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Evaluation of the robustness of optical density as a tool for estimation of biomass in microalgal cultivation – the effects of growth conditions and physiological state

Running title: Use of optical density as biomass-proxy

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Abstract

We evaluate the robustness of optical density (OD) as a tool in the assessment of the biomass of the cryptophyte *Rhodomonas salina* under different growth conditions. We measure the OD under three different wavelengths, 550, 665 and 750 nm.

We find, as expected, that growth rates of the microalga depend strongly on growth conditions, being highest in saturating light conditions under non-limiting nitrogen availability.

OD – cell count relationships are strong and well defined regardless of growth conditions and wavelength used for measurement. However, measuring within the absorption range of chlorophyll, at 550 and 665 nm, does give higher regression coefficients under conditions leading to a high cell chlorophyll content, while the coefficients of determinations are slightly higher when cell chlorophyll content is low.

We conclude that to use OD as a proxy for biomass under large-scale production of microalgae, it is important to take into account that the precise relationship between OD and algal biomass does depend on factors such as irradiance and nutrient availability, and hence the physiological state of the microalgae as well as the production conditions. Errors in estimation of biomass may range from 44 % to 95 %, if these factors are not taken into consideration.

Keywords

Biomass, Large-scale microalgal production, Microalgal aquaculture, Nutrients, Pigments

Introduction

Large-scale production of microalgal biomass is becoming increasingly important for a number of purposes ranging from biofuel production, over feedstock, to production of pharmaceuticals (Guedes et al., 2011; Sasso et al., 2012; Skarka, 2012). Regardless of the purpose, a fast and efficient method for determining the microalgal biomass and growth rate is needed. Measurement of optical density (OD) is probably the best-suited method for use in large-scale production of microalgal biomass (Sarrafzadeh et al., 2015). It can be done in-line, alleviating the need for opening and sampling in the production system, and it provides data in real-time, without the delay caused by taking and analyzing samples manually. In addition, due to the high degree of automation that can be achieved by using OD measurements, it is also the cheapest of the available methods. Alternative methods include extremely time-consuming manual cell counting by microscopy, chlorophyll analysis involving sampling, extraction and measurement, or automated counting on e.g. an electronic particle counter, which is faster, but still necessitates taking samples from the production system.

However, it is necessary to take into consideration that OD is a proxy for biomass, rather than a measurement of biomass itself. OD is not only determined by the biomass present, but is also affected by various characteristics of the biomass, especially the color or other parameters affecting the opaqueness of the microalgal biomass (Griffiths et al., 2011). The relationship between biomass and OD can therefore be expected to vary depending on the physiological state of the microalgae (Griffiths et al., 2011), and hence on production conditions. OD is also sensitive to contamination of the production system. The presence of dead algae, other organisms, other types of particles, and CDOM (colored dissolved organic matter) from microalgal metabolites will contribute to OD (Kirk, 1994). It is therefore

65 necessary to be able directly to relate OD measurements to the cell concentration or biomass
66 of the microalgae under the actual culture conditions in the production system.
67 The wavelength at which OD is measured will also affect measurements. In
68 fermentation, using bacteria or fungi, OD is usually measured at 750 nm (Shuler & Kargi,
69 2005). However, microalgae universally contain the photosynthetic pigment chlorophyll *a*
70 and 750 nm is outside its absorption range. This has several implications. On one hand, using
71 750 nm means that measurements will be independent of variations in the chlorophyll content
72 of the microalgal biomass. On the other hand, measurements at this wavelength will not be
73 microalgae specific. If instead measurements were done within the absorption range of
74 chlorophyll, measurements would be more sensitive to changes in microalgal biomass and be
75 less affected by other kinds of particles in the production system. However, measurements
76 would be sensitive to changes in the chlorophyll content of the microalgal biomass
77 independent of concomitant changes in biomass. Measuring OD within the absorption range
78 of chlorophyll therefore has both advantages and disadvantages compared to the more
79 common practice of using 750 nm for measurement of OD (Becker, 1994). Several
80 wavelengths within the absorption range of chlorophyll have been suggested for
81 measurement of OD of microalgal cultures, including measuring at wavelengths close to one
82 of the absorption peaks of chlorophyll e.g. around 665 nm, as well as measuring close to the
83 absorption minimum of chlorophyll around 550 nm (Becker, 1994; Griffiths et al., 2011). The
84 former would maximize sensitivity towards microalgal biomass; the latter would minimize
85 effects of changes in chlorophyll content not related to changes in biomass.
86 Changes in microalgal pigmentation depend on external environmental parameters as
87 well as on the physiological state of the algae. It is well know that microalgae compensate for
88 limiting light availability by increasing their cell specific chlorophyll concentration (e.g.

Antoine & Benson-Evans, 1983). Because chlorophyll, as well as the rest of the photosynthetic machinery of algae, contain nitrogen, the nitrogen availability also affects cell chlorophyll concentration (Seip et al., 1992). In this study, we work with the cryptophyte *Rhodomonas salina* (Wislouch) D.R.A.Hill & R.Wetherbee, which is an important feed alga in the production of live feed for aquaculture (Vu et al., 2016). *Rhodomonas*, in addition to chlorophyll *a* and *c*, also contain the pigment phycoerythrin, giving the algae a reddish color. It can be expected that this additional pigment will add further complexity to the establishment of relationships between OD and microalgal biomass, here determined as cell count, as well as carbon biomass and nitrogen biomass. We examine and establish OD – cell count relationships under two different irradiances as well as under two different nitrogen regimes together with growth rates and cell pigment concentrations, and we establish relationships between carbon biomass and nitrogen biomass and cell count. We expect that the OD of the microalgae will vary not only according to their cell density, but also be affected by both light and nitrogen availability, influencing the pigment content of the cells. Furthermore, we expect that changes in cell count will cause a larger increase in OD when measured at 550 and 665 nm than at 750 nm, but also that measuring at 550 and 665 nm will cause an increase in the 'noise' caused by variations in pigment content. We conducted the experiments as short-term batch experiments even though this means that the algal cultures were not in so-called balanced growth. We did this because this type of experiments are relevant to many types of microalgal production, where the algae are rarely in balanced growth.

Materials and Methods

Algal strain and culture conditions

In this study, we used the cryptophyte *Rhodomonas salina*, obtained as SCCAP K-1487 of the Scandinavian Culture Collection of Algae and Protozoa (University of Copenhagen, Denmark). Cultures of *R. salina* were maintained in acid washed 5 L round-bottom glass flasks containing autoclaved 0.2 µm filtered seawater (salinity 30 ‰) enriched with B1 medium (1 mL L^{-1} of seawater, (Hansen, 1989)). The cultures were maintained under a continuous irradiance of 80 µmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) in a thermostatted room at 20 °C. The flasks were gently aerated with atmospheric air (400 ppm CO_2) provided through 0.45 µm filters to mix the cultures to avoid temperature stratification, algal sedimentation, CO_2 depletion and O_2 accumulation.

Experimental design

For the experiments, the microalgae were grown in a Multicultivator MC1000 OD (Photon Systems Instruments, Czech Republic) with eight 100 mL test tubes. The test tubes were immersed in a 5 L rectangular glass container in which water was circulated by a pump through a cooling unit to maintain a stable temperature of 20 °C in all test tubes. Each test tube was bubbled individually with atmospheric air. The test tubes were illuminated by cool-white LEDs. These LEDs emit in the range 400 – 665 nm, with a main peak in the blue area (445 nm) and a secondary peak in the range 535 – 570 nm. Two levels of irradiance were used: 140 µmol photons m^{-2} s^{-1} (PAR) (saturating light) and 20 µmol photons m^{-2} s^{-1} (PAR) (limiting light) (Vu et al., 2016). For the experiments, nutrient medium with two different levels of nitrogen content were used. For all experiments we used the standard B1 medium (Hansen, 1989), except that nitrogen deplete conditions were achieved by using the B1

136 medium with a nitrogen content of $1/10^{th}$ of the standard B1 medium. All other nutrients were
137 kept at surplus concentrations as defined by the standard B1 medium. The effects of light and
138 nitrogen availability were tested in a 2x2 factorial design with four replicates (n = 4).
139
140 *Growth rate measurement*
141 The cell density of *R. salina* was determined twice per day with approximately 12 h intervals
142 during the experimental runs. The algae left the exponential growth phase and entered the
143 stationary phase 35 – 200 h after the initialization of the experimental run, depending on the
144 growth conditions. For calculation of growth rates, only data from the exponential growth
145 phase were included. The cell density as well as the cell biovolume were measured on a
146 Beckman Multisizer3 Coulter Counter (Beckman Coulter Inc., USA). All particles with a
147 diameter in the range 5 – 12 µm were considered algal cells.
148
149 *Cell density – OD relationships*
150 After measurement of cell number and biovolume on the Coulter Counter, the optical density
151 of the same samples was measured without any extraction on a spectrophotometer
152 (GENESYS 6, ThermoFisher, USA) at 550, 665 and 750 nm to achieve corresponding
153 measurements of cell count and optical density in the same samples. It should be noted that
154 the built-in OD measurement facility of the Multicultivator MC-1000 OD was not used in this
155 study, as it does not operate at the wavelengths we wanted to use in measuring OD.
156
157 *Carbon and nitrogen biomass*
158 After measurement of OD as described above, the algal samples were filtered on to glass
159 fiber filters (Whatman GF/C). The filters were dried at 105 °C to constant weight, after which

160 the filters with the retained algae were analyzed on a CHN-analyzer, using cystine and
161 acetanilide as standards, to yield the carbon and nitrogen content of the microalgal biomass as
162 well as the C/N-ratio.
163
164 *Calculation of coefficients for the OD – carbon biomass and OD – nitrogen biomass*
165 *relationships*
166 From the measurements described above, the relationship between OD and cell count (CC) is
167 established as OD = a*CC, the relationship between carbon biomass (CB) and cell count as
168 CB = b*CC, and the relationship between nitrogen biomass (NB) and cell count as NB =
169 c*CC. From it follows that $CB = \frac{b}{a} OD$ and $NB = \frac{c}{a} OD$.
170
171 *Algal pigments*
172 Pigment samples were taken at the conclusion of the cultivation period. Chlorophylls *a* and *c*
173 were extracted using standard methods (Jeffrey & Humphrey, 1975; Ritchie, 2006). Filter
174 samples were lyophilized before extraction. Each of these filters was placed in a glass vial
175 where 3.3 mL of 90 % acetone was added. Samples were shaken on a whirly mixer. Then
176 samples were placed in the dark for 24 h at 5 °C. The extraction solvent in each vial was
177 transferred into a quartz cuvette through a 0.2 µm pore size syringe filter and the absorbance
178 was measured at 664 and 630 nm on a spectrophotometer (GENESYS 6, ThermoFisher,
179 USA). The concentration of chlorophyll *a* and *c* was expressed as pg $cell^{-1}$.
180 Phycoerythrin (PE) was extracted based on procedures described in the literature
181 (Bennett & Bogorad, 1973; Zimba, 2012), and modified in our own laboratory (Thoisen et
182 al., 2017). After lyophilization, each of the filter samples for PE extraction was placed in a
183 glass vial together with 3 mL phosphate buffer (0.1 mol pH 7, 0.05 mol K_2HPO_4, 0.05 mol

184 KH_2PO_4). Samples were sonicated in an ice water bath for 15 min and then left refrigerated
185 for 12 h. Extraction solvent was filtered through a 0.2 µm pore size syringe filter into a
186 cuvette for spectrophotometric measurement of the absorbance at 455, 564 and 592 nm. The
187 concentration of PE was calculated according to the literature (Bennett & Bogorad, 1973),
188 and expressed as pg $cell^{-1}$.
189
190 *Statistical analyses*
191 Growth rates were calculated by ln-transforming the cell counts, followed by linear
192 regression analysis of the increase in cell count over time (Sokal & Rohlf, 1995). For the two
193 N-replete treatments a simple least-squares linear regression was performed. The two N-
194 deplete treatments showed a two-phased growth response due to the onset of N-limitation, so
195 for these treatments sequential least-squares linear regression was used. Only the slope of the
196 first linear sequence, corresponding to exponential growth, is reported in Table 1. GraphPad
197 Prism 8 was used for the linear regression analyses.
198 The relationships between cell number and optical density were evaluated using least-
199 squares linear regression analysis (Sokal & Rohlf, 1995), as described above, using GraphPad
200 Prism 8.
201 Cell count – carbon biomass and cell count – nitrogen biomass relationships were
202 analyzed using linear regression analysis. As with the growth rates, simple least squares
203 linear regression was used for the two N-replete treatments, while sequential least-squares
204 linear regression was used for the two N-deplete treatments, that showed a saturation of the
205 nitrogen biomass at high cell counts due to N-limitation. As for the growth rate analyses, we
206 used GraphPad Prism 8.

The slopes of the resulting regression lines from the above analyses were tested against each other for statistically significant differences based on the procedures described in Sokal and Rohlf (1995), using the TestSlopes procedure of the RealStats package v. 5.4.2 with $\alpha = 0.05$.

Chlorophyll *a* and *c* as well as PE concentrations were subjected to one-way ANOVA with treatment as a fixed factor. Tukey's test was subsequently used to compare individual means across treatments. Data were tested for homogeneity of variance (Cochran's test) and normal distribution (Kolmogorov-Smirnoff goodness of fit test) before being analyzed by ANOVA (Quinn & Keough, 2002). All tests were carried out using SYSTAT v. 13 with $\alpha = 0.05$.

Results

Growth rates

Growth rates, as established through direct cell counts, varied between treatments (evaluation of 95 % C.I., Table 1, Fig. 1). The highest growth rate was seen under nitrogen replete conditions under saturating light, were a specific growth rate of 0.82 d^{-1} was obtained. The second highest specific growth rate was obtained under nitrogen deplete, saturating light, conditions and found to be 0.50 d^{-1}. Limiting light conditions yielded the lowest specific growth rates with a growth rate of 0.21 d^{-1} under both nitrogen conditions. It should be noted that for the two nitrogen deplete treatments, only the first part of the sequential regression lines are considered in the comparison of growth rates, as the sequential linear regression analysis show a decline in growth rate, probably due to nitrogen limitation, after 78 hours under light limitation and after 35 hours under light saturation.

231 *Cell count – OD relationships*

232 The relationships found between direct cell counts and optical density are shown in Figs. 2A
233 (nitrogen replete, limiting light), 2B (nitrogen replete, saturating light), 2C (nitrogen deplete,
234 limiting light) and 2D (nitrogen deplete, saturating light). The linear relationships are all
235 strongly statistically significant, with slopes in the range 2.3 – 3.6, all significantly different
236 from 0 (least-squares linear regression, $p << 0.05$). The slopes and coefficients of
237 determination (the R^2 – values) are further evaluated in Fig. 3 and Table 1. The slopes
238 measured at 665 and 550 nm for nitrogen replete, limiting light conditions are 3.5 – 3.6 and
239 significantly higher than the rest of the slopes (2.3 – 2.8) ($p << 0.001$), but not significantly
240 different from each other ($p > 0.05$, Fig. 3 and Table 1). This indicates that under nitrogen
241 replete limiting light conditions, the direct cell count is accompanied by a larger increase in
242 OD when OD is measured within the absorption range of chlorophyll than is the case for the
243 other treatments. Measurement at 665 and 550 nm tend to give a higher increase in OD per
244 change in cell count than measurement at 750 nm, although this is only statistically
245 significant under nitrogen replete conditions, independently of light level ($p << 0.001$, Table
246 1, Fig. 3), not at nitrogen deplete conditions ($p > 0.05$, Table 1, Fig. 3). The coefficients of
247 determination are all high, > 0.95 (Table 1), but with a clear tendency for the nitrogen deplete
248 saturating light treatment to give the highest determination coefficients, ≈ 0.98. This
249 treatment thus gives the strongest relationship between cell count and OD with some 98 % of
250 the variation in OD being explained by increases in cell count (Table 1, Fig. 3).

251

252 *Variation in carbon and nitrogen biomass and cell content*

253 The carbon biomass of all treatments showed a direct linear relationship to the increasing cell
254 counts (Table 1, Fig. 4). The slope of this relationship is significantly lower for algae grown

255 under nitrogen replete, but light limited conditions (4.53 x 10^{-5} µg C $cell^{-1}$), than for the other
256 three treatments that varied from 8.13 – 8.97 x 10^{-5} µg C $cell^{-1}$ with no statistically significant
257 differences among them (Table 1, Fig. 4). The nitrogen biomass also showed initial linear
258 increases with cell count in all four treatments (Table 1, Fig. 5). However, for the two
259 nitrogen deplete treatments, the nitrogen biomass leveled off at cells counts of 2.58 x 10^6
260 under light limitation and 2.86 x 10^6 under light saturation (Fig. 5), and did not show any
261 further increase despite continued increase in the cell counts. This is reflected in changes in
262 the cell C/N-ratios and cell nitrogen content over time (Fig. 6), were the two nitrogen deplete
263 treatments show a decline in C/N-ratio and cell nitrogen content over time, while the two
264 nitrogen replete treatments do not show any statistically significant changes in these
265 parameters over time. None of the four treatments shows any statistically significant changes
266 in cell carbon content over time.
267
268 *Pigment content*
269 Chlorophyll concentrations are shown in Table 1 and Fig. 7. Concentrations of chlorophyll *a*
270 are significantly higher under nitrogen replete limiting light conditions (4.3 pg $cell^{-1}$) than
271 under the other treatments (ANOVA followed by Tukey post-hoc test, $p < 0.0001$). There is a
272 tendency for chlorophyll *a* concentration to be higher under light limited conditions, although
273 is only statistically significant for the nitrogen replete treatment. Concentrations of
274 chlorophyll *c* were very low, but tended to follow the same overall pattern as for chlorophyll
275 *a*.
276 Phycoerythrin concentrations are shown in Fig. 8. Concentrations varied from 0.72 to
277 7.35 pg $cell^{-1}$. Concentrations were higher under nitrogen replete conditions than under

nitrogen deplete conditions (ANOVA followed by Tukey post-hoc test, $p < 0.0001$), with a non-significantly tendency to be higher under limiting than under saturating light (Fig. 8).

Discussion

Not surprisingly, the growth rates of *Rhodomonas* in our study depend on both irradiance and nitrogen availability. The highest growth rate is thus achieved with saturating light and high nitrogen availability and is 0.82 d^{-1}, which is in good accordance with growth rates reported in the literature for *Rhodomonas* growing under optimal conditions (Guevara et al., 2016). The lowest growth rates, 0.21 d^{-1}, achieved under limiting light, are among the lowest reported in the literature, and confirms that this set of conditions is strongly limiting for *Rhodomonas* growth (Hammer et al., 2002). Comparison across treatments indicate that light is the more important factor controlling growth rate in present study, as the growth rate drops a factor of almost four when lowering the irradiance with the same nitrogen availability. In contrast lowering nitrogen availability under the same irradiance only lowers the growth rate from 0.82 d^{-1} to 0.50 d^{-1} under saturating light and not at all under light limitation.

Chlorophyll does not vary in parallel with growth rate, indicating that growth rate is not directly controlled by cell chlorophyll content. The highest cell chlorophyll concentrations are achieved under low irradiance and high nitrogen availability, as is usually found to be the case in algae and plants (e.g. Horton et al., 1996); while the lowest cell chlorophyll concentrations likewise are found under high irradiance. Phycoerythrin shows a similar pattern, with the highest concentrations under nitrogen replete conditions and with a non-significant tendency to be higher under light limitation. Similar results have been reported before (Vu et al., 2016), and are no doubt caused by the fact that phycoerythrin in

301 *Rhodomonas* is a light-harvesting billi-protein pigment with a similar role in cryptophyte
302 photosynthesis as chlorophyll.

303 The OD – cell count relationships evince high R^2 - values and cell count always explain
304 more than 95 % of the variation in OD regardless of treatment and wavelength, indicating
305 that the contribution of changes in cellular properties to OD is always small. This means that
306 no matter the physiological state of the microalgae and regardless of which wavelength used,
307 within the absorption range of chlorophyll or not, it is possible to establish a good, strong
308 relationship between OD and cell concentration. Hence, OD seems a very robust proxy for
309 microalgal biomass in different physiological states. The regression slopes, however, clearly
310 depend on the physiological state of the algae, as well as on the wavelength used. They are
311 highest at low irradiance and high nitrogen availability, just like cell chlorophyll content, but
312 are lowest under low irradiance and low nitrogen availability This most likely is due to the
313 OD – cell count relationship being governed primarily by nitrogen availability and to a lesser
314 extent by irradiance. The regression coefficients of the two saturating light treatments are
315 intermediate. Interestingly, this pattern is seen for all three wavelengths, although it was
316 expected that only OD measured at the two wavelengths within the absorption range of
317 chlorophyll, 665 and 550 nm, would depend on cell chlorophyll content. It is possible that
318 cell chlorophyll content co-vary with other parameters contributing to the absorption at 750
319 nm (Griffiths et al., 2011).

320 Higher cell chlorophyll contents not surprisingly cause a higher OD for any given cell
321 concentration. This is obviously most pronounced at 665 and 550 nm where we find the
322 highest slopes of the cell count – OD relationships. The differences in R^2 - values are always
323 very small, ranging from 0.95 to 0.98.

All four treatments show strong, linear, relationships between cell counts and carbon biomass. Interestingly, the slope of the relationship for the nitrogen replete, light limited treatment is only approximately 50 % (4.53) of the slopes of the three other treatments (8.13 – 8.97). It is possible that this is caused by microalgal cells under light limited, but nitrogen replete, conditions investing more in nitrogen uptake and storage in e.g. amino acids and pigments than in increased carbon biomass. This hypothesis is in accordance with the observed patterns of chlorophyll and phycoerythrin contents in the cells. In accordance with the observed linear relationships between cell count and carbon biomass, the cell carbon content is constant in all four treatments. We also observe linear relationships between cell counts and nitrogen biomass, but for the two nitrogen limited treatments only up to a certain point, where nitrogen apparently becomes limiting, and the carbon biomass of the culture continues to increase, while the nitrogen biomass remains constant. This happens at approximately the same cell densities regardless of light conditions, at a cell count of about $2.6 * 10^5$ cells ml^{-1} under light limited conditions and $2.8 * 10^5$ cells ml^{-1} under light saturation. It is accompanied by a decrease in cellular nitrogen content and increasing C/N – ratios of the cells from both nitrogen limited treatments, while the cellular nitrogen content and C/N – ratios remain constant for cells under nitrogen replete conditions.

We established coefficients for the relationships between OD and both carbon biomass and nitrogen biomass. These were highest when OD was measured at 750 nm, but only slightly lower when measured at the two other wavelengths, 665 and 550 nm. Obviously, these coefficients are affected both by the established relationships between OD and cell count and between cell count and carbon and nitrogen biomass, respectively.

We can conclude that it is indeed possible to use the convenient, fast and reliable OD measurement as a proxy for microalgal biomass during microalgal biomass production. The

three different wavelengths used in our study can all be used for measurements of OD, although the two wavelengths that lie within the absorption range of chlorophyll give stronger OD signals for any increase in microalgal biomass, especially under nitrogen replete conditions, which may be advantageous in some situations. The R^2 – values are always high, evincing very small differences, indicating that the 'noise' caused by cell pigment content is minimal. It is, however, important to take into account that the precise relationship between OD and biomass, here measured as cell count, does depend on factors such as irradiance and nutrient availability. These factors determine the physiological state of the algae, which translates into their overall absorbance signal, which must therefore be established in each specific case and set of production parameters. Especially if the microalgae become nitrogen limited during growth the OD measurements need to be interpreted carefully as cell division will cease under these conditions, while an increase in carbon biomass can still be observed. Failing to consider these factors, may cause over- or under-estimation of the biomass present, which can have serious economic consequences further downstream in the production. Based on differences among slopes, errors in biomass estimation may vary from 44 % based on cell concentration, over 66 % for carbon biomass, to 95 % for nitrogen biomass.

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Data availability statement

All data in presented in this paper are original for this study. The data that support the findings of this study are available from the corresponding author upon request.

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Table 1

Data for growth rates (μ) of the microalga *Rhodomonas salina*, slopes and squared multiple correlation coefficients of cell count – optical density relationships; as well as slopes of cell count – carbon biomass and cell count – nitrogen biomass for the algae growth under the four different treatment conditions in this experiment. Results are expressed as means ± 1 SD.

Treatment	Growth rate, μ (d^{-1})	Slope of cell count – OD relationship ($\times 10^{-5}$)			R^2			Slope of cell count – carbon biomass relationship ($\times 10^{-5}$)	Slope of cell count – nitrogen biomass relationship ($\times 10^{-5}$)
		750 nm	665 nm	550 nm	750 nm	665 nm	550 nm		
Nitrogen replete, Limiting light	0.21 ± 0.01	2.79 ± 0.12	3.47 ± 0.14	3.62 ± 0.14	0.952	0.956	0.958	4.53 ± 0.36	0.91 ± 0.07
Nitrogen replete, Saturating light	0.82 ± 0.03	2.47 ± 0.09	2.72 ± 0.09	2.72 ± 0.084	0.964	0.966	0.971	8.13 ± 0.60	0.80 ± 0.24
Nitrogen deplete, Limiting light	0.21 ± 0.03	2.32 ± 0.15	2.62 ± 0.16	2.67 ± 0.14	0.958	0.965	0.974	8.97 ± 0.72	1.22 ± 0.62
Nitrogen deplete, Saturating light	0.50 ± 0.07	2.57 ± 0.12	2.71 ± 0.13	2.70 ± 0.13	0.979	0.978	0.978	8.27 ± 0.99	0.43 ± 0.07

Table 2

Calculated coefficients for the relationships between optical density and carbon and nitrogen biomass, respectively. Carbon biomass is expressed as µg C ml^{-1} and nitrogen biomass as µg N ml^{-1}. For the two nitrogen deplete treatments, coefficients for nitrogen-limited growth (Fig. 5) are given in brackets.

Treatment	**Carbon biomass - OD**			**Nitrogen Biomass - OD**		
	750 nm	**665 nm**	**550 nm**	**750 nm**	**665 nm**	**550 nm**
Nitrogen replete, Limiting light	1.62	1.31	1.25	0.33	0.26	0.25
Nitrogen replete, Saturating light	3.29	2.99	2.99	0.32	0.29	0.29
Nitrogen deplete, Limiting light	3.87	3.42	3.36	0.53 (-0.002)	0.47 (-0.002)	0.45 (-0.002)
Nitrogen deplete, Saturating light	3.22	3.05	3.06	0.17 (-0.004)	0.16 (-0.003)	0.16 (-0.003)

Figure legends

Figure 1

Growth in cell counts over time in the four treatments. A: Nitrogen replete, limiting light; B: Nitrogen replete, saturating light; C: Nitrogen deplete, limiting light; D: Nitrogen deplete, saturating light. The lines represent the fitted regression lines. For treatments A and B, simple least-squares regression lines are given. For treatment C and D, the lines represent sequential least-squares regression lines. Please note that X-axes have different scales for nitrogen replete vs. deplete treatments and that the Y-axes are logarithmic.

Figure 2

Relationship between cell count and optical density (OD), measured at three different wavelengths, 750, 665 and 550 nm. The lines represent the fitted least-squares regression lines. Panel A: Nitrogen replete, limiting light treatment. Panel B: Nitrogen replete, saturating light treatment. Panel C: Nitrogen deplete, limiting light treatment. Panel D: Nitrogen deplete, saturating light treatment.

Figure 3

Comparisons of slopes for the regression lines from the cell count – optical density relationships for the four treatments, measured at the three wavelengths, 750, 665 and 550 nm. A: Nitrogen replete, limiting light; B: Nitrogen replete, saturating light; C: Nitrogen deplete, limiting light; D: Nitrogen deplete, saturating light. Error bars represent 95 % C.I., allowing direct visual comparisons.

Figure 4

Increase in carbon biomass as a function of increasing cell count per ml. The lines represent the fitted least-squares regression lines. Panel A: Nitrogen replete, limiting light treatment. Panel B: Nitrogen replete, saturating light treatment. Panel C: Nitrogen deplete, limiting light treatment. Panel D: Nitrogen deplete, saturating light treatment.

Figure 5

Increase in nitrogen biomass as a function of increasing cell count per ml. Panel A: Nitrogen replete, limiting light treatment. Panel B: Nitrogen replete, saturating light treatment. Panel C: Nitrogen deplete, limiting light treatment. Panel D: Nitrogen deplete, saturating light treatment. The lines represent the fitted regression lines. For treatments A and B, simple least-squares regression lines are given. For treatment C and D, the lines represent sequential least-squares regression lines.

Figure 6

Variation in C/N-ratio (upper panel), cell carbon content (middle panel) and cell nitrogen content (lower panel). Circles: nitrogen replete treatments, squares: nitrogen deplete treatments, filled data points: limiting light, open data points: saturating light. Least-squares linear regression lines were fitted if the variation of the parameter over time was found to be statistically significant.

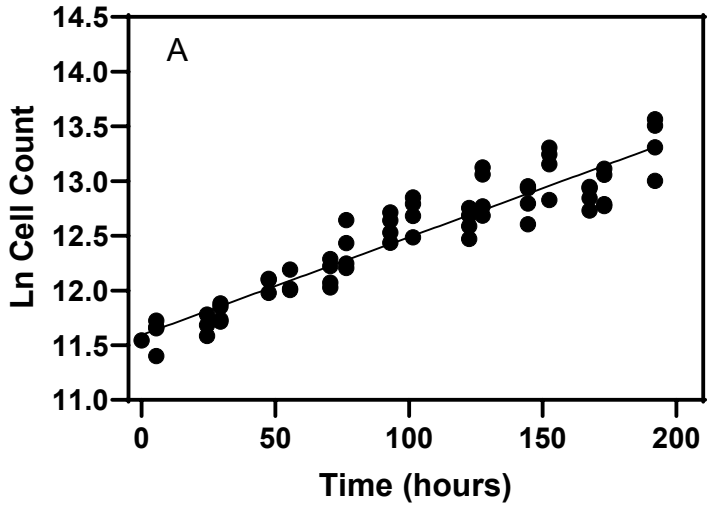
Figure 7

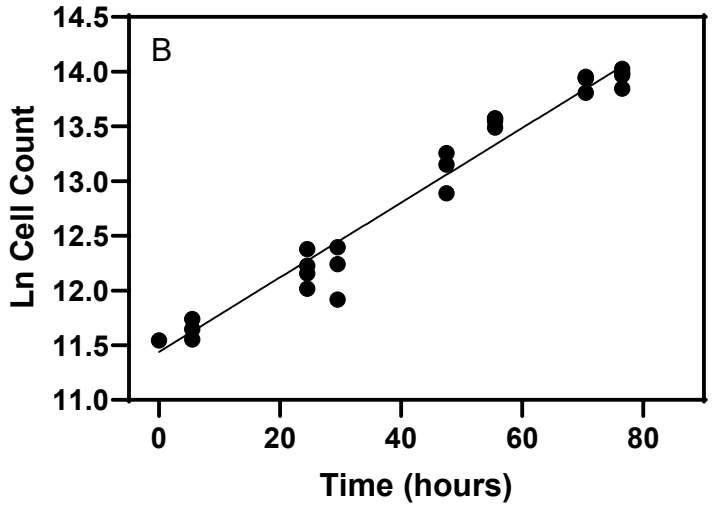
Chlorophyll concentrations (pg $cell^{-1}$) of algae from the four treatments. Values are given as means with error bars indicating 1 SD. Different letters indicate statistically significant

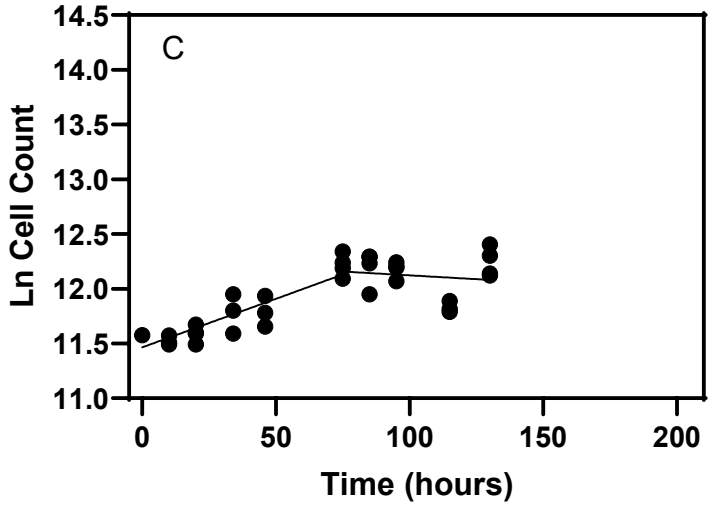
differences between treatments. Black bars and capital letters: Chl *a*, grey bars and lower case letters: Chl *c*, open bars indicate the sum of Chl *a* and *c* concentrations. A: Nitrogen replete, limiting light; B: Nitrogen replete, saturating light; C: Nitrogen deplete, limiting light; D: Nitrogen deplete, saturating light.

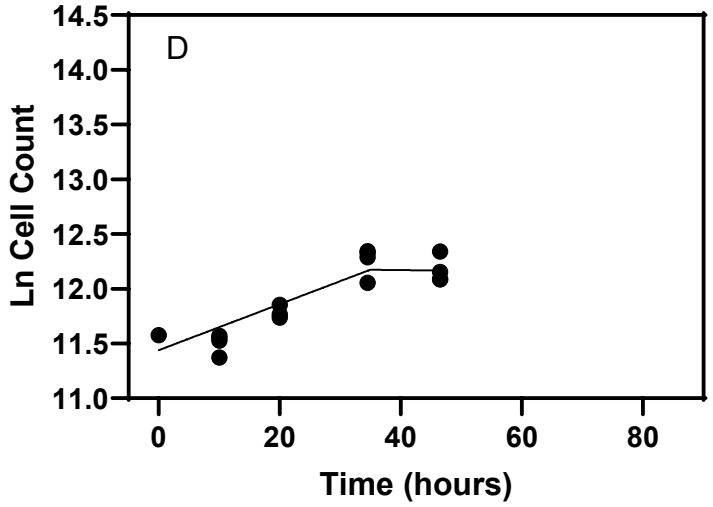
Figure 8

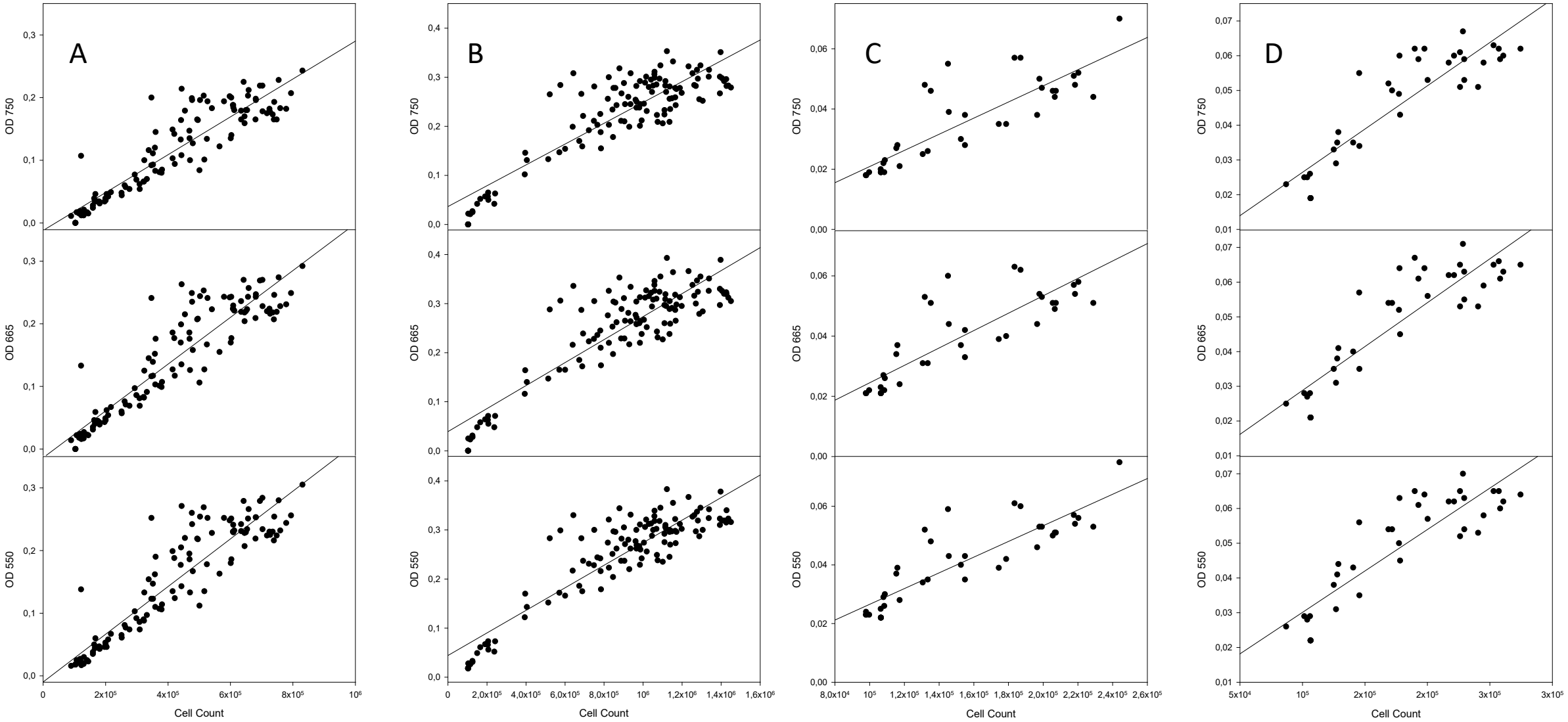
Phycoerythrin concentrations (pg $cell^{-1}$) of algae from the four treatments. Values are given as means with error bars indicating 1 SD. Different letters indicate statistically significant differences between treatments. A: Nitrogen replete, limiting light; B: Nitrogen replete, saturating light; C: Nitrogen deplete, limiting light; D: Nitrogen deplete, saturating light.

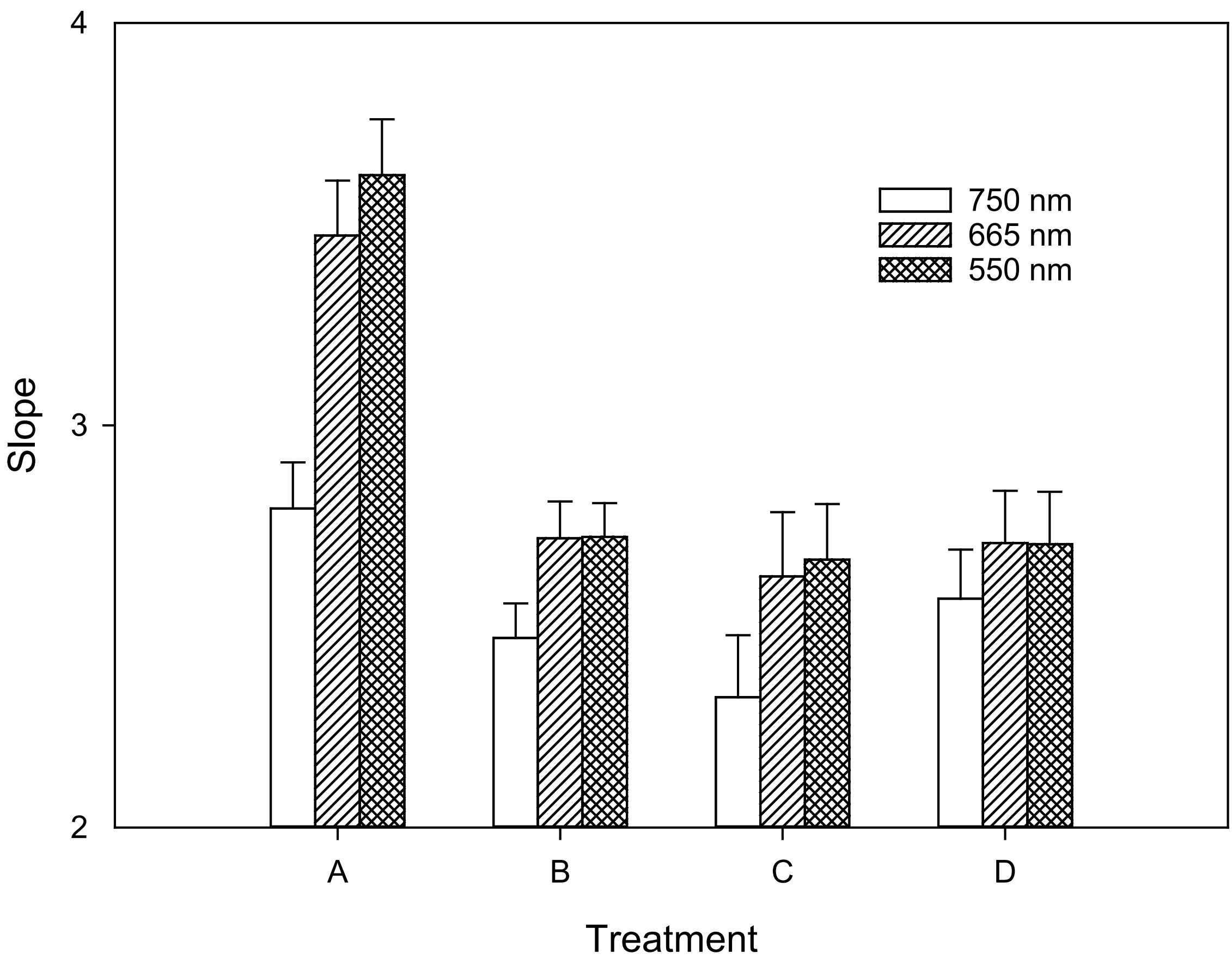

A
Ln Cell Count
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
0
50
100
150
200
Time (hours)

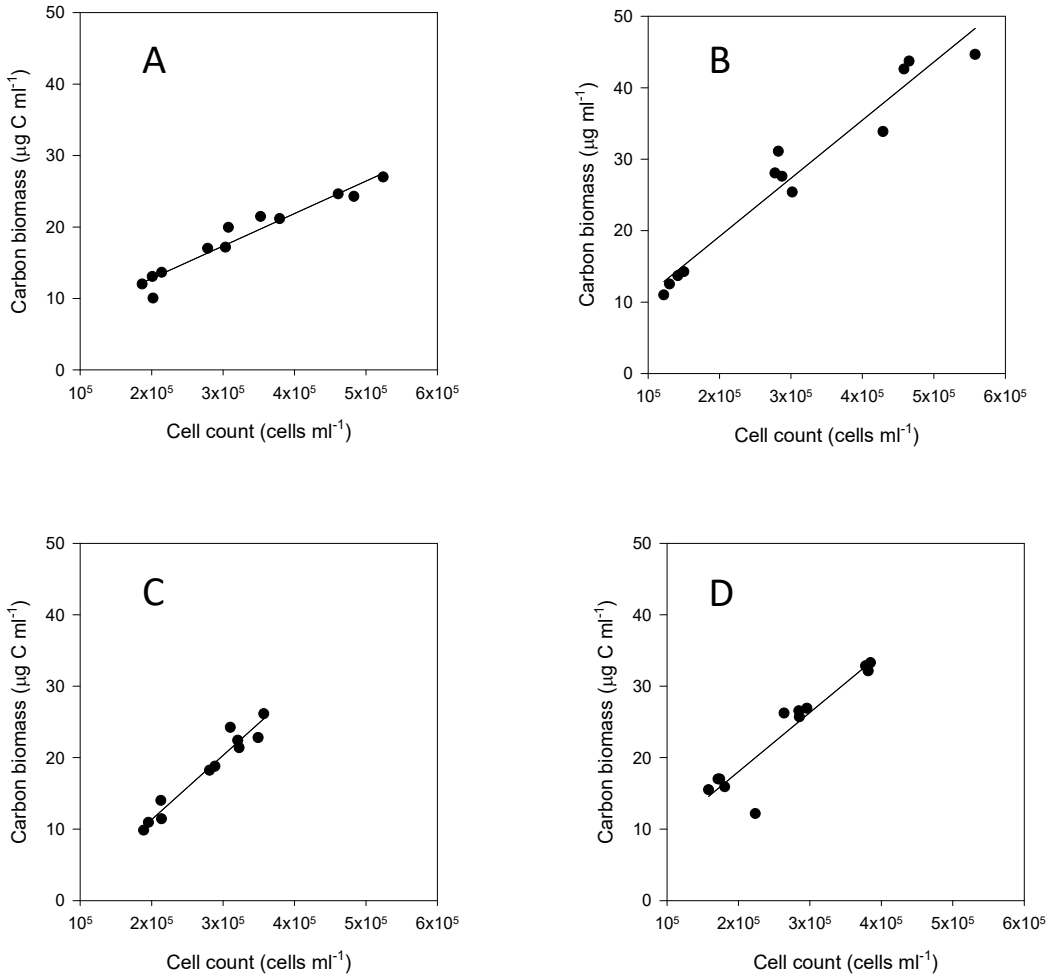

B
Ln Cell Count
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
0
20
40
60
80
Time (hours)

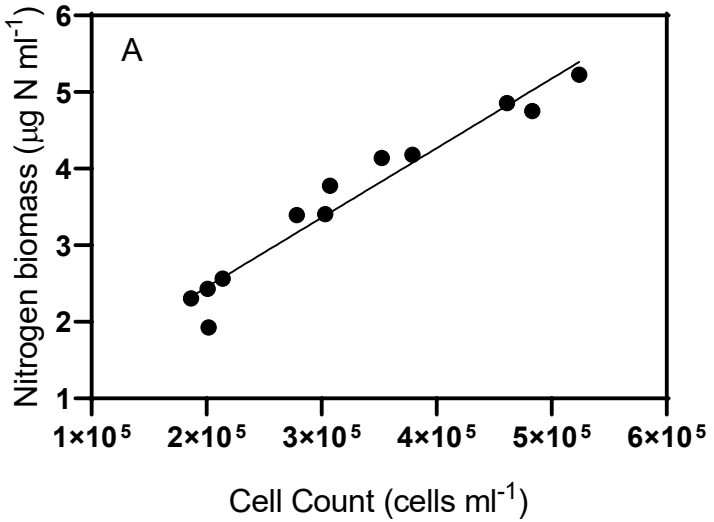

C
Ln Cell Count
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
0
50
100
150
200
Time (hours)

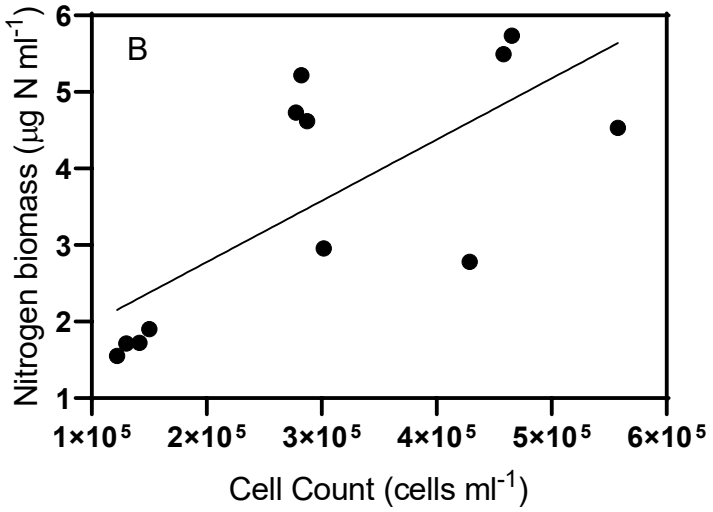

D
Ln Cell Count
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
0
20
40
60
80
Time (hours)

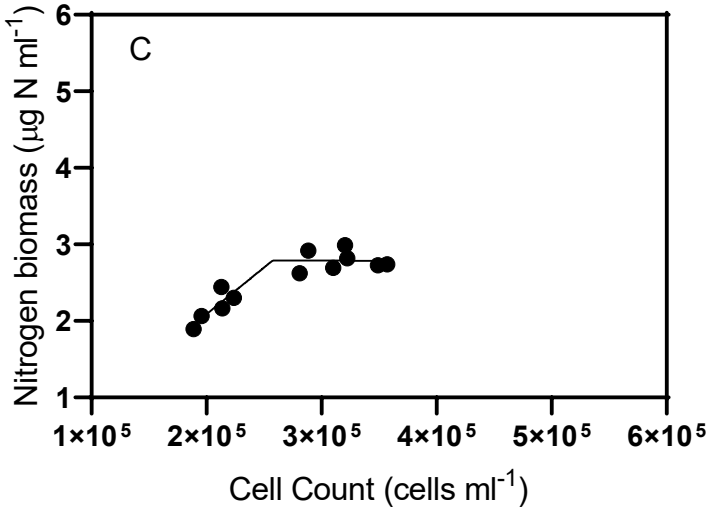

A
B
C
D
OD 750
OD 665
OD 550
Cell Count
0
2x10⁵
4x10⁵
6x10⁵
8x10⁵
10⁶
2,0x10⁵
4,0x10⁵
6,0x10⁵
8,0x10⁵
1,2x10⁶
1,4x10⁶
1,6x10⁶
8,0x10⁴
10⁵
1,2x10⁵
1,4x10⁵
1,6x10⁵
1,8x10⁵
2,0x10⁵
2,2x10⁵
2,4x10⁵
2,6x10⁵
5x10⁴
2x10⁵
3x10⁵

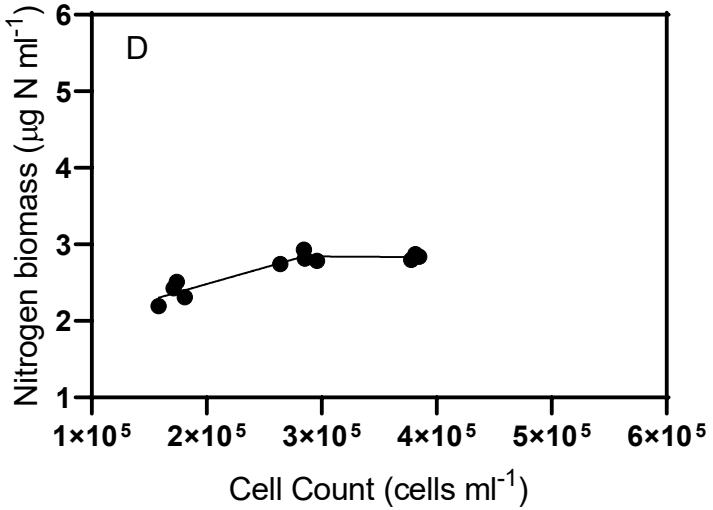

4
3
2
Slope
750 nm
665 nm
550 nm
A
B
C
D
Treatment

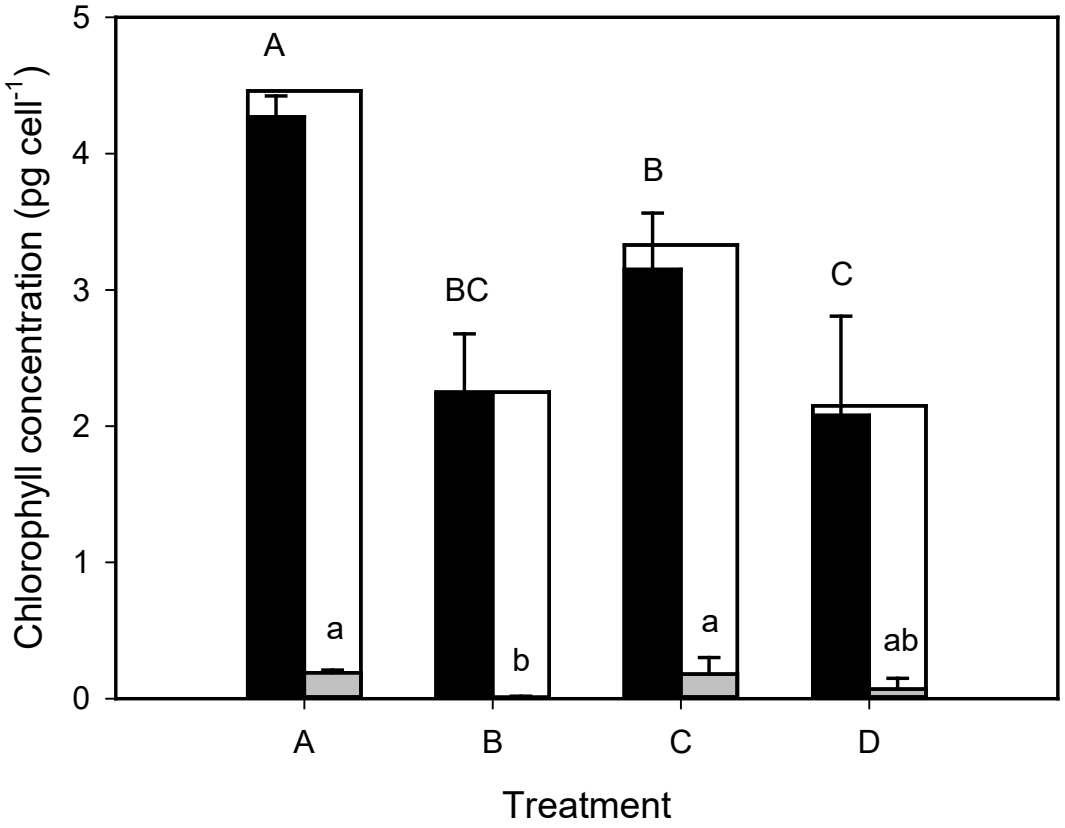

A
B
C
D
Carbon biomass (µg C ml-1)
Cell count (cells ml-1)
0
10
20
30
40
50
10^5
2x10^5
3x10^5
4x10^5
5x10^5
6x10^5

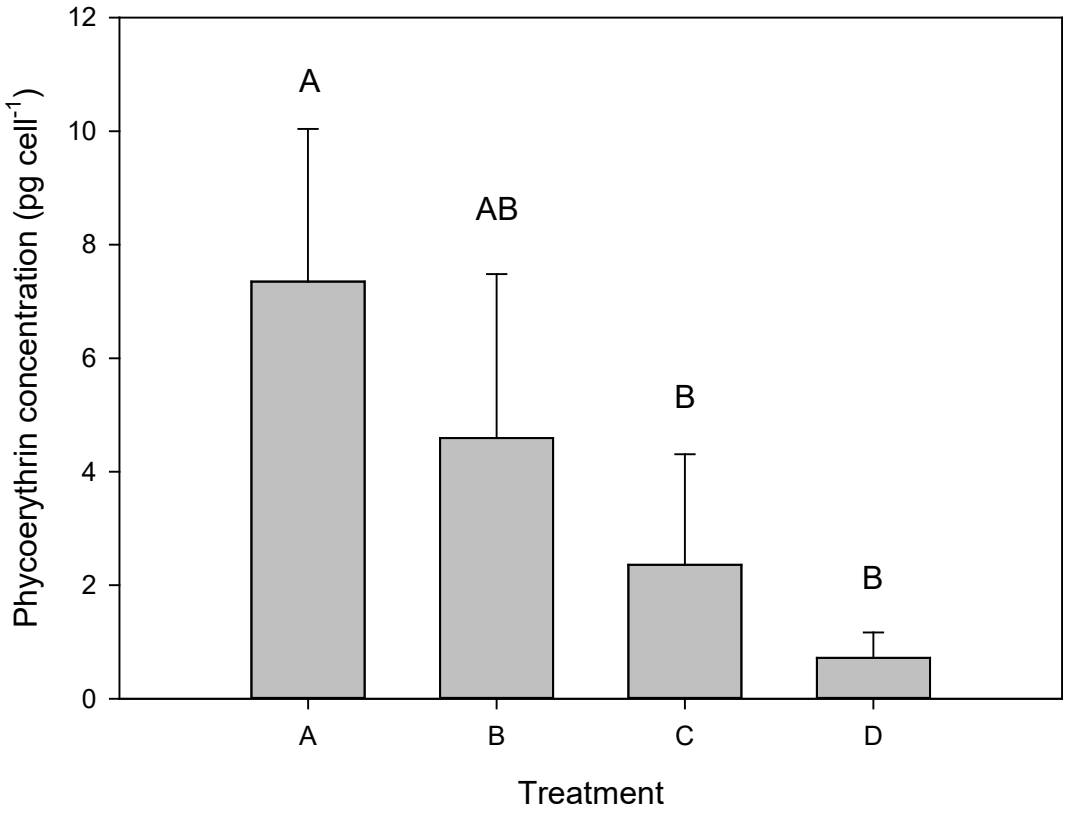

A
Nitrogen biomass (µg N ml-1)
Cell Count (cells ml-1)
1
2
3
4
5
6
1×10 5
2×10 5
3×10 5
4×10 5
5×10 5
6×10 5


B
Nitrogen biomass (µg N ml-1)
Cell Count (cells ml-1)
1
2
3
4
5
6
1×10 5
2×10 5
3×10 5
4×10 5
5×10 5
6×10 5


C
Nitrogen biomass (µg N ml-1)
Cell Count (cells ml-1)
1
2
3
4
5
6
1×10 5
2×10 5
3×10 5
4×10 5
5×10 5
6×10 5


D
Nitrogen biomass (µg N ml-1)
Cell Count (cells ml-1)
1
2
3
4
5
6
1×10 5
2×10 5
3×10 5
4×10 5
5×10 5
6×10 5


Chlorophyll concentration (pg cell-1)
5
4
3
2
1
0
A
BC
B
C
a
b
a
ab
A
B
C
D
Treatment


12
10
8
6
4
2
0
Phycoerythrin concentration (pg cell-1)
A
AB
B
B
A
B
C
D
Treatment