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Investigation of Effects of Nutrients and External Parameters on Kinetic Growth of Outdoor Microalgal Cultivation

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Microalgal productivity under changing conditions of light intensity and temperature is a crucial parameter for evaluating profitability and sustainability of an outdoor cultivation in cylindrical photobioreactors. The control of the principal outdoor variables, even for the more accurate systems, is subject to a wide number of factors that might influence the measures taken, thus falsifying their correlation with the microalgal growth. In this work two principal external variables (Temperature and Light) have been monitored and controlled, studying their variability for the reactors in a pilot plant having 10 column photobioreactors, by means of statistical analyses. Preliminary data obtained indicated how the position of the reactors in the pilot plant didn't affect light exposition, that was instead influenced by sampling position along reactors, time and data of the measurement. Temperature was significantly variable between outside and inside of the reactors, but the used control system successfully avoided excessive internal temperature increments.
Collection of growth data (cell count and biomass dry weight) for two different species: *Scenedesmus obliquus* and *Graesiella emersonii* at different initial $NaNO_3$ concentrations showed an increment in both maximum cell concentration and time to reach stationary phase when initial $NaNO_3$ concentration was increased. These tests permitted to obtain the best concentration of $NaNO_3$ at each inoculum: 0.35 g/L. However, these data were collected in different periods of the year. Further statistical analyses will be conducted to find also the principal input factors influencing microalgal growth parameters in order to develop an empiric and working model.

1. Introduction

Today, the cultivation of microalgae in phototrophy is still a subject of study for the high potential of cultured algae to provide a wide range of useful products exploiting their photosynthetic activity (Di Caprio et al., 2015a). Nevertheless, microalgae-based productions still present two main limits: the high costs (both fixed and operative), and the unfavourable energy balance, which is of particular relevance if biofuels are target products. Development of an integrated process in the bio-refinery view could be a valid way to overcome these limits (Visca et al. 2017). Microalgal autotrophic cultivation can be performed in indoor or outdoor systems; the outdoor cultivations are nowadays carried out in open ponds or in closed photobioreactors with different geometries and dimensions, making possible to choose the best solution to use. Each attempt to cultivate algal biomass in this way has to face principally with problems of light amount and distribution, but also with temperature variations and contaminations from competitive microorganisms (Acién-Fernández et al., 2013). Contaminations can be reduced using closed reactors that give the possibility of maintaining a strict control of operating variables, reaching generally higher productivities. Phototrophic cultivations are linked to light as an essential source for growth and photosynthetic activity; temperature also strongly affects the growth rate of every species of algae influencing cellular chemical composition, uptake of nutrients and CO_2 (Béchet et al., 2013). In addition to that, the role of nutrients in the culture medium (nitrogen, phosphorus, etc.) has to be controlled and optimized because they also influence accumulation of intracellular compounds (Altimari et al., 2014). In this work, the variability of light and temperature with respect to different sampling modalities for 10 column reactors, which make part of a pilot plant, has been measured and statistically analysed. In

addition, effect of different $NaNO_3$ initial concentration on microalgae growth has been investigated. All these data can give us information about how seasons, weather, nutrient concentrations and reactors' position inside pilot plant influence outdoor cultivation. Data presented in this work are part of a preliminary study having as aim the implementation of an empiric model for the estimation of microalgal growth in different outdoor conditions.

2. Materials and methods

2.1 Microalgae cultivation

Two different strains of algae named *Scenedesmus obliquus* and *Graesiella emersonii* were selected and maintained in a synthetic medium with this composition: 15 g/L Agar, 1.5 g/L $NaNO_3$, 30.5 mg/L K_2HPO_4, 75 mg/L $MgSO_4 \cdot 7H_2O$, 36 mg/L $CaCl_2 \cdot 2H_2O$, 20 mg/L $CaCO_3$, 6 mg/L ammonium Fe(III) citrate, 1 mg/L EDTA, 0.287 mg/L $ZnSO_4 \cdot 2H_2O$, 0.161 mg/L $MnSO_4 \cdot 2H_2O$, 0.0125 mg/L $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$, 0.061 mg/L H_3BO_3, 0.0025 mg/L $CuSO_4 \cdot 2H_2O$. Microalgae were firstly transferred from Petri dish to 500 mL flasks in a modified BG11 liquid medium (with a reduced $NaNO_3$ concentration to 0.35 g/L) (Di Caprio et al., 2015b) and then inoculated in the reactors of the pilot plant at different dilution ratio, using local tap water in place of distilled water. Tap water was used to have a more representative medium for industrial scale cultivation, considering that factors as the high calcium concentration can significantly influence microalgae growth (Di Caprio et al., 2018).

2.2 Determination of microalgae concentration

Microalgae concentration was determined daily both by cell count and dry weight. For dry weight measures, sample was firstly washed with 1 mL of sodium acetate buffer solution (sodium acetate 0.5 M at which concentrated HCl was added until reaching pH=4.8), dissolving any salt that could have misrepresented the measure. After that, 10 mL of sample were filtrated on 0.70 µm glass microfiber filters (VWR) (dried and weighted before for tare), then the filters were dried at 105 °C for half an hour and again weighted. Cell counting was performed with an optical microscope (Motic EF-N PLAN) in a 10^{-4} mL Thoma chamber.

2.3 Microalgae harvesting

When a certain concentration was reached (1-3 g/L), each reactor was emptied and the microalgal suspension collected and sent to a 95 L/h bucket centrifuge (Raw Power Centrifuge), then an aliquot of the concentrated phase was used for the subsequent inoculum.

2.4 Pilot plant

The pilot plant has been built in Bio-P s.r.l., a company located in Rome (Via di Vannina, 88). It was taken for most parts from pilot plant built for the project Alghe Energetiche (Ecoone, Di Caprio et al., 2016). The pilot plant is constituted by 10 column photobioreactors (used for both *Scenedesmus* and *Graesiella* cultivation) with a useful volume of 21 L (internal diameter=14 cm, useful height=150 cm), anchored to a structure of innocent tubes (Figure 1).

Figure 1: Pilot plant located inside Bio-P s.r.l.; in this particular test, five reactors were inoculated with Scenedesmus obliquus (right) and five with Graesiella emersonii (left).

Every reactor has been connected to air and CO_2 feeding system, making possible both mixing and pH control. The air flux was granted by a membrane compressor (AIRMAC 40W), and the mixing inside reactors was obtained by using toroidal sparger designed and realized ad hoc, while the CO_2 was injected on demand as pure CO_2. Both air and CO_2 had their respective flowmeters for a better regulation of flowrates.

2.5 Measurements and control of pH and temperature

During the experiments pH and temperature were continuously monitored, controlled by an active feedback control system, by means of probes inside reactors. The pH was maintained at its set point (pH=8) with the use of CO_2, that was injected on demand directly inside the reactors. The temperature was kept lower the chosen value (T=30°C), by a water spray system, designed and built for the purpose over the reactors. Both pH and temperature data were continuously registered and showed on a PC interface, by using LABVIEW sofware, for further data analysis.
For temperature measures, two probes were placed inside and outside the reactors, to measure internal and external temperature and their variations during day/night cycle, days and seasons.

2.6 Light analysis

The Illuminance was manually measured every day (three times per day) by using a luxmeter (LM-8000, LT-Lutron) and transformed to the corresponding value of Photosynthetic Photon Flux Density (PPFD) (μE m^{-2} s^{-1}) multiplying for the conversion factor (0.0185) for sunlight light source. For a more accurate analysis, every measure was taken on three points for each reactor, at different heights from the ground: at the bottom (20 cm), at the top (140 cm) and at a medium height (80 cm). In Table 1 the factors hypothesized to influence the measures are resumed together with how measures were taken.

Table 1: Light factors vs. Data collection

Considered Factor	Data collection
Position along the axis of each reactor	Measurements were taken at three positions (Top, Medium, Bottom)
Time of measurements' uptake (during the day)	Measurements were taken three times (10 a.m., 2 p.m., and 5:30 p.m) for each working day
Reactors' position inside the plant	Measurements were taken on each reactor of the pilot plant
Time of measurements' uptake (different days)	Measurements were taken at different days

2.7 Effect of nutrients

In addition to the external factors described above, also the effect of $NaNO_3$ concentration on biomass growth was studied. Three experiments with three different concentrations of $NaNO_3$ (0.175 g/L; 0.35 g/L; 0.7 g/L) but with same inoculum concentration of C_0=0.3 g/L were carried out.

2.8 Statistical analysis

The effect of the considerd factors on temperatures and light measures, was investigated by using a statistical analysis. In particular, for light studies two ANOVA tests were realized considering for each one two factors with replicates:

1) The factors: "position along the axis of each reactor" and "reactors' position inside the plant". Measures taken at different days were considered as replicates.
2) The factors: "time of measurements' uptake (part of the day)" and "time of measurements' uptake (different days)". Measures taken on different reactors were considered as replicates.

For temperature analysis a paired t-test was conducted comparing internal and external temperatures in time. Data were divided in three time intervals: temperature increment, internal temperature constant (system control active) and temperature decrement. For all analyses a value of α=0.05 was choosen and Microsoft Office Excel software was used.

3. Results

3.1 Growth results

In Figure 2 the growth can be seen, both in terms of biomass and cellular concentration, both for *S. obliquus* and *G. emersonii* species for an inoculum concentration C_0=0.3 g/L. This particular experiment was carried out in a summer period with the cooling system activated (10-27 July 2017), showing as expected that both species had a very similar trend comprising all the steps of a microbial growth (lag, exponential, stationary and decay phase). Furthermore, in the Figure 2 is showed that *G. emersonii* reached higher values of growth in terms of cellular concentration than *S. obliquus*. This difference can be due to the higher thermophilic features for *G. emersonii* with respect to *S. obliquus*, making it more suitable for hot climates.

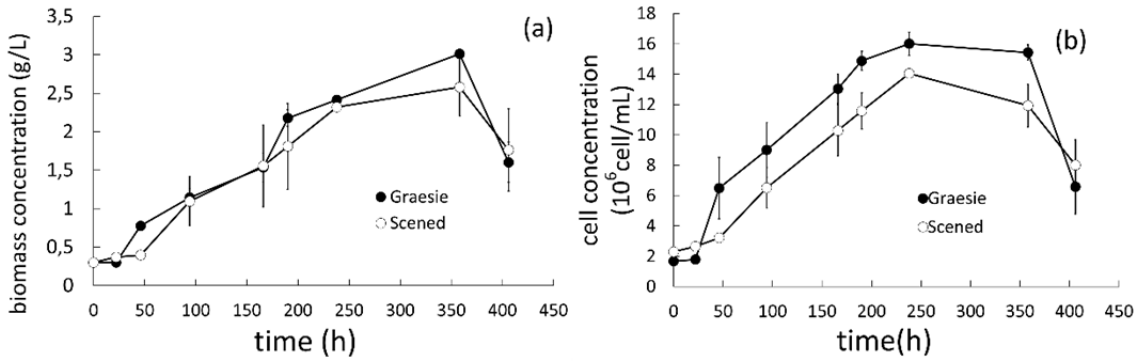

Figure 2: Growth curves of Scenedesmus obliquus and Graesiella emersonii in terms a) dry weight (g/L) and b) cellular concentration (10^6 cell/mL), with an inoculum concentration of C_0=0.3 g/L.

3.2 Light data results

In Table 2 the results of ANOVA test for the factors written in Table 1 are reported. The results show that the factors with $F>F_{crit}$ (null hypothesis rejected) give a significant influence on the amount of incident light on surface of the reactors. The only factor that didn't influence the collected data was the position of the reactors inside the plant, so each reactor inside the plant could be considered as a replicate for light exposition.

Table 2: Results of ANOVA test for the different considered factors which can influence light exposition of the reactors. SS=Sum of Squares; df=degree of freedom; MS=mean square; F= Fisher statistic.

Factor	SS	df	MS	F	P-value	F_{crit}
Position along the axis of each reactor	0.586	1	0.586	49.76	$7.34*10^{-11}$	3.91
Reactors' position inside the plant	0.097	6	0.016	1.37	0.229	2.16
Time of measurements' uptake: Part of the day	$5.55*10^{8}$	2	$2.77*10^{8}$	$2.29*10^{3}$	$1.40*10^{-119}$	3.05
Time of measurements' uptake: Different days	$3.03*10^{7}$	8	$3.79*10^{5}$	31.31	$2.88*10^{-29}$	1.99

The other factors influenced significantly light data collected, and the causes can be resumed in the shadow's effect between reactor-reactor and reactor-building and in the day-night cycle, weather and seasonal variability of the light.

In Figure 3 an example of light data collected during the experiment described above (10-27 July 2017) is reported. The vertical axes represent the value of the normalized PPFD with respect to the value collected at the reactor's top, measured for each reactor along the three positions, assuming the top measure as the highest because more lighted than the others. The results represented below confirmed the hypothesis of considering the top value as the highest, showing values below the unit, mostly between 0.4-0.9. Top Level isn't reported because it would give only unitary value for the Normalized PPFD.

Results show how to collect light data in a casual position of the reactor will be not accurate in describing light exposition, thus affecting the value of forecast growth in a future empiric model.

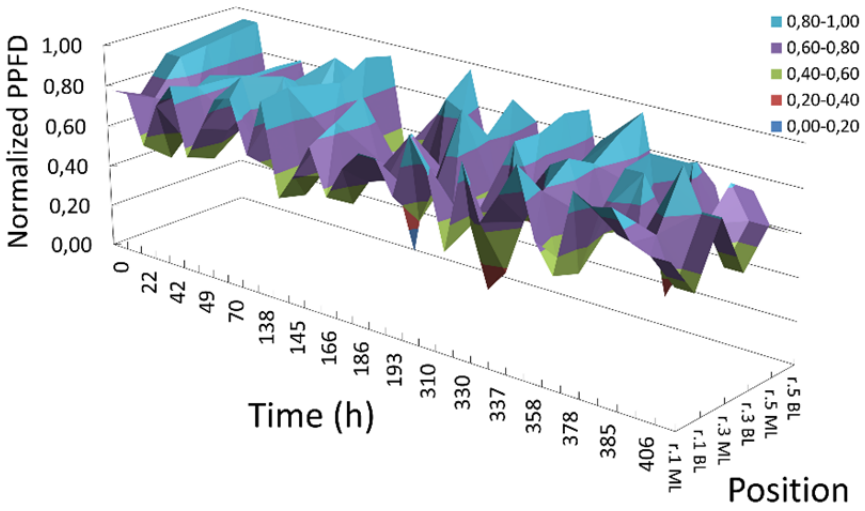

Figure 3: Example of normalized light data in function of time for different reactors and axis position during an experiment. The vertical axes represent the value of the normalized PPFD with respect to the value collected at the reactor's top; the deep axis (Position) takes into account the position along reactors (Medium: ML, Bottom: BL) for each considered reactor (r.1,r.2,r.3); the horizontal axes represent the time of the experiment (10-27 July 2017).

3.3 Temperature data results

Statistical analysis for temperature (T) indicates a significant difference between temperature inside and outside the reactors, with p-value that were 0.01, $1 \cdot 10^{-19}$ and 0.08 respectively for T increment phase, internal T constant phase and T decrement phase. Temperature in the reactors increased and decreased slower inside reactors with respect to external temperature and remained constant in the central hours of the day, because of the control system. In Figure 4 the profiles of internal and external temperature during a part of the experiment described above are plotted. It is evident that the cooling system reached its goal, maintaining the internal temperature at its set-point, except for a small peak at the end of the experiment due to a maintenance phase done to the spray system. External temperature reached higher values because the absence of an active control, but showing the same oscillation due to the circadian cycle. The statistical analysis used for temperature data, confirmed the necessity of using two probes, because internal and external temperatures should be considered as two different input parameters influencing the microalgal growth.

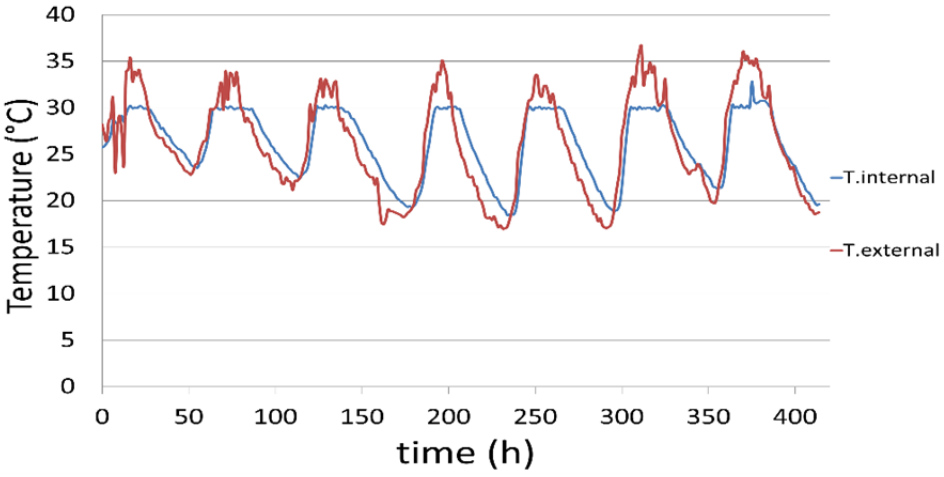

Figure 4: internal and external temperature profiles during half of the experiment (10-27 July 2017)

3.4 Effect of the nutrients

The variation of nutrient concentration described at paragraph 2.7 showed us that doubling or halving the initial nitrogen content of 0.35 g/L brought to doubling or halving both the final microalgal concentration (in terms of cell counts) and the time of its reaching.
With these results, the concentration of $NaNO_3$ at 0.35 g/L was chosen as the best starting condition, with a nutrient ratio of 1.17 g $NaNO_3$/g inoculum, allowing reaching a good equilibrium between cellular growth and metabolites accumulation. This deduction was done considering as only source of variation the nutrient concentration, assuming the other factors constant and irrelevant between the different tests. The validity of this assumption will be validated by further analyses, which will be done to investigate effect of nutrient coupled with the other external factors effect (light and temperature) on microalgal growth.

4. Conclusions

In this work, a preliminary analysis of the effect of several factors which could influence microalgae growth in the reactors of an outdoor pilot plant was done. These preliminary results allowed us to know how to collect data. Position of the reactors inside the pilot plant was the only factor which didn't influence light exposition, allowing considering every reactor as a replicate. Other factors investigated showed a significant influence on the measured light. Temperature results indicated the functionality and utility of the system control and a difference between external and internal temperature also when system control wasn't working.
In this preliminary investigation, the factors were considered singly, assuming no interaction between them. Further studies are currently running, using multivariate statistic tools and other data analysis' techniques to find how much the input factors (light, temperature, nutrients) will influence the output ones (growth rate, etc.) with a relative scale of influence. All these considerations will be connected together for the realization of an empiric model for growth and metabolites production in function of external parameters monitored.

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