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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

Nielsen, Søren Laurentius; Hansen, Benni Winding

1   Evaluation of the robustness of optical density as a tool for estimation of biomass in microalgal

2   cultivation – the effects of growth conditions and physiological state

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4   Running title: Use of optical density as biomass-proxy

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6   Søren Laurentius Nielsen[*]

7   Orcid ID: 0000-0003-4309-5153

8   Benni Winding Hansen

9   Orcid ID: 0000-0003-1145-561X

10   Department of Science and Environment

11   Roskilde University

12   Universitetsvej 1

13   DK-4000 Roskilde

14   Denmark

15

16   [*]: Corresponding author, e-mail: nielsen@ruc.dk; phone: +45 4674 2722

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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.

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.

89   Antoine & Benson-Evans, 1983). Because chlorophyll, as well as the rest of the

90   photosynthetic machinery of algae, contain nitrogen, the nitrogen availability also affects cell

91   chlorophyll concentration (Seip et al., 1992). In this study, we work with the cryptophyte

92   *Rhodomonas salina* (Wislouch) D.R.A.Hill & R.Wetherbee, which is an important feed alga

93   in the production of live feed for aquaculture (Vu et al., 2016). *Rhodomonas*, in addition to

94   chlorophyll *a* and *c*, also contain the pigment phycoerythrin, giving the algae a reddish color.

95   It can be expected that this additional pigment will add further complexity to the

96   establishment of relationships between OD and microalgal biomass, here determined as cell

97   count, as well as carbon biomass and nitrogen biomass. We examine and establish OD – cell

98   count relationships under two different irradiances as well as under two different nitrogen

99   regimes together with growth rates and cell pigment concentrations, and we establish

100   relationships between carbon biomass and nitrogen biomass and cell count. We expect that

101   the OD of the microalgae will vary not only according to their cell density, but also be

102   affected by both light and nitrogen availability, influencing the pigment content of the cells.

103   Furthermore, we expect that changes in cell count will cause a larger increase in OD when

104   measured at 550 and 665 nm than at 750 nm, but also that measuring at 550 and 665 nm will

105   cause an increase in the 'noise' caused by variations in pigment content. We conducted the

106   experiments as short-term batch experiments even though this means that the algal cultures

107   were not in so-called balanced growth. We did this because this type of experiments are

108   relevant to many types of microalgal production, where the algae are rarely in balanced

109   growth.

110

111

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 $CB = \dfrac{b}{a}OD$ and $NB = \dfrac{c}{a}OD$.

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$_2$PO$_4$). 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.

207   The slopes of the resulting regression lines from the above analyses were tested against

208   each other for statistically significant differences based on the procedures described in Sokal

209   and Rohlf (1995), using the TestSlopes procedure of the RealStats package v. 5.4.2 with $\alpha =$

210   0.05.

211   Chlorophyll *a* and *c* as well as PE concentrations were subjected to one-way ANOVA

212   with treatment as a fixed factor. Tukey's test was subsequently used to compare individual

213   means across treatments. Data were tested for homogeneity of variance (Cochran's test) and

214   normal distribution (Kolmogorov-Smirnoff goodness of fit test) before being analyzed by

215   ANOVA (Quinn & Keough, 2002). All tests were carried out using SYSTAT v. 13 with $\alpha =$

216   0.05.

217

218   **Results**

219   *Growth rates*

220   Growth rates, as established through direct cell counts, varied between treatments (evaluation

221   of 95 % C.I., Table 1, Fig. 1). The highest growth rate was seen under nitrogen replete

222   conditions under saturating light, were a specific growth rate of 0.82 d^{-1} was obtained. The

223   second highest specific growth rate was obtained under nitrogen deplete, saturating light,

224   conditions and found to be 0.50 d^{-1}. Limiting light conditions yielded the lowest specific

225   growth rates with a growth rate of 0.21 d^{-1} under both nitrogen conditions. It should be noted

226   that for the two nitrogen deplete treatments, only the first part of the sequential regression

227   lines are considered in the comparison of growth rates, as the sequential linear regression

228   analysis show a decline in growth rate, probably due to nitrogen limitation, after 78 hours

229   under light limitation and after 35 hours under light saturation.

230

Cell count – OD relationships

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

Variation in carbon and nitrogen biomass and cell content

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

255   under nitrogen replete, but light limited conditions (4.53×10^{-5} µg C cell^{-1}), than for the other

256   three treatments that varied from $8.13 – 8.97 \times 10^{-5}$ 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 2.58×10^6

260   under light limitation and 2.86×10^6 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

12

278   nitrogen deplete conditions (ANOVA followed by Tukey post-hoc test, $p < 0.0001$), with a

279   non-significantly tendency to be higher under limiting than under saturating light (Fig. 8).

280

281   **Discussion**

282   Not surprisingly, the growth rates of *Rhodomonas* in our study depend on both irradiance and

283   nitrogen availability. The highest growth rate is thus achieved with saturating light and high

284   nitrogen availability and is 0.82 d^{-1}, which is in good accordance with growth rates reported

285   in the literature for *Rhodomonas* growing under optimal conditions (Guevara et al., 2016).

286   The lowest growth rates, 0.21 d^{-1}, achieved under limiting light, are among the lowest

287   reported in the literature, and confirms that this set of conditions is strongly limiting for

288   *Rhodomonas* growth (Hammer et al., 2002). Comparison across treatments indicate that light

289   is the more important factor controlling growth rate in present study, as the growth rate drops

290   a factor of almost four when lowering the irradiance with the same nitrogen availability. In

291   contrast lowering nitrogen availability under the same irradiance only lowers the growth rate

292   from 0.82 d^{-1} to 0.50 d^{-1} under saturating light and not at all under light limitation.

293   Chlorophyll does not vary in parallel with growth rate, indicating that growth rate is not

294   directly controlled by cell chlorophyll content. The highest cell chlorophyll concentrations

295   are achieved under low irradiance and high nitrogen availability, as is usually found to be the

296   case in algae and plants (e.g. Horton et al., 1996); while the lowest cell chlorophyll

297   concentrations likewise are found under high irradiance. Phycoerythrin shows a similar

298   pattern, with the highest concentrations under nitrogen replete conditions and with a non-

299   significant tendency to be higher under light limitation. Similar results have been reported

300   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

348 three different wavelengths used in our study can all be used for measurements of OD,

349 although the two wavelengths that lie within the absorption range of chlorophyll give

350 stronger OD signals for any increase in microalgal biomass, especially under nitrogen replete

351 conditions, which may be advantageous in some situations. The R^2 – values are always high,

352 evincing very small differences, indicating that the 'noise' caused by cell pigment content is

353 minimal. It is, however, important to take into account that the precise relationship between

354 OD and biomass, here measured as cell count, does depend on factors such as irradiance and

355 nutrient availability. These factors determine the physiological state of the algae, which

356 translates into their overall absorbance signal, which must therefore be established in each

357 specific case and set of production parameters. Especially if the microalgae become nitrogen

358 limited during growth the OD measurements need to be interpreted carefully as cell division

359 will cease under these conditions, while an increase in carbon biomass can still be observed.

360 Failing to consider these factors, may cause over- or under-estimation of the biomass present,

361 which can have serious economic consequences further downstream in the production. Based

362 on differences among slopes, errors in biomass estimation may vary from 44 % based on cell

363 concentration, over 66 % for carbon biomass, to 95 % for nitrogen biomass.

364

371

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 $(x\ 10^{-5})$			R^2			Slope of cell count – carbon biomass relationship $(x\ 10^{-5})$	Slope of cell count – nitrogen biomass relationship $(x\ 10^{-5})$
		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.