Light attenuation by *Nannochloropsis salina* cultures growing in bioreactors.

A Thesis

by

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

The availability of photosynthetic radiant energy within microalgae cultures is a primary factor determining the synthesis of biomass. As light penetrates the aqueous medium of algae cultures its flux density decreases exponentially with depth, and this decrease with depth intensifies with increasing cell density. This phenomenon is described by the Beer-Lambert's Law, and it provides a method to calculate the availability of photosynthetic radiant fluxes within algae cultures. Estimates of photosynthetic photo flux densities within microalgae cultures can be used to estimate microalgae photosynthetic rates (PPFD) and, therefore, the potential growth rate of an algae culture. The objective of this research was to quantify the attenuation of photosynthetic photon fluxes as they penetrate cultures with a wide range of cell populations of the microalgae species *Nannocholoropsis salina* growing in flat bioreactors. The ultimate goal of this research was to obtain a mathematical expression of the dependency of the Beer-Lambert's coefficient of attenuation on a wide range of incident light flux densities and cell populations of this unicellular species with potential applicability in computerized modeling tools developed for research and/or management of production systems using this microalgae species. The attenuation of a wide range of incident photosynthetic photon flux densities (PPFD) passing through 0.1016-m deep microalgae cultures with cell populations ranging from about 10 x 10^6 mL^{-1} to about 275 x 10^6 mL^{-1} was characterized using four controlled-environment flat panel bioreactors operating in the Microalgae Physiology Laboratory at the Texas A&M AgriLife Research and Extension Center in Corpus Christi, Texas. The wide range of incident PPFD levels was generated by the combination of 1) differences in spatial distribution of incident light over the lighted side of the bioreactor, 2) various distances between the light source and the bioreactor, and 3) the addition of light-attenuating shades between the light source and the

bioreactor. The global equation representing the Beer Lambert's Law coefficient of attenuation across different light intensities and bioreactors was determined as $y = 2 * 10^{-06} x^3 - 0.0019x^2 + 0.5912x + 7.0564$, where x, ($x>0$) is the culture's cell population in millions per mL.

DEDICATION

I want to dedicate this thesis to my mother. You prepared me for so much in the short time you were with me. When you left this world, your absence truly left an emptiness in my heart. Now I realize that you were here on earth just long enough to teach me everything I needed to know. Which was the only thing that matters on this earth is family. My family is truly a blessing, and I know I can always draw strength from them regardless of the difficulties I may encounter. Every time I look at my boys, I get a glimpse of you, which inspires me to become a better father. I miss you with all my heart!

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I would also like to take this opportunity to express my gratitude and appreciation to my family, who encouraged and supported me on this educational journey. My wife, Alondra Flores, and my children, Erick, Jason and Noah, who believed in me and followed me every step of this long journey.

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1. INTRODUCTION

Microalgae cultures have the potential of being important contributors to the production of renewable biomass. This potential of microalgae is intrinsically related to their ability to capture radiant energy, dissolved CO_2, and nutrients, and to convert these into carbohydrates, proteins, and lipids. The terms PPFD (photosynthetic photon flux density) and PAR (photosynthetic active radiation) refer to the spectral range of radiant energy (light) from 400 to 700 nm that is used by terrestrial and aquatic plants, including algae, in photosynthesis (Thimijan and Heins, 1983). The availability of PAR or PPFD within the culture is of particular interest since it is considered the main limitation for the synthesis of biomass (Fernandes, 2010; Wu and Merchuk, 2004). The availability of PAR (and PPFD) within microalgae cultures can accurately be measured directly or be accurately estimated along a gradient in the water column (Lee, 1999). To be able to estimate PAR (or PPFD) within the culture, it is necessary to gather optical properties information within the culture's water column using the Beer-Lambert's Law (Juad et al., 2012). The Beer-Lambert's Law describes the attenuation of light as it is transmitted through an homogeneous medium (Ingle and Crouch, 1988).

When a beam of light (photon flux) reaches an aquatic layer a fraction of it is reflected and photons penetrating the layer are attenuated with depth in two ways: by particulate scattering and by absorption (Kirk, 1994; Benson and Rusch, 2006). The sum of the absorption and the scatter represents the attenuation of the photon flux penetrating the aquatic media (Kirk, 1994). As light penetrates the aqueous medium of algae cultures its flux density decreases exponentially with depth, and this decrease with depth intensifies with increasing cell density (Sutherland et al., 2015). This phenomena is described by the Beer-Lambert's Law. This law provides, a) a method to calculate the distribution of light at depths (Blanken et al., 2016; Béchet et al., 2013), and b) a

method for calculating the coefficient of attenuation (Acien et al., 1997). Some researchers consider the Beer-Lambert's Law a complicated solution due the effects of light scattering, absorption, and package effect (Myers et al., 2013, Jaud et al., 2012; Lee, 1999; R. J. Geider 1 and B. A. Osborne, 1997), which may result in an overestimation of light attenuation (Myers et al., 2013). Notwithstanding, several studies have demonstrated the feasibility of using the Beer-Lambert's Law to calculate its coefficient of attenuation for comparison of variations produced by microalgae cultures' biomass concentrations (Myers et al., 2013; Acien et al., 1997; Jerez et al., 2015; Lee, 1997). Quantitative analyses in microalgae cultures have found an exponential decay rate of the coefficient of attenuation through a culture media to be proportional to cell population biomass (Murphy et al., 2015; Wu and Merchek, 2004). It has been noted that cultures of unicellular species like *Nannochloropsis salina* are particularly apt to apply the Beer-Lambert's Law, since cultures of this unicellular species are less effected by packaging at high cell densities (Kromkamp et al., 2009). Preliminary estimates of the light attenuation coefficient obtained from scalar PPFD measurements made within *N. salina* cultures growing in bioreactors ranged from 0.98 m^{-1} to 53.85 m^{-1}, depending on the culture biomass density, with the lowest value corresponding to only saline water and the higher value to a culture with a biomass density of 0.783 g L^{-1} (Fernandez et al., 2015).

Estimates of PAR (or PPFD) within microalgae cultures can be used to estimate microalgae photosynthetic rates (Sakshaug et al., 1999) and cell population growth (Blanken et al., 2016, Lee, 1999), and their inclusion in algae growth simulation models can lead to a powerful method for the optimization of microalgae production systems (Acien et al., 1997; Lee, 1999, B.C. Benson et al., 2016; M. Huesemann et al., 2016). Moreover, a major determining factor to increase microalgae biomass production yield appears to be the implementation of an efficient light

utilization system (Grobbelar, 2011). Quantifying the Beer-Lambert's Law coefficient of attenuation for a particular algae culture becomes essential for estimating the availability of PPFD at particular depths and algae cell densities. The Beer-Lambert's law coefficient of attenuation can then be applied to calculate the depth of photic zone when the cell concentration measurements are known (Myers et al., 2013). Algae culture depth has been manipulated to maintain the culture within the photic zone as a mechanistic solution to increase the efficiency of light utilization (Wu and Merchuk, 2004). Depth manipulations studies contend, however, that high densities must be maintained to sustain high productivity (Jeon et al., 2005). Further research found that the increase in productivity also shares an inverse relationship to water depth (Murphy et al., 2015) and as a result a decreased net photosynthesis (Frankovich et al., 2017). The potential of culture depth manipulation for improvement of algae culture production offers a logical approach to increasing photosynthetic efficiency and it has been investigated using thin panel photo bioreactors and shallow outdoor raceways (Sforza et al., 2012; Lee, 1997).

The importance of quantifying the Beer-Lambert's Law coefficient of attenuation has been clearly documented. It has been emphasized that this parameter is especially useful in algae growth simulation models, which can become useful management tools for maximizing the productivity of outdoor algae production systems. However, more information is needed to better quantify the changes in the coefficient of attenuation in growing microalgae cultures under variable light environments, as they occur in outdoor production systems.

1.1. Objective

The objective of this research was to quantify the attenuation of a wide range of photosynthetic photon fluxes as they penetrate cultures of the microalgae species *Nannochloropsis salina* growing in flat bioreactors. The ultimate goal of this research was to obtain a mathematical expression of

the dependency of the Beer-Lambert's coefficient of attenuation on a wide range of incident light flux densities and cell populations of this unicellular species with potential applicability in computerized modeling tools developed for research and/or management of production systems using this microalgae species.

2. MATERIALS AND METHODS

2.1. Brief description of research procedures

The attenuation of light produced by different populations of microalgae cultures was characterized in controlled-environment bioreactors operating in the Microalgae Physiology Laboratory at the Texas A&M University AgriLife Research and Extension Center in Corpus Christi, Texas. The study was superimposed on a larger ongoing study quantifying the effects of combinations of incident light and culture temperature on the growth of a unicellular microalgae. In this larger ongoing study, monocultures of the microalgae were grown from low cell populations until their growth stabilized or started to decline after a maximum population was reached. This set up created an excellent opportunity for quantifying the attenuation of light passing through 0.1016-m deep cultures with cell populations ranging from about $10x10^6$ mL^{-1} to about $275x10^6$ mL^{-1}. Light attenuation caused by a wide range of culture cell populations was calculated using Beer-Lambert's Law applied over an also very wide range of incident light levels. The wide range of incident light levels was generated by the combination of 1) differences in spatial distribution of incident light over the lit side of the bioreactor, 2) various distances between the light source and the bioreactor, and 3) the addition of light-attenuating shades between the light source and the bioreactor.

2.2. Microalgae species and culture growing conditions

The unicellular microalgae species used in the larger ongoing study was *Nannochloropsis salina* (CCMP1776). This strain of *N. salina* was obtained from the Biogelow Laboratory for Ocean Sciences at the National Center for Marine Algae and Microbiota in Eastbooth Bay, Maine. Typical adult cell sizes of *N. salina* range from 1 to 2 microns (Beacham et al., 2014). In this larger

ongoing study, monocultures of *N. salina* were exposed to combinations of constant incident light and culture temperature and allow to grow until a maximum cell population either remained stable for several days or showed a clear declining trend. Four levels of culture temperature (15, 19, 23, 27 C) and 6 levels of incident light (average photosynthetic photon flux densities at the bioreactor's lit surface of 75, 160, 270, 380, 590, and 840 µE m^{-2} s^{-1}) supplied at 12-hr photoperiods rendered 20 test growing conditions (Table 1). Salinity of the aquatic medium, pH, nutrients supply, and air supply to each bioreactor were maintained uniform during the studies. Salinity of the culture media was artificially maintained at about 30,000 ppm by mixing purified city water and Instant Ocean sea salt (Aquarium Systems, Inc., USA). Carbon dioxide was injected as needed to control culture's pH at 8.2. The daily addition of a modified Hoagland nutrient solution provided macro and micronutrients. Filtered outdoor air was injected into the culture at the bottom of the bioreactor at flow rates of 3 to 4 L min^{-1}. Mixing of the culture was attained by continuous operation of a centrifugal pump re-circulating the medium from top to bottom.

At the end of each test and to secure the maintenance of the same algae strain, the cultures were retrieved and diluted to about the initial low cell population of $10x10^6$ mL^{-1}. To prevent culture contamination, the bioreactors were cleaned and disinfected between tests before being refilled with the new initial low cell population.

2.3. Physical characteristics of microalgae bioreactors

The microalgae bioreactors are integrated in a fully automated system of four 68.95-L vertical prismatic controlled-environment bioreactors (outside dimensions: 1.213x0.597x0.127 m) made of 0.0127-m thick clear acrylic. The culture media is re-circulated from top to bottom with a ½ HP, 113.6 L min^{-1} centrifugal pump (Dayton Model LTAA21SA), which provides a whole-volume

refreshment every 34.6 s. Outdoor filtered air is injected at the bottom the culture volume by means of two 0.3048x0.0381x0.0381 m paired prismatic ceramic air diffusers.

Experimental culture temperature level was monitored by a thermistor sensor and controlled automatically by circulating refrigerated coolant through each bioreactor's internal heat exchanger in line with the culture medium re-circulation system. Experimental light flux density levels were supplied to each bioreactor by two floodlight fixtures each equipped with a 400-watt metal halide lamp (Model FZH400PSQ, RAB Lighting Inc., Northvale, NJ) controlled by an electronic timer. These two light fixtures were placed equidistant and centered facing the top and bottom halves of the bioreactor's back side (lit side). Different levels of incident photosynthetic photon flux density (PPFD) was attained by manually adjusting the distance between the light source and the lit side of the bioreactor (0.1016, 0.254, and 0.4064 m) and the addition of one, two, or three layers of insect fiberglass screens between light source and the bioreactor's lit side when the light source was at the farthest position. The maximum average incident PPFD on the lit side of the bioreactor was 840 µE m^{-2} s^{-1} with the light source positioned closest to the bioreactor. Positioning the light source 0.254 and 0.4064 m from the bioreactor decreased the average PPFD 30% and 55%, respectively. The addition of 1, 2, and three screens further decreased the average PPFD 68%, 81%, and 91%, respectively. To minimize the heat load on the bioreactors lit side, a rectangular 1.213x0.597-m vertical 0.0635-m thick tempered clear glass was positioned 5 inches in front of the light fixtures.

2.4. Measurement of light flux density: Incident on and transmitted across the bioreactor

Because of the uneven spatial distribution of light produced by the two floodlight fixtures (Fig. 1), a measurement system was created for assessing the variation of the photosynthetic photon flux density (PPFDs) received on the lit surface of the bioreactor. The hardware of this system consisted

of a battery of 5 quantum sensors (Model LI190SB, Campbell Scientific INC. Logan, Utah) installed on a 0.5969-m wide by 0.5588-m high rectangular 0.0127-m thick plywood board (Fig. 2). This quantum sensor's frame was designed to cover half of either side (lit and non-lit) of the bioreactor, but with the 5 quantum sensors uniformly distributed within one half triangular section of the board or, by design, one fourth triangular section of the bioreactor side. By alternatively rotating the board 180° degrees, the quantum sensor arrangement made it possible to obtain equally spaced PPFD measurements covering the whole lit or non-lit surfaces of the bioreactor. The board could be placed in four positions at either the lit side or the non-lit side of the bioreactor. Positions 1 and 2 (rotated), covering the top half side, and positions 3 and 4 (rotated) covering the bottom half side. With this system, PPFD could be measured at 20 fixed locations in both the lit and non-lit surfaces of the bioreactor. Since the board was pivoted around sensor 5 at the center (Fig. 2), two of the 20 measurements were obtained from the same central location at each half (top and bottom) surfaces. This sensor positioning system required the development of a measurement identification method to facilitate the analysis of PPFD measurements (Fig. 3).

PPFD measurements from the five quantum sensors were obtained sequentially from 1 to 5 at 1-second intervals and averaged over 4 minutes using an automated data-logger (Model CR1000, Campbell Scientific Inc., Logan, Utah). These PPFD measurements were taken continuously and the quantum sensor board remained at each position during 12 minutes. The 4-min averages allowed for sufficient stabilization of the PPFD measurements, which minimized potential "data noise" caused by light output fluctuations. The 12-minute span for measurements at each position secured the collections of at least one whole 4-minute average at each position. Since the quantum sensor board was changed manually between positions there was likelihood that data collected during the first and last 4-minute periods of the 12-minute span could be

"tainted" with data from the previous and/or the following board position. Upon downloading the light flux measurements collected by the datalogger, the data was imported onto a spreadsheet (Microsoft Excel™ 2016 v16) for extracting each sensor's middle 4-min averages within the 12-min span of measurements at each sensor frame position (Table 1). All extracted light flux data, including incident on lit side and attenuated on the non-lit side for each light source setting and for each microalgae cell population sampled and each bioreactor, were then incorporated and organized onto another spreadsheet for further processing.

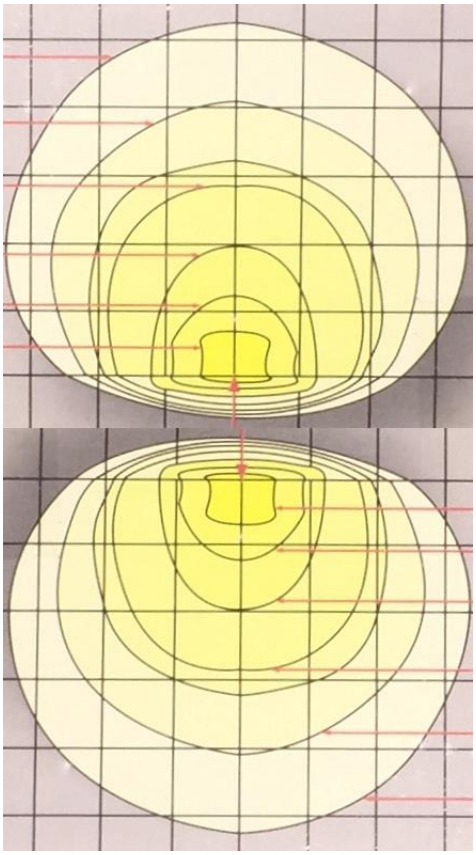

Figure 1. Spatial light distribution pattern produced by the two floodlight fixtures equipped each with a 400-watt metal halide lamp used as light source for bioreactors according to specifications provided by the manufacturer.

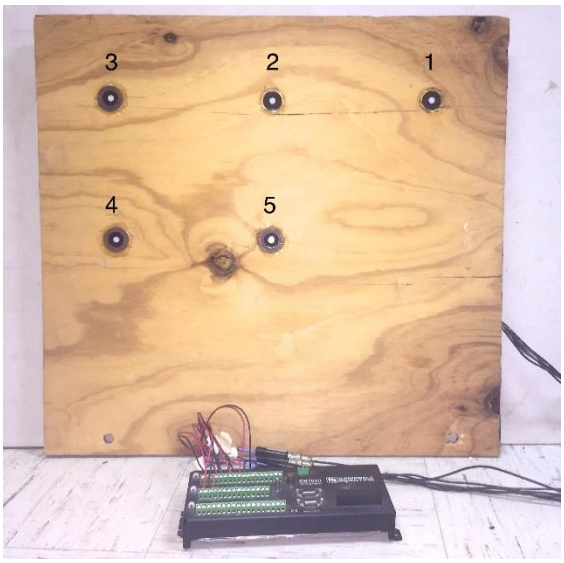

Figure 2. Five-quantum sensor outfit used to collect incident and transmitted photosynthetic photon flux density data on the lit and unlit sides of bioreactors.

Array	Year	Julian Day	Clock Time	PPFD sensor 1	PPFD sensor 2	PPFD sensor 3	PPFD sensor 4	PPFD sensor 5	Bioreactor ID	Light source setting	Sensor frame position
101	2012	236	800	13.13004	18.19922	17.70393	20.97055	21.21408			
101	2012	236	804	13.34388	16.25851	18.14392	20.95007	21.94372			
101	2012	236	808	13.35977	16.46919	18.09789	21.00661	22.08752	CAB1	16shade1	1
101	2012	236	812	13.31001	16.30445	18.04734	20.96806	22.248			
101	2012	236	816	19.86284	21.31373	12.45872	12.15641	22.41153			
101	2012	236	820	23.09225	22.80573	9.957506	8.432508	21.9909	CAB1	16shade1	2
101	2012	236	824	23.00509	22.70077	9.876142	8.613288	22.32996			
101	2012	236	828	17.90879	18.11407	14.72409	14.92153	18.82199			
101	2012	236	832	14.09096	13.96676	18.17009	19.32252	16.75324	CAB1	16shade1	3
101	2012	236	836	14.05851	13.98484	18.24026	19.21765	16.38902			
101	2012	236	840	9.799798	9.624733	11.47029	16.13779	16.27589			
101	2012	236	844	7.0437	6.950978	8.205165	13.87217	16.48805	CAB1	16shade1	4
101	2012	236	848	7.058155	6.969002	8.15721	14.07847	16.54819			
101	2012	236	852	11.65287	11.81925	14.44767	21.62087	23.90462			
101	2012	236	856	10.21871	17.78264	16.80863	24.65337	27.04836	CAB2	16shade1	1
101	2012	236	900	8.539052	19.76452	16.32163	24.41812	26.97762			
101	2012	236	904	18.18675	21.86141	13.4807	17.08927	26.97377			
101	2012	236	908	24.34893	24.23954	11.55852	11.67144	26.76855	CAB2	16shade1	2
101	2012	236	912	24.25526	24.13169	11.35942	11.78738	26.51419			
101	2012	236	916	24.33488	29.5117	23.31296	24.70407	28.97234			
101	2012	236	920	24.03864	32.74025	28.87273	31.23649	30.83609	CAB2	16shade1	3
101	2012	236	924	24.04253	32.55686	28.78822	31.37438	30.74449			
101	2012	236	928	22.41016	25.40443	24.05877	27.24171	30.35591			
101	2012	236	932	21.43491	21.40465	21.28438	24.84586	30.41609	CAB2	16shade1	4
101	2012	236	936	21.44571	21.57397	21.40241	24.9544	30.0836			
101	2012	236	940	21.53901	21.59136	21.45712	24.91024	30.29138			

Table 1. Sample of raw data of light flux measurements downloaded from datalogger and data rows highlighted for extraction and transfer to another spreadsheet for further processing.

Since the dimensions of the quantum sensor board fit half of the bioreactor's lit or non-lit side, and the locations of the 5 sensors in the board were fixed, each one of the 20 measurements

obtained on the lit side could be paired with those obtained on the non-lit side of the bioreactor. This design allowed for the study of the attenuation of light passing through 18 equally distributed point transects across the bioreactor (18 points considering that 2 of the measurements from sensor #5 are repeated).

2.5. PPFD measurements for estimating light attenuation by microalgae cell populations

Light attenuation by monocultures of unicellular *N. salina* was estimated from a large set of PPFD measurements of incident fluxes and of fluxes transmitted across the bioreactors containing a wide range of cell populations. These measurements were obtained from cultures growing in the bioreactors as part of a larger ongoing study quantifying the effects of constant combinations of six incident light levels (average incident PPFDs of 840, 590, 380, 270, 160, and 75 $\mu E\ m^{-2}\ s^{-1}$) and four culture temperature levels (15, 19, 23, and 27 C) on the growth of *N. salina*. In this larger ongoing study, monocultures of *N. salina* were grown from low cell populations ranging from $10x10^6\ mL^{-1}$ to $14x10^6\ mL^{-1}$ until their growth stabilized or started to decline after a maximum cell population was reached. The length of these tests ranged from 16 to 32 days depending on the growing conditions created by the particular combination of incident light and culture temperature being tested. To quantify the effect of cell population levels on the attenuation of light, the flux densities of light incident on and transmitted through a bioreactor were measured at various times during the progressive growth of the *N. salina* cultures. At each of these particular times with distinct cell populations, the level of incident light flux density was momentarily and sequentially changed during about 5 to 6 hours of the 12-hr photoperiod to produce average PPFD levels of 840, 590, 380, 270, 160, and 75 $\mu E\ m^{-2}\ s^{-1}$ (i.e. the same levels of incident light flux density treatments in the ongoing study) by adjusting the distance of the light source to the lit side and adding light-attenuating screens between the light source and the lid side of the bioreactors (this

procedure was explained *supra*. This procedure of collecting incident and transmitted light flux density measurements at the various levels of incident light during 5 to 6 hours was designed to minimize adverse effects on the overall growth of microalgae in the larger ongoing growth study due to the momentarily changes of the incident light level. To separate the attenuation effect of the microalgae populations from that of the culture medium, the light flux measurements also included a set collected from the bioreactors operating only with re-circulated saline water and the injection of outdoor air. Light flux measurements were collected from all four bioreactors used in the ongoing larger study.

The large data comprised of incident and transmitted PPFDs collected all four bioreactors at various combinations of light source distances and light attenuating screens, at various levels of cell populations were input onto a MS Excel spreadsheet and aligned onto columns separated by bioreactor and sample day (corresponding to different cell populations) from both the light flux measurements taken from the lit and non-lit side of the bioreactors. Each column consolidated 120 measurements (twenty sensor measurements times six light source setting positions) for each incident (lit side) or attenuated light fluxes (non-lit side). The number of columns ranged from 1 to 6 columns for each bioreactor depending on the number of sampling days taken throughout the growth of the cultures.

Unlit side					Lit side				
Top	10	11	12	***Position 1***	**Top**	30	31	32	***Position 5***
		14	13				34	33	
	18	19		***Position 2***		38	39		***Position 6***
	17	16	15			37	36	35	
Bottom	20	21	22	***Position 3***	**Bottom**	40	41	42	***Position 7***
		24	23				44	43	
	28	29		***Position 4***		48	49		***Position 8***
	27	26	25			47	46	45	

Figure 3. System of keys for identifying quantum sensors relative positions for measuring incident (lit side) and transmitted (unlit side) light flux densities. The number sequence from 10 to 49 identify the 40 possible sensor positions by combining the 5 quantum sensors in the board with the 8 positions of the board (4 on lit side and 4 in the unlit side of the bioreactor). In the diagram, as a visual aid, each of the 5 quantum sensors was assigned a particular color. The red colored cells correspond to sensor number 5, as shown in Fig. 2, which always overlaps its position when the quantum sensors board is flipped vertically from position 1 to 2, from position 3 to 4, from position 5 to 6, and from position 7 to 8.

2.6. Quantifying the effects of culture medium, cell population, and incident PPFD on light attenuation

A first step to visualize the attenuation of light passing through the bioreactors consisted in quantifying the influence of incident light flux density (measured on bioreactor's lit side) on the light flux density transmitted across the bioreactor (attenuated light flux density measured on the bioreactor's non-lit surface). This quantification consisted in obtaining linear regression equations for each of the individual sets of light flux density measurements collected in each of the bioreactors at different culture cell populations including the only saline growing medium. The linear regression equations were obtained using the "Add Trendline..." tool in Microsoft Excel's Chart menu. Since the regression coefficients in these equations represent the numerical response of light flux transmission through the culture medium to incident light flux density, a further

interpretation of the data consisted in obtaining the trend of this response as a function of the culture's cell population.

Although this first analytical step was considered useful for an initial assessment of how much light fluxes are attenuated by the culture medium and its different cell populations, a more detailed characterization of light attenuation by microalgae cultures was deemed necessary for developing more "universal" parameters usable in computer models simulating light environments in other cultural conditions such as outdoor production systems.

A second step in the analysis of the attenuation of light as it penetrates cultures of *N. salina*, focused on obtaining a more "universal" parameter usable to characterize the light environment in cultures of this microalgae species. The approach followed in this second analytical step involved the use of the Beer-Lambert's Law (also known as Beer-Lambert Law). Although this law (eq. 1) describes the transmission of a parallel beam of mono-chromatic radiation through a homogeneous medium, it was nevertheless chosen to help us quantify and characterize the light attenuation effects of populations of *N. salina*. The equation representing Beer-Lambert's Law states that the flux density of radiation at any depth ($I_{(x)}$) can be calculated from the incident radiation flux density ($I_{(0)}$) at x=0 and by assuming that the attenuation of radiation in a thin layer of depth=x is proportional to both x and $I_{(0)}$, where the constant of proportionality (α) is a coefficient of attenuation.

$$I_{(x)} = I_{(0)}\, e^{-\alpha x} \qquad \text{(eq. 1)}$$

In this study, $I_{(0)}$ is the photosynthetic photon flux density (PPFD in $\mu E\ m^{-2}\ s^{-1}$) incident on any of the twenty measurement locations on the lit side of the bioreactor, $I_{(x)}$ is the PPFD measured on any of the corresponding twenty measurement locations at the non-lit side of the bioreactor, x is the path length of the light (thickness of the bioreactor or distance from lit surface to non-lit

surface, in m), and α is the coefficient of attenuation at that location path. Equation 1 is rearranged to solve for the coefficient of attenuation as shown in eq. 2.

$$\alpha = LN(I_a / I_o)/-x \qquad \text{(eq. 2)}$$

The fundamental premise of this second analytical step is that the coefficient of attenuation in this equation can be a parameter with potential use to describe the light environment in cultures of this unicellular species grown under other cultural conditions.

As in the first step of the analysis described above, a total of 3,673 coefficients of attenuation were calculated from the individual paired measured values of incident and transmitted PPFD using equation 2. As indicated in section 2.5, this large data set was comprised all measurements of incident and transmitted PPFDs collected from all four bioreactors at various combinations of light source distances, light attenuating screens, and various levels of cell populations, including the non-populated saline medium.

For the sake of simplicity, it was assumed that the two primary factors of variation affecting the coefficient of attenuation are the magnitude of the incident PPFD and the magnitude of *N. salina* cell population. Therefore, the approach followed in this second analytical step involved first quantifying the effect of incident PPFD on the coefficient of attenuation and second quantifying the effect of cell population on the coefficient of attenuation and the possible interactive effects of incident PPFD and cell population. The ultimate outcome pursued from this second analytical step was to develop an "universal" equation to estimate the light attenuation of *N. salina* cultures under different light environments. As done in the first analytical step, the regression equations calculated in this second step were obtained using the "Add Trendline..." tool in Microsoft Excel's Chart menu.

The effect of incident light level (independent variable) on the coefficient of attenuation (dependent variable) was quantified from data sets separated by culture's cell population and bioreactor. Each of the separated independent variable data sets included the wide range of incident light levels measured at each of the 20 lit-surface locations for the various light source distances and attenuation screens. For each of the separated data sets, the effect of incident light level on the coefficient of attenuation was assessed by means of best-fit regression equations. To furthering the interpretation of the effects incident PPFD on the coefficient of attenuation and the interaction with the cultures' cell population, the collection of coefficient of regressions and regression intercepts were regressed on the culture's cell populations.

The effect of culture's cell population (independent variable) on the coefficient of attenuation (dependent variable) was quantified from data sets grouping the wide range of incident light levels measured at each of the 20 lit-surface locations for the various light source distances and attenuation screens and separated by bioreactor. For each of the separated data sets, the effect of cell population on the coefficient of attenuation was assessed by means of best-fit regression equations. Depending on the similarity of the effects of cell populations on the coefficient of attenuation among bioreactors, the data sets would be eventually grouped across bioreactors.

As described *up supra* the approach followed to quantify the light attenuation caused by *N. salina* cultures involved the culture's cell population and not the culture's biomass. However, the approach followed by most of other related research work published elsewhere involved the culture's biomass. Therefore, to provide a common ground with other studies, a relationship was developed for converting *N. salina* culture's cell population measurements to ash-free dry weight. This relationship was developed from a collection of 180 paired discrete cell population and ash-free dry weight data obtained from the larger ongoing study quantifying the effects of

combinations of incident light and culture temperature on the growth of *N. salina*. Some short timespan interpolations were calculated to generate same-day values of these two state variables whenever the date of measurements did not coincide. A regression analysis performed on these paired data yielded the following best-fit significant (R^2 = 0.94406 **) quadratic polynomial equation:

$$AFDW = -6E\text{-}06P^2 + 0.0059P \qquad \text{(eq. 3)}$$

Where AFDW is the culture's ash-free dry weight in g L^{-1} and P is the culture's cell population in millions per mL. This equation was applied to the final outcome of this study to express light attenuation in terms of the culture's ash-free dry weight (Fig. 4).

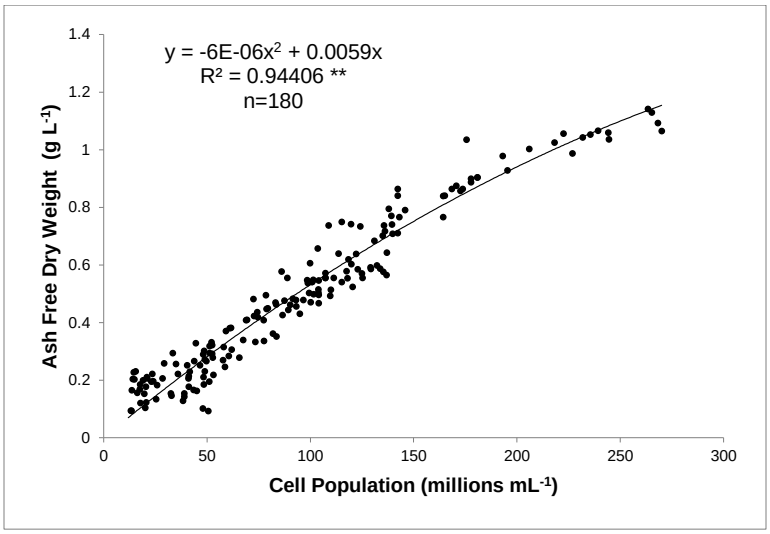

Figure 4. Quadratic polynomial equation describing the relationship between ash free dry weight and cell population density of *Nannochloropsis salina* estimated from discrete data measured in all four environmentally controlled bioreactors.

3. RESULTS AND DISCUSSION

3.1. First analytical phase: visualizing the effects of incident PPFD and cell population on the transmitted PPFD transmitted across the bioreactors

As expected, the amount of photosynthetic photon flux transmitted across the bioreactor walls (lit and unlit faces) and the culture medium was linearly dependent on the PPFD incident on the bioreactor's lit face, the liquid culture medium, and the culture's cell population, as shown in Figs. 5 to 9. Overall, the data was very consistent across the four bioreactors. Except for two of the high cell populations (Fig. 9), the linear regressions of transmitted PPFD measured at the unlit face on incident PPFD measured at the lit face resulted highly significant with positive regression coefficients. The maximum regression coefficients were obtained with the recirculating air-injected saline medium without microalgae culture, averaging 0.4356±0.04369. The magnitude of the regression coefficients indicates that more than 50% of the incident light was not transmitted but absorbed and/or dispersed by the bioreactor walls, the saline medium, and the air injection bubbles. The increasing culture's cell population had a large exponentially decreasing effect on the fraction of incident photosynthetic flux transmitted across the bioreactors as shown in Fig. 10. The fraction of incident photosynthetic photon flux transmitted across the bioreactors was practically nil after a culture's cell population of about 80 million cell mL^{-1}.

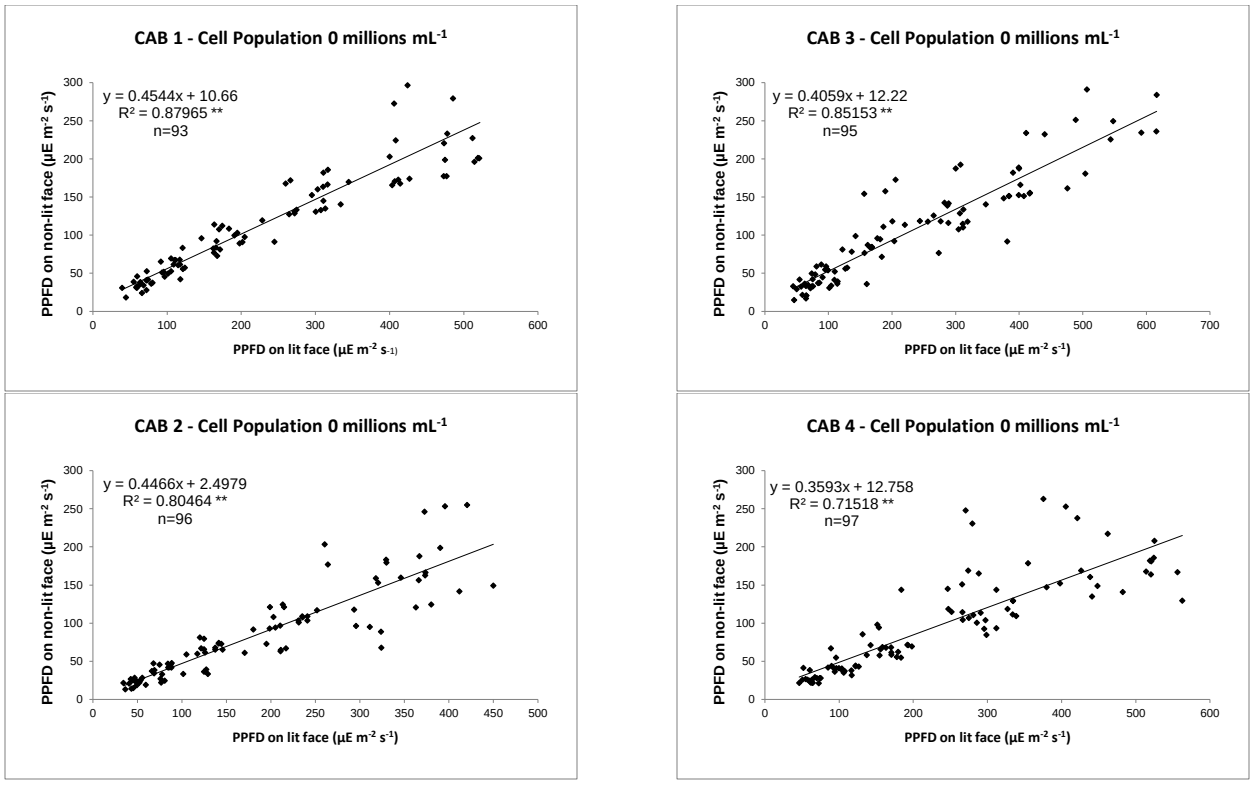

Figure 5. Linear regressions obtained for bioreactors #1-4 of transmitted photosynthetic photon flux density (PPFD) measured at the unlit face on incident PPFD measured at the lit face. Bioreactors operating with air-injected recirculating saline water and nil cell population of *Nannochloropsis salina*.

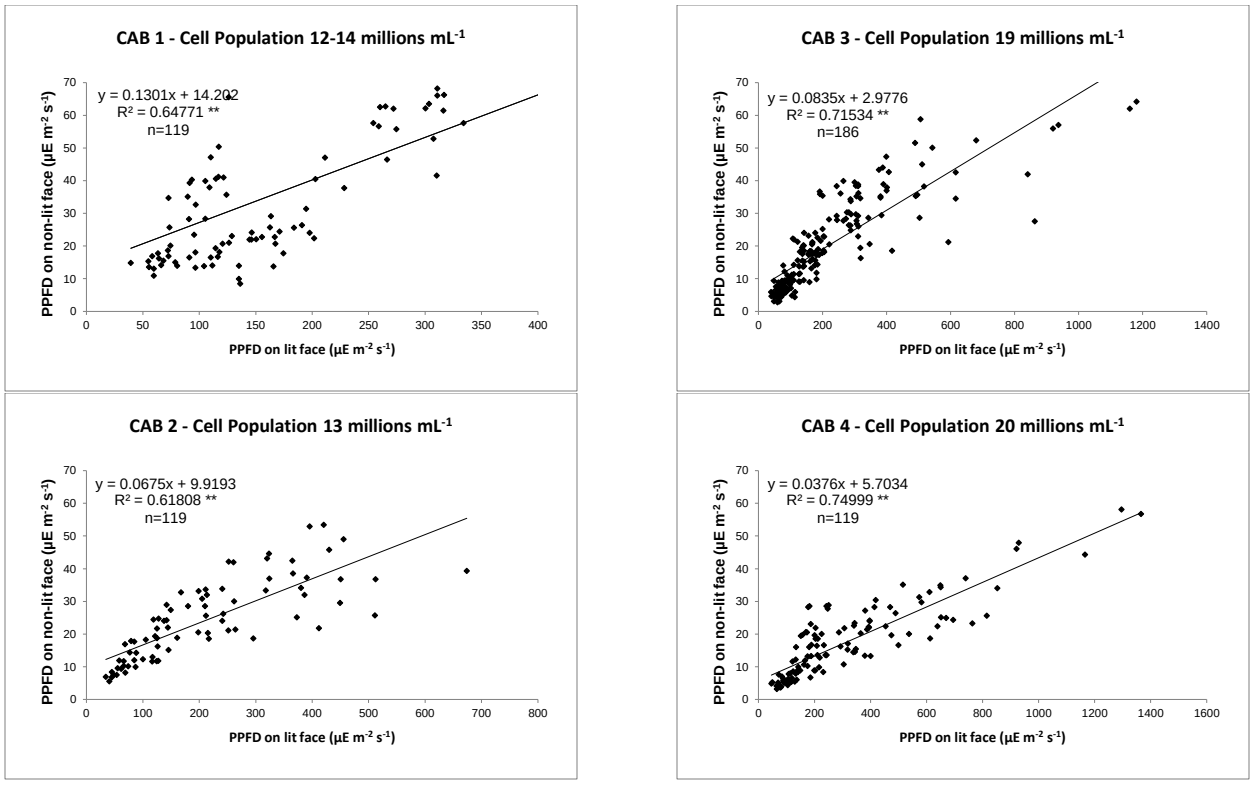

Figure 6. Linear regressions obtained for bioreactors #1-4 of transmitted photosynthetic photon flux density (PPFD) measured at the unlit face on incident PPFD measured at the lit face surface. Bioreactors operating with air-injected recirculating saline water and low culture cell populations of *Nannochloropsis salina* ranging from 12 to 20 million cells per mL^{-1}.

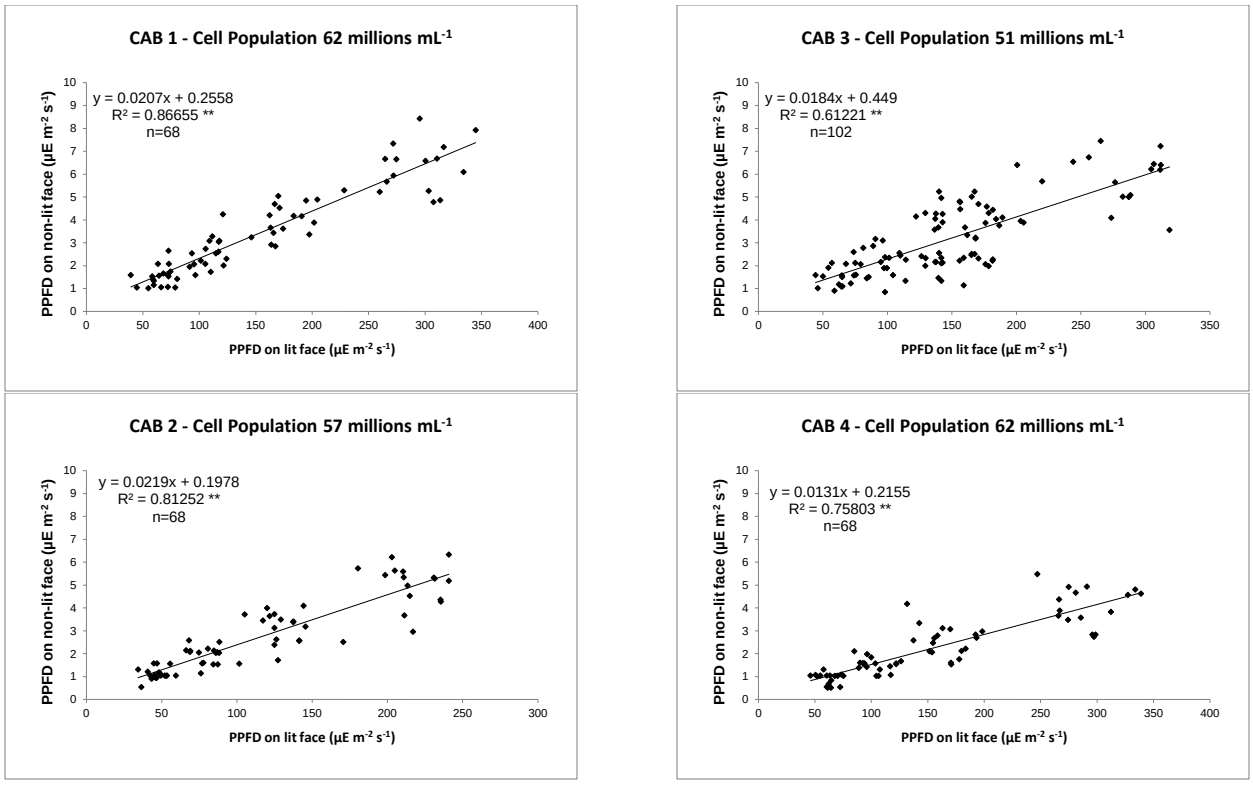

Figure 7. Linear regressions obtained for bioreactors #1-4 of transmitted photosynthetic photon flux density (PPFD) measured at the unlit face on incident PPFD measured at the lit face surface. Bioreactors operating with air-injected recirculating saline water and medium-low culture cell populations of *Nannochloropsis salina* ranging from 51 to 62 million cells per mL^{-1}.

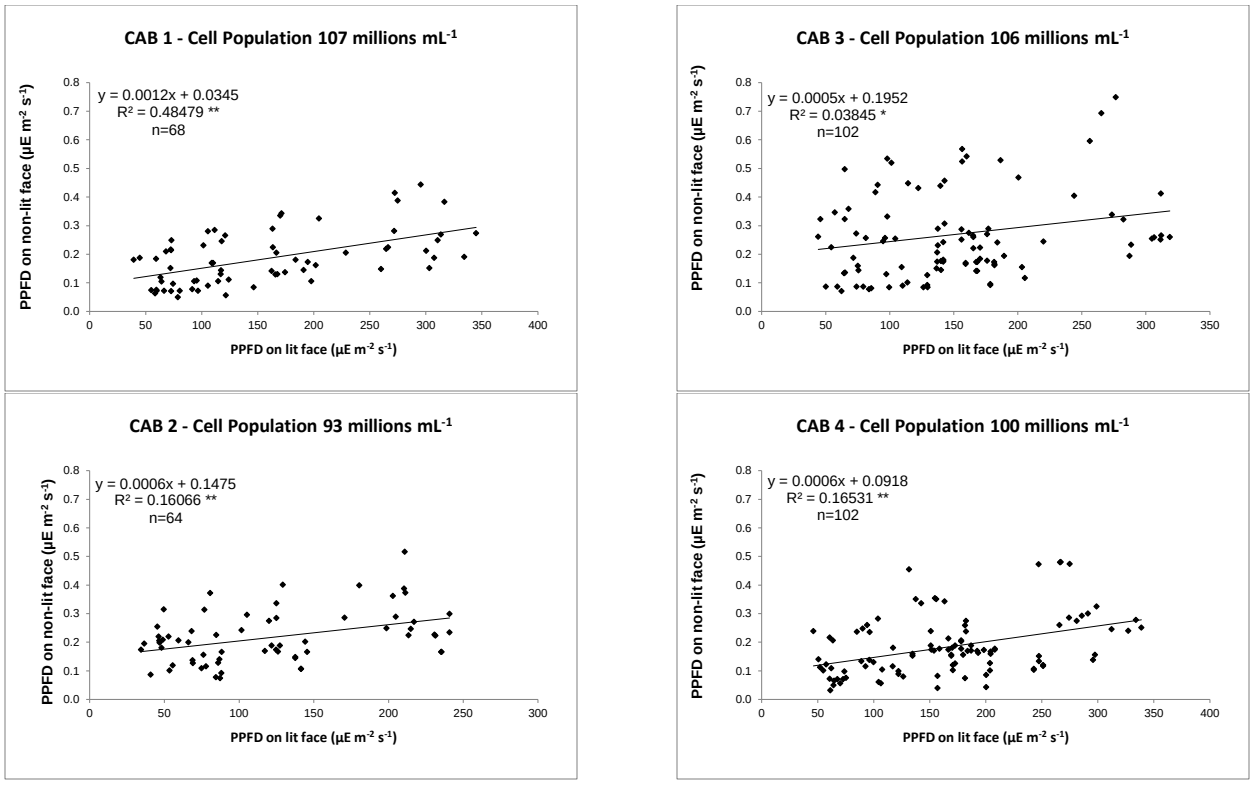

Figure 8. Linear regressions obtained for bioreactors #1-4 of transmitted photosynthetic photon flux density (PPFD) measured at the unlit face on incident PPFD measured at the lit face surface. Bioreactors operating with air-injected recirculating saline water and medium culture cell populations of *Nannochloropsis salina* ranging from 93 to 107 million cells per mL^{-1}.

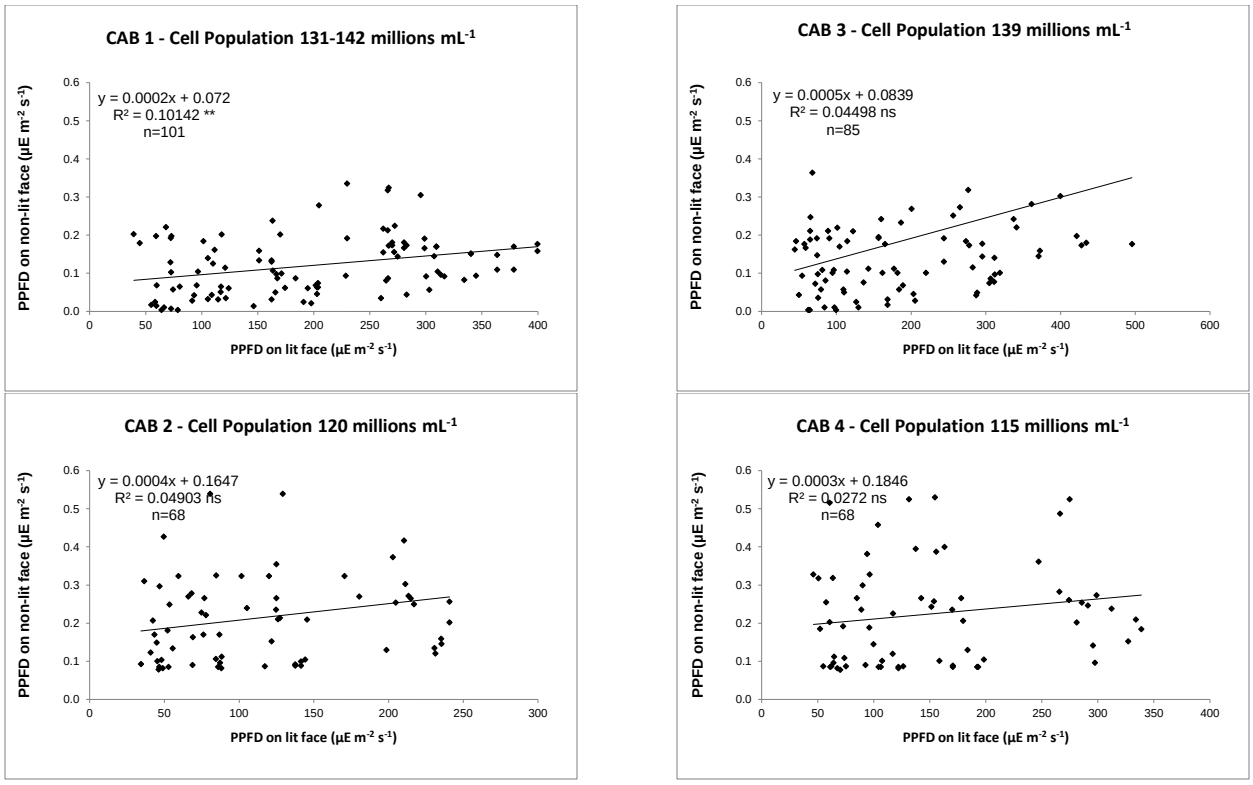

Figure 9. Linear regressions obtained for bioreactors #1-4 of transmitted photosynthetic photon flux density (PPFD) measured at the unlit face on incident PPFD measured at the lit face surface. Bioreactors operating with air-injected recirculating saline water and medium-high culture cell populations of *Nannochloropsis salina* ranging from 115 to 142 million cells per mL^{-1}.

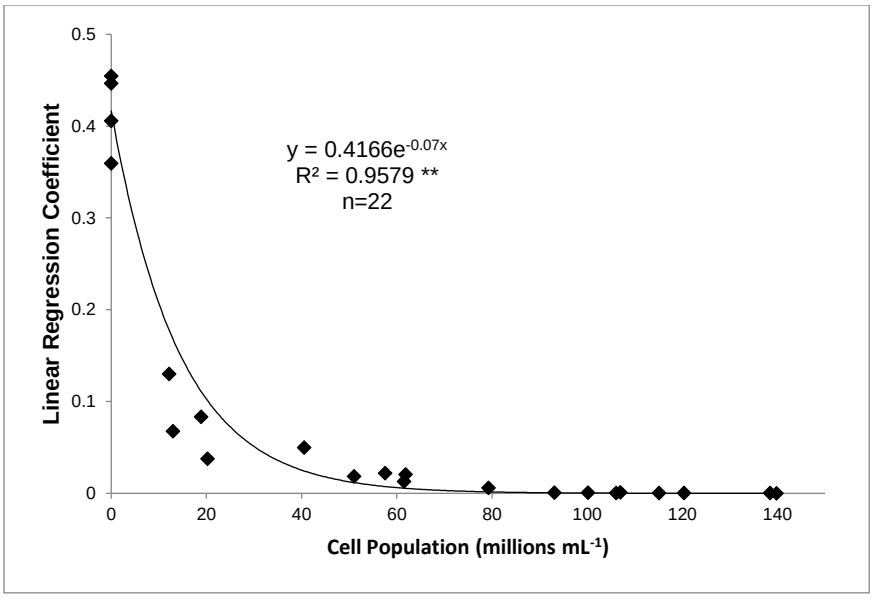

Figure 10. Exponential equation describing the dependency of the transmittance of photosynthetic photon flux density (PPFD) on cell population density of *Nannochloropsis salina* across all four bioreactors. The linear regression coefficients (Y axis) were obtained from linear regression analyses of transmitted PPFD at non-lit face on incident PPFD at lit face performed in all four bioreactors shown in Figures 5 to 9.

Although the results obtained in the first analytical step were descriptive about the effects of incident PPFD and culture's cell population on the amount of photosynthetic photon flux transmitted across the bioreactors, the equations obtained for quantifying these effects cannot be directly applicable to other *N. salina* cultures growing conditions outside the bioreactors. The second analytical step that follows below provides a more detailed characterization of light attenuation by *N. salina* cultures focusing on the development of more "universal" parameters, such as the light attenuation coefficient included in the Beer-Lambert's Law (eq. 1) , which can be applicable in computer models simulating light environments in other cultural conditions such as outdoor productions systems.

3.2. Second analytical phase: quantifying the effects of incident PPFD and cell population on Beer-Lambert's Law coefficient of attenuation.

The Beer-Lambert's Law attenuation coefficient, called from now on attenuation coefficient throughout the text below, was increased by both the incident PPFD and the culture's cell population, as shown by the regression equations in Figs. 11 to 15. Similarly to the graphics displayed in the first analytical phase, and for the sake of simplifying illustration, Figures 11 to 15 show only a selection of data sets over the range of cell populations. Overall, the data were very consistent across the four bioreactors. The effect of incident PPFD on the attenuation coefficient was much smaller than that of the culture's cell population. The increase in the attenuation coefficient associated with the increase in incident PPFD, as assessed by the regression coefficients, averaged 1.9% per µE m^{-2} s^{-1}, ranging from a minimum of 0.3% to a maximum of 3.5%, as measured across bioreactors and cultures with cell populations ranging from 0 to 142 million cells per ml. This small response may be an artifact from the bioreactor design resulting from a higher concentration of aeration bubbles and higher incident PPFD in about the middle 0.3-m section of the bioreactor. The increased concentration of aeration bubbles likely enhanced the scattering of light and this coincided with higher values of incident PPFD. On the other hand, the increase in the attenuation coefficient associated with the increase in the culture's cell population, as assessed by the regression intercepts, was about 8.5 fold across bioreactors from about 4.5 m^{-1} at zero cell population to 46.9 m^{-1} at cell populations between 115 and 142 million cells per ml.

The linear regressions of the coefficient of attenuation on incident PPFD obtained for the 4 bioreactors operating with only the saline medium (0 cell populations; Fig. 11) would show, in principle, the combined attenuation effects of the bioreactor walls and the circulating bubbling saline medium on the transmission of light going through the bioreactors. However, the light attenuation caused by the acrylic walls of the same bioreactors used in this study, as assessed from

scalar PPFD data measured with a bulb quantum sensor immersed in the culture medium, was found to be negligible (Fernandez et al., 2015). Therefore, the linear regressions of the coefficient of attenuation on incident PPFD displayed in Fig. 11 would show only the attenuation effects of the circulating bubbling saline medium on the transmission of light going through the bioreactors. The intercept of the linear regression equations averaged 5.821±0.8077 m^{-1} across bioreactors, while the linear regression coefficients showed that the average increase of the coefficients of attenuation across bioreactors with the increase in incident PPFD was 0.0027±0.00065 per µE m^{-2} s^{-1}. This increase in the coefficient of attenuation with increasing incident PPFD is small in practical terms and, as explained elsewhere above, an apparent artifact of the bioreactors' light and aeration supply. The significance of these linear regression parameters is that they could be utilized to globally remove the effects of the bioreactors' walls and saline medium on light attenuation, thus isolating the attenuation effect of *N. salina* culture's cell populations.

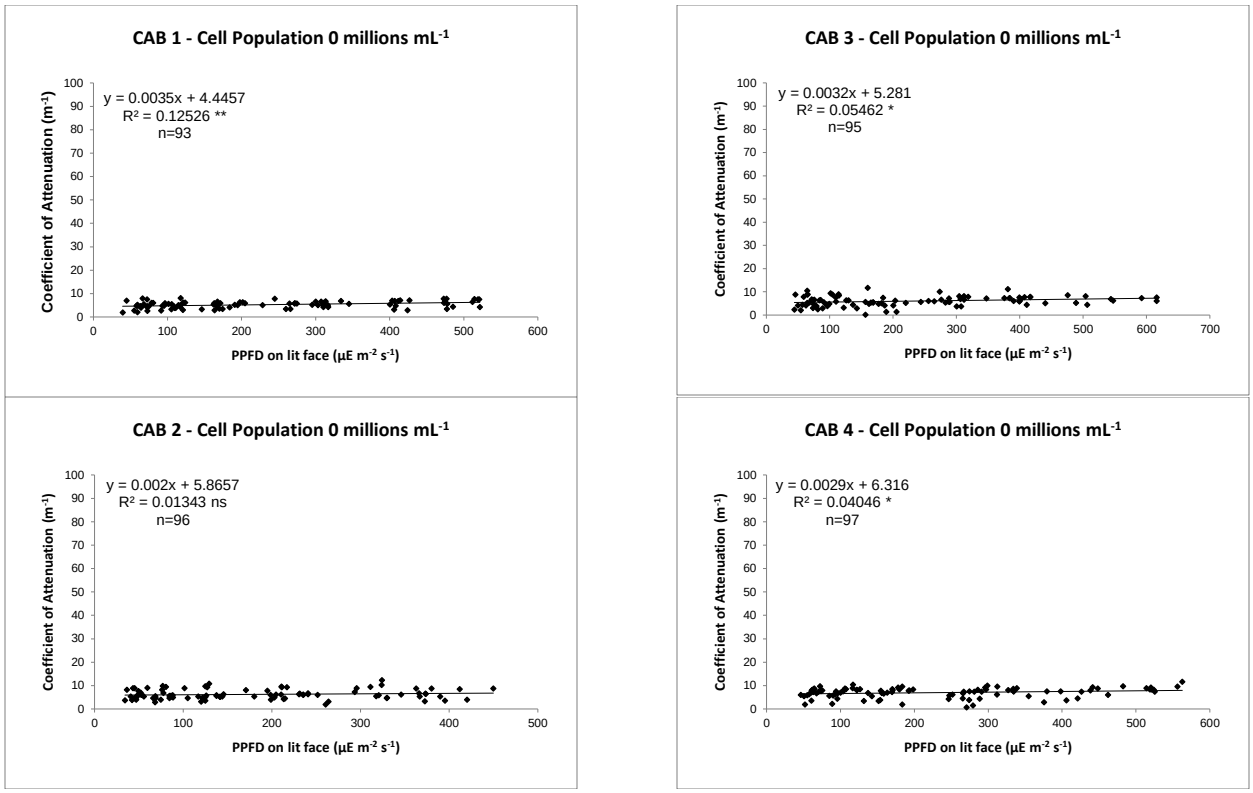

Figure 11. Linear regressions obtained for bioreactors #1-4 of Beer's Law attenuation coefficient on incident photosynthetic photon flux density (PPFD) measured at the lit face. Bioreactors operating with air-injected recirculating saline water and nil cell population of *Nannochloropsis salina.*

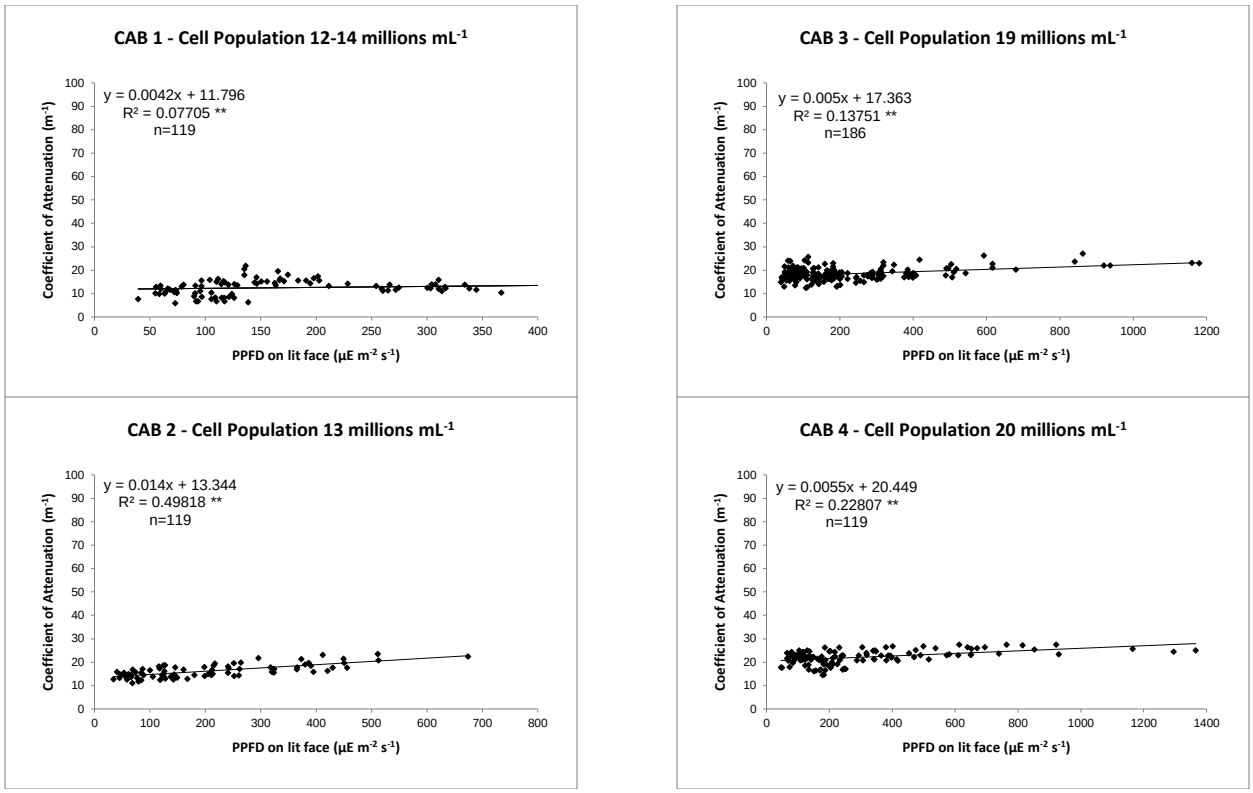

Figure 12. Linear regressions obtained for bioreactors #1-4 of Beer's Law attenuation coefficient on incident photosynthetic photon flux density (PPFD) measured at the lit face. Bioreactors operating with air-injected recirculating saline water and low culture cell populations of *Nannochloropsis salina* ranging from 12 to 20 million cells per mL^{-1}.

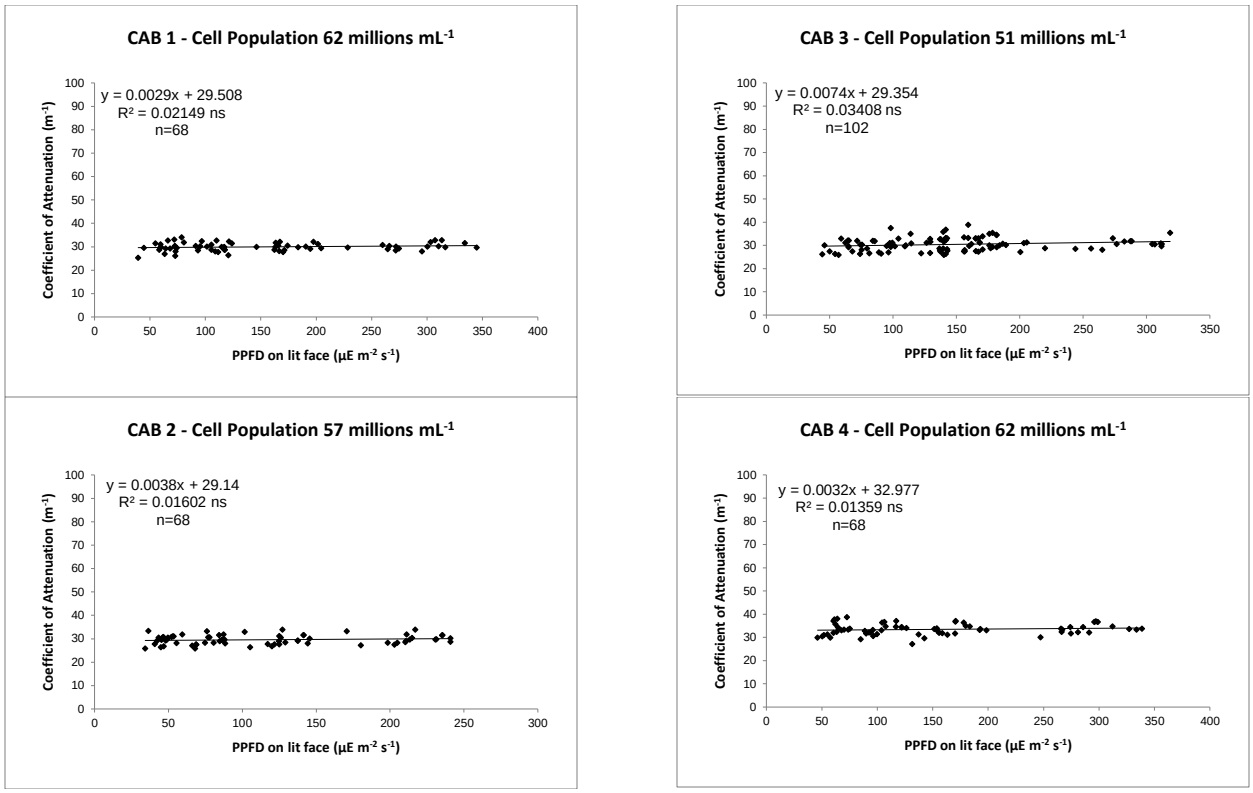

Figure 13. Linear regressions obtained for bioreactors #1-4 of Beer's Law attenuation coefficient on incident photosynthetic photon flux density (PPFD) measured at the lit face. Bioreactors operating with air-injected recirculating saline water and medium-low culture cell populations of *Nannochloropsis salina* ranging from 51 to 62 million cells per mL^{-1}.

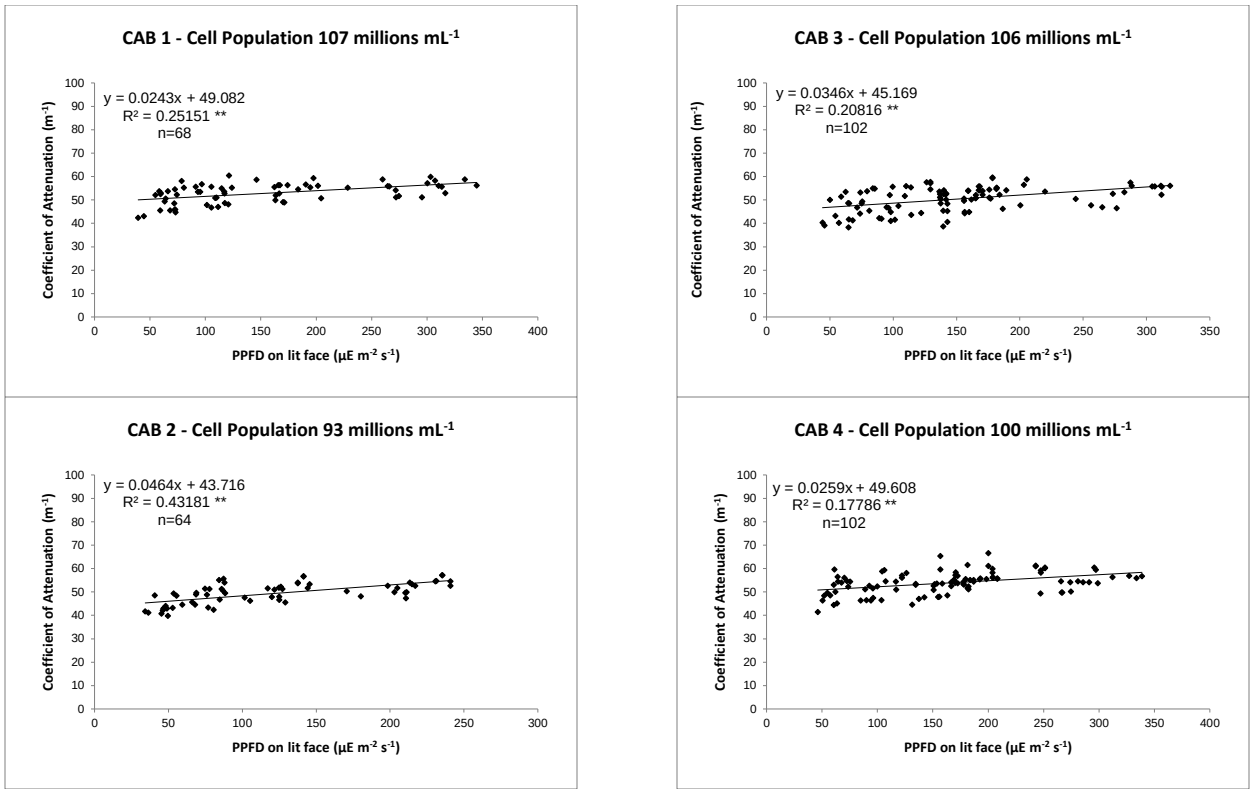

Figure 14. Linear regressions obtained for bioreactors #1-4 of Beer's Law attenuation coefficient on incident photosynthetic photon flux density (PPFD) measured at the lit face. Bioreactors operating with air-injected recirculating saline water and medium culture cell populations of *Nannochloropsis salina* ranging from 100 to 107 million cells per mL^{-1}.

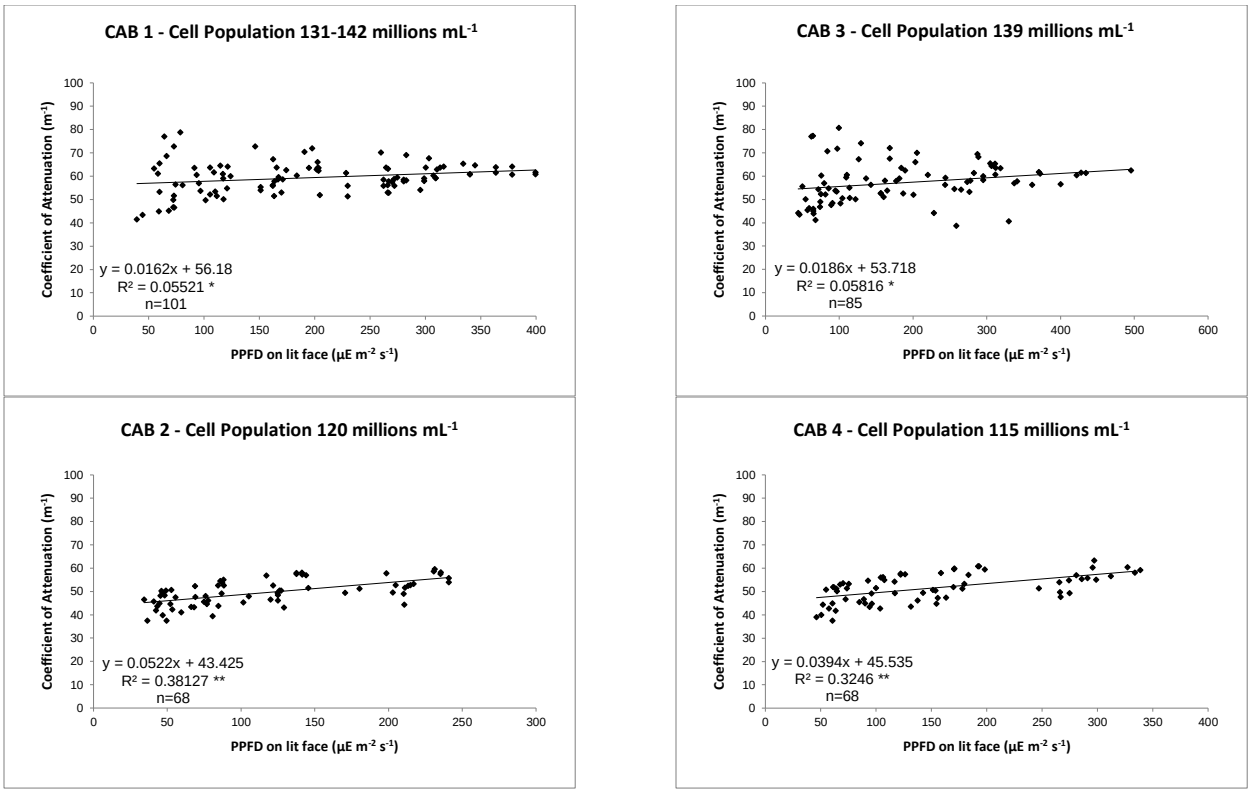

Figure 15. Linear regressions obtained for bioreactors #1-4 of Beer's Law attenuation coefficient on incident photosynthetic photon flux density (PPFD) measured at the lit face. Bioreactors operating with air-injected recirculating saline water and medium-high culture cell populations of *Nannochloropsis salina* ranging from 115 to 142 million cells per mL^{-1}.

The effects of incident PPFD and *Nannochloropsis salina* culture's cell population on the coefficient of attenuation (Figs. 11 to 15) are better summarized in Figs. 16 and 17. The overall response of the coefficient of attenuation to incident PPFD averaged over the range of culture's cell population was assessed by a linear regression analysis of 22 coefficients of regression (20 of them shown in Figs. 11 to 15) *N. salina* culture's cell population. The resulting linear regression (Fig. 16) shows a small increasing response of the coefficient of attenuation to incident PPFD with increasing cell population (0.02% per µE m^{-2} s^{-1}). This small increased response of the coefficient of attenuation to incident PPFD may likely be related to an increased scattering of light produced by the higher density of *N. salina* cells.

Unlike the small response of the coefficient of attenuation to incident PPFD, the overall response of the coefficient of attenuation to increasing culture's cell population was much larger. A near-overall response of the coefficient of attenuation to culture's cell population was assessed by calculating a regression of 22 intercepts of the linear regression equations of the coefficient of attenuation on incident PPFD on *N. salina* culture's cell population (20 of them shown in Figs. 11 to 15). The resulting quadratic regression equation (Fig. 17) shows a large increasing response of the intercepts to cell population with a slight moderate quadratic decline with increasing cell population (52.8% per million cells per mL with a 0.14% quadratic decline). The use of the linear equation intercepts to assess the effect of cell population was justified as the slopes of the regression equations of the coefficient of attenuation on incident PPFD (Figs. 11-15) were particularly small (average 0.02% per µE m^{-2} s^{-1}). The quadratic regression equation obtained shows that the cell population explained 97% of the variation of the linear equation intercepts.

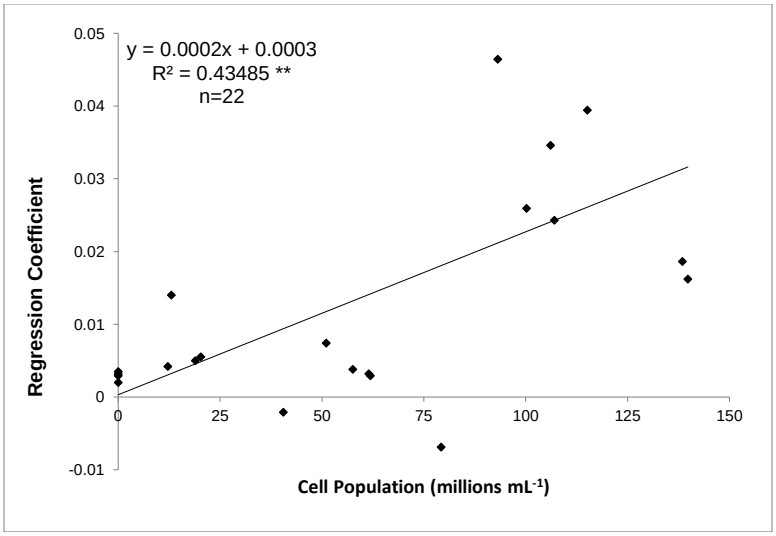

Figure 16. Linear equation describing the dependency of the response of Beer's Law coefficient of attenuation to incident photosynthetic photon flux density (PPFD) on the cell population density of *Nannochloropsis salina* across all four bioreactors. The regression coefficients (Y axis) were obtained from linear regression analyses of the coefficient of attenuation on incident PPFD at the lit face performed in all four bioreactors as shown in Figures 11 to 15.

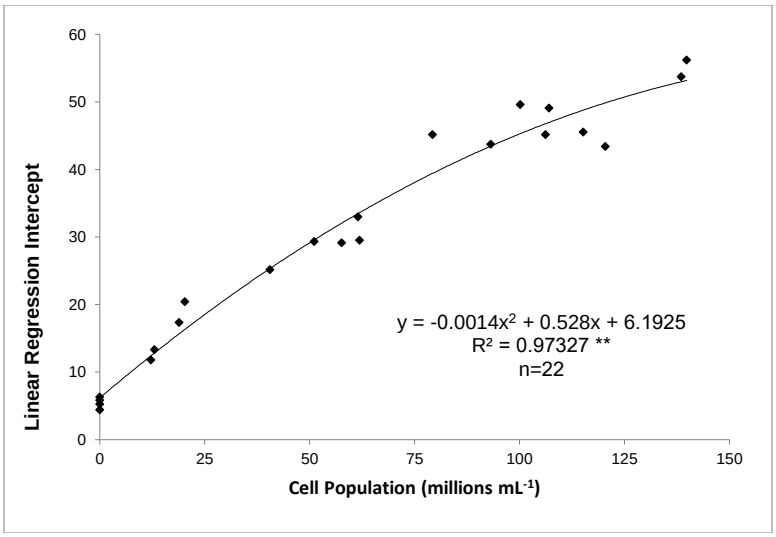

Figure 17. Quadratic polynomial equation describing the dependency of the near-overall response of Beer's Law coefficient of attenuation on the cell population density of *Nannochloropsis salina*. The near-overall response of the coefficient of attenuation to cell population was assessed by the magnitude of the intercept of the linear regression equation of the coefficient of attenuation (Y axis) on incident photosynthetic photon flux density (PPFD) obtained for a range of cell populations across all four bioreactors as shown in Figures 11 to 15.

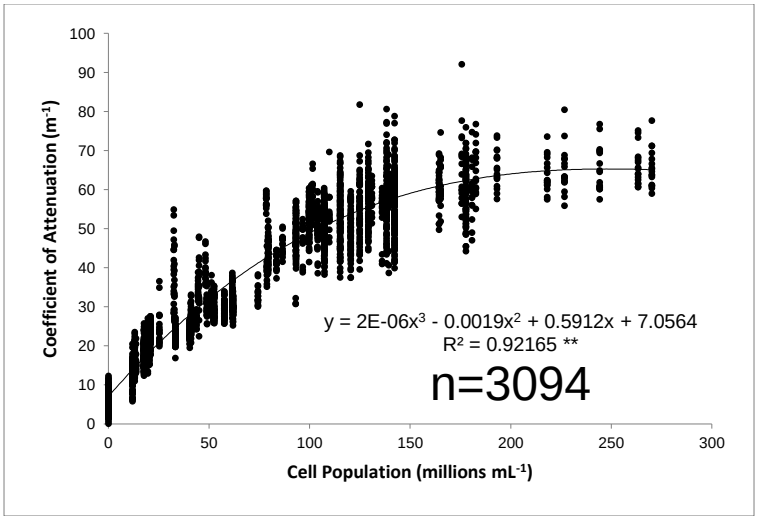

Figure 18. Cubic polynomial equation describing the comprehensive response of the Beer's Law coefficient of attenuation to cell population density of *Nannochloropsis salina* estimated from the large set of widely ranging incident and transmitted photosynthetic photo flux densities (PPFDs) measured at various cell populations of *Nannochloropsis salina* cultures growing in four environmentally controlled bioreactors.

The overall response of the coefficient of attenuation to *N. salina* culture's cell population across incident PPFD and bioreactors (a total of 3094 pairs of data points) is best quantified by a cubic polynomial equation (Fig. 18). For practical considerations, we'll call this equation the "global attenuation equation". This global attenuation equation explains 92.2% of the variation observed in the calculated coefficient of attenuation values. The intercept term in this global attenuation equation (7.0564) represents the value of the coefficient of attenuation at nil cell population, which represents, as explained elsewhere above, the attenuation effect of the air-infused recirculating saline medium. The value of this intercept is higher than the value of 0.0016 cm^{-1} (0.16 m^{-1}) reported by Gallegos and Kenworthy (1996) for seawater, which likely may have resulted from the dispersion of light caused by the bubbles in the air-infused recirculating culture medium. As shown in Fig. 18, the coefficient of attenuation increased at an initial rate of 59.1% per million cells mL^{-1} increase in culture's cell population, followed by a slow decline (quadratic and cubic terms of the equation) until it practically flattens at a value of approximately 66 m^{-1} beyond a cell population of about 225 million cells mL^{-1}.

Since most of the published research on light attenuation by algae cultures involved the culture's biomass, and for the sake of providing a common ground with those other studies, cell population data was converted to ash-free dry weights using eq. 3 to quantify the overall light attenuation response to *N. salina* culture's ash-free dry weight (Fig. 19). As expected, both cubic polynomial curves representing the responses of the coefficient of attenuation to *N. salina* culture's cell population and ash-free dry weight are similar. Consistent with the response to biomass, Benson et al. (2016), studying the light dynamics in a blend of native microalgae species typically found in sugar-mill ponds, reported also a curvilinear (although quadratic) increasing response of the coefficient of attenuation to increasing biomass concentration.

Clearly, the most direct applicability of any of these equations pertains to characterizing the availability of photosynthetic radiant energy within *N. salina* cultures grown in the bioreactors as part of larger studies quantifying the growth responses of this species to various environmental conditions. In particular, numerical estimates of available photosynthetic PPFD or PAR at different culture's depths, different levels of incident PPFD or PAR, and different culture's cell populations or biomass densities are essential for estimating algae's photosynthetic and growth rates. An extended applicability of any of these equations would be pertinent to similar characterization of available PPFD or PAR within cultures of *N. salina*, and possibly cultures of other unicellular microalgae species with comparable cell dimensions, grown in outdoor ponds exposed to diurnal and seasonal variation of solar radiation. Such characterization would be essential for estimating photosynthetic and growth rates in outdoor growing cultures. However, the applicability of any of these two equations for outdoor growing cultures would require a slight modification to minimize the attenuation effects caused by the relative dense air-infused bubbles in the recirculating culture medium, which are particular to the bioreactors used in this study. A simplistic attempt to remove the attenuating effects of the bioreactor's air-infused saline medium from either equation describing the coefficient of attenuation response to cell density would be to just disregard their regression intercept. However, it can be reasonably argued that it is unlikely that the value of this intercept is nil. Benson et al., 2016, studying the response of the coefficient of attenuation to the biomass concentration of a blend of native microalgae found in sugar-mill ponds, reported an intercept of 0.27 m^{-1}, while Gallegos and Kenworthy (1996), reported an intercept of 0.16 m^{-1} for microalgae growing in seawater.

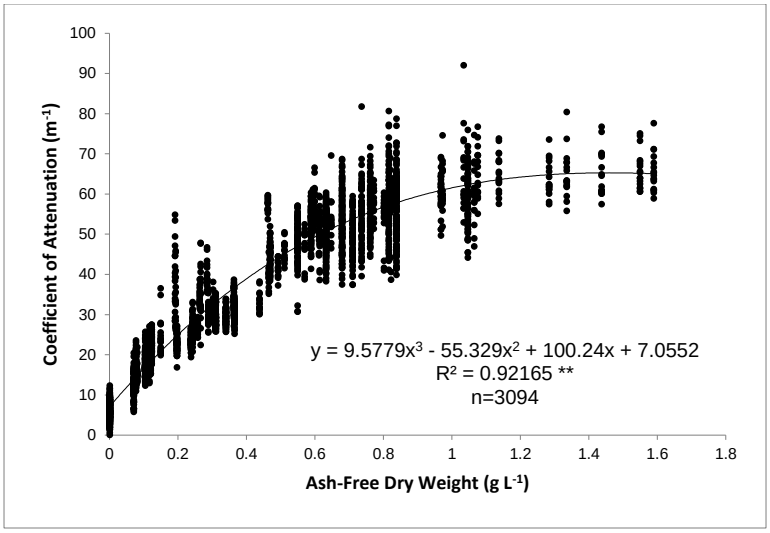

Figure 19. Cubic polynomial equation describing the comprehensive response of the Beer's Law coefficient of attenuation to ash free dry weight of *Nannochloropsis salina* estimated from the large set of widely ranging incident and transmitted photosynthetic photo flux densities (PPFDs) measured at various cell populations of *Nannochloropsis salina* cultures growing in four environmentally controlled bioreactors.

4. CONCLUSION

This research study, which involved an extensive collection of more than 3000 pairs of incident/transmitted PPFD data points across cultures of *N. salina* growing in flat panel controlled-environment bioreactors, provided a useful base for quantifying the light-attenuation effects of a wide range of cell population densities of this microalgae unicellular species. While at a given cell population density the PPFD transmitted across the bioreactors increased with the PPFD incident on the lit surface, this response decreased exponentially with the increase in the culture's cell population density becoming practically nil beyond about 80 million cell mL^{-1}. This light attenuating phenomena caused by *N. salina* cultures was well captured and summarized by cubic polynomial equations describing the effect of a wide range of cell population densities in terms of both, million cell counts per ml or ash-free dry weights in g L^{-1}, on the coefficient of attenuation in the Beer-Lambert's Law.

The most direct applicability of any of these two equations pertains to characterizing the availability of photosynthetic radiant energy within *N. salina* cultures grown in the same bioreactors as part of larger studies quantifying the growth responses of this microalgae species to various environmental conditions. However, with minor adjustment of the equation intercept, which reflects the attenuation effects of the bioreactors' high density air-infused bubbles, the applicability of these equations could be extended to characterize also the availability of PPFD within outdoor growing cultures of *N. salina*.

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