (Ma et al., 2016). Carbohydrate content was found to be 10.3% by mass in chu and 28.5% by mass in the M+ treatment. Our growth rates are similar to another study that employed F2 as the standard media, however our biomass yields were lower, and our carbohydrate yields were higher. More specifically when cultivated on F2 media and F2 media supplemented with nutrient rich wastewater, *N. oculata*'s growth rate was found to be 0.37 and dry weight to be 2.41 g/L with a carbohydrate content of 1% ww (Reyimu & Özçimen, 2017). When Reyimu and Ozcimen (2017) supplemented nutrients with 25 to 100% wastewater their biomass yield decreased to 1.19-0.975 g/L and the growth rate was between 0.1248 and 0.5430 with a carbohydrate content ranging between 2.11 and 2.39% depending on the percentage of wastewater added (Reyimu & Özçimen, 2017).

For our control cultures we let the cultures grow for 12 days. During that time, *P. tricornutum* produced a dry biomass of 30.03 g/L. Other experiments found that dry biomass produced by PT was 0.50 g/L (Polishchuk et al., 2015). One explanation as to why our work shows such a dramatic increase in dry mass could be due to the differences in experimental conditions and in the methods employed to determine biomass yields. Methods used for gathering dry weight and ash-free dry weight also differed between our study and others. Song et al. used glass microfiber filters to filter the biomass from the

cultivation media prior to measuring AFDW. Whereas, in our study, we employed an AFDW method that involved drying down an entire sample and measuring AFDW on the dried sample. The method we employed can retain salt from the media, which can have a hygroscopic character after the drying step. The net effect can result in an apparently higher measure of AFDW. Growth rate of *P. tricornutum* tends to vary between experiments but tends to be slower in struvite-based media, both in our experiments and in work reported by others. One study found a growth rate in F2 media to be  $0.68 \text{ d}^{-1}$  (Song et al., 2020) while ours were  $0.23 \text{ d}^{-1}$ . Another study that looked specifically at how *P. tricornutum* would grow using struvite found productivity to be around 0.2 in L1 medium. While in crude struvite productivity was approximately 0.4 while we measured it to be 0.27 (Davis et al., 2015).

### **Media Effects on Lipid Production**

The form and amount of available nitrogen present in a growth medium can affect the type and amount of lipids produced by numerous algal strains, including those tested here (Y. Li et al., 2008). In this experiment, the M+ media and the two standard media contained similar initial nitrogen concentrations but differed in the available nitrogen source. The primary nitrogen source in the M+ treatments was ammonium ( $\text{NH}_4^+$ ), while the two standard media contained nitrate ( $\text{NO}_3^-$ ). There was a species dependent response to this difference in available nitrogen species that influenced the type and amount of lipids produced. For example, in *N. oculata* linoleic acid production, one of our PUFAs of interest, was not influenced by media type (Figure 5, Supplementary Figure 10). Further, the overall lipid profile of *N. oculata* did not differ between the two media types (Figure 5). Similar results have been observed in experiments using wastewater

generated from paper production to cultivate *N. oculata* (Polishchuk et al., 2015). More specifically, and similar to our study, there was no difference in linoleic acid production, and the overall lipid profile between the wastewater media and the artificial saltwater media, which contained  $\text{NH}_4^+$  and  $\text{NO}_3^-$  as the primary N-sources, respectively (Polishchuk et al., 2015).

Conversely, when *C. reinhardtii* was grown in M+ media with  $\text{NH}_4^+$  as the primary nitrogen source, there was an increase in linoleic acid (Figure 5). However, total lipid content was only 1.84% of the total cell dry weight. This is far lower than what others have observed with the same organism and N-source. For example, when swine wastewater, which is rich in  $\text{NH}_4^+$ , was used to cultivate *C. reinhardtii* the final biomass had a lipid content of 21.7% (Hasan, 2014). One potential reason for our lower than expected lipid yield was that, unlike Hasan, where total lipids were measured, we only performed a FAME analysis due to having limited biomass to analyze. As such our estimates of total lipid yield in each of our treatments was generated by summing the total detected FAMES and is therefore likely an underestimate of what was present in the *C. reinhardtii* treatments.

*P. tricornutum* had a higher overall lipid content in standard media (1.85% DW) compared to the M+ (0.83% DW). This could also be due to the fact we didn't directly measure total lipids. Looking at the lipid profile of cultures grown in standard media *P. tricornutum* had 25.5  $\mu\text{g}/\text{mg}$  of palmitic acid (C16:0) while the M+ cultures had 8.30  $\mu\text{g}/\text{mg}$ . Wang et. al. compared F2 and wastewater mixed with seawater as cultivation media for *P. tricornutum*. They found that total lipid yields between the two treatments were not significantly different. When quantifying the lipid profile between the two



treatments they found C16:0 was higher in standard F2 compared to the wastewater-based media. They also found that other lipids, that we weren't able to quantify, were higher in wastewater media compared to F2 (Wang et al., 2019). This difference in lipid profile due to lipids that we weren't able to quantify and our inability to directly measure total lipids could explain the difference we see between standard and M+ media in this experiment.

### **Effects of Nutrient Stress on Lipid Yield and Lipid Profile**

Under N-limitation, algae often accumulate carbon storage compounds (either lipids or carbs) (Benvenuti et al., 2015). Similarly, under N or P limitation *C.reinhardtii*, *N. oculata*, and *P. tricornutum* have been shown to also shift biomass productivity away from growth (i.e., increase in cell number) and towards accumulation of lipids (Sharma et al., 2012). In our experiment, the nutrient stress we applied in our struvite-based media treatment necessarily limited both N and P simultaneously. As our nutrient stress was applied by removing the cells from their cultivation media, washing them, and resuspending them in the same media composition without struvite. Given that struvite is a mineral that contains both N and P, our nutrient stress treatments limited both N and P simultaneously. The combined limitation of N and P in our treatments may be one of the drivers of the varied species and treatment-specific responses in individual lipid production we observed. For example, in *C. reinhardtii* the only fatty acid that showed a significant response to the nutrient stress was palmitic acid (C16:0). Other studies that looked into how a combined nutrient stress (i.e. simultaneous limitation of nitrogen and phosphorus) on *C. reinhardtii* found that the lack of these nutrients does not necessarily increase lipid production as is commonly observed with single nutrient limitation

(Kamalanathan et al., 2016). Nutrient stress had a varied effect on *N. oculata* where in certain lipids such as linoleic acid had a positive effect. Oleic acid had a significantly negative response to nutrient deprivation. Similar results have been seen in other studies looking at nitrogen stress where there is an increase in certain fatty acids and not others (Converti et al., 2009); (Roleda et al., 2013). Nutrient stress didn't seem to influence lipid production in *P. tricornutum*. This could be due to a lack of phosphorus as other studies have shown that just a N deficient media *P. tricornutum* was able to accumulate a high oil content (Yodsuwan et al., 2017).

### **Effects of Temperature Stress on Lipid Yield and Lipid Profile**

Prior work has demonstrated that exposure to temperatures below the optimum growth temperature can induce elevated levels of PUFAs in certain algal species. More specifically, when *P. tricornutum* was grown at 15°C for 16 days it had significantly higher DHA/EPA content than control cultures grown at 20°C and 25°C (Qiao et al., 2016). Conversely, others have demonstrated that a short duration increase in cultivation temperature can increase the lipid mass fraction in *C. reinhardtii* by 74% when cultures were placed in 30°C for 6 days (Li et al., 2021). In our work we were primarily interested in determining if we could modulate cultivation conditions to induce higher levels of PUFA production in the three algal strains tested here. Based on the work by Qiao et al (2016) we applied a cold stress of 15°C for 24 hrs. We applied the same treatment level and duration to all three algal species to enable a direct comparison of treatment effects across all three species tested. The short-duration temperature shock employed here was intended to determine if a similar response as noted by Qiao et al, could be induced with a shorter duration exposure and to determine if the response varied by algal species.

Similar to the nutrient stress treatment, we observed a species and media-dependent response to our temperature shock treatment. More specifically, there was no treatment effect on any of the fatty acids produced by *C. reinhardtii* or *N. oculata*. The amount of time the cultures were exposed to the cold could be a factor as to why we didn't see a significant response in these two green algae. Indeed, one study found that gene expression for lipid production in *C. reinhardtii* tends to take longer than the 24 hrs treatment duration we applied (L. Li et al., 2020). Conversely, *P. tricornutum* showed a media-dependent response to the temperature stress, where certain fatty acids in F2 had a positive response while others in M+ significantly decreased. Based on our results and those reported elsewhere, it is likely that a 24 hrs cold shock treatment was not a sufficient duration of reduced temperature exposure to induce an increase in lipid production in any of the strains we tested. Future work should explore different temperature regimes and durations of exposure to determine if struvite-based cultivation of these three algal species can be tuned to produce increased lipid contents and/or differential distributions of lipids based on cultivation temperature.

### **Annualized Biomass Production Projections**

The ultimate goal of our laboratory-scale investigation was to identify algal strains and cultivation conditions that can induce high levels of production of targeted lipids (e.g., PUFAs). To evaluate the outcome of our investigations thus far, we extrapolated our small-scale observations to a hypothetical commercial-scale operation. To do this we calculated if the treatment effects observed here would influence the estimated gross economic potential of a commercial scale operation for each algal strain tested. Beyond projecting annualized biomass yields we also calculated the gross

economic value of the produced biomass for a suite of identifiable commodities for which current market rates were available (e.g. PUFAs, high protein cattle feed, and bio-oil). Our hypothetical commercial scale system consisted of a 1 million gallon cultivation volume operated for 270 days/year, a common framework for estimating annualized yields from commercial scale algal cultivation systems (Pate et al., 2011). By applying our observed growth rates and yields to a system of this size, we estimated an annualized biomass production of 9,460 kg/year, and 3,520 kg/year for *C. reinhardtii* in chu 13 and M+, respectively; 7,040 kg/year and 3,300 kg/year for *N. oculata* in chu 13 and M+, respectively; and 56,650 kg/year and 6-720 kg/year for *P. tricornutum* in F2 and M+, respectively. This was calculated using equation (1) where  $X_t$  = final biomass  $X_0$  = Beginning biomass,  $V$  = volume, and  $h$  = number of harvests over the 270-cultivation period. It is important to note that in this calculation we estimated the initial biomass ( $X_0$ ) to be negligible relative to the final biomass.

$$\text{growth kinetics single batch } (X) = (X_t - X_0) \times v \times h \quad (1)$$

If the biomass generated by each species were to be used for bio-crude we could estimate the value of the bio-oil using equation (2) where  $X_y$  = biomass yield,  $L\%$  = lipid%, and  $V_l$  = bio-crude value. In this case the bio-crude is valued at the current market rate for West-Texas intermediate crude oil (WTI). The current market rate of which is \$0.61/L WTI (up.com). The value of the dry biomass from each hypothetical commercial-scale system was estimated as high protein cattle feed using equation (3) where  $X_y$  = biomass yield,  $P\%$  = protein %, and  $V_p$  = protein value of \$230 per ton (Jiao, 2022). Finally, the economic value of the high value lipid content of the biomass (e.g. PUFA income potential) was estimated by using the same equation as the for determining

economic value as bio-crude with a modification to the value term ( $V_{\text{PUFA}} = \$/\text{mg}$ ) multiplied by the fractional component of the lipid content of the biomass that consisted of C18:2, and C20:5 (Equation 4). For *P. tricornutum*  $V_{\text{PUFA}} = \$20.46/\text{mg}$  as it had produced C18:2, and C20:5. For *C. reinhardtii* and *N. oculata* which produced C18:2  $V_{\text{PUFA}} = \$8.18/\text{mg}$ . Economic projections can be seen on Table 4.

$$\text{oil income potential}(I_o) = X_y \times L_{\%} \times V_l \quad (2)$$

$$\text{protein income potential}(I_p) = X_y \times P_{\%} \times V_p \quad (3)$$

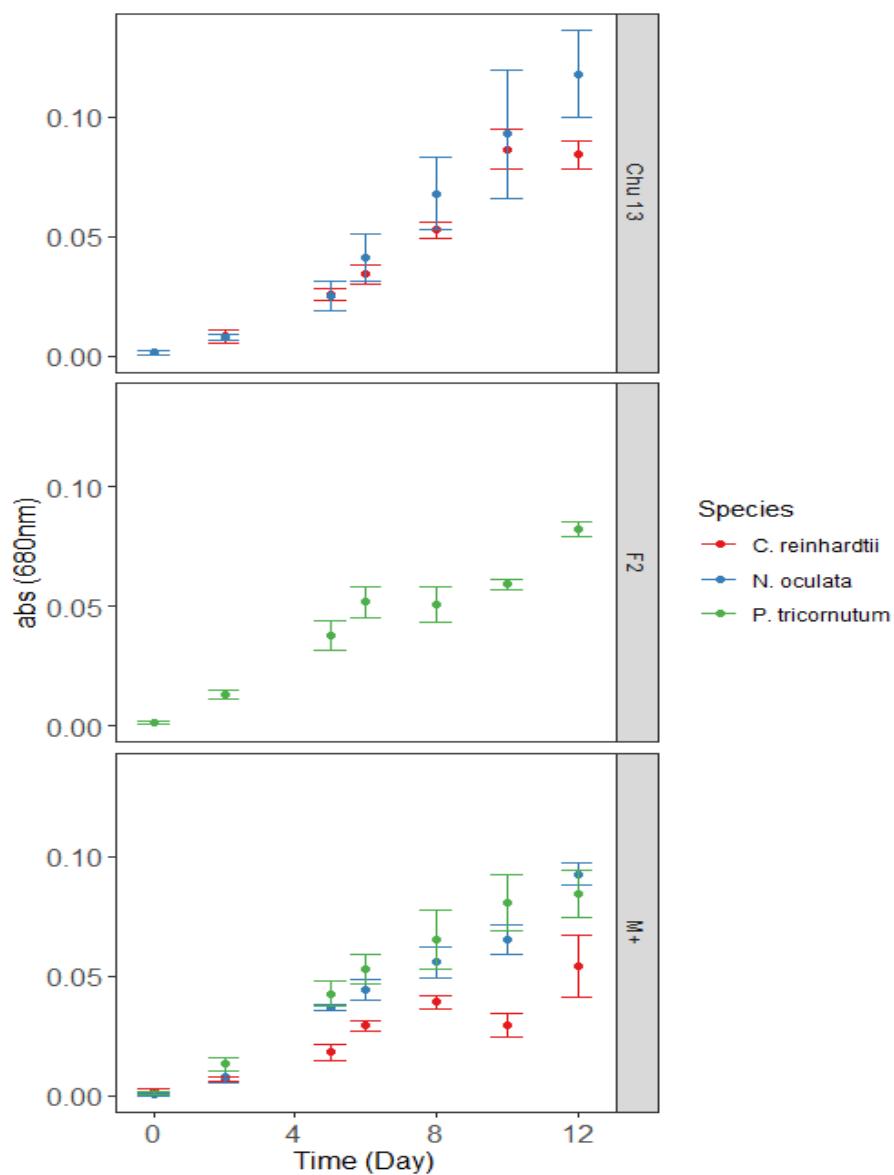
$$\text{PUFA income potential} = X_y \times V_{\text{PUFA}} \times \%L_{\text{PUFA}} \quad (4)$$

All three strains under normal conditions have a higher dollar value being sold as high protein cattle feed compared to bio-crude or PUFA supplements. The highest value would come from *P. tricornutum* where in M+ where we could expect to see around \$927,487.62. In the bio-crude market *P. tricornutum* in standard media exposed to temperature stress would generate approximately \$87,050.17 vs no stress \$63,929.53 (Table 2). Selling our biomass in the nutraceutical market our highest return would be \$337,808.27 from *N. oculata* under nutrient stress conditions (Table 4). Although our data did not detect a significant difference in biomass yields if we extrapolate the mean yields from our experiments across an annual level of production there is the potential that the gross economic value of these treatments may differ. Also, it is important to note these projections reflect the gross income we would expect and do not take into account any production costs.

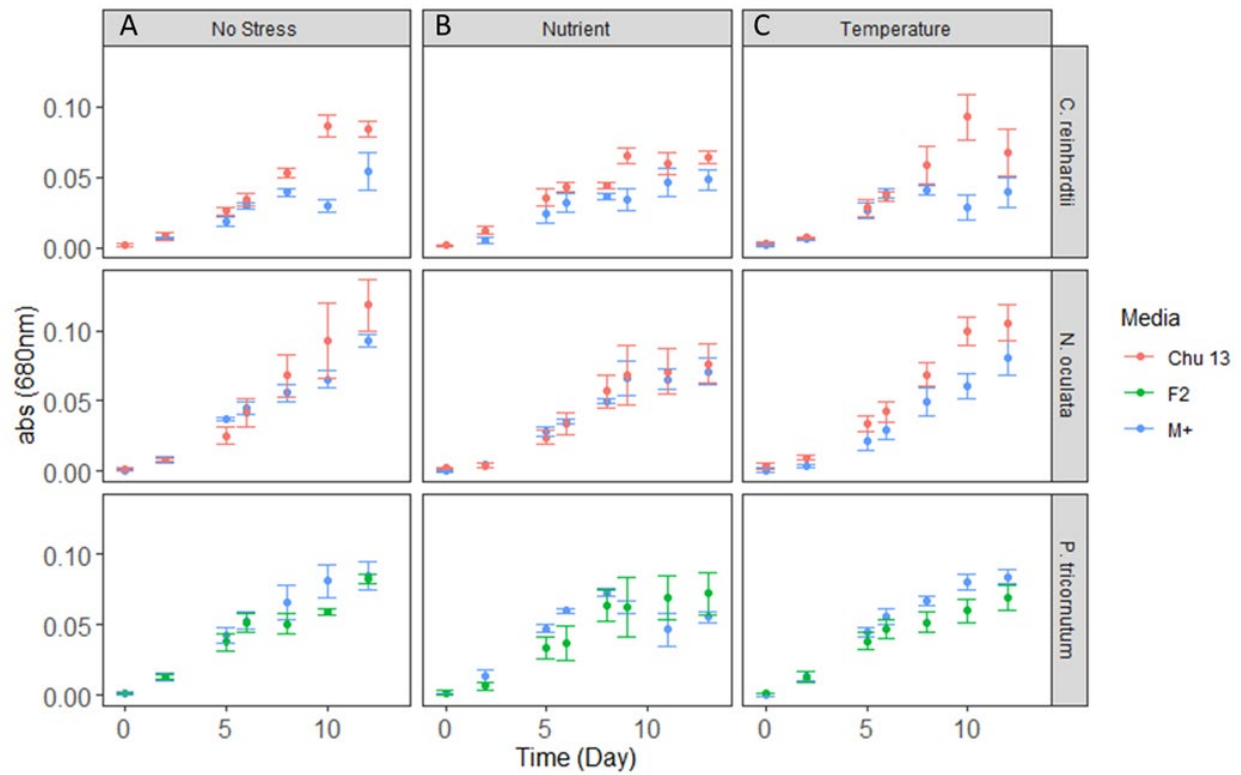
## CONCLUSION

All three algal strains tested here produced the same level of biomass in M+ struvite amended media compared to standard Chu 13 or F2. Indicating that struvite can be used as a supplementary source of nutrients for microalgal growth. Additionally, we demonstrated that temperature and nutrient stresses affected the macromolecular composition of each strain differently. Our results indicate that utilizing struvite as the primary source of nutrients for growth and then applying either a nutrient, or temperature stress to different strains of microalgae can influence the yield of biomass and targeted components of that biomass. Our results show that the highest economic potential would come from selling the biomass on the high protein cattle feed market as the average strain, media, and stress combination had the highest dollar value in this market compared to the bio-crude and nutraceutical markets. Again our results show that we can influence the different lipids of the biomass. However, more work is required to fine-tune the approach to maximize yields of specific lipids of interest.

## FIGURES

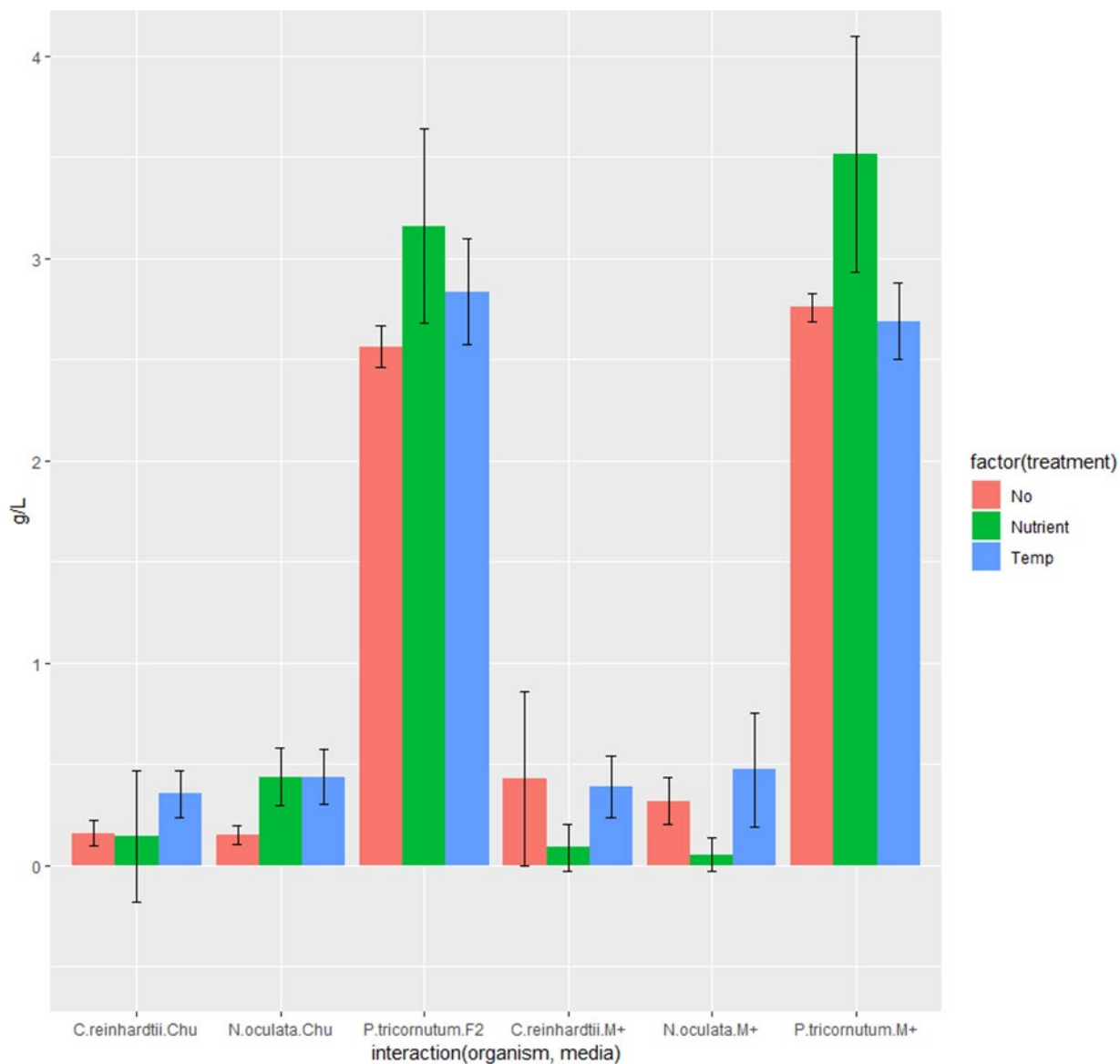


**Figure 1.** Mean culture density vs. time by species and media. Culture density was determined by measuring absorbance at 680nm (A680) (n = 4). Error bars = +/- standard deviation.

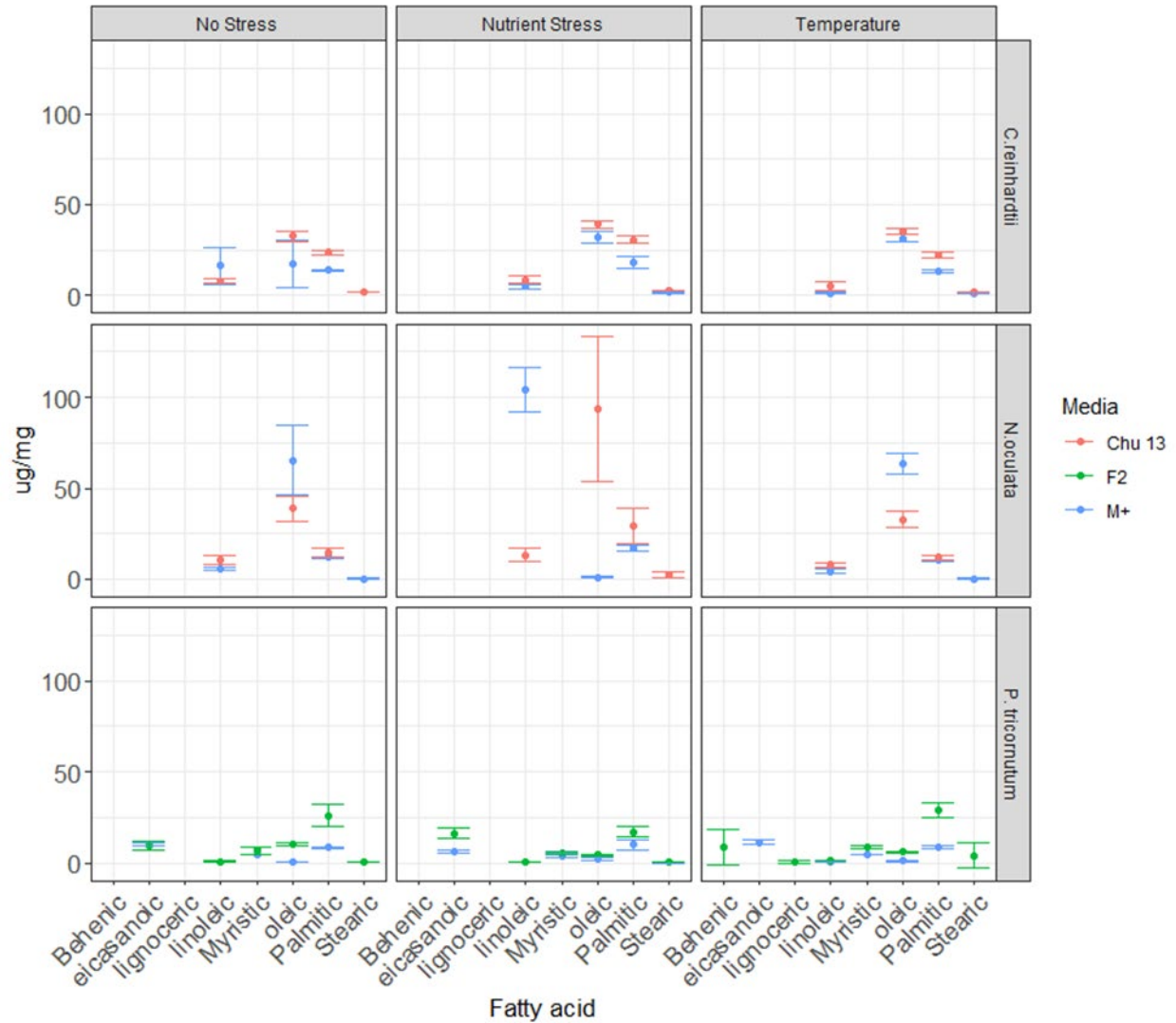


**Figure 2.** Stress treatment effects on growth patterns as measured by absorbance at 680 nm (A680) vs. time (dots = mean, error bars = +/- std. dev., n = 4). Column A = No stress; Column B = Nutrient stress; Column C = Temperature stress. Media treatments: Red dots = Chu 13, Green dots = F2, and Blue dots = M+.

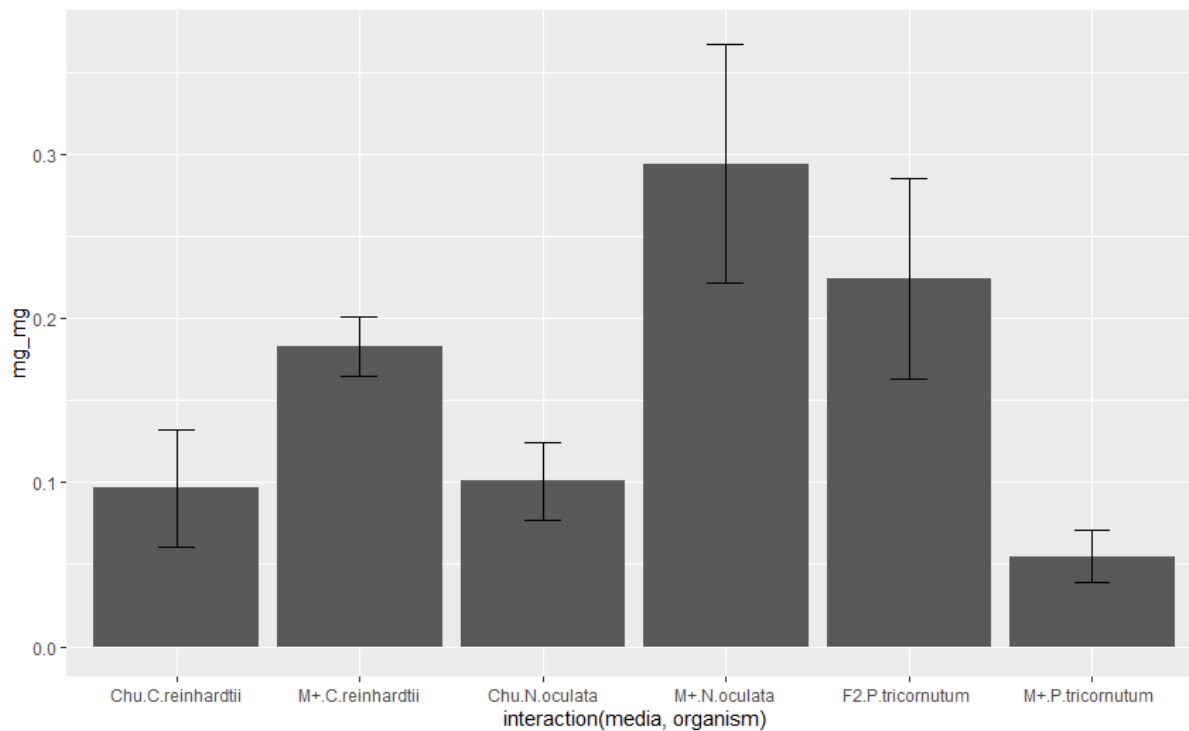




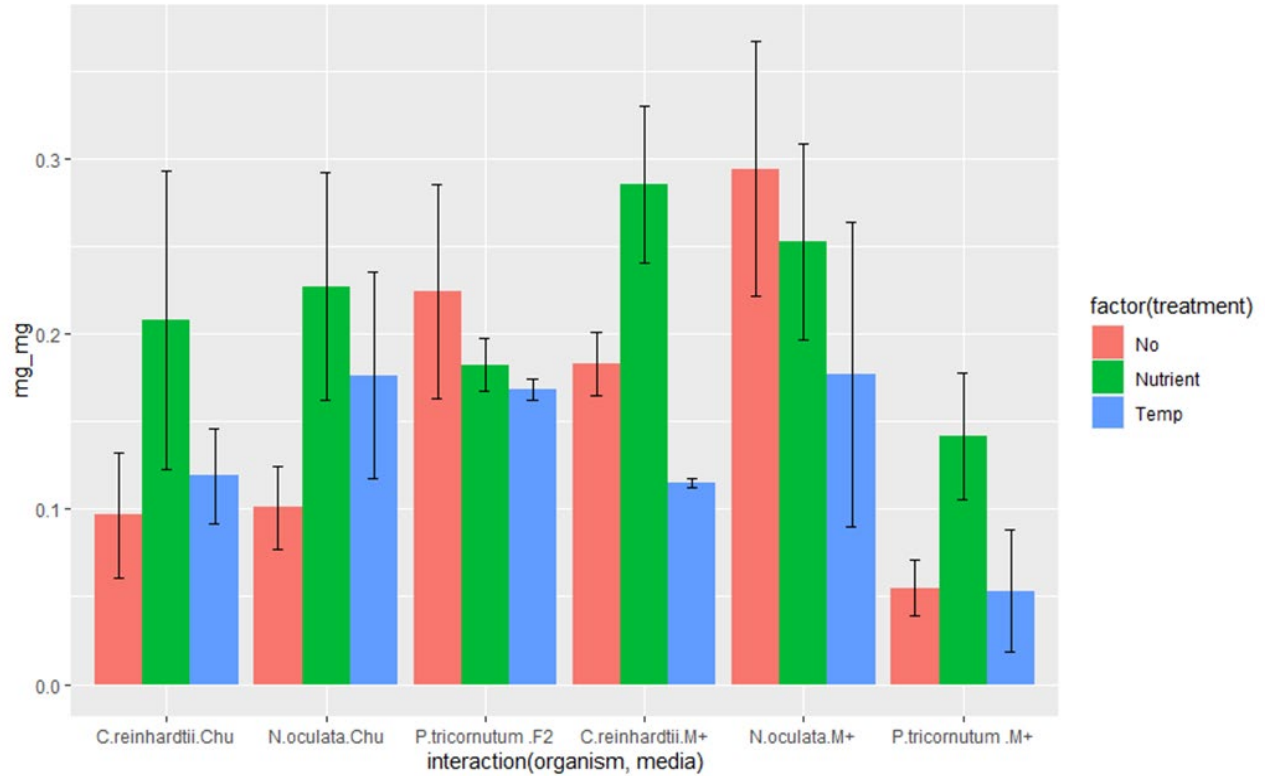
**Figure 3.** Ash Free dry weight of *C. reinhardtii*, *N. oculata*, and *P. tricornutum* under stress conditions and media type (n=4). Cultures exposed to nutrient stress for 4 days and temperature for 24 hours at 15° C. Colored bars represent treatment and black bars represent standard deviation (+/-).



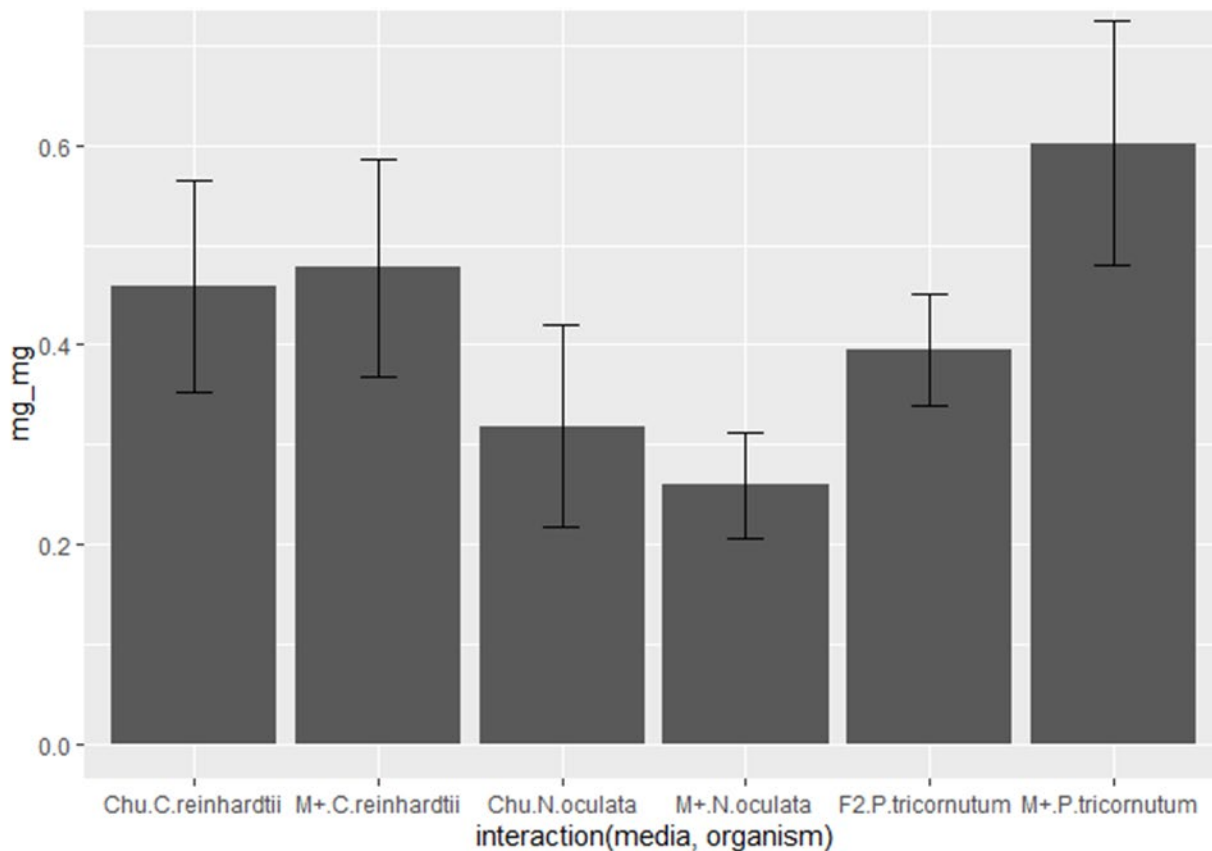
**Figure 4.** Species and stress treatment effect on lipid production measured by GC-MS  $\mu\text{g}/\text{mg}$  vs lipid type (dots= mean, error bars =  $\pm$  standard deviation,  $n=4$ ). Media treatments: Red dots=Chu 13, Green dots=F2, and Blue dots=M+ struvite amended.



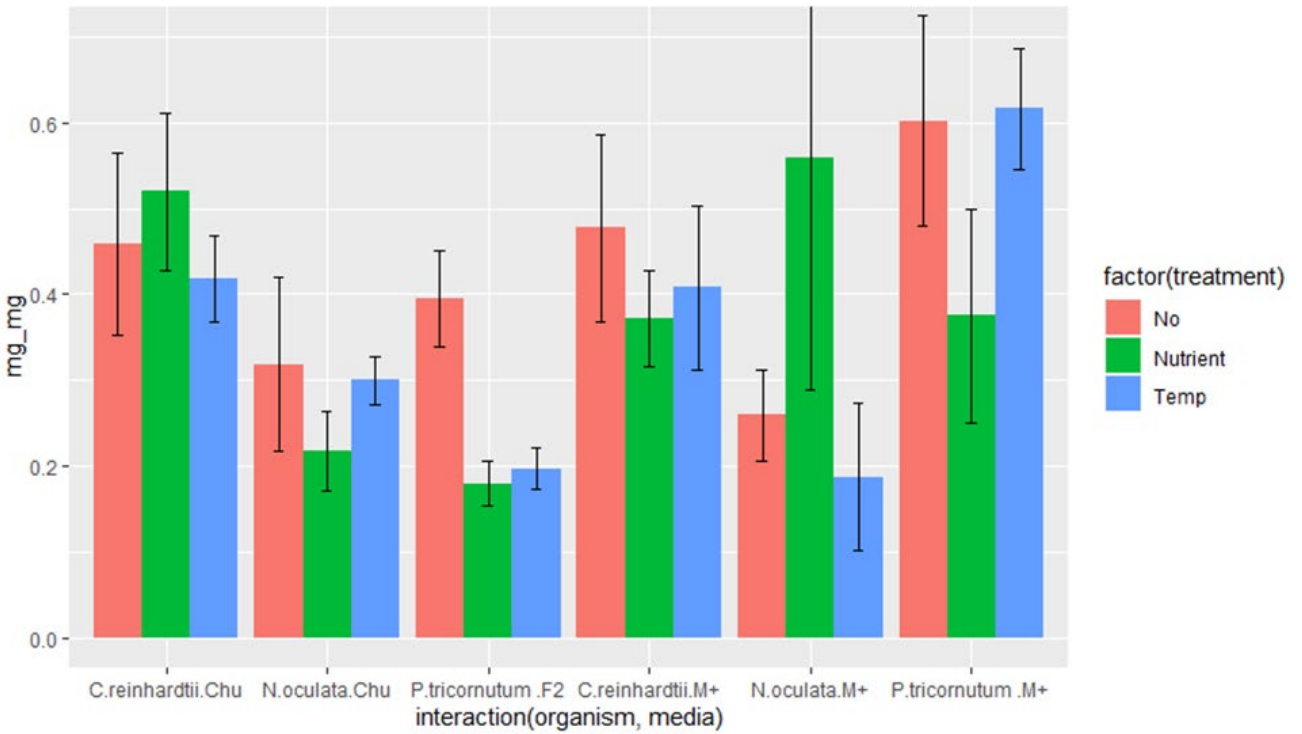
**Figure 5.** Average carbohydrate yield by organism and media type measured by phenol-sulfuric acid assay absorbance (nm 490) (mg of carbohydrate per mg of algal dry mass). Bars represent mean (+/-) standard deviation (n=4).



**Figure 6.** Maximum carbohydrate production measured by phenol-sulfuric acid assay absorbance (nm 490) of *C. reinhardtii*, *N. oculata*, and *P. tricornutum* under stress conditions along with media type (n=4). Cultures exposed to nutrient stress for 4 days and cultures exposed to 15°C temperature stress for 24 hours. Colored bars represent treatment and bars representing standard deviation (+/-).



**Figure 7.** Maximum protein produced measured by Lowry assay absorbance (nm 750) of *C. reinhardtii*, *N. oculata*, and *P. tricornutum* under no stress in standard media and struvite supplemented media M+ (n=4). Bars represent mean (+/-) standard deviation.



**Figure 8.** Maximum protein production measured by Lowry assay absorbance (nm 750) of *C. reinhardtii*, *N. oculata*, and *P. tricornutum* under stress conditions along with media type (n=4). Cultures exposed to nutrient stress for 4 days and cultures exposed to 15°C temperature stress for 24 hours. Colored bars represent treatment and bars representing standard deviation (+/-).

## TABLES

**Table 1. Average growth rates of *C. reinhardtii*, *N. oculata*, and *P. tricornutum* in different media and under different stress conditions.**

|                       | Standard Media |                 |                    | M+ Media  |                 |                    |
|-----------------------|----------------|-----------------|--------------------|-----------|-----------------|--------------------|
|                       | No Stress      | Nutrient Stress | Temperature Stress | No Stress | Nutrient Stress | Temperature Stress |
| <i>C. reinhardtii</i> | 0.309          | 0.238           | 0.355              | 0.287     | 0.331           | 0.319              |
| <i>N. oculata</i>     | 0.362          | 0.432           | 0.330              | 0.324     | 0.400           | 0.441              |
| <i>P. tricornutum</i> | 0.227          | 0.386           | 0.236              | 0.266     | 0.278           | 0.267              |

**Table 2. Yearly economic projections of bio-crude at \$0.61.**

|             | C. reinhardtii |            | N. oculata  |             | P. tricornutum |             |
|-------------|----------------|------------|-------------|-------------|----------------|-------------|
|             | Chu 13         | M+         | Chu 13      | M+          | F2             | M+          |
| No Stress   | \$14,657.32    | \$3,929.38 | \$10,693.06 | \$5,535.75  | \$63,929.53    | \$30,742.54 |
| Nutrient    | \$3,671.71     | \$4,203.14 | \$4,435.98  | \$29,583.05 | \$68,491.65    | \$33,963.34 |
| Temperature | \$14,073.62    | \$8,813.59 | \$15,362.55 | \$17,655.35 | \$87,050.17    | \$35,377.80 |



**Table 3. Yearly economic projections of high protein cattle feed at \$230 per ton**

|             | C. reinhardtii |             | N. oculata  |              | P. tricornutum |              |
|-------------|----------------|-------------|-------------|--------------|----------------|--------------|
|             | Chu 13         | M+          | Chu 13      | M+           | F2             | M+           |
| No Stress   | \$110,031.27   | \$42,593.80 | \$56,889.08 | \$21,686.21  | \$567,647.67   | \$927,487.62 |
| Nutrient    | \$26,117.67    | \$30,100.76 | \$6,695.10  | \$137,173.25 | \$317,765.81   | \$736,126.70 |
| Temperature | \$92,025.87    | \$80,727.14 | \$79,552.46 | \$46,045.26  | \$313,150.44   | \$925,001.26 |

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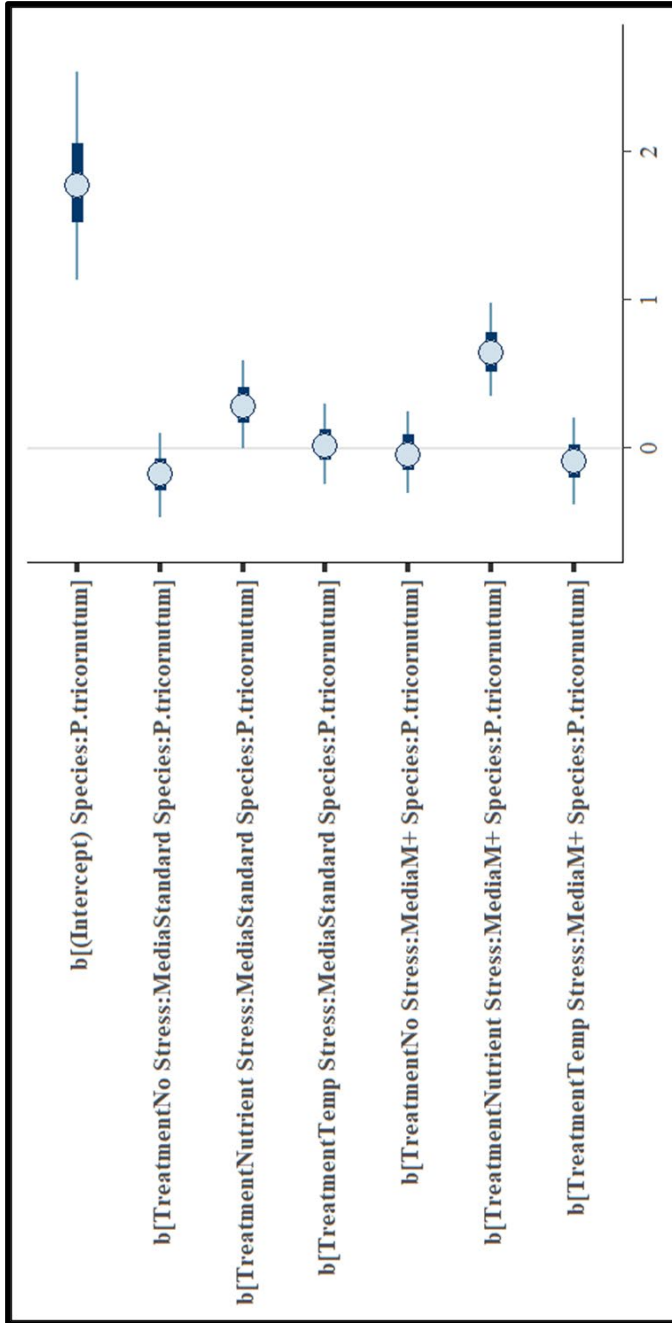
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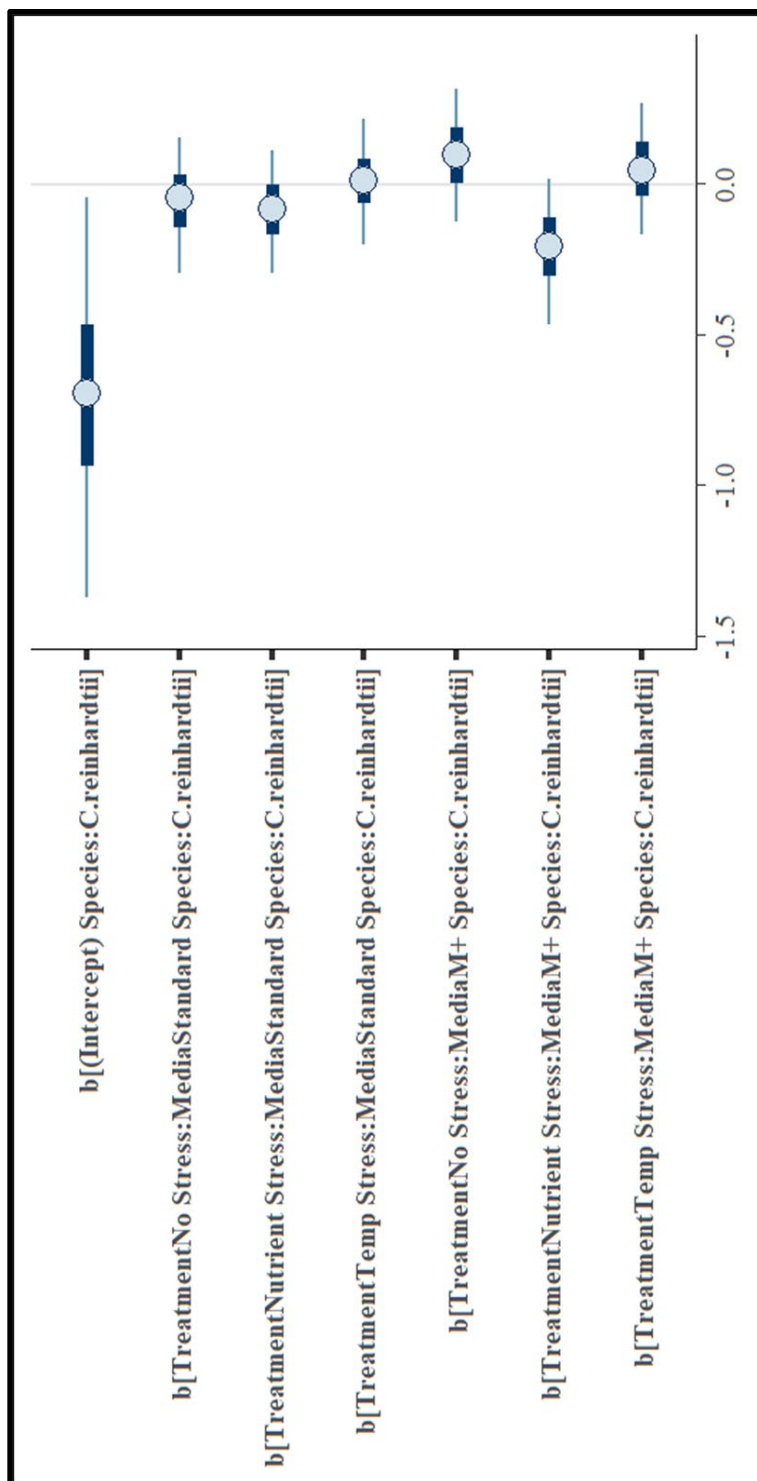
## APPENDIX

## Supplementary Figures

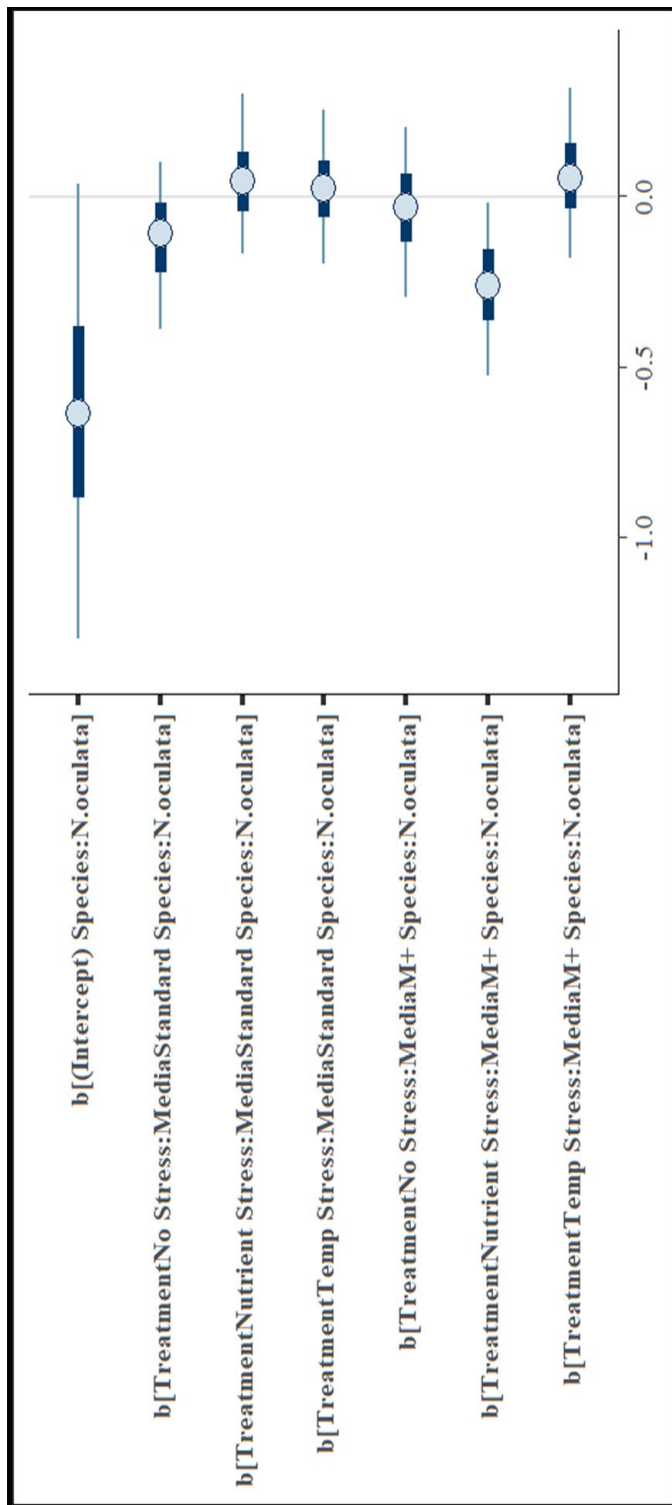


Supplementary Figure 1. 90% confidence interval (line) and median (circle) of *P. tricornutum* Ash Free Dry Weight

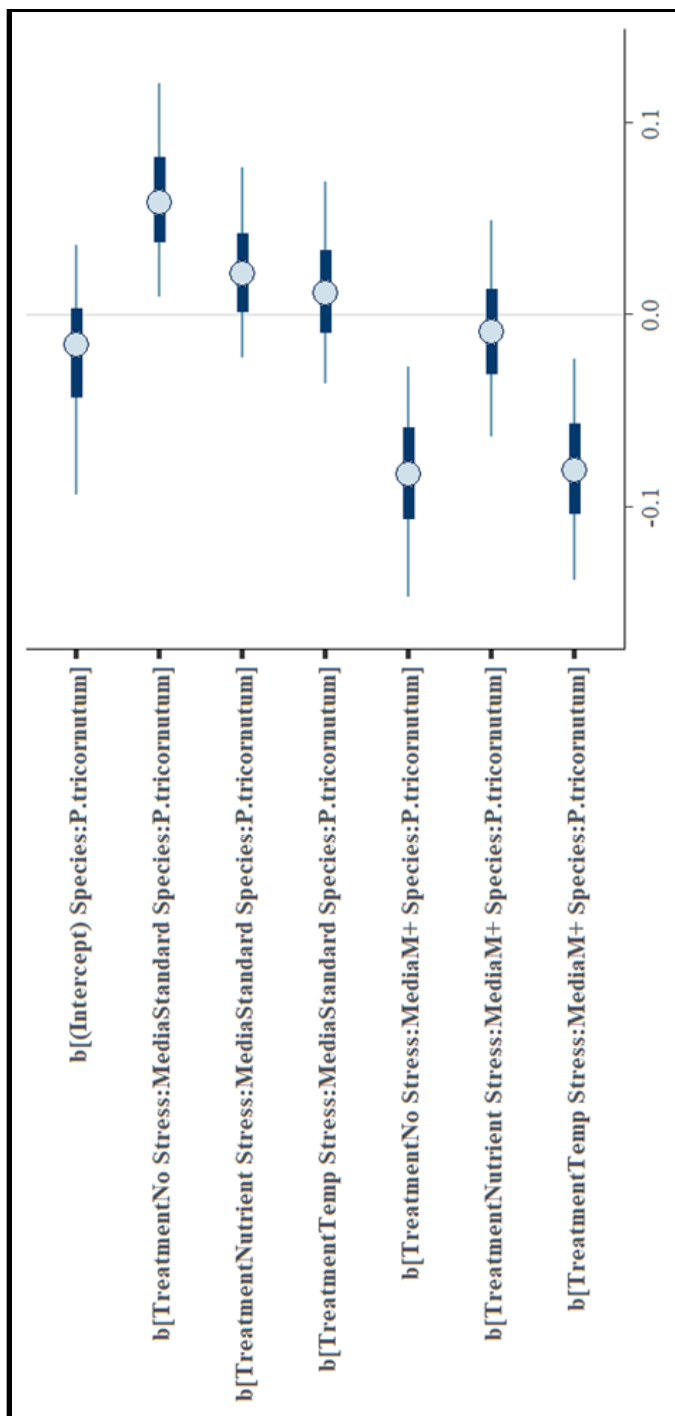




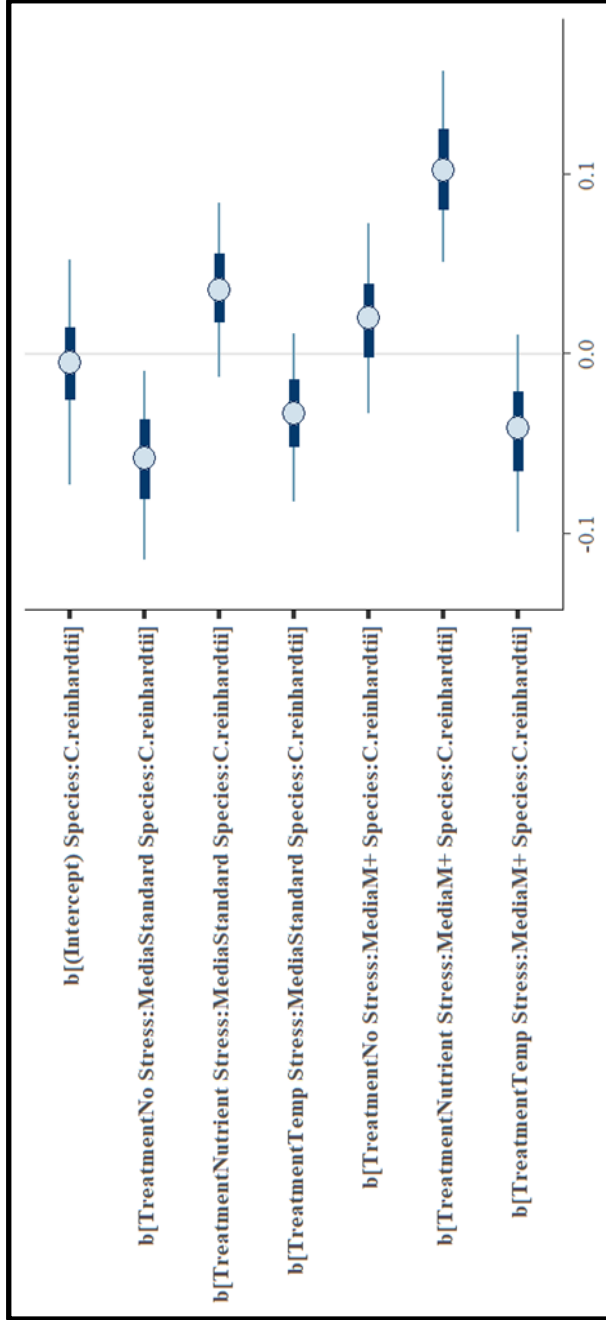
Supplementary Figure 2. 90% confidence interval (line) and median (circle) of *C. reinhardtii* Ash Free Dry Weight



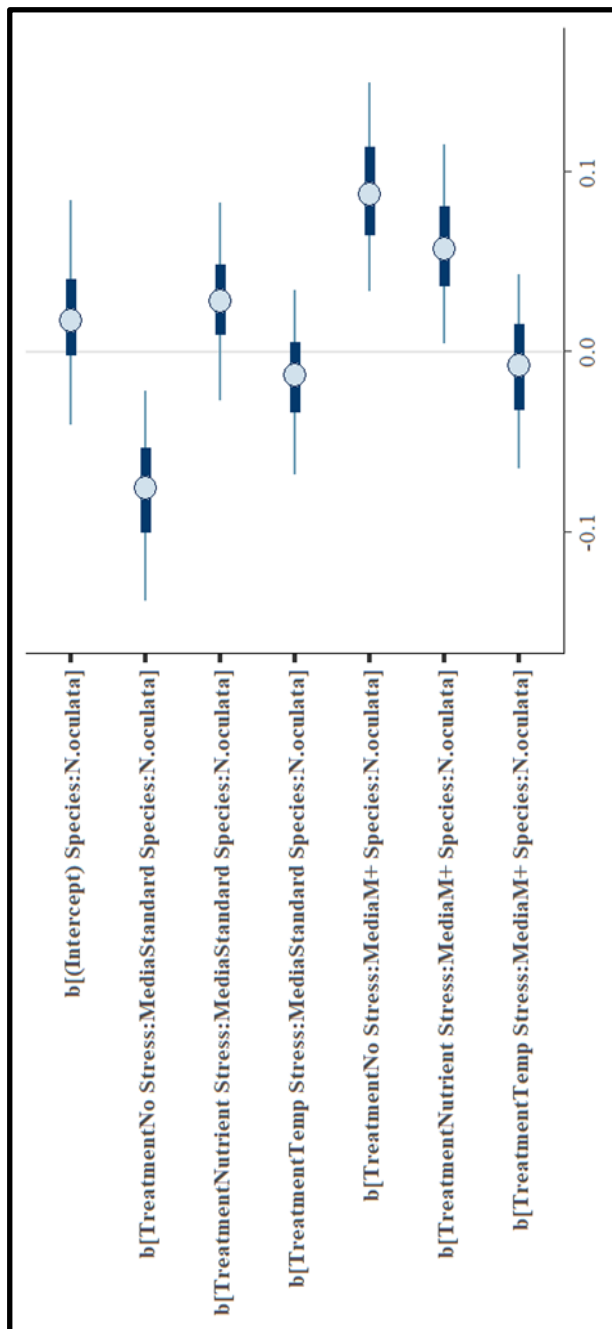
**Supplementary Figure 3.** 90% confidence interval (line) and median (circle) of *N. oculata* Ash Free Dry Weight



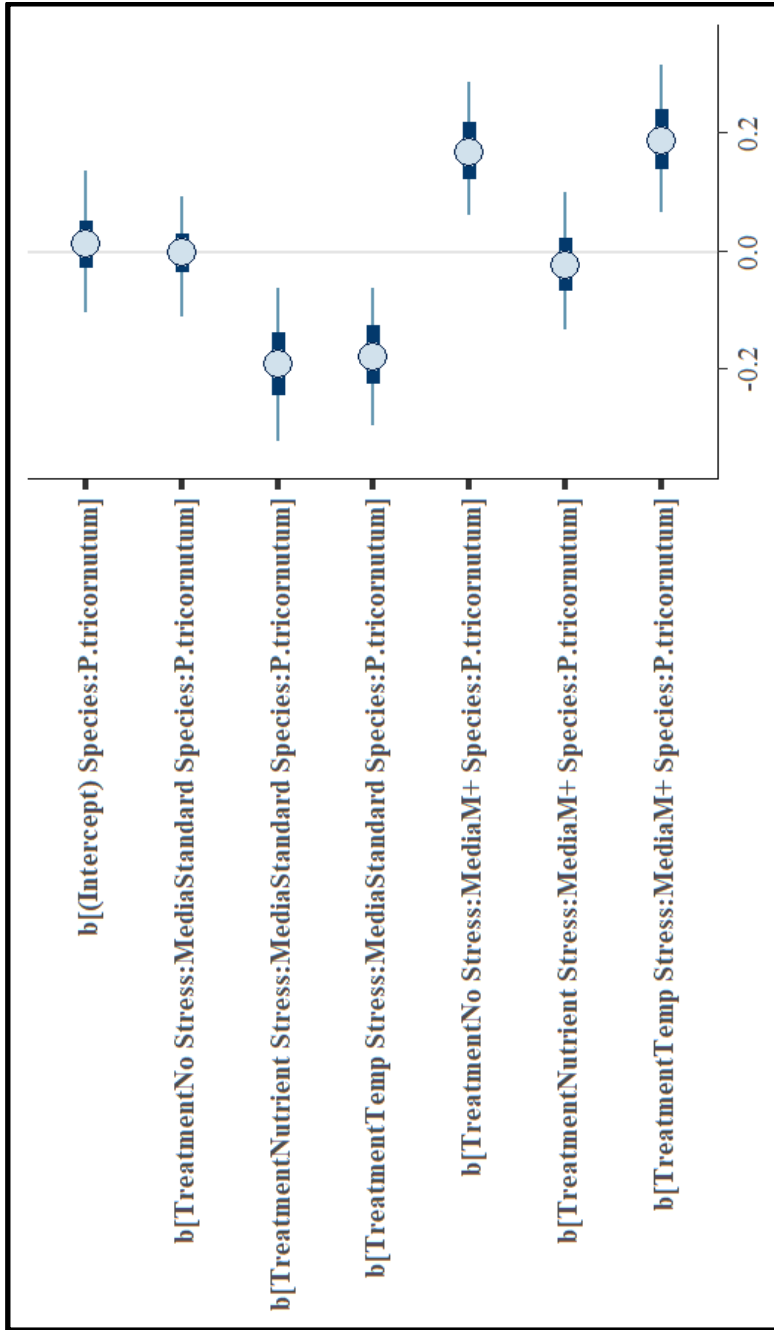
Supplementary Figure 4. 90% confidence interval (line) and median (circle) of *P. tricornutum* Carbohydrate



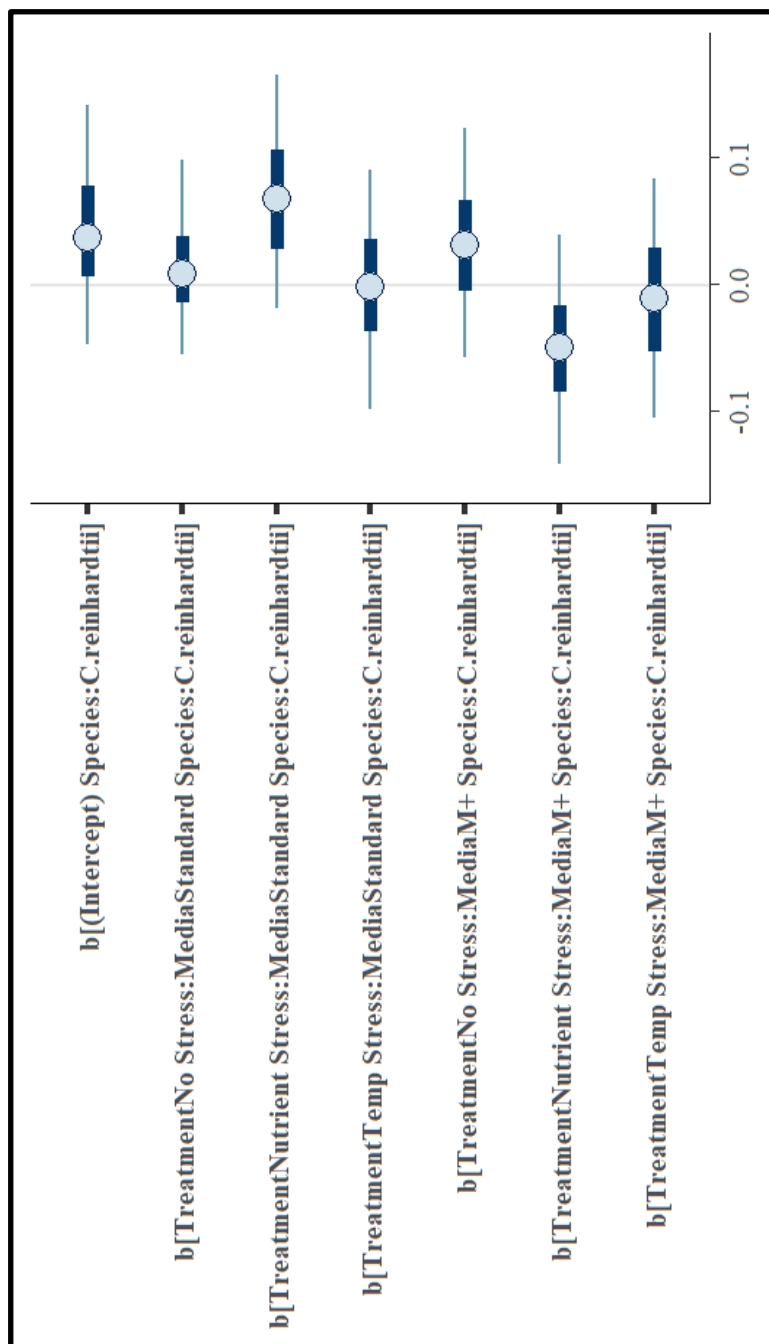
Supplementary Figure 5. 90% confidence interval (line) and median (circle) of *C. reinhardtii* Carbohydrate



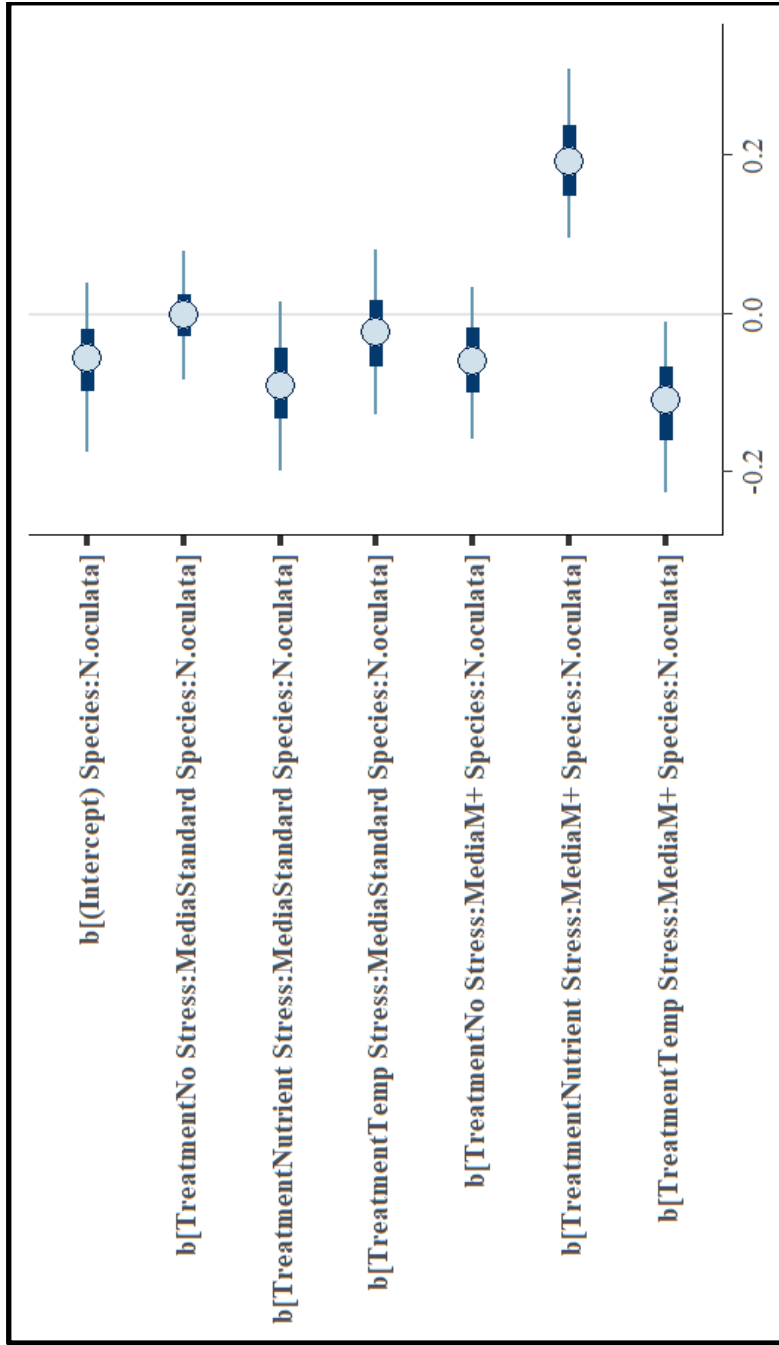
Supplementary Figure 6. 90% confidence interval (line) and median (circle) of *N. oculata* Carbohydrate



Supplementary Figure 7. 90% confidence interval (line) and median (circle) of *P. tricornutum* Protein

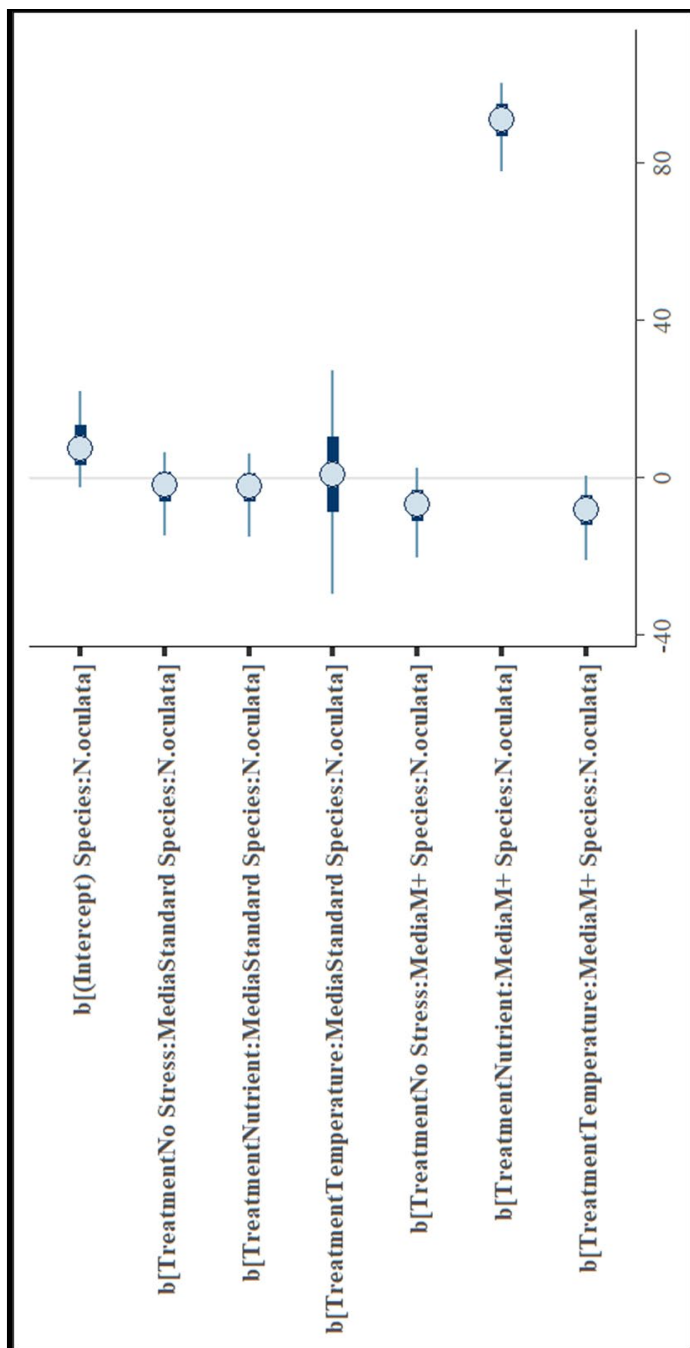


Supplementary Figure 8. 90% confidence interval (line) and median (circle) of *C. reinhardtii* Protein

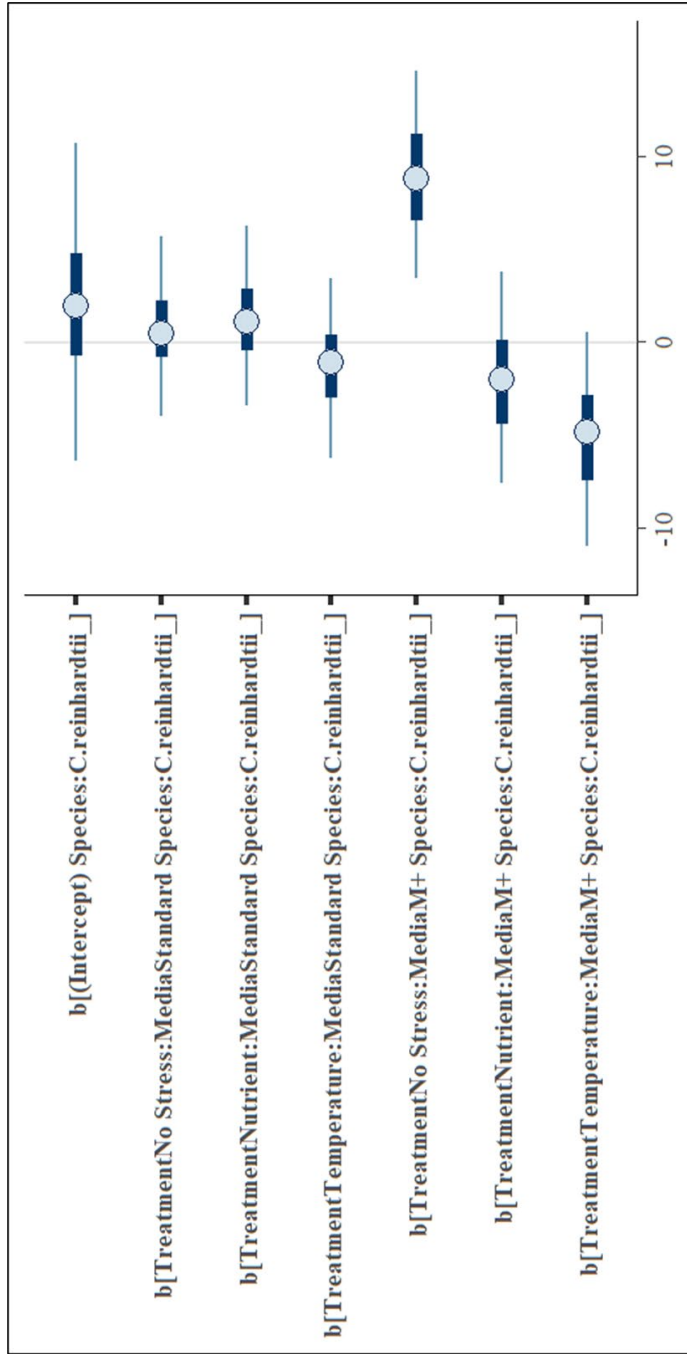


Supplementary Figure 9. 90% confidence interval (line) and median (circle) of *N. oculata* Protein

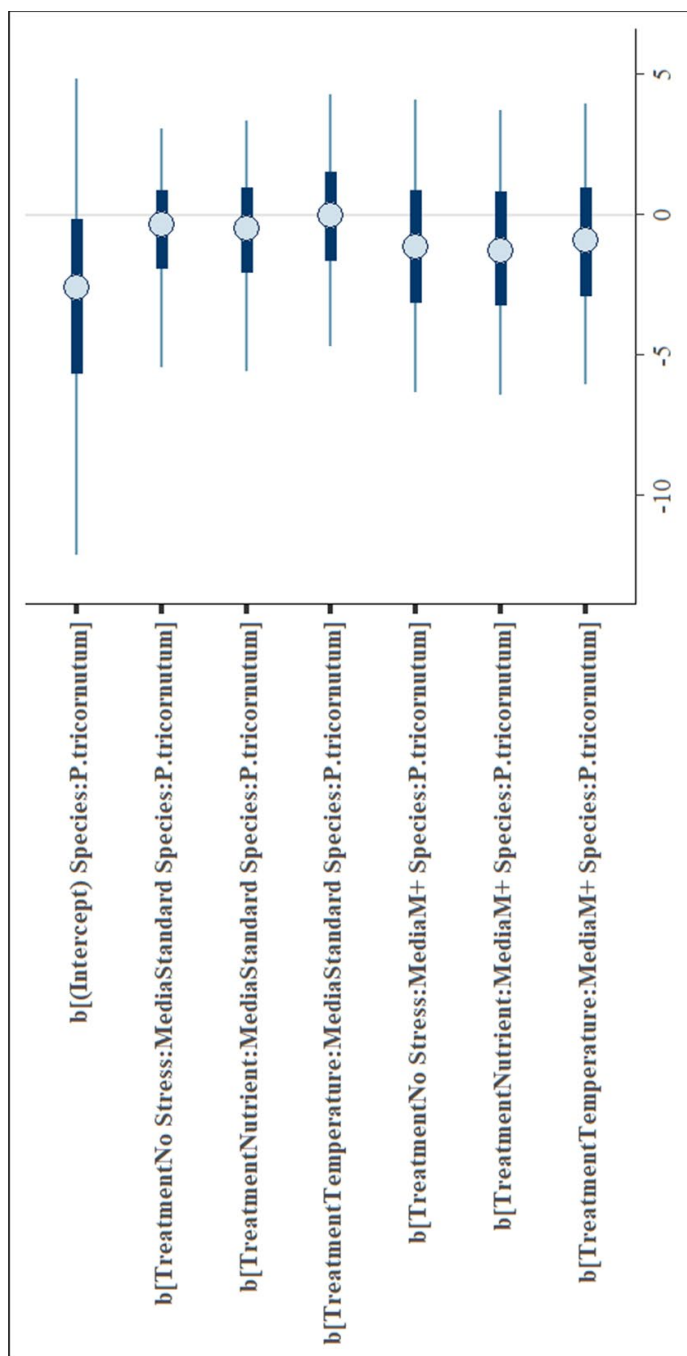




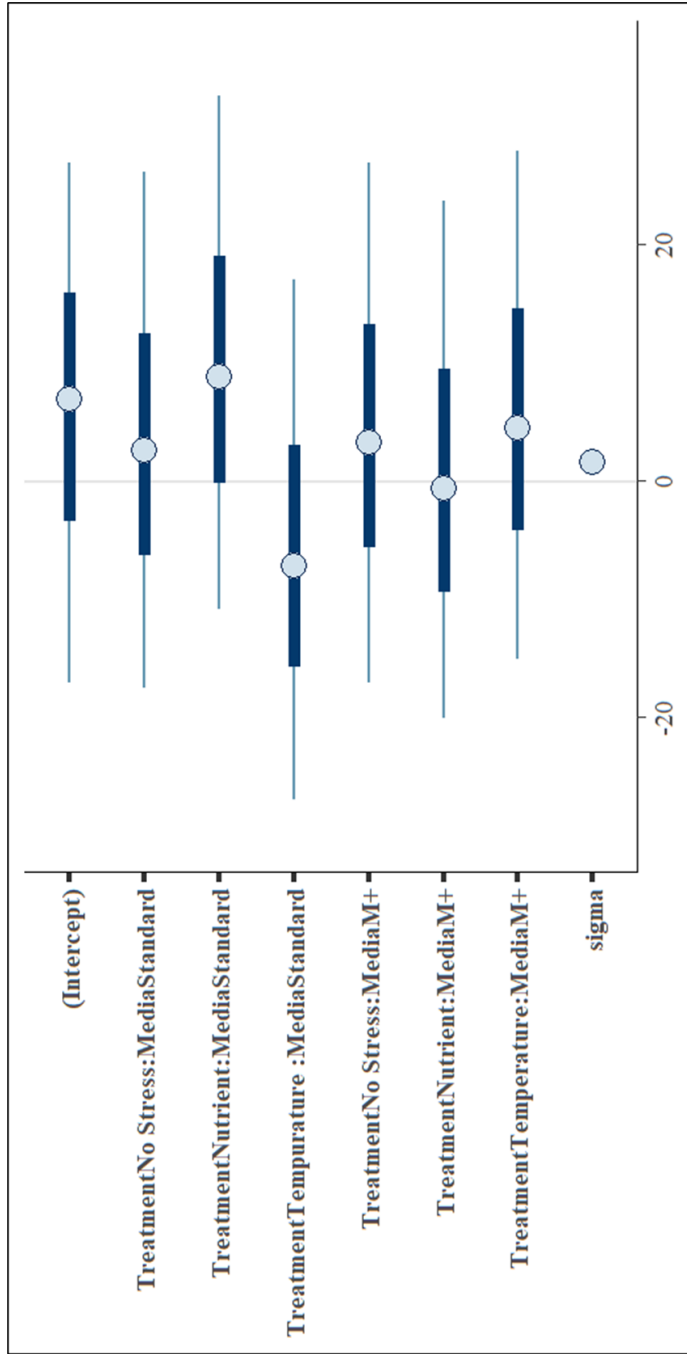
Supplementary Figure 10. 90% confidence interval (line) and median (circle) of *N. oculata* linoleic acid



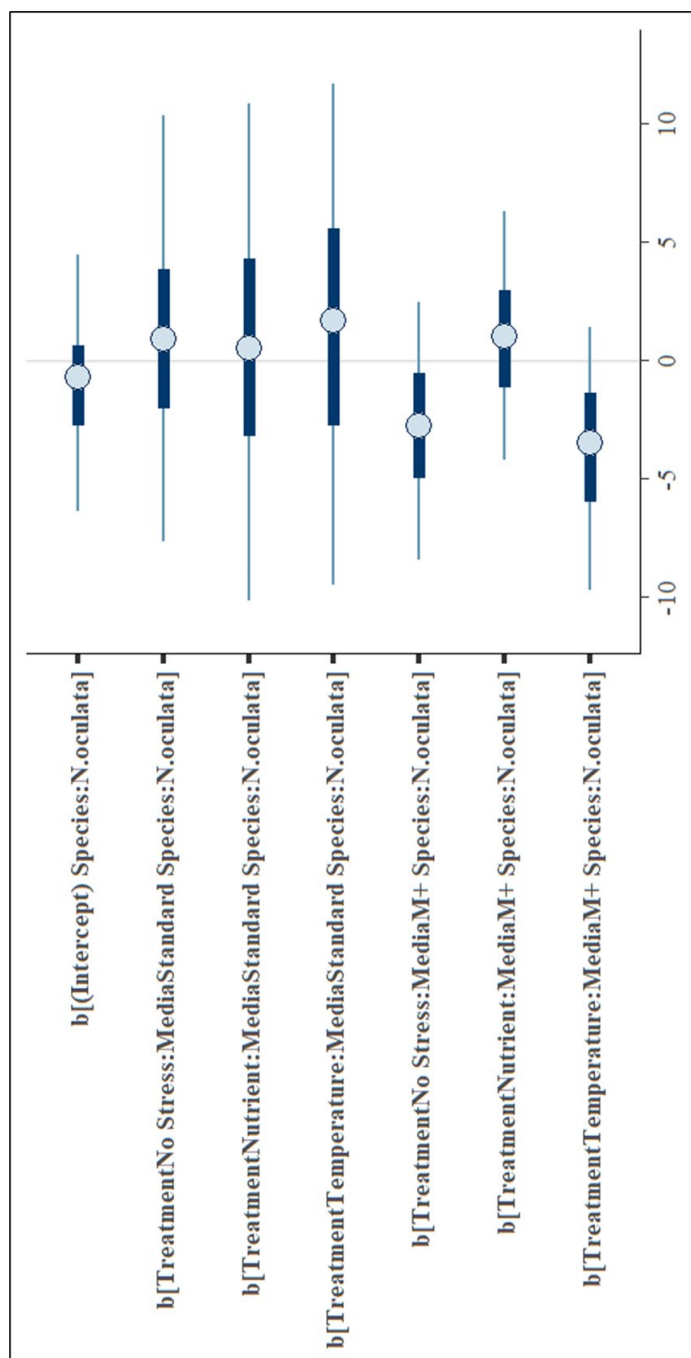
**Supplementary Figure 11.** 0% confidence interval (line) and median (circle) of *C. reinhardtii* linoleic acid



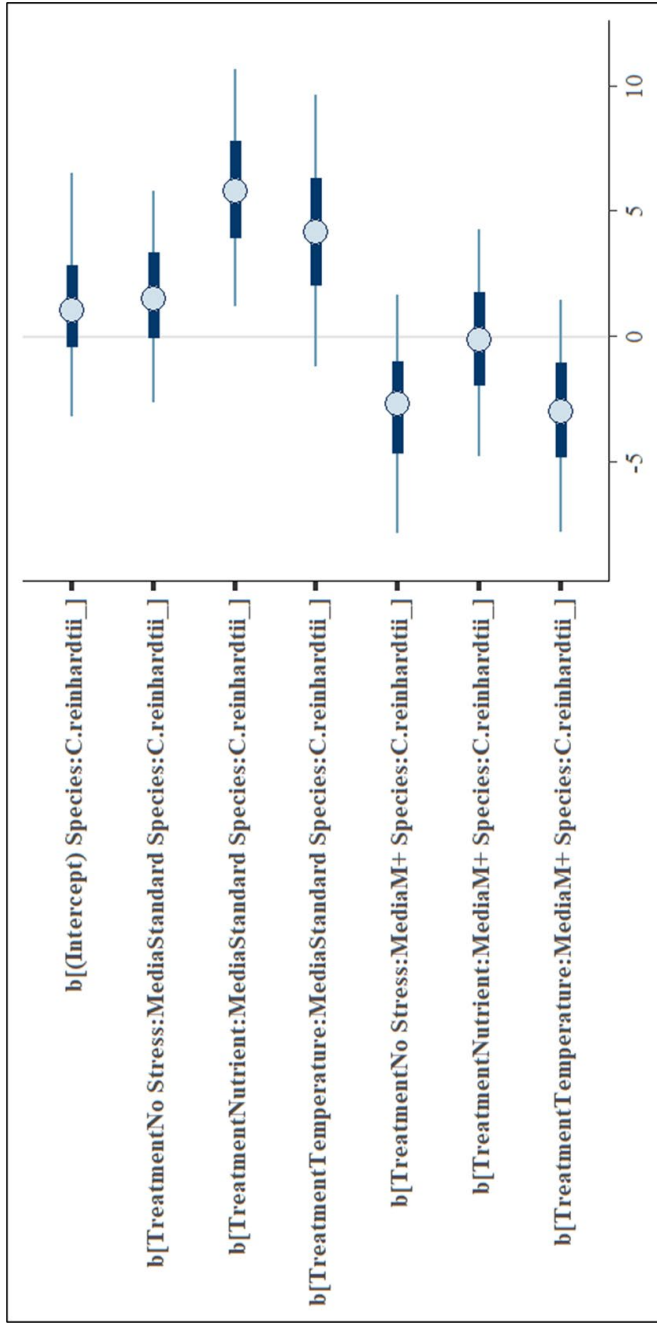
Supplementary Figure 12. 90% confidence interval (line) and median (circle) of *P. tricornutum* linoleic acid



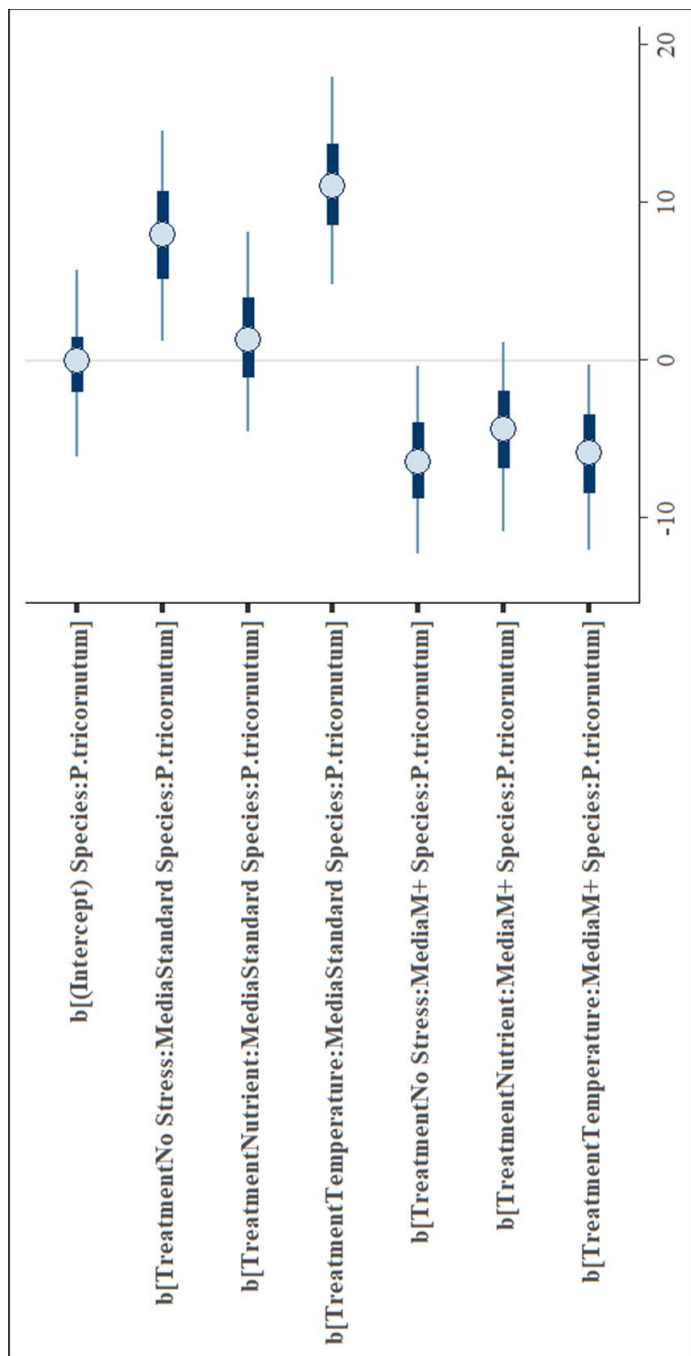
Supplementary Figure 13. 90% confidence interval (line) and median (circle) of *P. tricornutum* Eicosapentaenoic acid



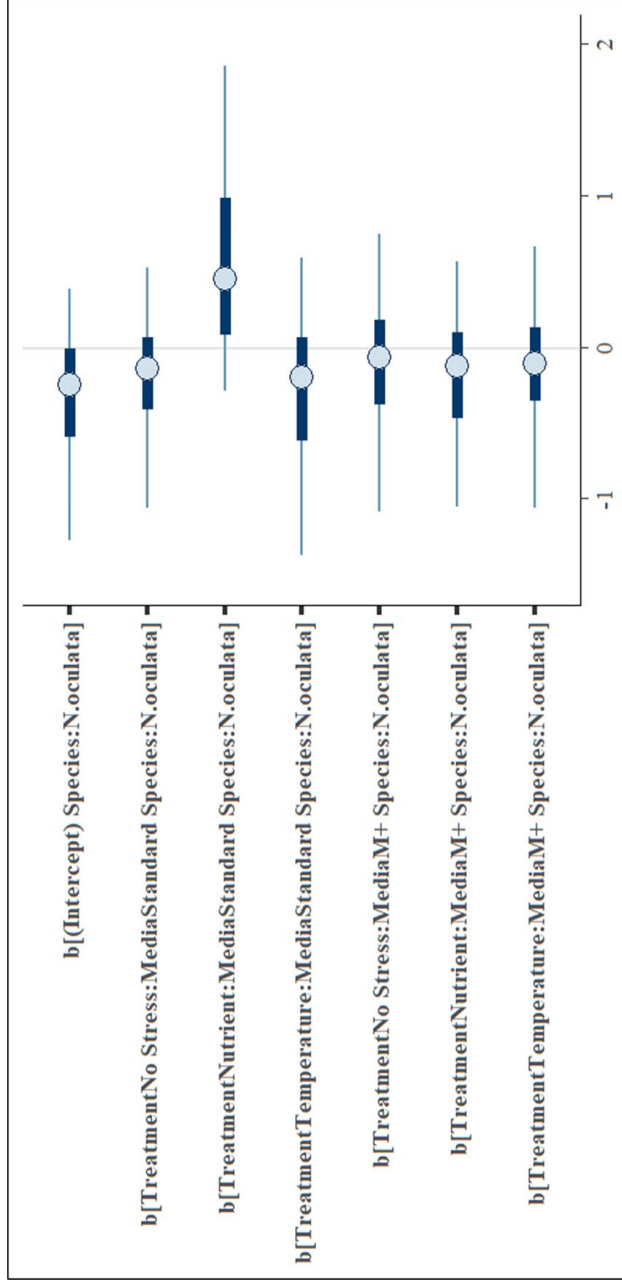
**Supplementary Figure 14. 90% confidence interval (line) and median (circle) of *N. oculata* Palmitic acid**



Supplementary Figure 15. 90% confidence interval (line) and median (circle) of *C. reinhardtii* Palmitic acid

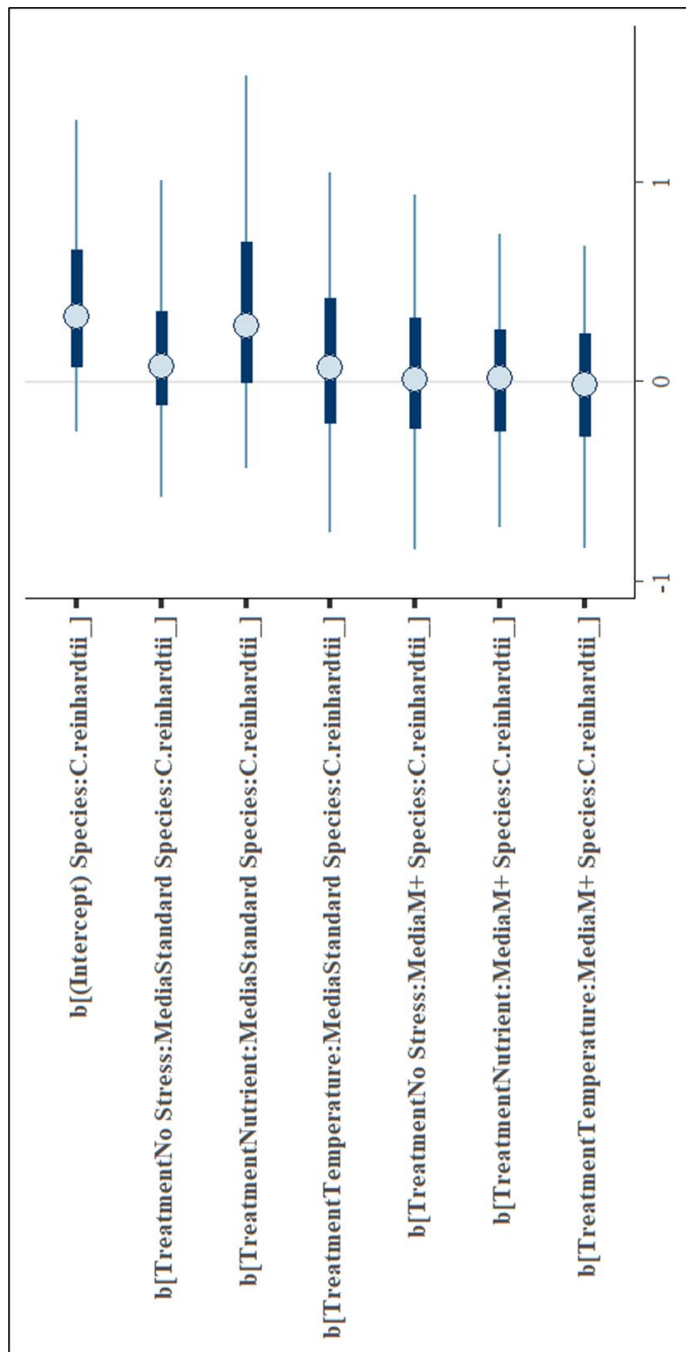


Supplementary Figure 16. 90% confidence interval (line) and median (circle) of *P. tricornutum* Palmitic acid

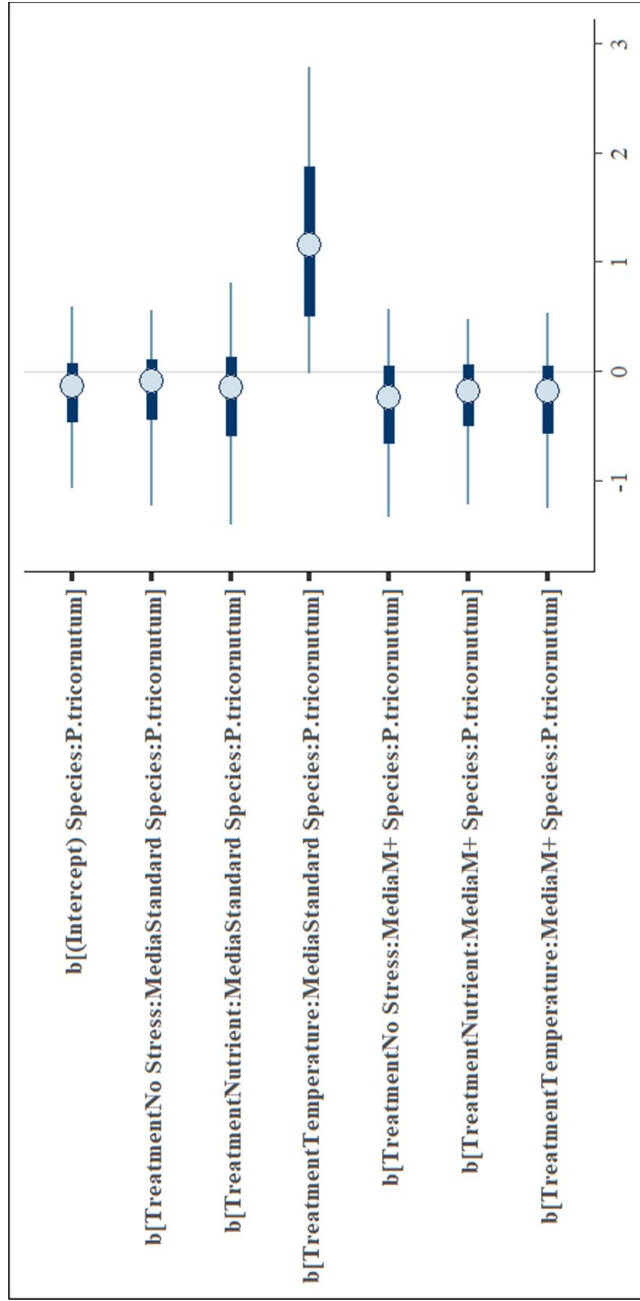


**Supplementary Figure 17. 90% confidence interval (line) and median (circle) of *N. oculata* Stearic acid**

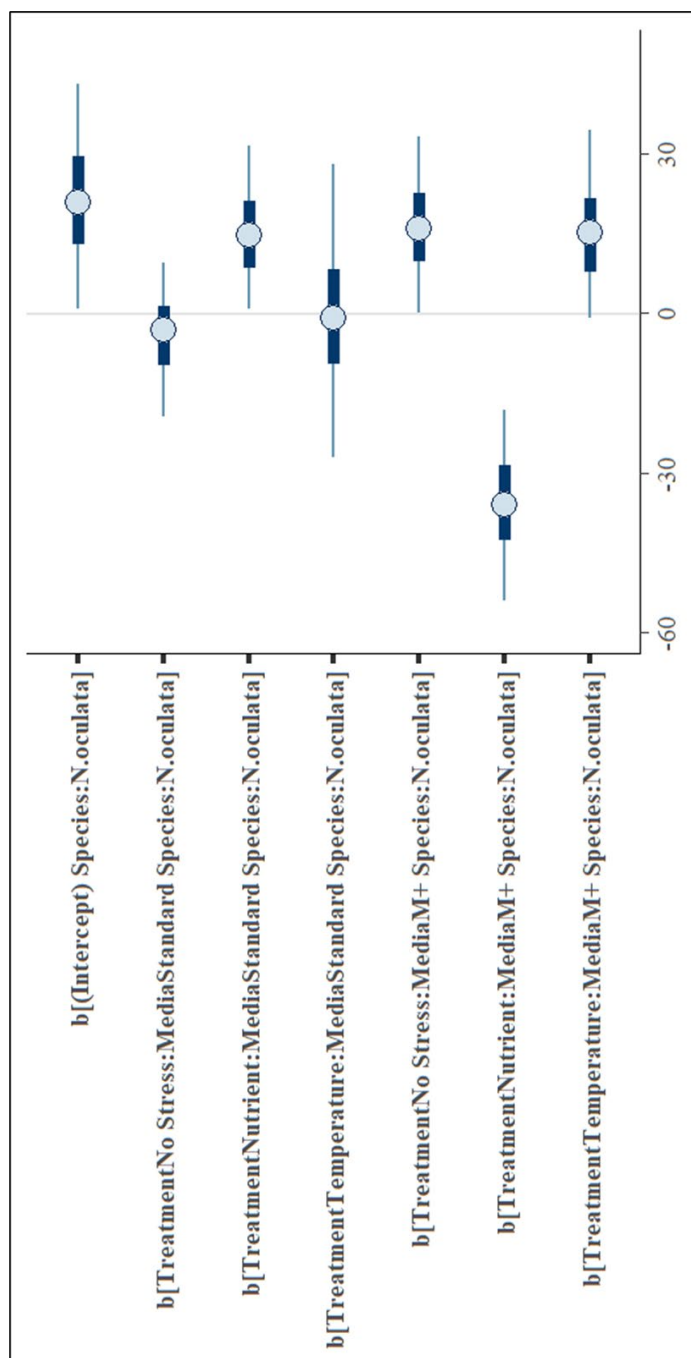




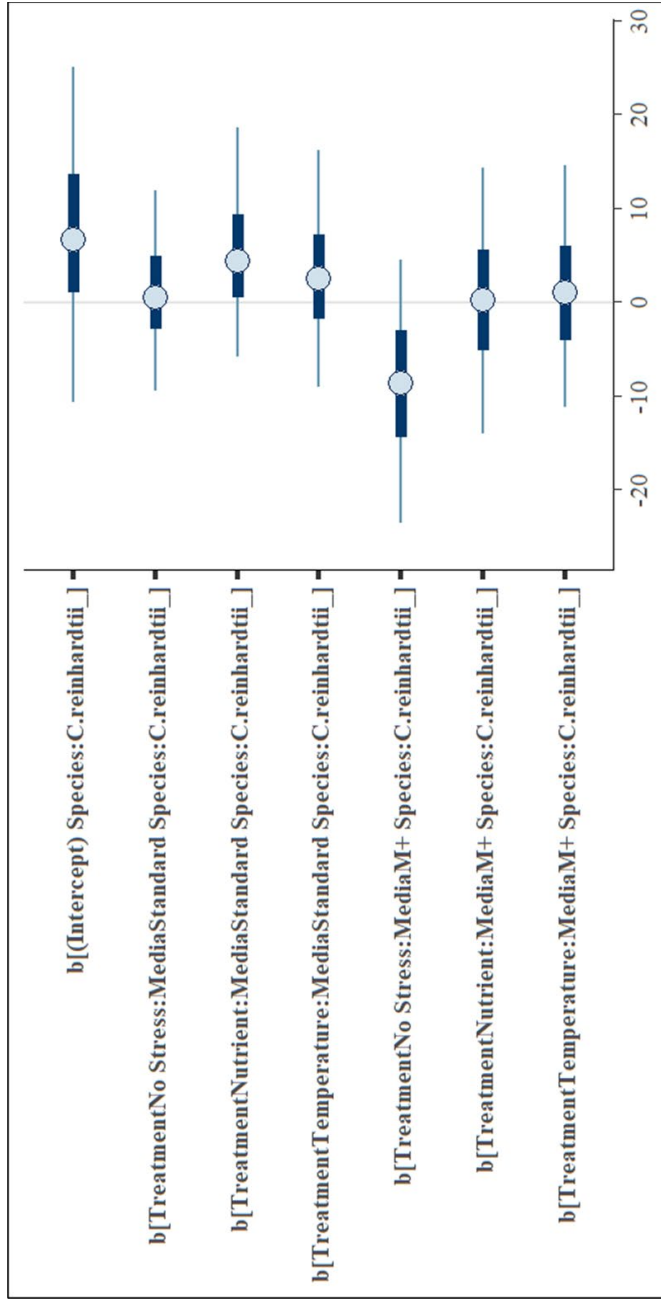
**Supplementary Figure 18. 90% confidence interval (line) and median (circle) of *C. reinhardtii* Stearic acid**



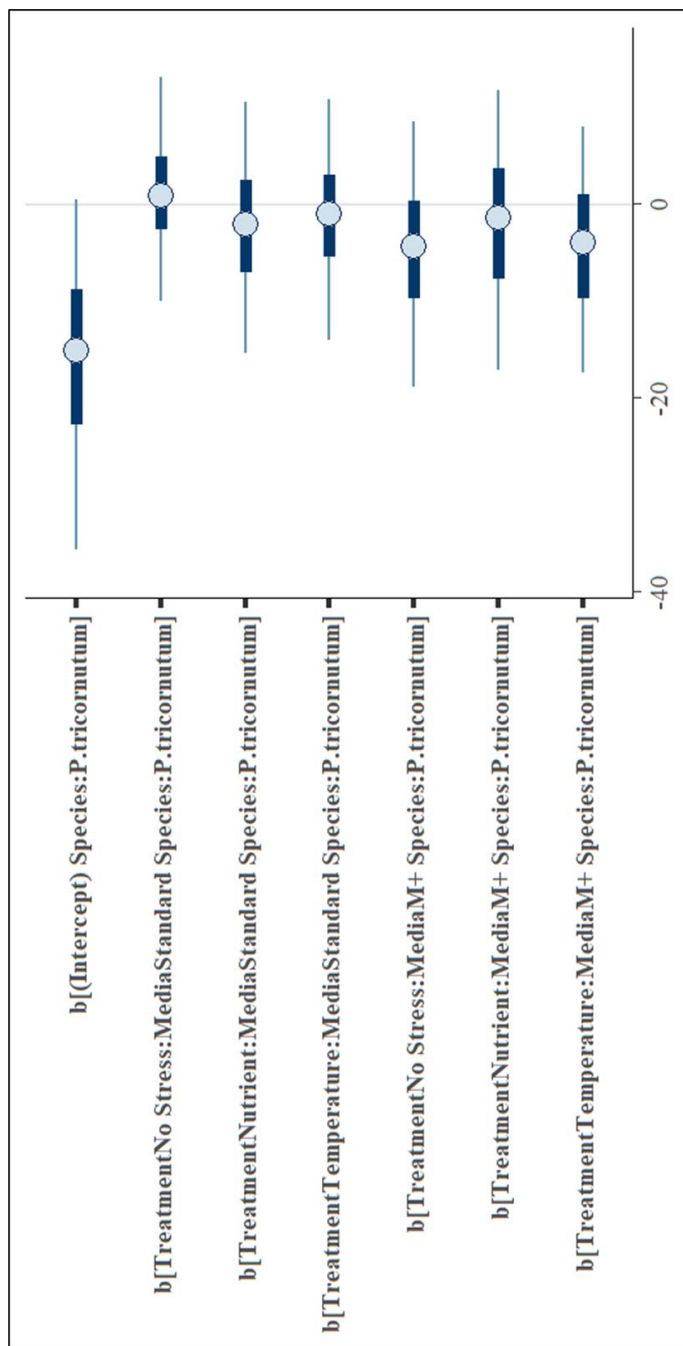
Supplementary Figure 19. 90% confidence interval (line) and median (circle) of *P. tricornutum* Stearic acid



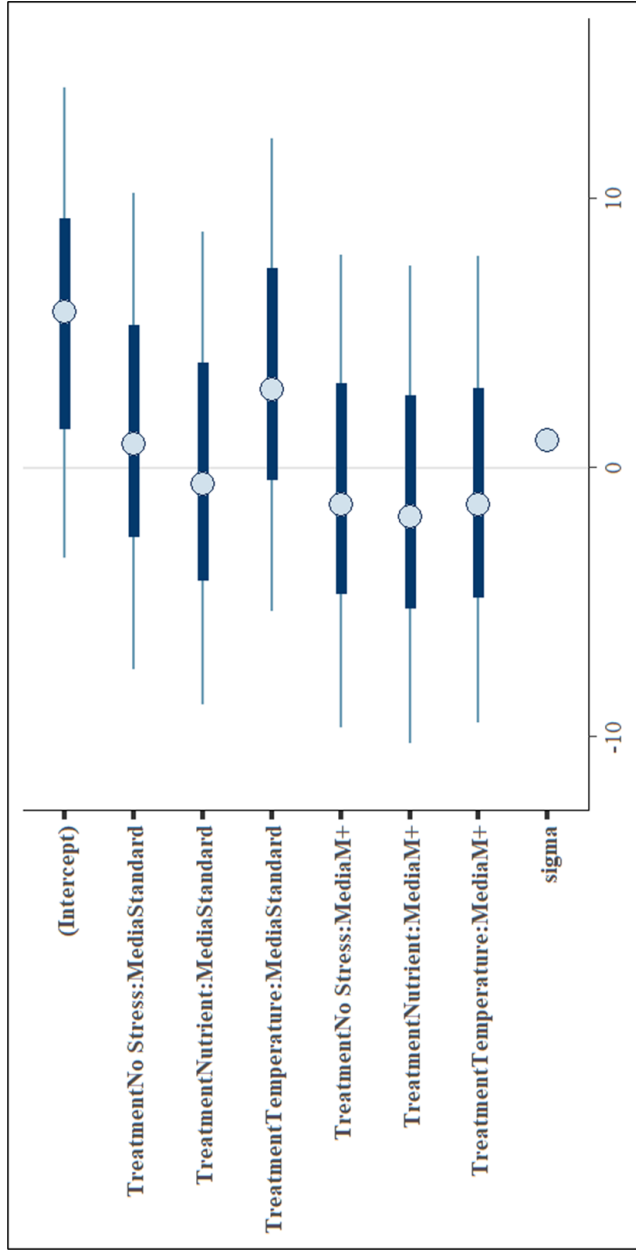
Supplementary Figure 20. 90% confidence interval (line) and median (circle) of *N. oculata* Oleic acid



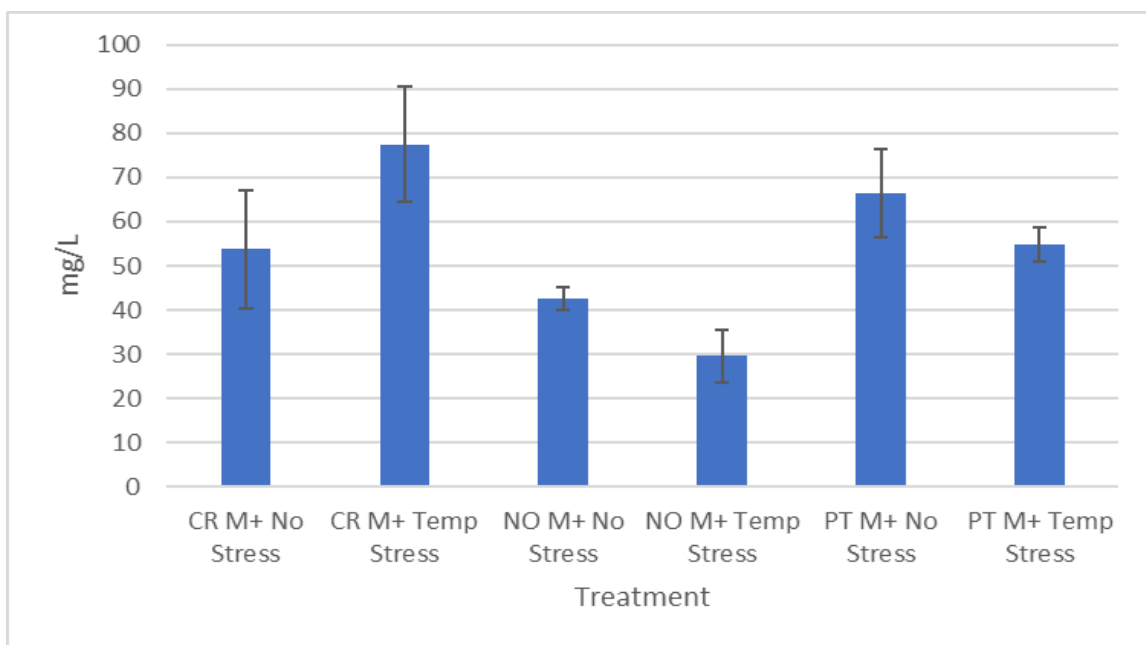
**Supplementary Figure 21. 90% confidence interval (line) and median (circle) of *C. reinhardtii* Oleic acid**



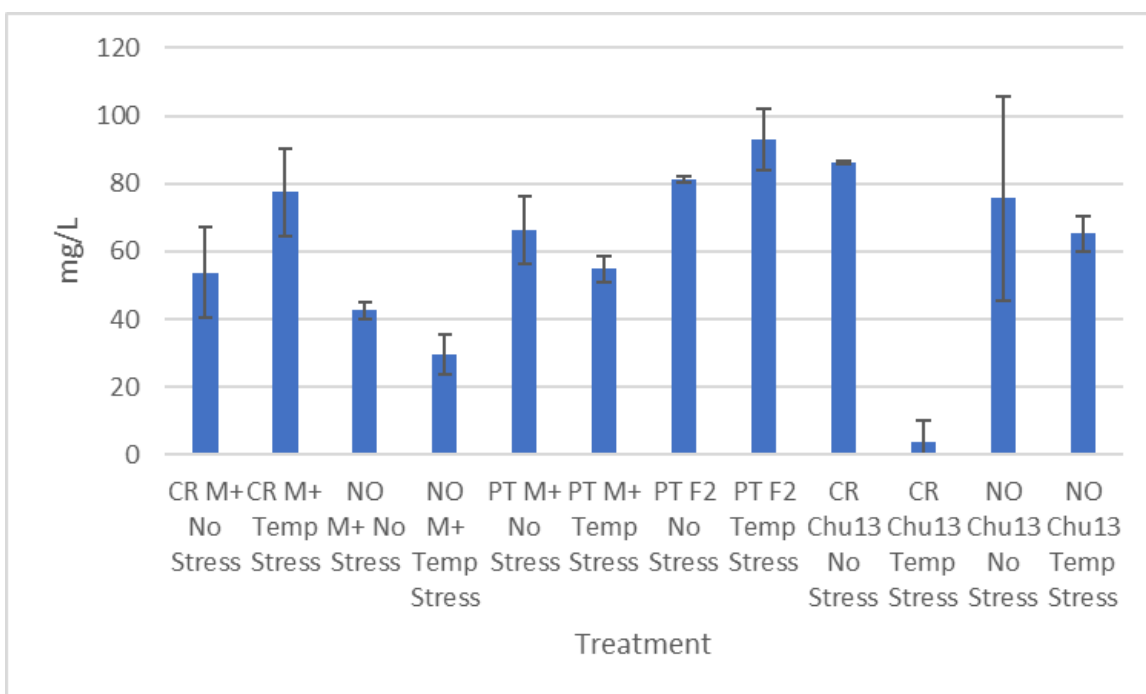
Supplementary Figure 22. 90% confidence interval (line) and median (circle) of *P. tricornutum* Oleic acid



Supplementary Figure 23. 90% confidence interval (line) and median (circle) of *P. tricornutum* Myristic acid



**Supplementary Figure 24.** Maximum Percent recovery of ammonium ( $\text{NH}_4^+$ ) of *C. rienhardtii*, *N. oculata*, and *P.tricornutum* under stress conditions (n=4). Cultures exposed to temperature to a 15°C for 24 hours. Bars represent (+/-) standard deviation.



**Supplementary Figure 25.** Maximum Percent recovery of phosphate ( $\text{PO}_4^{3-}$ ) of *C. rienhardtii*, *N. oculata*, and *P.tricornutum* under stress conditions (n=4). Cultures exposed to temperature to a 15°C for 24 hours and a nutrient stress for 4 days. Bars represent (+/-) standard deviation.