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Calibration Curves of Culture Density Assessed by Spectrophotometer for Three Microalgae (*Nephroselmis* sp., *Amphidinium carterae* and *Phormidium* sp.)

George N. Hotos, Despoina Avramidou, and Vlassoula Bekiari

ABSTRACT

This study concerns the finding of an effective operational equation relating the measured absorpbance (or O.D. - optical density) in a spectrophotometer of the suspended cells of microalgae to their cell density (c.d.) in culture in order to construct calibration curves for use in culture operations. The microalgae examined were the chlorophyte *Nephroselmis* sp., the dinophyte *Amphidinium carterae* and the filamentous cyanobacterium *Phormidium* sp. Wavelengths of 430 and 680 nm were selected that correspond to chlorophyll-a peaks of their absorption spectra were used and additionally 750 and 570 nm where absorbance was not peaked. From all equations extracted best fitness with strong predictive values were those of a logarithmic type: O.D.=0,9328*ln(c.d.)-14,108 (R^2=0,9943) at 680 nm for *Nephroselmis*, a power equation: O.D.=0,0000009*(c.d.)^{0,9195} (R^2=0,9936) at 680 nm for *Amphidinium* and a polynomial second order: O.D.=0,9869(c.d.)^2+2,4393(c.d.)+0,2666 (R^2=0,9737) at 570 nm for *Phormidium*.

Keywords: Absorbance, cell density, spectrophotometry, calibration curve, microalgae, *Nephroselmis*, *Amphidinium*, *Phormidium*.

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I. Introduction

With the modern world facing increasing carbon dioxide levels in the atmosphere already reaching 450 ppm [1] that could contribute to global warming as stated by the IPCC, a remedy can only ensue from trapping as much CO_2 as possible by natural means. Photosynthesis is the answer to this issue and algae can be the key element to this procedure. Microalgae especially possess a central role in CO_2 sequestration globally as they are 10-50 times more efficient in trapping it from the atmosphere as compared to terrestrial plants [2]. Additionally, microalgae are mass cultured for biofuels, feedstock and value added products, wastewater treatment and nutrient removal to mention their main uses [3]. The list of the cultured microalgae for various uses is continuously enlarging and knowledge accumulates concerning their particular needs for efficient production. Culture techniques for various species are adapted to their particular requirements concerning environmental conditions (light, salinity, temperature), chemicals (nutrients, pH, toxic substances) and special conformation of culture support (e.g. aeration, CO_2 addition, vessel configuration). The aim is to produce maximum algal biomass using in the most economical way the necessary labor, energy, space, and time. Common practice to any type of microalgal culture is the continuous monitoring of the cultured algal density in terms of cells/ml or dry weight (g/L) to mention but the most common of them. To accomplish that, frequent sampling of the cultured biomass is a prerequisite and a small amount of liquid is examined microscopically for counting the cells, or an amount of liquid is filtrated and then dried for hours in an oven and then weighted in order to calculate the dry weight. Both are time consuming procedures especially when a lot of culture vessels are employed. Monitoring of culture density can be greatly simplified by just recording optical density (absorbance) of culture samples and deducing the algal density using a reference regression line of optical density (O.D.) to cell density. This is accomplished by measuring one time several samples with different densities and counting with a haematocytometer the number of cells per ml. Then each cell density is assigned to the relevant O.D. in a spectrophotometer at an appropriate wave length. Afterwards only O.D. need to be measured and the cell density is calculated by the regression equation. There are in the literature [4]-[9] various regression equations of this type for several microalgae species. However, there are also many microalgae with culture potential for which this relation has never been attempted. This is exactly where our work aims, that is to present reference regression equations of O.D.'s to cell densities for selected microalgae that are promising for producing value products after being mass cultured in a feasible way.

Among the microalgae the dinoflagellates are a source of

several bioactive substances [10], [11]. Amphidinols and karlotoxins are produced from *Amphidinium carterae* using simple culture processes [12]-[14] the only constrain being its sensitivity to hydrodynamic stress [15]. Additionally, bioactive production oriented mass culture of *Amphidinium* (being a dinoflagellate) can yield other high-value chemicals such as carotenoids (e.g. peridinin) and fatty acids (EPA and DHA) that are used in numerous nutraceutical and pharmaceutical applications [16], [17]. So, the mass culture of *A. carterae* that have been successfully attempted using LED-illuminated photobioreactor [13], [14] will much benefit from a guide reference relation of O.D.-cells per ml.

Data on culture of the chlorophyte *Nephroselmis* sp. are scarce. Its mass production has been successful [18], [19] and it has been recorded as an efficient producer of lipids and C16-C18 FAME in the order of 39,4% and 77,8% respectively in yields from 0,4116 to 0,5468 g/L dry cell weight. So, for this species also a relation of optical density to cells per ml would serve as a useful tool in its culture.

Phormidium sp. is a filamentous non heterocytous cyanobacterium that has potential of producing several useful compounds like antioxidant carotenoids and phycobilins and novel bioactive toxins for pharmaceutical applications [20]-[22]. Its initial cultures in our laboratory exhibited a high growth rate in various salinities and an impressive sedimentation rate that could serve in its after-culture concentration. So, it was selected as a promising alga for our experimentation.

Motivated by the fact that although monitoring and control of algal mass growth are an essential tool in the microalgae culture its detailed investigation has not drawn the attention it deserves [23], we attempt here to offer useful regression models for predicting algal growth of selected species by means of spectrophotometrically measured optical density (O.D.) at certain wave lengths.

II. Materials and Methods

The microalgae species used (Fig. 1) were of two kinds, single-celled (*Amphidinium carterae* and *Nephroselmis* sp.) and filamentous (*Phormidium* sp.). All are marine species collected from the lagoons of W. Greece and *Phormidium* especially from the evaporation pond of Messolonghi salterns (salinity 70 ppt). They were isolated from raw samples by means of serial dilutions and kept in pure non-axenic cultures in Erlenmeyer vials of 500 ml at the appropriate salinity in which previously exhibited best growth (40 ppt for *Amphidinium* and *Phormidium*, 60 ppt for *Nephroselmis*). The microalgae were cultivated in the salinities mentioned in triplicate 1 L Erlenmeyer glass flasks using sterilized and salinity adjusted seawater fertilized with all minerals contained in a Walne formula. Temperature was kept at 20±1 ºC and illumination was provided by 20 watt 1600 lm LED lamps with an intensity of 7500-8000 lux at the surface of the culture vessels (measured by a Bioblock LX-101 lux meter) generating an active radiation of 150-160 μmol photons m^{-2} s^{-1}. Continuous aeration was supplied to the vessels via 1ml glass pipettes that created small bubbles of filtered air through 0,45 μm cut-off cartridge fed by a 2.5 hp blower with an average flow of 60 cm^3 s^{-1}.

Spectrophotometric measurements were performed using a Shimadzu UVmini-1240 UV-visible spectrophotometer. For each of the 3 species analyzed the absorption spectrum in the range of 400-800 nm was first inspected to find the absorption peaks in order to serve as a guide for the wave length to be selected in the regression model of O.D.-cell density.

Cultures were started with inoculum of 100 ml taken from a mature culture of each species and filling the experimental vessels with 900 ml sterilized and fertilized seawater of the appropriate salinity. Every day aliquots of each vessel fixed with Lugol solution were measured for cellular density in a Neubauer haemocytometer using Leica DM-1000 microscope at 40X magnification. The O.D. of an untreated aliquot was measured in the spectrophotometer at the wave lengths selected. Care was taken before measurements for the aliquots to be properly agitated to avoid sedimentation of cells (most prominent in *Phormidium*) that could distort their actual density calculation.

APHA method (APHA, 2012) indicates 750, 664, 647 and 630 nm as the wavelengths most appropriate for measuring O.D. in microalgae cell density calculations. From these we selected 750 nm for all species as it is a wave length well beyond the absorbance peak of the chlorophylls in all species examined. For *Phormidium* we selected also 430 and 680 nm (chlorophyll-a peaks) and additionally 570 nm based on the fact that this is a wave length compromising the absorbance of the sum of its accessory pigments (carotenoids and phycobilins) present in cyanobacteria. For *Nephroselmis* and *Amphidinium* 430 and 680 nm that coincide with the lower and higher chlorophyll-a peaks were also selected. Measurements were done on a daily basis following the population growth of the cultures. At the peak of the growth phase of each species (~ 60 x 10^6 cells/ml for *Nephroselmis*, ~5 x 10^6 cells/ml for *Amphidinium* and ~1,2 g D.W./L for *Phormidium*) aliquots were taken and their cell density was measured. Subsequently by serial dilutions in 10% steps of the initial aliquot of each species 9 different concentrations were made and their O.D. was recorded in order to construct the calibration reference curve of O.D. - cell density. However, in the case of *Phormidium* cell density was measured in terms of cells dry weight per liter as counting its long filaments in haematocytometer was impossible. To accomplish measurement a quantity of 100 ml of culture was filtered in a Heto-SUE-3Q vacuum generator with Whatman paper (pores ~11 μm, Edevor Nr 20, 9 cm), washed with distilled water to remove salt and then by drying the filter in an oven (3 hours at 110 ºC) its dry weight was recorded to the 3rd decimal in a KERN-EG 620-3NM electronic balance. This procedure was performed 3 times during the 10 days period that culture lasted and O.D. measurements were also taken. Additionally, O.D. was also measured daily. At day 10 the experiment terminated, a quantity of *Phormidium* was filtrated and its dry weight calculated while its O.D. was also measured. From the same vessel serial dilutions of 10% steps were prepared and their O.D.'s recorded. The dry weights allocated to these O.D.'s were simply calculated and so a calibration curve was constructed. Finally, the calibration curve presented here for *Phormidium* was based on the pooled data of O.D.-dry

weight relation from any kind of measurements taken in the experiment. Similarly, pooled data were also used for the other two algae as well. All data were analyzed with Excel software to extract the functions relating O.D. to cell density. Of all possible equations relating O.D. to cell density for a particular wavelength it was selected that with the greatest Pearson correlation coefficient (R^2).

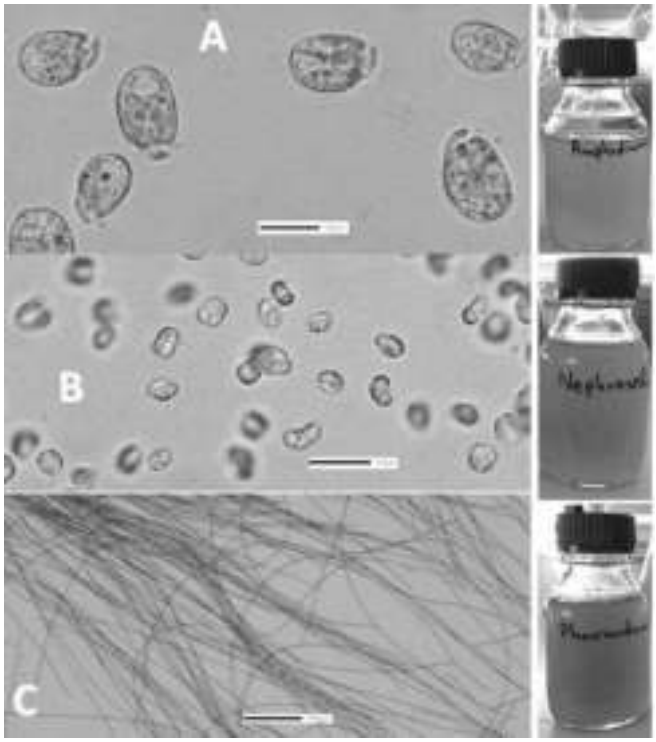

Fig. 1. Photonic microscope images of the microalgae cells of this study with a sample of their culture aside in order to show their color in their exponential phase of growth. A: *Amphidinium carterae*, B: *Nephroselmis* sp., C: *Phormidium* sp.

In order to validate the efficiency of the constructed equations for each species, the percentile deviation for the measured and predicted O.D.'s was calculated using the equation [24]:

$$\text{Percentile deviation} = \left(\frac{O.D._{obs.} - O.D._{pred.}}{O.D._{obs.}}\right) \times 100.$$

Following that, the data were analyzed and the wavelength at which the pooled percentiles presented the least Standard Error and Standard Deviation was suggested for use judged to be of the highest accuracy among the others.

III. Results and Discussion

The absorbance spectra of the microalgae examined are shown in Fig. 2, 3 and 4, PAR range (400-700) of wavelengths is included in them. The spectra were measured at different growth stages of the algae (that is: induction, exponential and stationary phases) and showed almost identical pattern so the selected spectrum of each species presented here is that corresponding to the end of the exponential phase. All three species exhibited two prominent peaks at the regions of ~430 and ~680 nm corresponding to the absorbance maxima of chlorophyll-a. Slight differences of the maximum absorbance for the microalgae tested and for other species found in the literature can be attributed to differences in the particular contents of chlorophylls (a & b for chlorophytes, a & c for dinoflagellates and cyanobacteria) and carotenoids or billiproteins present in the cells [25], [26]. For the convenience of similarity in the presentation of a useful wavelength for all the tested algae we smoothened out the small peak differences among them and choose 430 and 680 nm as the working wavelengths for all 3 algae. Presumably at the above two wavelengths (430 and 680 nm) representing the wavelengths of maximum sensitivity to quantity for the microalgae examined, the relationship between absorbance and cell density should be of the highest accuracy. Such a conclusion would be meaningless without, for the sake of comparison, testing additional wavelengths at which absorbance is minimum. So the chosen wavelengths of 750 nm for all species and additionally 570 nm for *Phormidium* were tested to see if they can also be useful to predict cell density on the ground that at these wavelengths absorbance will express nothing but turbidity alone.

The wavelengths tested resulted in very high overall Pearson correlation coefficients (R^2 >0,97) for all algae indicating that the functions chosen were strong meaning that measured cell density shows high positive correlation with absorbance explaining more than 90% of the variance [24]. Instead of a simple linear relation of O.D. to cell density a power or polynomial or logarithmic function were chosen depending on the highest R^2 resulted. With power equations it was evident that b coefficients were different from unity an indication that absorbance does not increase linearly with cell density. If b was unity, then a linear model would be justified. In *Nephroselmis* and *Amphidinium* where power equations were selected on the basis of best fitness, b values were lower from unity indicating that absorbance decreases with cell density due presumably to some kind of a cell to cell shading effect. In the case of *Phormidium* the situation was different as the best fitness in all wavelengths tested resulted from application of a polynomial equation of 2nd order with R^2 values close to 0,98.

For *Nephroselmis* (Fig. 2) measurements at 430 nm gave the highest regression coefficient (R^2=0,9903) using the power equation: $O.D._{430}$ =0,000005 x (cell dens.)^{0,7467} with the b value (0,7467) indicative of a decreasing rate of absorbance (O.D.) with cell density. On the contrary at 680 and 750 nm the higher R^2 (0,9943 and 0,994 respectively) ensued from the logarithmic equations: $O.D._{680}$ = 0,9328 x ln(cell dens.)-14,108 and $O.D._{750}$ = 0,6829 x ln(cell dens.)-10,115 respectively.

For *Amphidinium* (Fig. 3) at the wavelengths of 430 and 680 nm power equations resulted in best fits with R^2 very high all above 0,99. At the wavelength of 750 nm a linear regression exhibited the best fitness. The equations are: $O.D._{430}$ =0,0000009 x (cell dens.)^{0,9199}, $O.D._{680}$ =0,0000009 x (cell dens.)^{0,9195} and $O.D._{750}$ =0,0000002 x (cell dens.) + 0,0673. The power equations have b values close to unity meaning that absorbance (O.D.) is not decreasing substantially with cell density.

For *Phormidium* (Fig. 4) being a filamentous cyanobacterium whose cell density calculation is somewhat

cumbersome due to the multistep method of calculation (filtration paper weight, filtration of culture, drying of the concentrate, final weighing) with inherent intrusion of bias errors, the plotted values of O.D. to cell density presented greater variation than the other 2 algae. Nevertheless the relating equations were satisfactory with the best fit for the polynomial ones: $O.D._{430}=0,9893(c.d.)^2+3,132(c.d.)+0,3298$ ($R^2=0,9855$), $O.D._{680}=1,051(c.d.)^2+2,874(c.d.)+0,292$ ($R^2=0,9841$), $O.D._{750}=0,8945(c.d.)^2+2,2587(c.d.)+0,2233$ ($R^2=0,9748$) and $O.D._{570}=0,9869(c.d.)^2+2,4392(c.d.)+0,2666$ ($R^2=0,9737$).

Rather limited studies monitored algal culture growth using spectrophotometry [9], [27]. [28] and in all of them wavelengths of around 680 nm was a key value for measurements. Obviously 680 nm is a compromise value of the slight variations of absorbance peaks in the range of 675-686 that different algae exhibit due to their absorbance maxima of their chlorophyll-a. In all studies 680 nm proved a good predictive wavelength for biomass monitoring. This was also the case of the present study as for all 3 algae examined its predictive use is strong. This is of a particular importance as the 3 algae belong to different taxa (chlorophyta, dinophyta, cyanobacteria) and probably it is a clue that 680 nm can be a universal tool for use in any taxonomic group of microalgae culture. Strangely enough the other chlorophyll-a peak of 430 nm has not been used in relevant studies. In the present study 430 nm proved to be equally effective to 680 nm in constructing the calibration curves in all 3 algae. So on the ground of selecting wavelengths based on absorption spectra and in particular those of the chlorophyll-a maxima, irrespectively of the slight differences of maximum absorbance among species in the areas of ~430 and ~680 nm, selecting anyone of them (430 or 680) will be correct.

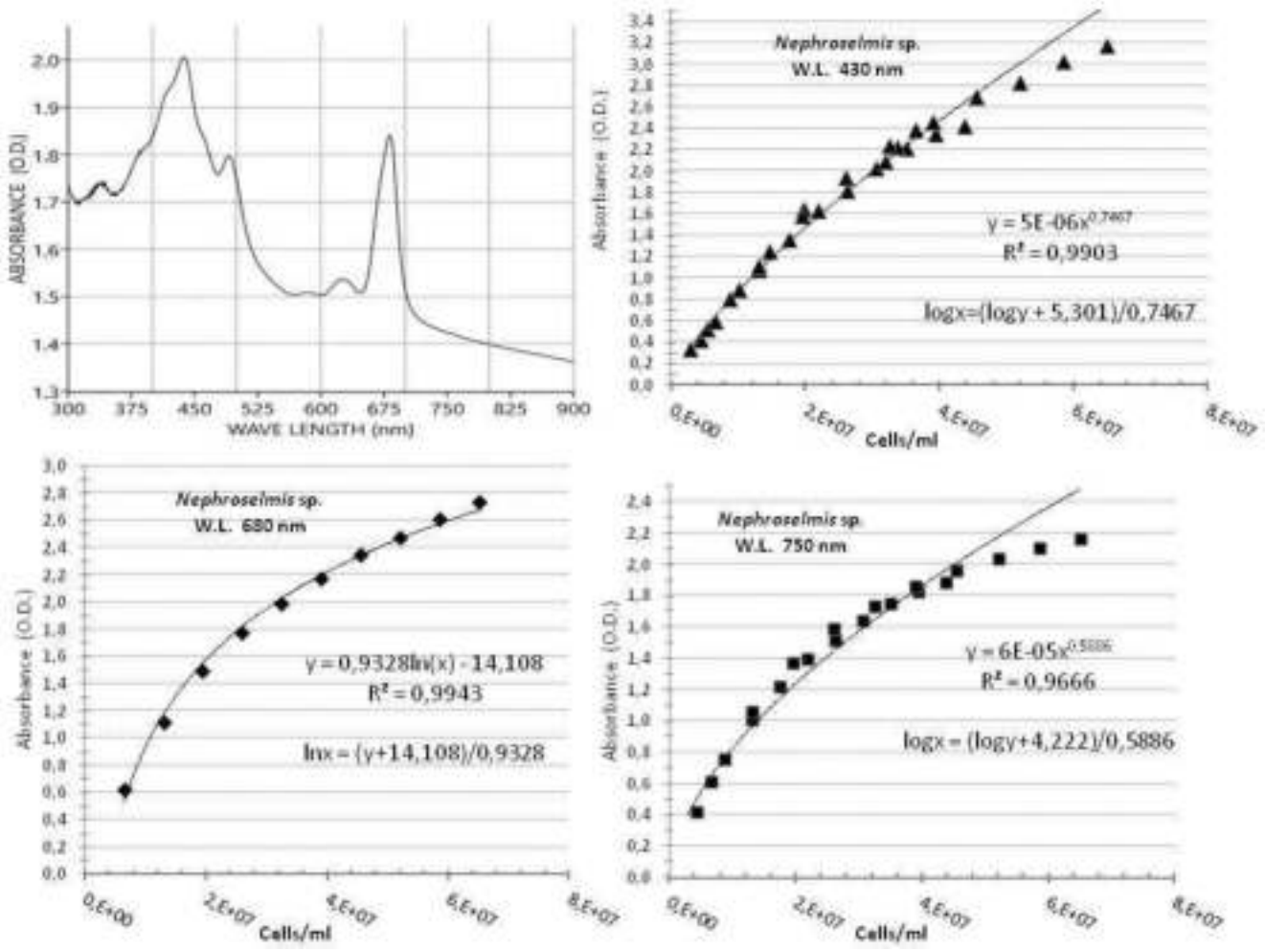

Fig. 2. The absorbance spectrum (upper left) and the relationship between absorbance and cell density for *Nephroselmis* sp. at three wavelengths (430, 680 & 750 nm).

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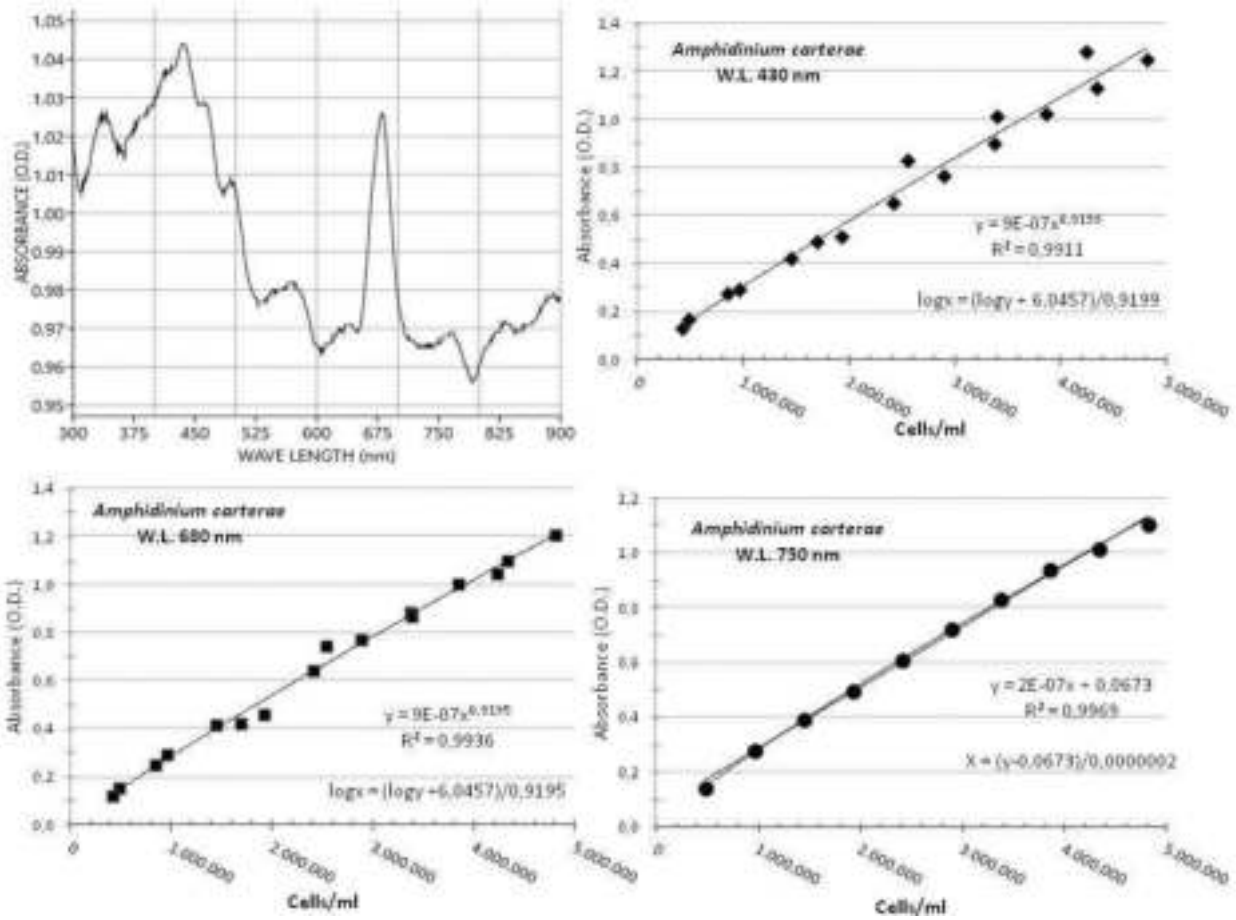

Fig. 3. The absorbance spectrum (upper left) and the relationship between absorbance and cell density for *Amphidinium carterae* at three wavelengths (430, 680 & 750 nm).

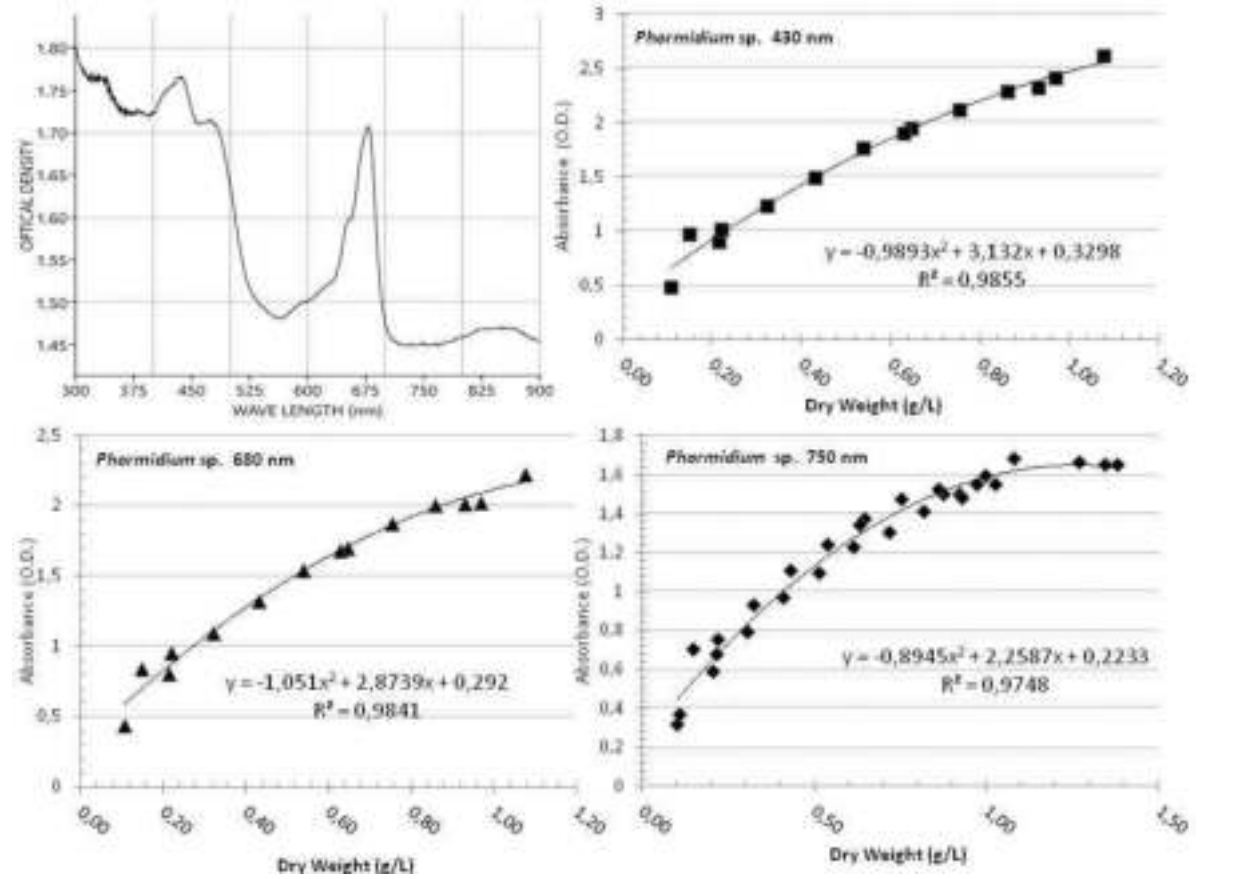

Fig. 4. The absorbance spectrum (upper left) and the relationship between absorbance and cell density for *Phormidium* sp. at three wavelengths (430, 680 & 750 nm).

On the other side, that of selecting wavelengths that do not correspond to any absorbance peak in a spectrum, the findings of the present paper indicate towards the equally to 430 and 680 nm usefulness of the wavelengths of 750 nm (for all 3 species) and 570 nm for *Phormidium*. It seems that there is not a necessity to use exclusively wavelengths that correspond to a spectrum peak in order to get a calibration curve of O.D.-cell density. Practically any wavelength can be used as the spectrophotometer detects turbidity levels caused by the suspended cells in the vial. In the present study where for the first time we present constructed equations relating O.D. to cell density for the particular species of microalgae examined, future culturists can rely upon the equations presented here and use them to monitor their cultures provided they use exactly the wave length they choose and its relevant equation. However, in order to increase the accuracy and usefulness of the predicting equations presented here and as a final selection of the best wavelength for each species we checked the percentile deviation of each wavelength and selected those with the least standard error and deviation (Table 1). Based on the summary statistical data of the percentiles (Table 1) we indicate 680 nm for *Nephroselmis* and *Amphidinium* and 570 nm (Fig. 5) for *Phormidium* as the most reliable wavelengths for the estimation of their cell density.

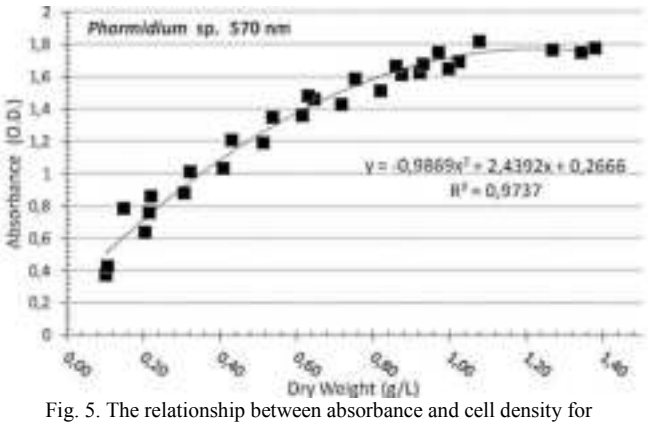

Fig. 5. The relationship between absorbance and cell density for *Phormidium* sp. at the wavelength of 570 nm.

TABLE 1. SUMMARY STATISTICS OF THE PERCENTILE DEVIATION FOR THE WAVELENGTHS TESTED IN ALL 3 SPECIES OF MICROALGAE

	Nephroselmis			*Amphidinium*			*Phormidium*			
	430 nm	**680 nm**	750 nm	430 nm	**680 nm**	750 nm	430 nm	680 nm	750 nm	**570 nm**
N	28	10	20	16	16	10	14	14	29	29
Min	-7,82	-4,99	-16,98	-6,175	-17,75	-12,02	-36,59	-33,33	-39,45	-33,45
Max	15,049	15,439	14,674	15,681	6,639	11,02	19,779	17,324	23,232	22,652
Sum	107,544	5,858	71,413	41,026	-60,38	72,706	-18,46	-16,26	-37,55	-31,56
Mean	3,84085	0,5858	3,57065	2,5641	-3,77	7,2706	-1,318	-1,161	-1,29	-1,088
Std. error	1,10256	1,7969	1,80172	1,598	1,504	2,202	3,1287	2,9123	1,978	1,8134
Variance	34,0383	32,292	64,9243	40,90	36,19	48,50	137,0	118,7	113,5	95,37
Stand. deviation	5,83423	5,6826	8,05756	6,395	6,016	6,964	11,70	10,89	10,65	9,765
25 prcntil	-1,239	-2,564	-0,682	-2,193	-6,26	6,862	-1,454	-2,189	-4,61	-4,69
75 prcntil	7,51525	1,4962	9,46075	8,579	-0,19	10,85	1,985	1,507	4,001	3,37
Skewness	-0,3203	2,2584	-0,9951	0,765	-0,83	-2,864	-1,916	-1,803	-1,54	-1,044
Kurtosis	-0,3382	6,1508	0,9221	-0,459	1,094	8,577	7,551	6,572	6,116	4,466
Coeff. variation	151,899	970,05	225,66	249,4	-159,	95,79	-887,7	-937,8	-822,8	-897,1

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