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Study of the Growth Parameters of the *Nannochloropsis Oculata* for the Nitrogen and Phosphorus Removal from Wastewater through Design of Experiment Approach

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Microalgae are a promising renewable energy source because of their capability to produce a large amount of oil, which can be directly used as a fuel or converted into biodiesel through transesterification processes. Microalgae growth needs a certain amount of nutrients such as nitrogen and phosphorus and, with this regard, the wastewater arising from aquaculture and many others agro-industrial processes can be a suitable and cheap source of these elements. Moreover, nitrogen and phosphorus are responsible for the eutrophication of the waters basins, therefore microalgae can be proficiently used to remove these pollutants. In this work *Nannochloropsis oculata* was tested to verify the nitrogen removal capability from a synthetic wastewater. The medium was composed of marine water added with appropriate macro and micronutrients mixtures. The experiments were carried out by means of lab-scale, completely mixed bubble-columns, photo-bioreactors. A Design of Experiments (DoE) approach was here used in order to assess whether the input factors (i) light intensity, (ii) different nitrogen concentration sources and (iii) carbon dioxide have a statistically significant impact on the microalgae growth. It was demonstrated that the only factors statistically significant are the light intensity and its interaction with the nitrate concentration, at least for the parameter ranges here investigated. As a final remark, it was found that nitrate removal were completed from 3 to 5 days depending on the medium composition, whereas urea and phosphates needed more time.

1. Introduction

Algal biomasses represent a promising source of oil for the biofuels production, but microalgae cultivation requires a high amount of water *resources* and inorganic nutrients. This is a significant cost item for the feedstock production and affects importantly the oil cost. A solution to overcome the high costs is to couple the biodiesel production with waste water treatment based on microalgae, since the secondary effluent of plants contains high amounts of nitrogen and phosphorous (Xin *et al.,* 2010). The treatment of waste water with microalgae to remove the nitrogen and phosphorous is well consolidated in the literature and it was originally proposed 50 years ago by Oswald and Gotaas (Abdel-Raouf *et al.*, 2012). This was followed by several studies on laboratory and pilot scale (see e.g. Shelef *et al.*, 1980, Oswald, 1988, Shi *et al.*, 2007, Zhu *et al.*,2008). Microalgae growth and nutrient uptake depend on many factors such as pH, light intensity and wavelength, CO_2 concentration, temperature and algal density (Dvoretsky, 2015). As CO_2 concentration increases, it determines a biomass and total lipid productivity decrease, for some algal species (Chiu *et al.,* 2009).The algal density strongly affects the nutrient removal performance and the photosynthetic efficiency (Lau *et al.,* 1995, Darley, 1982). The nutrient removal efficiency is also affected by the light wavelengths and wavelengths mixing ratio. The latter parameter increases the microalgae production rate and the nitrogen and phosphorous removal rates (Kim *et al.,* 2013). It should be remarked that these results were obtained with the

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single factors that were analyzed one at time. On the other hand, a thorough analysis of the impact of these factors jointly varied in the analysis is still lacking in literature and a complete understanding of the growth process should require the knowledge of the interactions among them. *Nannochloropsis oculata* is a marine *eustigmatophyte* widely used in aquaculture as a food for rotifers. In this sector *N. oculata* plays a significant role for its robust nature and the ability to accumulate intracellular oil (Kromkampet *et al.*, 2009). In this work the growth of the *N. oculata* in seawater reinforced with the *Guillard medium* at different concentration levels of urea and nitrates was studied. The experiments were carried out using bubble column annular photobioreactors with a culture volume of six liters. The light intensity, carbon dioxide, nitrates and urea at two levels of concentration were the parameters that were jointly varied in the Design of Experiment (DoE) (Montgomery, 2013).The aim of this work was to analyze the best condition to improve nitrogen removal from wastewater by microalgae, in the range explored and for the parameters chosen.

2. Materials and methods

2.1 *Algae* species

Nannochloropsis is a genus of *algae* mostly found in marine environment. This *algae* is an eukaryotic photosynthetic microorganism that grows rapidly due to its simple structure (Horsman *et al.*, 2008) and because of its small size, ranging from 2 to 4 μm, it is considered to be part of phytoplankton. The microalgae cultures, were permanently kept in Erlenmeyer flasks with a solution of marine water and Guillard F/2 medium (Guillard *et al.*, 1962-1975), under constant illumination and aeration at 22 °C temperature in the laboratories of the IMC Foundation-International Marine Centre, located in Torregrande, Oristano, Sardinia, Italy. Each experiment started from this culture as inoculum, that was previously cultivated to reach a desired concentration and then inoculated into the photobioreactors.

2.2 Medium

To simulate a saline aquaculture wastewater, marine water has been modified adding the *Guillard medium* and different levels of urea and nitrate salts. The *Guillard medium* composition is reported in Table 1, urea and sodium nitrate used were analytical grade, and all the compounds listed were from *AppliChemPanreac*. A one ml/l of trace metals and 0.5 ml/l of mix-vitamins were added to complete the nutrients and micronutrients profile of the medium, to ensure the microbial growth. Nitrate and urea were added in the medium when required as further explained in the experimental section.

Table1: Modified Guillard medium

Compound	Conc [mol/l]	Compound	Conc [mol/l]
$NaH_2PO_4 * H_2O$	3.62 x 10-5	**Trace metals:**	
$Na_2SiO_3 * 9H_2O$	1.06 x 10-4	$CoCl_2 * 6H_2O$	4.20 x 10-8
Trace metals:		$CuSO_4 * 5H_2O$	3.93 x 10-8
$FeCl_3 * 6H_2O$	1.17 x 10-5		
$Na_2EDTA * 2H_2O$	1.17 x 10-5	**Vitamins:**	
$MnCl_2 * 4H_2O$	9.10 x 10-7	Thiamine HCl	2.96 x 10-7
$ZnSO_4 * 7H_2O$	7.65 x 10-8	Biotin	2.05 x 10-9
$Na_2MoO_4 * 2H_2O$	2.60 x 10-8	Cyanocobalamin	3.69 x 10-10

2.3 Parameter measurements

Monitoring of the *algae* growth was obtained by means of optical density measurements, which were taken using a dual beam spectrophotometer (UV-VIS Jasco V-530), acquiring both 680 and 750 nm wavelengths. In particular, the first wavelength can be related to the α-chlorophyll and the second to the biomass. Also a portion of the UV-Visible spectrum from 300 to 800 nm was acquired, to allow further studies. Two samples a day were withdrawn from the photobioreactors and frozen, to search the following parameters: dry weight, nitrates and urea. During the experiments pH, temperature and electrical conductivity were monitored and recorded. Different measurements of photosynthetically active radiation (PAR) were also undertaken, to obtain a complete profile of light irradiation in the different sections of the photobioreactors (LI-COR quantum sensor, model LI-190).

2.4 Chemical Analysis

In addition to the indirect estimation of the biomass amount, through direct OD measurements, dry weight analysis (Clasceri et al, 1999) were done for each sample withdrawn (twice a day) during the experiments. Forty ml of sample were filtered through 0.45 μm Whatman membranes, under vacuum. After complete filtration, each filter was washed with 20 ml of distilled water under vacuum, to remove the salts from the

saline water, and dried at 105 °C. The membranes were then brought to constant weight. The liquid fraction of the sample was used for the nitrate, urea and phosphorous analysis. To obtain the N-NO_3 and P-PO_4 profile over time and for each experiment, an high performance ion chromatography unit from Thermo Scientific™ (Dionex™ ICS-2000) was used. The instrument was equipped with a Dionex™ Ion Pac ™ AS19 Column, operating in the following conditions: constant temperature, 30°C, eluent KOH 10mM from 0 to 10 min, gradient from 10 to 45 [mMol] from 10 to 25 minutes. The sample was previously treated with a Dionex™ ON GUARD II Ag 1cc cartridge, to remove the excess of Cl^-, Br^- and I^- ions that could interfere with the nitrate analysis. The calibration curve, with five points, was done every day before the analysis using a multiparametric certified standard mix (F^-, Cl^-, NO_2^-, NO_3^-, Br, SO_4^{2-} and PO_4^{3-}). In this working conditions, the retention time for nitrates was 11 min and for phosphates 20.4 minutes. Urea consumption was analyzed by means of an adapted colorimetric method (Mulvenna, 1991). The 0.45 micron filtered samples were treated at acid pH with Diacetylmonoxime and Thiosemicarbazide, incubated at 85 °C for 20 minutes, then refrigerated with a bath of cold water for 5 minutes and, finally, the absorbance at 520 nm was acquired in a Varian Cary®50 UV-VIS spectrophotometer. The calculated detection limit was 1.5 mg/l, uncertainty ±1.5 mg/l (almost constant in the range of calibration curve), according to the UNICHIM statistical procedures, and the calibration points ranged from 0 to 150 mg/l.

2.5 Photobioreactors

The experiments were carried out using two lab scale, completely mixed, bubble column, photo-bioreactors of six liter capacity (each). The two reactors are equipped with temperature and pH monitoring and regulation control system. The lighting system is composed of four neon daylight lamp (four fluorescent lamps type cool day light, OSR FQ 24W/865).The gas supply is ensured by a blower and the pure carbon dioxide is fed into the air stream. As known, during the CO_2 fixation by photosynthesis, the *microalgae*, in presence of NO_3^- or NH_4^+, form OH^- ions increasing the pH of the medium. The pH control system operate in order to keep the pH at set point value, opening the CO_2 valve when pH exceed 7.60 (optimal pH growing conditions for *N.O.*). The carbon dioxide was also used as integrated carbon source and to evaluate if its presence enhanced the nitrogen removal. The growth conditions were: Temperature 22°C, pH 7.60 ± 0.2. Air volumetric flow 2 l/min, CO_2 volumetric flow 0.2 l/min.

2.6 Experimental design

In order to evaluate the behavior of the *algae* growth due to the different parameters, four factors that may influence the *algae* growth were chosen and were jointly varied by means of design of experiment approach. Light intensity, carbon dioxide, nitrate and urea were selected to perform the experiments (Spolaore *et al.,* 2006, Faria *et al.,* 2012). A fractional factorial design (indicated as 2_{IV}^{4-1}) consisting of two level, four factors and half fraction of the full factorial, with resolution IV, was used (for further details see Montgomery, 2013) with the aim of reducing the number of experiments. In fact, due to the long term duration of the experiments, the fractional factorial design was an obliged choice to describe the process under investigation in reasonable times. Table 2 reports the levels adopted for each factor varied.

Table 2: Levels at low and High concentration/number

Factor/Level	Low	High	Units
A= Lamps	**2**	**4**	***number***
B=N_aNO_3	**60**	**120**	***mg/l***
C=Urea	**0**	**120**	***mg/l***
D=CO_2	**0**	**1**	**-**

The fractional factorial design has been created using the *generators* and *defining relations* reported on Table 3, along the complete alias structure, for more details see Montgomery, 2013. As it can be seen in the Table 3 the single effects from the two way interactions can be resolved because of the resolution IV of the chosen fractional factorial design.

Table 3: Generators, defining relations and complete alias structure

Terms				
Generators	D=ABC			
Defining relation	I=ABCD			
Alias structure	I+ABCD	A+BCD	B+ACD	C+ABD
	D+ABC	AB+CD	AC+BD	AD+BC

2.7 Growth model

The *algae* growth is mathematically modelled by resorting to well-known population models (Seber *et al.*, 2003). Amongst the others, the Gompertz model (eq.1) revealed to be the most proper to fit the experimental results in terms of S (standard deviation of the error, eq.2), compared to the other models (e.g. Logistic equation). The choice was taken by considering the mean square error of the fit. In the equation 1, A is the *algae* population, μ is the specific growth rate and k is the carrying capacity.

$$\frac{dA}{dt} = \mu \cdot A(\log k - \log A) \qquad (1) \qquad\qquad S = \sqrt{\frac{SSE}{N-P}} \qquad (2)$$

The equation 1 was used to systematically obtain the scalar μ for each experimental run, considering the specific growth rate a representative parameter of the growth process. The specific growth rate was used to evaluate the fractional factorial design. Eq. 2 was also used to compare the goodness of fit (S) among the nonlinear regressions performed for each run. The parameter SSE is the Sum of Squared Errors ($\sum(y_i - \hat{y}_i)^2$), *N* is the number of observations and *P* is the number of parameters.

3. Results and discussion

3.1 Nitrate and Urea consumption

As described in Table 2, the $NaNO_3$ concentration at t_0 ranged from 60 to 120 mg/l, and its degradation was accomplished in 3-5 days (e.g. Figure 1), depending on the initial concentration and on the experimental conditions. On the other hand, degradation of urea revealed to require more time, probably because the *microalgae* started degrading first the nitrate, more easily degradable than the urea. However, the presence of urea, that ranged from 0 to 120 mg/l, slightly improved the microbial growth in terms of specific growth rate μ. Figure 1 shows the complete depletion of nitrate (almost in all the runs) and the partial degradation of urea and phosphorous reached in ten days.

Table 4: Growth rates, removal rates (R-xx) and percentage consumption (%C) for Nitrate, Urea (when present) and Phosphorus.

Run	R-NO_3 [mg/l/Die]	%C NO_3	R-Urea [mg/l/Die]	%C Urea	R-PO_4 [mg/l/Die]	%C PO_4
1	14.9	99.3	-	-	0.27	30.8
2	20.0	100.0	-	-	1.01	35.0
3	30.0	100.0	-	-	0.19	26.6
4	29.9	99.7	-	-	0.39	36.2
5	12.0	100.0	4.8	57.7	0.57	48.4
6	10.5	100.0	9.0	72.6	0.53	59.5
7	19.4	96.9	5.2	44.7	0.23	52.1
8	24.0	100.0	3.8	47.8	0.21	40.6

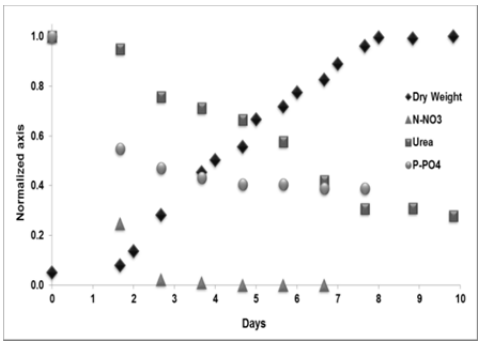

Figure 1: Dry Weight Vs. Urea, N-NO_3 and P-PO_4 consumption (Run 6 – t_0: Urea & NO_3 120 mg/l)

Table 4 reports the removal rates (R-xx) for nitrates, urea and phosphorus, and the amount of the nutrients removed during the experiment period (10 days) on a percentage basis, for each experiment performed. The *algae* biomass, in terms of dry weight, ranged from 0.366 to 1.097 g/l, depending on the experimental conditions.

3.2 Statistical analysis

As already introduced in Section 2.7, the impact of the four factors chosen has been evaluated by means of the representative scalar μ. By use of the Yates algorithm (Nelson *et al*., 2003) we were able to assign an effect to each factor in order to quantify its impact on the system. In figure 2, the Pareto chart shows the absolute value for the effects computed. The dashed line reported in figure 2 represents the threshold value for the statistically significant terms (light and light-N-NO_3 interaction on the right) from the not significant, on the left. As described by Montgomery, the not significant factors could be neglected (threshold value: α=10%), and the experimental campaign could be projected to a 2^2 full factorial design, with two replicates (urea and

CO_2 discarded). This procedure allowed to re-process the data obtained from the experimental campaign, without losing significant information, and to perform the analysis of variance (ANOVA), that is summarized into Table 5. The Light, as expected, is confirmed as significant factor, p-value 0.01, and interaction light-$NaNO_3$ is statistically significant as well, p-value 0.028. The nitrates have a mild negative impact on the system, but are not statistically significant (p-value 0.762).

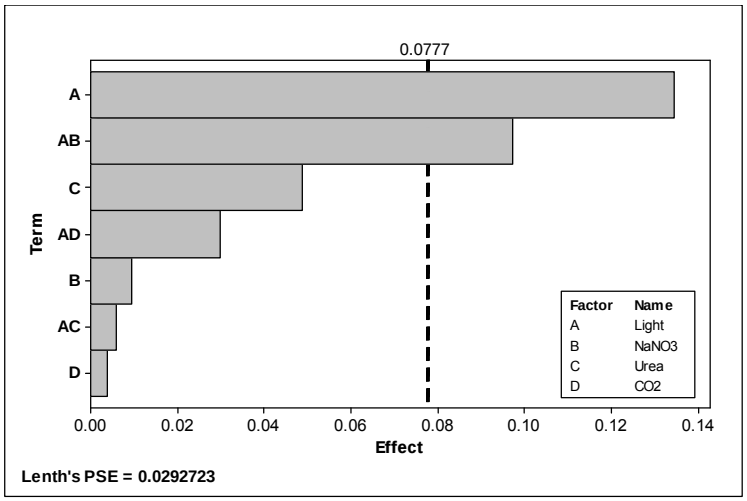

Figure 2: Pareto chart for computed effects [dimensionless]

Table 5: Effects and coefficients for each significant factor (coded unit) – ANOVA table (dimensionless)

Term	Effect	Coef	SE Coef	DoF	SSE	MSE	F-ratio	p-value
Constant		0.31642	0.01438	2	0.0362	0.0181	10.97	0.024
Light	0.13440	0.06720	0.01438	1	0.0361	0.0361	**21.83**	**0.010**
NaNO3	-0.00931	-0.00466	0.01438	1	0.0001	0.0001	0.10	0.762
Light/NaNO3	-0.04855	-0.04855	0.01438	1	0.0189	0.0189	**11.40**	**0.028**

In figure 3 is reported the growth curve for the run 6 (4 lamps, 60 mg/l N-NO_3, 120 mg/l Urea), in terms of dry weight, and the non-linear Gompertz equation, that well represents the data, along with the confidence intervals (MSE=0.00154 and S=0.03929). Picture 4 clearly shows the interactions plot for light and N-NO_3. Indeed, a marked curvature on the interactions lines is evident. The fastest growth was found for high levels of light end low levels of N-NO_3.

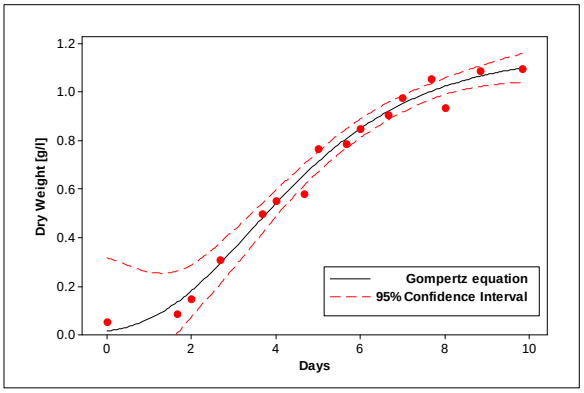

Figure 3: growth curve in terms of Dry Weight and fitted Gompertz equation

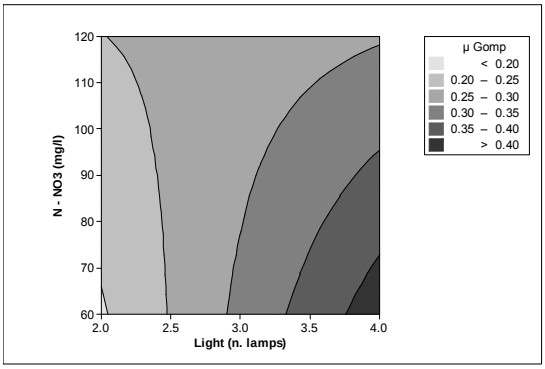

Figure 4: Interaction plot for Light and Nitrates, computed with μ [day-1] as output

4. Conclusions

The DoE analysis applied to the experimental campaign showed that the light was the most statistically significant parameter for the *algae* growth. It was also found that the interaction between light and N-NO_3 was

statistically significant as well. Indeed, the main value for the specific growth rate was found for the experimental run 6 (4 lamps, 60 mg/l N-NO_3, 120 mg/l Urea), whereas the lower value was found in the run 3 (2 lamps, 120 mg/l N-NO_3, 0 mg/l Urea). This confirms that, as found with the DoE analysis, the light has the main impact on the system. The total N-NO_3 degradation was accomplished in 3-5 days, while the Urea consumption reached removal rates ranging from 44.7 to 72.6% in 10 days. The P-PO4 removal, in the same operating conditions, achieved values between 27 to 60 %. Urea and CO_2, in the range here explored, were found not statistically significant.

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