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Factorial analysis of the biokinetic growth parameters and CO_2 fixation rate of *Chlorella vulgaris* and *Botryococcus braunii* in wastewater and synthetic medium

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ABSTRACT

Microalgae strains, *Botryococcus braunii* and *Chlorella vulgaris* were cultured in urban wastewater as monoalgal cultures and together in co-cultures; the same experiments were performed in synthetic growth medium to establish comparisons between both media. A fully crossed factorial design was used to design and carry out the experiment, resulting in 18 tests, and this procedure allowed the development of regression models that defined experimental factors and their interactions. Results indicated that both strains were able to grow in wastewater, but productivities in this medium were halved respective to those obtained in the synthetic medium. Specific growth rates presented higher values in wastewater than in synthetic medium. *B. braunii* was the most productive strain, but when both strains were grown together in co-cultures *C. vulgaris* dominated the reactor. The use of microalgae in wastewater treatment systems demonstrates to minimize anthropogenic environmental pollution load and to generate valuable biomass.

Keywords: Microalgae; Wastewater; Factorial design; Co-cultures; *Botryococcus brauni*; *Chlorella vulgaris*

1. Introduction

Anthropic activities from human-developed communities have a large disturbing effect on natural ecosystems. Through industrialization, agricultural practices, and urbanization, humans have increased the input of elements and compounds into biogeochemical cycles [1].

Utilization of microalgae for inorganic nutrient removal has been studied for more than 50 years [2]. One of the major advantages of microalgae in wastewater treatment systems is that wastes may be recycled into potentially valuable biomass [3]. The versatility of microalgae culture systems allows them to participate in different processes, such as production of biofuels, foods, feeds, and high-value bioactives [4]. The most widely used microalgae cultures for nutrient removal and wastewater sanitation are species of *Chlorella* [1,5].

The Earth is continuously bombarded by energy from the Sun. An average of 236 W for every square

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meter reaches the surface of the Earth. Part of this energy might be used through microalgae, as CO_2 capture, for exhaust flue carbon recycling and a tool for greenhouse gas mitigation [6].

Lipids from microalgae can be extracted and chemically processed to make biodiesel [7]; recently many research efforts have been placed on lipid-producing algae, where studied microalgae strains range from 10 to 60% lipids [8] on dry weight depending on the strain and the culturing conditions. The green alga *Botryococcus braunii* has high hydrocarbon content, ranging from 15 to 75% of dry weight, as long-chain unsaturated hydrocarbons. This alga is, therefore, a potentially good renewable lipid source for fuel [9,10].

Mixed cultures of several microalgae species can be substantially different from those of monocultures; they could affect the growth characteristics, biochemical compositions, nutritional compositions, and other growth factors of the strains [11]. Co-cultures of microalgae are similar to mixed cultures, but there is a unique difference in cultivation. In co-cultures, the quantity and type of organisms in the culture are all defined at inoculation, whereas in naturally occurring mixed cultures different organisms may become dominant during the cultivation period [12].

Several studies had focused on mixed cultures to explain ecological mechanisms of algal population dynamics [13–15], but there are no references about co-cultures in wastewaters.

This study aims to determine the biomass production and CO_2 biofixation of two microalgae strains, *B. braunii* and *C. vulgaris*, and their ability to use wastewater as feedstock. Candidate strains were grown in single culture and co-culture under a 2^k experimental design. Besides the use of wastewater, a synthetic medium was used to allow comparisons and detect eventual interactions between the strains and the culture media.

2. Materials and methods

2.1. Microorganism

B. braunii (SAG-30.81) and *C. vulgaris* (SAG 211–12) were obtained from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut, (Universität Göttingen, Germany). Stock cultures were maintained routinely on both liquid and agar slants of CHU 13 medium [9] by regular subculturing at two-week intervals. Cultures were maintained at 20 ± 1˚C temperature with 143 μmol/m^2 s light intensity under 14/10 light/dark cycle.

2.2. Culture medium

The feedstock used was the wastewater effluent from the wastewater treatment plant located in Arcos de la Frontera (Cádiz, Spain), which receives about 5,500 m^3 wastewater per day, mainly from domestic sources and presents the following processes: preliminary screening, primary sedimentation, activated sludge, and secondary sedimentation processes. The wastewater was collected prior to discharge. The characteristics of wastewater effluent are shown in Table 1.

Wastewater was filtered by 1 μm nominal pore Glass Fiber Filter (Pall Corporation. Type A/E) and frozen at −20˚C ± 2˚C aiming to maintain repetitive conditions during the experiment replicates. Wastewater was also autoclaved before each set of experiments to avoid bacterial growth in the medium.

CHU 13 was utilized as synthetic culture medium. It contains the following components (g/L): KNO_3 (0.2), K_2HPO_4 (0.04), $MgSO_4{\cdot}7H_2O$ (0.1), $CaCl_2{\cdot}2H_2O$ (0.08), Ammonium ferric citrate (0.011), citric acid (0.1), and micro elements (mg/L): B (0.5), Mn (0.5), Zn (0.05), Cu, Co, and Mo (0.02) in distilled and acidified water which included, AEDT (0.436).

2.3. Experimental set-up

The experiments were conducted in batch, by using 2,000 ml borosilicate flasks as photobioreactors (12.5 cm diameter × 14.5 cm height). Illumination was provided from the top of the flasks by using eight fluorescent lamps (four PHILIPS Master TLD 58 W/840 and four SYLVANIA Gro-Lux F 58 W/GRO-T8) with 143 μmol m^{-2} s^{-1} light intensity and 14/10 light/dark cycle. Light intensity was measured by a digital light meter (Hansatech QRT1 Quantitherm light meter). The experiments were conducted at (20 ± 1˚C) in a thermostatic chamber. Aeration was supplied from the bottom

Table 1
Output wastewater characterization, COD (chemical oxygen demand); SS (suspended solids) and nutrients

	COD (mg/L)	SS (mg/L)	N–NH_4^+ (mg/L)	N–NO_2^- (mg/L)	N–NO_3^- (mg/L)	*N*-total (mg/L)	P–PO_4^{3-} (mg/L)	*P*-total (mg/L)
Output wastewater	55.67	6.80	17.42	0.25	0.68	18.60	1.82	1.82

of the flask by an air compressor at a flow rate of 1.5 L/min. At the beginning of each series of experiments, 1,500 mL of culture medium was inoculated with 90 mL suspension of pre-cultured cells to obtain similar initial biomass concentration in all reactors. Co-cultures were inoculated with 45 ml of each strain culture. Initial biomass average and standard deviation of all experiments was 0.104 ± 0.057 g dry biomass/L.

2.4. Experimental design

A multilevel factorial design, including all the possible combinations among three levels of the factor algae (*B. braunii*, *C. vulgaris*, and co-culture of both strains) and two levels of the culture medium factor (wastewater and synthetic medium), was applied. The experimental domain is given in Table 2. The study was set up in triplicate (three different runs of six experiments).

Factorial design was used to screen factors that may have significant effects on response(s). It allowed examining, how an experimental response changes due to the effect of factors via regression model [16]. These models can be used to make predictions and to increase research efficiency.

Descriptive analysis, non-linear regressions using the Quasi-Newton estimation method, multifactorial analysis of the variance (ANOVA), Kolmorogov–Smirnov's test for normality, Levene's test for homocedasticity, and Tukey's multiple comparison tests for significant differences were carried out on the experimental data. All these statistical analyses were performed with the STATISTICA Program (Statsoft, Inc. Version 7.0, 2004).

2.5. Biomass growth

Biomass concentration was daily measured through the correlation between the optical density and the dry weight of algal biomass (Eqs. (1)–(3)).

Table 2
Experimental domain. Factor strain has three levels (+1, 0 and −1) and factor medium has two levels (+1 and −1)

Experiment	Strain	Medium	Resulting combination
1	−1	−1	*C. vulgaris* in wastewater
2	0	−1	Co-culture in wastewater
3	+1	−1	*B. braunii* in wastewater
4	−1	+1	*C. vulgaris* in synthetic medium
5	0	+1	Co-culture in synthetic medium
6	+1	+1	*B. braunii* in synthetic medium

Optical density (OD_{680}) was measured at 680 nm by means of a spectrophotometer (Thermo GENESYS 10-Vis) and algal biomass dry weight was determined gravimetrically as suspended solids (SS), according to the standardized method 2540-D [17].

$$B.\ braunii\ \mathrm{SS(g/L)} = (\mathrm{OD}_{680} - 0.0495)/1.0889 \quad R^2 = 0.9991 \tag{1}$$

$$C.\ vulgaris\ \mathrm{SS(g/L)} = (\mathrm{OD}_{680} - 0.061)/2.0284 \quad R^2 = 0.9919 \tag{2}$$

$$\text{Co-culture SS(g/L)} = (\mathrm{OD}_{680} - 0.292)/1.6356 \quad R^2 = 0.9853 \tag{3}$$

Batch growth biokinetics parameters of the cultures were obtained according to the method proposed by [18], this method is based in a non linear regression of the experimental data of biomass concentration vs. time to the Verhulst's model [19] (Eq. (4)).

$$\frac{dX}{dt} = \mu X_{(t)}\left[1 - \frac{X_{(t)}}{X_m}\right] \tag{4}$$

where "X" is the concentration of biomass at "t" time (g-dry biomass/L), "X_m" the maximum biomass concentration reached (g-dry biomass/L), and "μ" the maximum specific growth rate (d^{-1}).

Then, the volumetric biomass productivity (P) from batch experiments is calculated based on the expression,

$$P = \frac{X_m - X_0}{t_m - t_0} \tag{5}$$

where "t_0" is time at the beginning of the experiment, "X_0" is the initial biomass concentration, and "t_m" is the time spent in reach "X_m".

These authors consider that the time spent in the lag phase and in the late stationary phase of the cultures must not be included in calculations, in order to reduce sources of variation that can hide productivities (initial biomass concentration of the inoculum or its preservation conditions), as can be seen in Fig. 1(b). Then, authors arrive at the following expression considering only the biomass generated once initial biomass has increased in a 10% and until 90% of the maximum biomass is reached, Fig. 1(c).

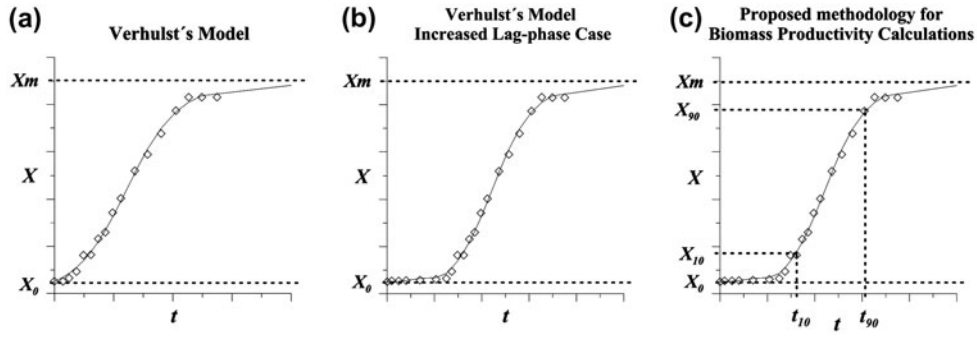

Fig. 1. Example of adjustment of experimental data (points) to the Verhulst's model (line) as proposed by Ruiz et al. [18]. (a) Verhulst´s model adjustment, (b) increased lag phase case, and (c) proposed methodology for biomass productivity calculations including X_{10}, X_{90}, t_{10}, and t_{90}.

$$P = \frac{\mu(0.9X_m - 1.1X_0)}{Ln\left(\frac{9(X_m - 1.1X_0)}{1.1X_0}\right)} \quad (6)$$

2.5.3. Carbon content

At the end of the experiments, biomass was harvested by centrifugation at 4,000 r.p.m. for 10 min, washed with deionized water twice and lyophilized. The percentage of elementary carbon content (%C) of dry biomass was determined in triplicate using an elementary analyzer (LECO-CHNS-932).

2.5.4. CO_2 fixation rate

For the CO_2 fixation rate (P–CO_2), it was considered that all carbon fixated into biomass was directly taken from the atmosphere. Then, the P–CO_2 was calculated based on the stoichiometric molecular adjustment of the %C via the atomic mass ratio of the C and the CO_2 (44/12) and multiplied by P.

$$\text{P–CO}_2 = \frac{\text{P} \cdot (\%\text{C}) \cdot (M_{CO_2}/M_C)}{100} \quad (7)$$

where M_{CO_2} is the molar mass of CO_2; and M_C is the molar mass of carbon.

3. Results and discussion

3.1. Preliminary analysis

Experimental results for all the studied variables are plotted in Fig. 2. Biomass concentration data from the nonlinear regression fitted very well to the Verhulst's Model, ranging the regression coefficient between 0.98 and 0.99.

To verify all descriptive observations, a factorial analysis of the variance (ANOVA) was applied to the experimental data (Table 3). That test determines the presence of significant differences between groups of data within a variable and allows discrimination of factors, strain or medium, that significantly affect results. If results are affected by the interaction effect of both factors, only the interaction effect should be considered and not the isolated factors independently [20].

As can be observed in Fig. 2, the culture medium factor affected all the variables. This observation was verified by the ANOVA analysis (Table 3), as the results grouped by the medium factor presented significant differences to the rest of groups by factors.

The highest X_m value (1.917 g/L) was reached by *B. braunii* in the synthetic medium. All X_m values in experiments involving wastewater were lower, Fig. 2 (a). *C. vulgaris* and co-culture showed similar values in both media, becoming approximately half of the values reached by *B. braunii*. The ANOVA analysis detected differences in groups of results related to the alga factor, the culture medium factor, and the interaction of both of them (Table 3). Considering only the interaction effect, differences in X_m values due to the alga factor were not the same for each culture medium. Further statistical analysis must be performed to establish the magnitude of variations for each factor.

Related to the μ, the highest value was reached by *C. vulgaris* and the co-culture when they were grown in wastewater (0.741 d^{-1}). This value is similar to those presented by Ruiz et al. [5] (0.434–0.702 d^{-1}), in a set of experiments that changed the initial nitrogen and phosphorus content of the wastewater medium, and their most similar conditions to these of this study is implied by a μ of 0.672 d^{-1}. Those μ values were higher of that presented by Wang et al. [21] (0.343 d^{-1}) for a *Chlorella* species in a wastewater med-

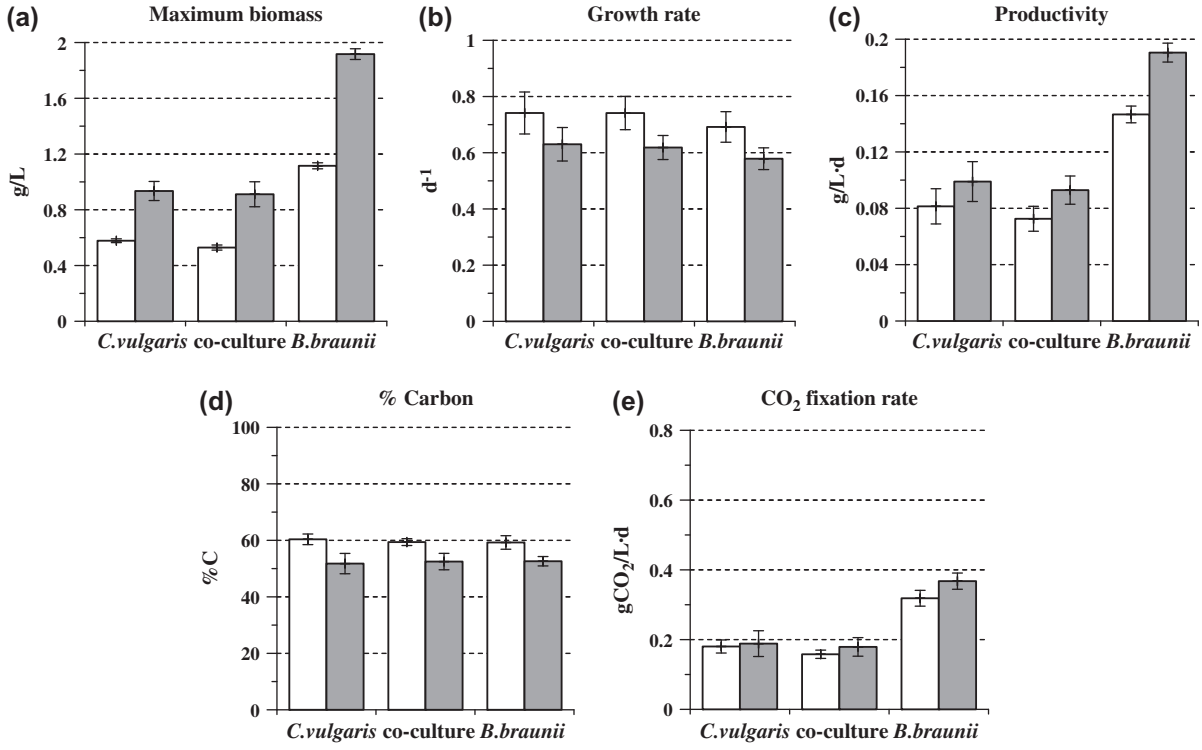

Fig. 2. Descriptive plots of all the variables studied. (a) Maximum biomass; (b) growth rate; (c) productivity; (d)% carbon; (e) CO_2 fixation rate. Error bars represent confidence limits ($p < 0.05$). (□) Wastewater medium; (■) synthetic medium.

ium, although their cultures were not aerated and initial total phosphorus was lower.

The value of this variable tended to be higher for both species in all the experiments conducted in wastewater, Fig. 1(b). The ANOVA analysis confirmed that the medium factor presented a significant effect on this variable.

As can be observed in Fig. 2(c), *B. braunii* was the most productive strain and CHU 13 was the most productive medium. The highest P values were reached in experiments with *B. braunii* in synthetic medium (0.191 g/L d). When *B. braunii* was cultured in wastewater, it reached values 76.9% lower (0.147 g/L d) and these results are in accordance with those of [22] that described higher biomass production in CHU 13 than in secondary treated wastewater. On the other hand, *C. vulgaris* and co-cultures showed similar P between them and these were approximately 50% of the productivity of *B. braunii* in both media.

The ANOVA analysis established significant effects on P results related to the factors algae, culture medium, and the interaction of both of them, as the case of X_m. It has to be noted that P is directly dependent on X_m (Eq. (6)), thus the effect of the strain factor is transmitted from the X_m to its derived variables.

The $\%C$ content of the biomass did not present significant variations related to the strain factor (Fig. 2 (d)), but they did for the culture media factor. Biomass grown in wastewater presented perceptibly more $\%C$ than in synthetic medium. That variable ranged from the lower value of *C. vulgaris* in synthetic media (51.78%) to the higher value (60.37%), when *C. vulgaris* was grown in wastewater. The ANOVA analysis validated these observations for $\%C$.

Results of P–CO_2 are clearly related to the P results, as there is a direct dependence between both variables. *B. braunii* grown in CHU 13 medium was the most productive case and also the case that fixed more CO_2 (0.368 gCO_2/L d). As can be appreciated in Fig. 2(g), when *B. braunii* was grown in wastewater P–CO_2 slightly descended (0.319 gCO_2/L d). There were non-appreciable differences on P–CO_2 between *C. vulgaris* and the co-culture. The ANOVA analysis verified that both factors, alga strain and culture medium, significantly affected P–CO_2, each one in an independent way.

As can be seen in all plots of Fig. 2, *C. vulgaris* and co-cultures presented appreciably similar results for all the studied variables. This fact, suggests the hypothesis of an interspecific competitive relationship between the *C. vulgaris* and *B. braunii* strains when

Table 3
ANOVA summary table. Significant factors at p-values under 0.05 are represented in bold

Variable	Factor	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
X_m	**Alga**	2	2.425	1.212	612.504	**$<10^{-4}$**
	Medium	1	1.186	1.186	599.383	**$<10^{-4}$**
	Alga × Medium	2	0.187	0.093	47.233	**$<10^{-4}$**
	Error	12	0.024	0.002		
	Total	17	3.822			
μ	Alga	2	0.009	0.005	1.861	0.198
	Medium	1	0.060	0.060	24.442	**$<10^{-4}$**
	Alga × Medium	2	0.000	0.000	0.023	0.977
	Error	12	0.030	0.002		
	Total	17	0.099			
P	**Alga**	2	0.027	0.014	229.482	**$<10^{-4}$**
	Medium	1	0.003	0.003	56.606	**$<10^{-4}$**
	Alga × Medium	2	0.001	0.000	5.307	**0.022**
	Error	12	0.001	0.000		
	Total	17	0.032			
%C	Alga	2	0.080	0.040	0.009	0.991
	Medium	1	244.918	244.918	53.865	**$<10^{-4}$**
	Alga × Medium	2	3.379	1.690	0.372	0.697
	Error	12	54.563	4.547		
	Total	17	302.939			
P–CO_2	**Alga**	2	0.112	0.056	118.583	**$<10^{-4}$**
	Medium	1	0.003	0.003	6.512	**0.025**
	Alga × Medium	2	0.001	0.001	1.388	0.287
	Error	12	0.006	0.000		
	Total	17	0.122			

they are grown together, leading *C. vulgaris* as the best competitor in both culture media. The greater presence of *C. vulgaris* over *B. braunii* was observed through microscopic observations.

A Post Hoc Tukey's, Honestly Significant Difference (HSD), a multiple comparison test was carried out to find if there were significant differences between culturing a single strain or a co-culture (Table 5). The test was carried out with data corresponding to the P–CO_2 variable, which satisfied the statistical requirements of independency, normal distributed, and homoscedasticity (Table 4). Results of the Tukey´s HSD analysis identified two clearly differentiated groups involving *C. vulgaris* strain and co-culture in the same homogeneous group.

Those results indicated that, under these experimental conditions, when both strains were grown in the same reactor, *C. vulgaris* was the most competitive strain and prevailed over *B. braunii*. This fact could be explained through two hypotheses, a faster growth or a faster nutrient uptake of *C. vulgaris*, both hypotheses would lead an absence of a limiting nutrient for *B. braunii*. As μ of both strains did not present significant differences for the factor alga strain (Table 3), the hypothesis of the faster nutrient uptake becomes stronger. Tilman et al. [23] described a similar process in a silicate-limited competition experiment between two species of diatoms. According to all resource-based theories, when two or more species compete for the same limiting resource, they reduce the ambient level of the resource until only that species with the lowest resource requirement survives [24]. If this effect has to be controlled to promote the prevalence of one strain over the other, several authors as Yamasaki et al. [14] and Huang et al. [15] established that this could be solved by controlling the initial culture inocula of both strains.

This competitive process of strains with similar feeding requirements could carry out several implications concerning the operation of large-scale monospecific photobioreactors, such as contamination with more competitive strains. For example, in this case the most competitive strain was not the most productive in terms of biomass, Fig. 2(c). This species contamination could be solved with adequate pretreatment of the culture media, choosing the most competitive

Table 4
Results of Kolmogorov–Smirnov test (normality) and Levene test (homoscedasticity) of P–CO_2 data

	Kolmogorov–Smirnov			Levene	
	D	*p*-value	*C. vulgaris* vs.	*F*	*p*-value
C. vulgaris	0.172	$p > 0.20$	Co-culture	0.119	0.738
Co-culture	0.257	$p > 0.20$			

Table 5
Post Hoc Tukey´s HSD test with homogeneous groups for P–CO_2 data, *p*-values under 0.05 are represented in bold

Tukey's Honestly significant differences comparison test						
(*p*-values)				Mean	Homogeneous groups	
Algae level	*C. vulgaris*	Co-culture	*B. braunii*	(gCO_2/L d)	A	B
C. vulgaris		0.441	**$<10^{-4}$**	0.184	****	
Co-culture	0.441		**$<10^{-4}$**	0.169	****	
B. braunii	**$<10^{-4}$**	**$<10^{-4}$**		0.343		****

strain for a determinate purpose or controlling the initial culture inoculum of a selected strain.

It is generally assumed the faster growth of the *Chlorella* strains compared with the *Botryococcus* strains, several authors reported data of both strains cultured in synthetic media [25]. However, Sydney et al. [26] reported faster μ of *B. braunii* than *C. vulgaris* in secondary treated wastewater while the results of this study show similar μ for both species. On the other hand, Chen et al. [27] reported *C. vulgaris* as the long-term dominant species in an outdoor raceway pond fed with anaerobically digested dairy farm wastewater, this strain resulted tolerant to high nutrient loadings in a five-month cultivation.

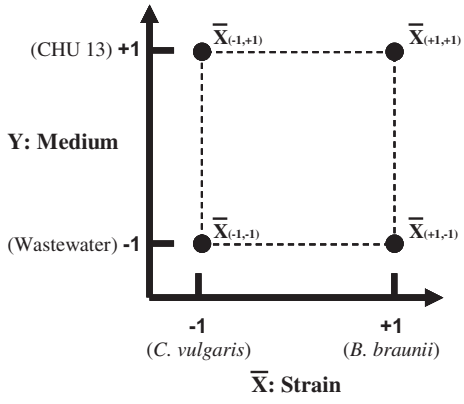

Fig. 3. Experimental domain for two factors, strain and medium. $\overline{X}$ are mean values of the effects.

3.2. Factorial analysis

In order to determine the main and interaction effects of factors analyzed a deeper factorial analysis was performed and regression models were developed. Due to the assumption of prevalence of *C. vulgaris* in co-cultures, data from the co-culture level were excluded of the factorial analysis. The experimental domain was finally set in a two-level factorial design (2^k), with two factors ($k = 2$) at two levels: low (−1) and high (+1), resulting in four experiment combinations ran in triplicate ($n = 12$). The square plot in Fig. 3 shows the experimental domain for each variable, vertices of the square are the mean values of the effects related to the combinations of factors.

One of the outputs obtained from the statistical analysis of the experiments are the equations presented in Table 6. These are regression models ($\alpha = 0.05$) that relate all the studied variables to the experimental factors (strain and medium).

The main and interaction effects of factors are shown in Fig. 4. "Main effect" is defined as the average differences in response between the effects of a factor at high (+1) and low (−1) levels, while the "interaction effect" is defined as the average difference in response between the effects of one factor at high and low levels of other factors [16,28]. Main and interaction effect plots were derived from the regression models. Only factors having significant effects were considered for discussion.

As shown in Table 6, three of the seven variables analyzed, presented a model that fitted very well to

Table 6
Developed models of each variable studied, "x" is the factor algae, "y" is the factor medium and "$x \times y$" is the interaction between both factors, coefficients in bold mean that their factors presented significant effects ($p < 0.05$) on the variables

Variables	Units	Model	R^2 (adjusted)	Standard error coefficient	Eq.
X_m	(g/L)	$X_m = \mathbf{1.136} + \mathbf{0.380} \times \boldsymbol{x} + \mathbf{0.289} \times \boldsymbol{y} + \mathbf{0.111} \times \boldsymbol{x} \times \boldsymbol{y}$	0.996	0.011	(8)
μ	(d^{-1})	$\mu = \mathbf{0.660} - 0.025 \times x - \mathbf{0.056} \times \boldsymbol{y} - 0.000 \times x \times y$	0.562	0.015	(9)
P	(g/L d)	$P = \mathbf{0.129} + \mathbf{0.039} \times \boldsymbol{x} + \mathbf{0.015} \times \boldsymbol{y} + \mathbf{0.007} \times \boldsymbol{x} \times \boldsymbol{y}$	0.968	0.002	(10)
%C	(%)	$\%C = \mathbf{56.000} - 0.072 \times x - \mathbf{3.810} \times \mathbf{y} + 0.488 \times x \times y$	0.751	0.638	(11)
P–CO_2	(g CO_2/ L d)	$P\text{–}CO_2 = \mathbf{0.246} + \mathbf{0.079} \times \boldsymbol{x} + 0.000 \times y + 0.008 \times x \times y$	0.928	0.007	(12)

experimental observations (X_m, P and P–CO_2,), with an adjusted coefficient of determination higher than 0.9. One variable (%C) presented a coefficient between 0.75 and 0.80. Finally, μ was the variable that presented the lower adjusted coefficient of determination (0.56).

3.3. Maximum biomass concentration

Fig. 4(a) shows how the strain factor affected the X_m, reached in the reactors, regardless of the culture media used. As can be extracted from the plot, experiments with *B. braunii* led higher X_m than experiments with *C. vulgaris*. In the case of the culture medium factor, Fig. 4(b) shows how synthetic medium gave higher X_m values regardless of the strain cultured. These results were expected since CHU 13 composition is designed to satisfy algae growth requirements, this medium also had higher concentrations of nitrogen and phosphorous than the wastewater.

However, these main effects must not strictly be taken into account due to the presence of an interaction effect. As can be seen in Fig. 4(c), there are substantial differences on the X_m between strains if experiments were conducted in wastewater rather than in synthetic medium. Both strains in CHU 13 presented higher magnitude of X_m than in wastewater, but the increase was more pronounced for *B. braunii* than for *C. vulgaris*. This indicates that when culturing conditions are less limited by nutrients (CHU 13), differences between *C. vulgaris* and *B. braunii* are more accentuated, while under more nutrient limited conditions (wastewater) the culture medium effect smoothes differences between species.

3.4. Specific growth rate

Concerning μ, the culture medium was the variable that exerted a significant effect, Fig. 4(d). It has to be taken into account that corresponding regression model (Eq. (9)) did not fit accurately. This implies that qualitative effects must be considered rather than quantitative effects; wastewater culture medium gave a significant higher μ than synthetic medium regardless of the strain cultured.

Considering the experimental data, the initial concentrations of essential elements in the wastewater, as nitrogen and phosphorus, were below for those in the synthetic medium and the presence of other limiting nutrients remained unknown, for example, iron is also considered as an essential element [7,29]. The complex matrix of elements and compounds in the wastewater could promote enhancement of the μ, until depletion of most limiting nutrient, then cultures reached the X_m of the system, which in wastewater was smaller than in synthetic medium.

3.5. Productivity

Related with productivity, Fig. 4(e) and (f) show that in the synthetic medium, higher P were obtained regardless of the strain cultured and *B. braunii* was the alga with higher P regardless of the medium used. Comparing both slopes in the plots, it can be observed that the effect of the species was more important on P rather than the effect of medium.

As P is influenced by the magnitude of X_m (Eq. (6)) and considering that P depends on both X_m and μ, these results indicated that X_m had a higher importance than μ on final P values following the methodology proposed by Ruiz et al. [18].

The interaction effect of both factors is presented in Fig. 4(g). As mentioned before, this interaction effect is transmitted from X_m (Fig. 4(c)). Comparing the interaction effects for both variables X_m and P (Fig. 4(c) and (g)), it can be observed that differences between slopes of the effects were less pronounced for

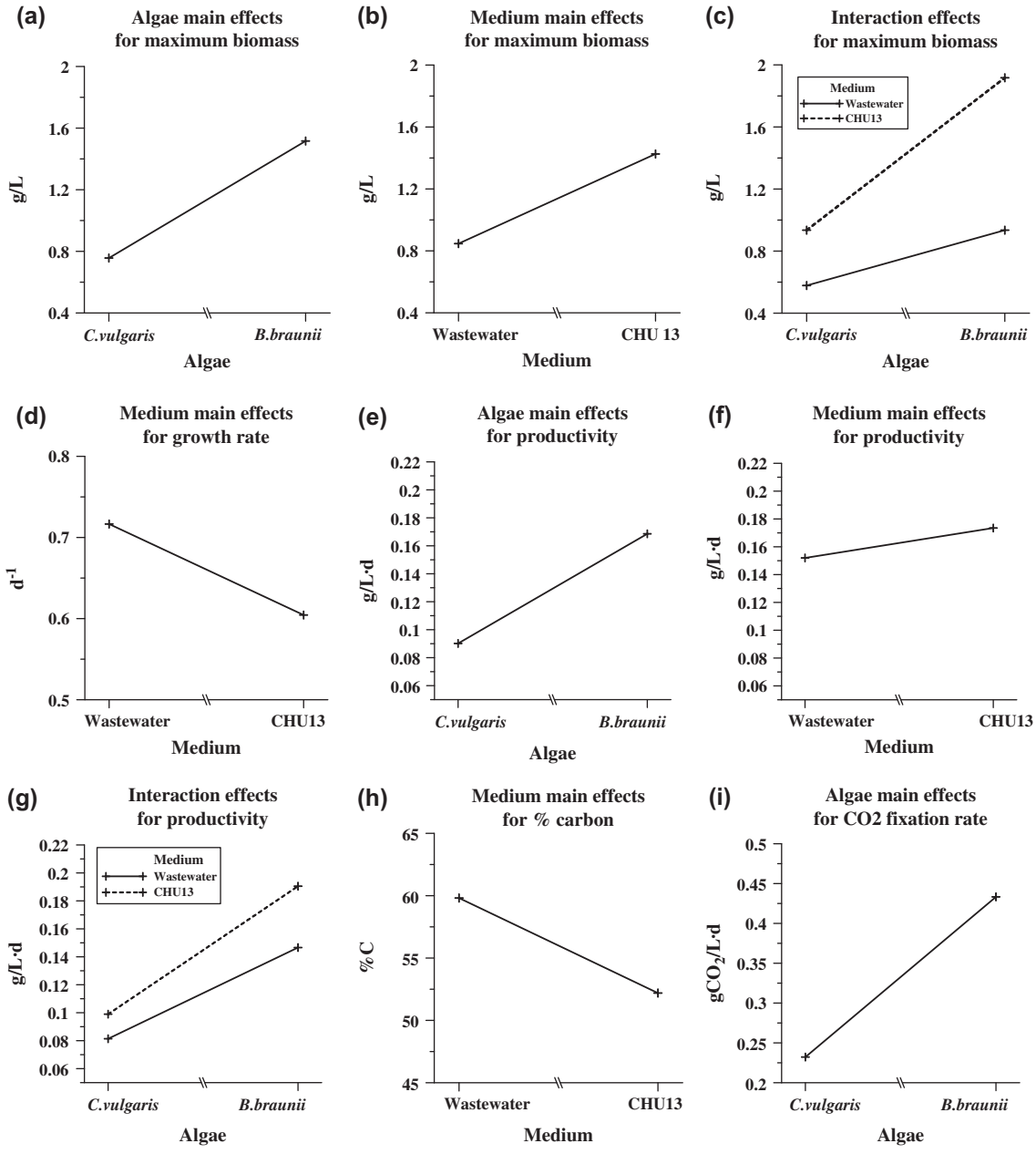

Fig. 4. Significant effects plots: (a) Algae main effects for X_m; (b) medium main effects for X_m; (c) interaction effects for X_m; (d) medium main effects for μ; (e) algae main effects for P; (f) medium main effects for P; (g) interaction effects for P; (h) medium main effects for %C; (i) algae main effects for P–CO_2.

P, Fig. 4(g) due to the higher μ in wastewaters (Fig. 4(d)).

3.6. Carbon content

Culture medium was the only factor that induced significant main effect on $\%C$, Fig. 4(h), the $\%C$ model (Eq. (11)) predicted that, independent of the strain, microalgae cultured in wastewater presented higher $\%C$ than in CHU 13.

3.7. CO_2 fixation rate

The rate of CO_2 biofixation depended on the strain, being higher in the case of *B. braunii* regardless of the culture media used, Fig. 4(i), even if the algae cultured in wastewater presented higher carbon content, the higher productivity of the synthetic medium was directly related to a higher P–CO_2.

It is important to evaluate the CO_2 sequestration capability of the strains, in order to reduce the carbon

Table 7
P–CO_2 of the experiments, P–CO_2 is calculated based on the elemental analysis and P–CO_2^* is calculated according to the formula proposed by Wang et al. [30]

Experiment	P–CO_2 (g CO_2/L d)	P–CO_2^* (g CO_2/L d)	Percent of differences (P–CO_2^*/P–CO_2) × 100
C. vulgaris in wastewater	0.180	0.153	84.83%
Co-culture in wastewater	0.158	0.136	86.30%
B. braunii in wastewater	0.319	0.276	86.51%
C. vulgaris in synthetic medium	0.189	0.186	98.67%
Co-culture in synthetic medium	0.179	0.175	97.48%
B. braunii in synthetic medium	0.368	0.358	97.42%

footprint of the wastewater treatment processes in an environment friendly and sustainable manner.

Several authors as Chisti [7], estimated the fixation of CO_2 in the basis of the approximate molecular formula of the microalgal biomass $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$. In this study, there are given rates of 183 tons of CO_2 fixed per 100 tons of algae biomass produced. Other authors as Wang et al. [30], calculated CO_2 fixation rate as (P–CO_2) = 1.88 × biomass P.

Comparing the estimation method, based on the formula proposed by Wang et al. [30], with the one applied in this study, based on elemental analysis of the biomass, resulted that the first one gave very accurate results for the synthetic medium but not for the wastewater (Table 7). When algae were grown in synthetic medium, underestimations in P–CO_2 varied from 2.88 to 1.44% respective to the calculations of this study, but underestimation became larger, from 13.30 to 15.45%, when algae were grown in wastewater. This fact is related to the higher %C of the biomass when cultured in wastewater, which implied changes in the molecular formula for microalgal biomass proposed by Chisti [7]. The higher %C of the biomass in wastewater (Fig. 4(h)) led to a higher P–CO_2. This gap in estimations must be considered for CO_2 balances in wastewaters.

4. Conclusions

Comparing the strains (*C. vulgaris* and *B. braunii*) and the culture media (wastewater and CHU 13), under these experimental conditions in batch photobioreactors, *B. braunii* was the most productive strain but *C. vulgaris* was the most competitive strain when they were grown together.

Reactor productivities in batch depended more on the strain cultured than on the culture media.

Specific growth rate and carbon biomass content depended on the culture medium regardless of the microalgae cultivated.

CO_2 fixation rate depended on the strain cultured and it was independent of the medium used.

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