The dark side of algae cultivation: Characterizing night biomass loss in three photosynthetic algae, *Chlorella sorokiniana*, *Nannochloropsis salina* and *Picochlorum* sp.

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**Abstract**

Night biomass loss in photosynthetic algae is an essential parameter that is often overlooked when modeling or optimizing biomass productivities. Night respiration acts as a tax on daily biomass gains and has not been well characterized in the context of biomass production for biofuels. We examined the night biomass loss in three algae strains that may have potential for commercial biomass production (*Nannochloropsis salina* — CCMP1776, *Chlorella sorokiniana* — DOE1412, and *Picochlorum* sp. LANL-WT). Biomass losses were monitored by ash free dry weight (AFDW mg L$^{-1}$) and optical density (OD$_{750}$) on a thermal-gradient incubator. Specific night biomass loss rates were highly variable (ranging from $-0.006$ to $-0.59$ day$^{-1}$), species-specific, and dependent on both culture growth phase prior to the dark period and night pond temperature. In general, the fraction of biomass lost over a 10 h dark period, which ranged from ca. 1 to 22% in our experiments, was positively correlated with temperature and declined as the culture transitioned from late exponential to linear to late linear phase. The dynamics of biomass loss should be taken into consideration in algae strain selection, are critical in predictive modeling of biomass production based on geographic location, and can influence the net productivity of photosynthetic cultures used for bio-based fuels or products.

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**1. Introduction**

Fossil fuels currently supply the majority of world energy demand; however the nonrenewable nature and accumulation of atmospheric carbon dioxide limit the suitability of fossil fuels as long-term energy sources [1]. One of the outstanding challenges of the current century is renewable, sustainable solar energy capture and storage [2]. Biomass, solar energy stored in chemical bonds, may play a significant role in providing renewable fuels compatible with the current fossil fuel infrastructure. Photosynthetic algae hold great promise in converting large quantities of solar radiation into biomass, which can be transformed into solid, liquid, or gaseous fuels [3]. Advantages of algae over terrestrial biomass crops include the capability to use non-potable water and non-arable land, while storing the majority of solar energy in relatively simple compounds such as polysaccharides, proteins and lipids, with low concentrations of recalcitrant structural compounds (e.g., lignin).

Algae as a polyphyletic group are extremely diverse [4] and therefore selecting candidates for industrial biomass production has been a formidable task. Algal strain development and characterization remain a challenge for improving the sustainability of algae based-biofuels [5]. Many physiological aspects can be considered when prospecting, breeding or engineering algae. Depending on the end use of the biomass, characteristics such as fatty acid profiles [6], protein content [7], or high value products [8] may be of interest as selection criteria. In many biofuel-focused screening efforts, lipids (primarily triacylglycerols) produced by algae were considered the primary feedstock for biofuel production and selection of strains hinged on the ability of the organism to accumulate oil [9–13]. However, in one of the first life cycle assessments of microalgal biofuels, Lardon et al. [14] demonstrated that energetic costs associated with lipid extraction (drying biomass, solvent use, etc.) accounted for 90% of the process energy consumption. Further techno-economic and life cycle assessments arrive at similar conclusions, regarding lipid extraction as an energy and capital intensive aspect of algae based biofuel production [15,16]. Recent developments in hydrothermal liquefaction (HTL) of algae biomass may improve the energy balance of algae generated biofuels when compared with biodiesel production after lipid extraction [17–21]. With HTL technological advances for whole algal biomass conversion into fuels, selection of lipid-producing algae becomes less critical and biomass productivity becomes the primary selection criterion.

The quantification of biomass productivity is not as straightforward as simply measuring the growth rate of an algal strain. Many environmental and physiological factors influence the ultimate yield of...
captured solar energy in algal biomass. Substantial respiratory losses at night, for example, can significantly impact the biomass production capacity of solar-based algae cultures and therefore the suitability of strains for biofuel production. Several studies report that greater than 30% of the biomass fixed during the day in outdoor, sunlit algae cultures (both ponds and photobioreactors) can be lost at night [22–24]. A commonly used method of assessing respiratory losses in algae cultures is the use of potentiometric oxygen electrodes, which can conveniently measure both the photosynthetic production of O₂ as well as the consumption of O₂ due to respiration. Interestingly, ratios of respiration to photosynthesis in algae can vary substantially across different algae taxa [25–27]. In extreme cases, taxonomic variability in respiration to photosynthesis ratios can span an order of magnitude (0.05 to 0.59 day⁻¹) [28]. Apart from metabolic variability among different algae, environmental factors such as temperature have been demonstrated to have marked effects on algal respiration [26–33]. Additionally, the physiological state of the culture (growth phase/cell density) can influence respiratory losses at night [23,29,30,34,35]. Selecting strains not only on cell composition or growth rate, but also on minimal biomass loss over the dark period can potentially improve biomass productivity by increasing the retention of captured solar energy as biomass.

Much of the previous work on characterizing algal respiration in the dark relied heavily on changes in oxygen concentrations as a proxy for biomass losses in the dark [25,28,29]. Despite the valuable insight provided by these studies, they typically used small volumes (ca. 1 mL), constant temperatures and short incubations (<10 min); these estimates of respiration are difficult to extrapolate to actual biomass losses in large volume ponds under fluctuating environmental conditions and lengthy night periods. Furthermore, pond temperatures at night can vary significantly from daytime pond temperatures [32]. Knowing the actual change in biomass concentration under variable night temperature conditions is a critical parameter that is often neglected in predictive phycological modeling attempts [36]. Determining biomass loss rates at a range of night temperatures and growth phases prior to the night can improve the accuracy of predictive modeling in outdoor environments.

In light of the species-specific, environmentally-mediated variability in algal respiration, it is the objective of the current study to evaluate biomass losses at a range of night temperatures and several growth phases prior to the dark period in three species of algae currently considered as possible candidates for large scale algae-based biofuels (Nannochloropsis salina — CCMP1776, Chlorella sorokiniana — DOE1412, and Picchularum sp. LANL-WT). These parameters are considered, based upon the current available literature, to be the most critical in describing the loss of biomass over the night period under conditions that would be encountered in outdoor algae cultivation. Assessing night biomass loss rates under variable environmental and physiological conditions supports a robust characterization of biomass productivity prior to field testing.

2. Materials and methods

2.1. Microalgae cultivation conditions and media

Cultures were grown in well-mixed 1 L Roux bottles filled with sterile media at an average surface light intensity of ca. 500 μmol photons m⁻² s⁻¹ and a 14:10 (day:night) photoperiod. Average surface light intensity was calculated based on five measurements across the Roux bottle surface using a LI-190 quantum sensor (LI-COR, Inc., Lincoln, Nebraska). Three microalgae strains were characterized: C. sorokiniana (DOE1412, obtained from Dr. Juergen Polle of City University of New York), Picchularum sp. (LANL-WT, obtained from Dr. Taraka Dale of Los Alamos National Laboratory), and N. salina (CCMP1776, obtained and confirmed by 18s rRNA molecular identification from the National Center for Marine Algae and Microbiota). The freshwater strain C. sorokiniana was cultivated in a modified BG-11 medium [37] with 17.6 mM NO₃ and 0.66 mM PO₄ at a day time temperature of 25 °C. The marine strain Picchularum was cultivated in a modified f/2-Si medium with 8.8 mM NO₃ and 0.25 mM PO₄ at a salinity level of 32 ppt and a day time temperature of 27 °C. N. salina was cultivated in a modified f/2-Si medium with 4.0 mM NO₃ and 0.1 mM PO₄ at a salinity level of 35 ppt and day time temperature of 23 °C [38]. Cultures were maintained at a pH of 7.0 for the freshwater medium and 7.5 for saltwater media using humidified, sterile filtered, CO₂-enriched air distributed through glass fritted gas diffusers (40–60 μm pore size). Roux bottles were placed on stir plates with stir bars to keep cells in suspension. Prior to inoculation, the cultures were checked by microscopic observation of pelletized samples to ensure no obvious bacterial, fungal, zoo-plankton, or algal contamination.

2.2. Biomass loss rates (µdark)

Three inoculation densities were used to assess dark loss at different biomass concentrations (AFDW of ca. 100, 200, and 500–600 mg L⁻¹; OD₇₅₀ of 0.4, 1.0, and 3.0) to represent growth stages in cell cultivation (i.e., late exponential, linear, and late linear). After inoculation, the cultures were grown in 1 L Roux bottles as previously described for 14 h under ca. 500 μmol photons m⁻² s⁻¹ light, divided into four 50 mL aliquots and placed in 125 mL Erlenmeyer flasks wrapped in aluminum foil. Covered flasks containing algae were placed on a shaking thermal gradient incubator for 10 h rotating at 70 rpm at four temperature set points (Fig. 1). Cultures were sparged with humidified, sterile filtered CO₂-enriched air to avoid oxygen depletion and to maintain the medium pH. Total culture volume was initially measured in a 100 mL graduated cylinder to the nearest 0.5 mL, and afterwards in an identical graduated cylinder. Volume lost was determined by difference and the evaporation due to sparging over the 10 h period was compensated by addition of sterile, deionized water. Biomass was measured as optical density at 750 nm (OD₇₅₀) and ash-free dry weight (AFDW) as described in Van Wagener et al. [39]. Biomass measurements were taken at the beginning and end of the 10 h dark period and used to calculate biomass loss (%) as a fraction of biomass prior to dark incubation. The specific dark loss rates (µdark day⁻¹) were calculated as:

$$\mu_{dark} = \frac{\ln B_f - \ln B_i}{\Delta t}.$$  

Where delta t is the dark incubation time period, B_f is the final biomass after the dark incubation, and B_i is the initial biomass.

![Fig. 1. Biomass growth and loss (AFDW mg L⁻¹) in Nannochloropsis salina CCMP1776 over a typical incubation (ca. 14 h in the light and 10 h in the dark), showing both diurnal growth (daytime temperature of 23 °C incident light intensity of 500 μmol photons m⁻² s⁻¹) and nocturnal biomass loss at the range of temperatures set on the thermal gradient incubator. Error bars represent the standard error of the mean, n = 3 at t0, n = 5 at t15, and n = 2 at t25.](image-url)
2.3. Biomass light absorption coefficient ($k_a$) and average light intensity ($I_{avg}$)

The biomass light absorption coefficient ($k_a$) of culture samples was measured as previously described by Huesemann et al. [40] and used to calculate the average light intensity ($I_{avg}$) to which the cells were exposed during the light period [41]:

$$I_{avg} = I_0 \cdot \frac{1 - e^{-k_a \cdot OD_{750} \cdot d}}{k_a \cdot OD_{750} \cdot d}$$

where $d$ is the horizontal Roux bottle culture depth ($d = 0.046$ m) and $I_0$ is the light intensity at the surface of the culture. The average light intensity was determined using the average $OD_{750}$ during the light period for each of the three represented growth stages, i.e., average $OD_{750} = \frac{1}{2} (\text{final } OD_{750} + \text{initial } OD_{750})$.

3. Results and discussion

Observed biomass loss rates in the dark ($\mu_{dark}$) and the extent of biomass loss (% of total biomass) of the three characterized organisms ($N. salina$ CCMP1776, $C. sorokiniana$ DOE1412, and $P. sp.$ LANL-WT) were highly variable. Loss rates over the temperature-controlled dark incubation spanned two orders of magnitude ($-0.006$ to $-0.59 \text{ day}^{-1}$). Aggregate data over all temperatures and biomass densities demonstrate that variability of biomass loss both between and within species was considerable (Fig. 2). Despite high inter- and intra-species variability, average biomass losses of the three organisms were relatively similar ($6.8 \pm 1.4\%$), as were rates of biomass loss ($-0.17 \pm 0.037 \text{ day}^{-1}$). Minimum loss rates of the three organisms were comparable and relatively close to zero ($-0.006$ to $-0.012 \text{ day}^{-1}$) and generally occurred at the lowest tested temperatures (10–15 °C). In contrast, maximum loss rates were highly variable between species ($-0.32$ to $-0.59 \text{ day}^{-1}$), similar to previously reported accounts of interspecies respiration [26–28]. $N. salina$ showed both the greatest variability and the greatest rate of biomass loss at 24 °C ($-0.59 \text{ day}^{-1}$), nearly twice the maximum loss rate observed by Michels et al. [34] for the marine strain Tetraselmis suecica at 20 °C (ca. $-0.3 \text{ day}^{-1}$). Although difficult to directly compare freshwater and marine organisms adapted to several media, the high variability of $\mu_{dark}$ rates and the extent of total biomass lost over a ten hour dark incubation indicate that the commonly held assumption of a fixed percentage night biomass loss due to respiration is over simplistic and as noted by Geider and Osborne [28] should be applied with caution.

![Fig. 2. Aggregated data over all experimental temperatures and biomass densities for N. salina CCMP1776 (n = 46), C. sorokiniana DOE1412 (n = 57), and P. sp. LANL-WT (n = 53). A) Biomass loss rates in the dark ($\mu_{dark}$ 1/day) and B) extent of biomass loss (% of total biomass). Lower and upper portions of the ‘box’ represent the second and third quartiles, respectively. The middle line of the ‘box’ represents the mean of the data set. Lower and upper ‘whiskers’ represent the minima and maxima of the data sets, respectively.](image-url)
This is especially true when modelling growth and loss in highly dynamic environments [36], such as outdoor algae cultivation ponds where temperature and light are in constant flux.

Apart from the species specific nature of respiration, environmental factors such as temperature and growth phase also impact the rate of biomass loss in algae cultures. It is generally acknowledged that temperature plays a role in night biomass loss in algae cultures [22,23,29,31,42]. In the current study, a general trend of increasing biomass loss corresponded with increasing dark incubation temperatures, although this was more pronounced for N. salina and C. sorokiniana than for P. belli (Fig. 3). Temperature is a major and practically uncontrollable (economically) driving force of outdoor pond productivity. Just as growth rate is known to be impacted by the water temperature of the medium [43], the dark biomass loss rates observed in this study were likewise impacted by temperature. It is postulated that increasing dark incubation temperature increases metabolic costs of maintenance and in accordance μdark rates at higher temperatures were generally greater than those observed at cooler temperatures, regardless of growth phase (Tables S1 and S2). An interesting exception to this trend was P. belli, which showed only a weak influence of temperature on μdark rates. While μdark in exponentially growing N. salina was 53% greater at 25 °C than at 15 °C, μdark in exponentially growing P. belli was only 22% greater over the same temperature range (Fig. 3). Responses to temperature shifts may be explained by presumed physiological differences between two unrelated organisms (N. salina is a Eustigmatophyte and P. belli sp. is a Chlorophyte). Reducing medium temperature at night may be an approach to limit biomass loss by slowing the metabolic rates of the algal cells, as suggested by Vonshef et al. and Hindersin et al. [42,44]. Although potentially practical in small ponds [45] or photobioreactors [44], lowering the night-time temperatures in large outdoor ponds is difficult and not economically feasible for fuel production.

Biomass density of an algae pond is a factor easily regulated by controlling the dilution rate of the pond with fresh or recycled medium. Growth phase (estimated by average light intensity) prior to dark incubation was a key driving factor in the rate and extent of biomass loss in the dark. Cells exposed to high average light intensities (late exponential phase) exhibited the highest rates of biomass loss in the dark (Fig. 4, Table S1), which is in agreement with previous studies on post-illumination respiration [35]. Both growth rates and dark loss rates increase in magnitude with more dilute, exponentially growing cultures (Fig. 5). This “light history effect” was also observed by Torzillo et al. [24], where Spirulina platensis cells exposed to higher light intensities (lower biomass concentration) had greater night biomass losses. Ogbonna and Tanaka [30] demonstrated that night biomass losses in Chlorella pyrenoidosa were greatest during the exponential phase of growth and least during the stationary phase of cultivation. This phenomenon was observed by Hu et al. and Michelis et al. [23,34], who both suggested that night biomass loss was related to diurnal biomass density preceding the dark period. Rapidly growing cells in exponential phase cultures have significantly increased metabolic rates, and are presumably penalized with high biomass loss rates over the subsequent dark period. In contrast, Le Borgne and Pruvost [31] did not observe a significant effect when examining biomass decay rates at different biomass densities. A possible explanation for this lack of a light history effect is the high biomass densities used in their experimental comparison (ca. 1 and 1.7 g/L), where average light intensities were very low and thus effects of growth phase may have been minor.

The effect of average light intensity on night biomass loss rates held true for all three organisms tested in the current study (Tables S1 and S2) and provides useful general insight into the management of large ponds where, in order to maximize productivity, a steady state biomass concentration should be set to a theoretically optimal cell culture density. In practice, steady state cell culture densities are either set at convenient volumetric dilutions or solely to maximize growth rates. An alternative approach would adjust the biomass density to minimize the ratio of μdark/μlight and therefore minimize the amount of biomass lost during the dark. As indicated from the data in Fig. 5, growth rates and biomass loss rates have an inverse relationship; the greater the estimated rate of growth during the day, the greater the observed loss rate over the subsequent night. In this study, ratios of μdark/μlight ranged from 2.2 to 38.7%. The range of this ratio is similar to the range found by Falkowski and Owens [27] using rates of respiration over photosynthesis (8.6–48.2%). The ratio of μdark/μlight for individual alga cultures could conceivably be used to determine the optimum average light intensity to maximize growth (solar gains) and simultaneously minimize biomass loss in the subsequent dark period.

Biomass losses over the dark period may also be limited by cultivation practices that support minimal dark respiration, such as reduced mixing at night. Reduced dissolved oxygen may suppress night biomass losses, and thus limited mixing of algae cultures at night may be a ‘best practice’ for minimizing dark losses. Ogbonna and Tanaka [30] assessed the influence of mixing on cell biochemical composition during the night. Without mixing, cells showed only a moderate decrease in biomass lost over the dark period (from 7 to 5.6%). These experiments were most likely O2 limited, regardless of mixing, as neither the control...
nor treatment was aerated during the dark period. Turning off mixing methods at night (e.g., paddlewheels, airlifts, and aerators) may also reduce power consumption and would be especially convenient for solar voltaic powered operations. However, reducing the amount of dissolved oxygen at night may have consequences for the composition of the algae biomass. For instance, in some species of algae, night is the primary time for synthesis of proteins [30]. If the production of high value products (such as proteins or pigments) requires night aeration, the reduced biomass productivity may be a reasonable cost. In the context of generating biomass for fuel production, the content of proteins or pigments may be negligible. Nevertheless, increased protein and pigment content may increase the growth rate of the culture during the next photoperiod, especially under adverse culture conditions such as high light and cold temperatures, where protein degradation by photodamage can outpace repair mechanisms [46,47] and auxiliary pigments can reduce the extent of photo-damage [48]. In addition to impacting the algal metabolism, dissolved oxygen can influence the metabolism of other microbial pond inhabitants. Because this study did not use axenic cultures, it is possible that heterotrophic bacteria influenced night biomass losses, however high bacterial populations were not observed before or after the dark incubation period. Dissolved organic carbon (DOC) released by algae is not captured in the quantification of AFDW. Heterotrophic bacteria could conceivably consume DOC increasing the total measured AFDW, a measure that does not readily distinguish algal and bacterial biomass, leading to an underestimation of microalgae biomass loss rates. Conversely, pathogenic bacteria could cause cell lysis, releasing cellular organics and exaggerating biomass loss rates. Heterotrophic–autotrophic interactions are expected to play a much larger role in outdoor, open pond cultivation scenarios and should be addressed by future research.

The two common methods of measuring biomass in algae cultures employed in this study, optical density measurements at 750 nm (OD750) and AFDW, were markedly different. Compared with AFDW measurements, OD750 significantly over predicted biomass loss rates in all three organisms tested (Fig. 6). This was most pronounced in N. salina, with rates determined by OD750 up to 57.4% greater than rates determined by AFDW. Changes in cell size and number over the dark period, due to cell division, likely explain the discrepancy in OD750 and AFDW measurements and therefore calculated rates of biomass loss. Cells grown under a light:dark photoperiod often divide in a circadian rhythm. Cell counts were performed for N. salina culture before and after the dark period to investigate this possibility. Cell number increased after dark incubation, which corresponded with smaller cell sizes, especially at higher temperatures. At the wavelength of 750 nm, optical density is mostly a measure of light scattering and therefore, in addition to measuring changes in cell number, this optical method is highly susceptible to changes in cell size [46]. We caution the use of optical methods to measure night biomass loss and, when possible, gravimetric methods are recommended.

4. Conclusions

In algae, significant fractions of daily photosynthetic productivity can be lost at night to respiration. Night biomass loss remains an under-appreciated aspect of optimizing algae productivity in outdoor pond cultivation and, as suggested by Hu et al. [23], potentially represents one of the most important limitations to productivity. From the collected data in this study, the rate and extent of biomass loss in the dark are a species-specific physiological characteristic mediated by environmental conditions. Two dominant environmental factors driving biomass loss in algae cultures are night pond water temperature and light exposure prior to the dark period. All three organisms characterized for night biomass loss were highly sensitive to growth phase prior to the dark period, which relates to biomass density and therefore average light intensity (i.e., late exponential = high light intensity, linear = medium light intensity and late linear = low light intensity, see also Tables S1 and S2).

![Fig. 5](image-url) The inverse relationship of specific growth rate (estimated over the 14 h light period) and specific loss rate (estimated over the ten hour dark period) at 24 °C. Error bars represent standard error of the mean.

![Fig. 6](image-url) Biomass loss in the dark during the exponential growth phase at ca. 24 °C in Picoclorhum sp. (LANL-WT), Nannochloropsis salina (CCMP1776), and Chlorella sorokiniana (DOE1412) using gravimetric (AFDW) and optical (OD750) measurement techniques. Error bars represent standard error of the mean.
Biomass loss over the dark period for individual phototrophs under variable night conditions represents a relatively poorly explored aspect of primary photosynthetic physiology. If algal biomass productivity is to be maximized, biomass losses at night should be minimized. Minimizing night biomass losses from a biological standpoint can involve either adjusting cultivation techniques to suit the algae of interest (optimum average light intensity, medium mixing/aeration, or temperature) or screening productive strains of algae for a more detailed understanding of biomass loss rates under variable conditions. Assuming a constant biomass loss for algae across species or strains is inappropriate for estimating or predictively modeling biomass productivity in dynamic environments. To improve predictive model accuracy and maximize algal biomass productivity, characterization of night biomass loss is essential.

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Appendix A. Supplementary data

Supplementary data to this article can be found at http://dx.doi.org/10.1016/j.algal.2015.10.012.

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Appendix A. Supplementary data

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