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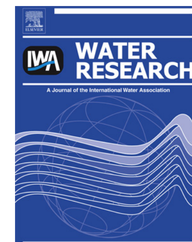

Capability of different microalgae species for phytoremediation processes: Wastewater tertiary treatment, CO_2 bio-fixation and low cost biofuels production

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ABSTRACT

Scenedesmus obliquus, *Chlorella vulgaris*, *Chlorella kessleri* and a natural Bloom were cultivated in batch experiments, under controlled conditions, in urban wastewater (WW) and synthetic wastewater (SW) under 5% CO_2 in air, with the object of estimating their capacity for nutrient removal, carbon dioxide biofixation, and generation of valuable biomass. In both culture media, the *Bloom* (*Bl*) and *Scenedesmus* (*Sc*) showed higher final biomass concentration (dried weight, dw) than the other species; the maximum yield obtained was 1950 ± 243 mg L^{-1} for *Bl* and the minimum 821 ± 88 mg L^{-1} for *Cv*, both in synthetic wastewater. Maximum specific growth rate values do not show significant differences between any of the 4 strains tested ($p \leq 0.05$), nor between the 2 culture media. A new homogeneous method of calculating productivities has been proposed. Nitrogen removal in all the reactors was higher than 90%, except for *Bl*SW (79%), and for phosphorus, the removal was higher than 98% in all trials. Maximum CO_2 consumption rates reached were 424.4 and 436.7 mg L^{-1} d^{-1} for *Sc*SW and *Sc*WW respectively.

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1. Introduction

Currently the high rate of urban population growth is generating very large amounts of waste that must be treated before discharge. Wastewater (WW) and greenhouse gases (GHG) – mainly carbon dioxide (CO_2) – are two of the main wastes that pose a major challenge to global environmental sustainability.

Considering first the problem of WW, a relatively new requirement for wastewater treatment plants (WWTP) is the capacity to remove high concentrations of nutrients, in particular, nitrogen and phosphorus, which otherwise present a serious risk of eutrophication when those nutrients accumulate in rivers and lakes. Eutrophication causes an accelerated growth of algae and plants, leading in turn to an undesirable disturbance to the balance of organisms present

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in the water, and to deterioration of water quality. The European Commission Directive 98/15/EEC established the requirements for water discharges from urban WWTPs to sensitive areas (those with high eutrophication risk), setting total nitrogen (TN) in 10 mg L^{-1} and total phosphorus (TP) in 1 mg L^{-1}. Currently a wide range of techniques is available for WW nutrient removal, such as Bardenphro, A2O, and UCT. Most of the studies are based on physical-chemical and mainly biological processes, where different combinations of anaerobic, aerobic, and anoxic zones are required. These processes generally entail high investment and operational costs, complex operations and large volumes of waste sludge production. Undoubtedly, the main disadvantage is the difficulty in simultaneously remove TN and TP with these processes.

Microalgae can simultaneously grow in WW and produce valuable biomass while they remove organic carbon and inorganic nutrients (nitrogen and phosphorus) from the WW, therefore microalgae can play a very important phytoremediation role, particularly during the final tertiary treatment phase of the WWTP (Oswald et al., 1957; De la Noüe and De Pauw, 1988; Tredici et al., 1992; Oswald, 1995; González et al., 1997; Mallick, 2002). The treatment of WW with microalgae offers many advantages over conventional treatments: (1) nitrogen and phosphorous can be converted into biomass without any external source of organic carbon; (2) Nitrogen and phosphorus are removed from WW simultaneously (3) the effluent discharged into receiving water bodies is oxygenated; and (4) high-value products can be extracted from the biomass generated such as protein and lipids.

From the other hand, GHGs in the air are dramatically accumulating in the atmosphere as a result of human activities and industrialization, CO_2 is the main GHG (IPCC, 2001). The increase in atmospheric CO_2 derived from fossil fuel combustion poses a very serious threat to worldwide environmental sustainability. Various CO_2 mitigation strategies have been investigated, including fixation technologies such as: (1) chemical reactions such as washing emissions with alkaline solutions; (2) deep injection of sequestered gas (underground, in the ocean depths); and (3) biological processes, by means of autotrophic organisms. The last one has attracted much attention as an alternative strategy; biological mitigation of CO_2 can be carried out by plants and photosynthetic microorganisms, but microalgae and cyanobacteria can grow much faster than the terrestrial plants, resulting in a CO_2 conversion efficiency about 10–50 times higher than terrestrial plants (Li et al., 2008). Microalgae are one of the most important bioresources that are currently receiving a lot of research attention, for many reasons. The organic matter produced by photosynthetic microalgae and cyanobacteria can be transformed into a wide range valuable products, such as pharmaceuticals, food additives and other health-care products (Pulz and Gross, 2004). Microalgae also represent an interesting alternative to the production of third generation biofuels (Brennan and Owende, 2010; Chisti, 2007).

Therefore, the aims of the study reported are to evaluate the growth, consumption of nutrients and carbon dioxide assimilation kinetics of three widely-used microalgae, *Chlorella vulgaris*, *Chlorella kessleri*, *Scencedesmus obliquus* and a natural bloom, each cultivated in two different culture media (urban wastewater and synthetic wastewater).

2. Material and methods

2.1. Microalgae strains

The microalgae strains used in this study are *Scenedesmus obliquus* (*Sc*) (SAG 276-10), *C. vulgaris (Cv)* (SAG 211-12), *Chlorella kessleri (Ck)* (SAG 211-11) from the Algae Culture Collection (SAG) of the University of Göttingen (Germany), and a natural algal bloom (*Bl*) isolated from the Guadalete river, downstream from the effluent of the urban WWTP of Arcos de la Frontera (Latitude: 36,749°, Longitude: −5.793°, Spain). *Bl* was obtained after filtering the river water sample through a fiber filter of 0.45 μm pore diameter (PALL Corporation, Type A/E) and enriching it with 10 folds Combo medium (Kilham et al., 1998). Single species inocula for the experiments were cultivated in Combo medium incubated at 20 ± 1 °C and 250 μmol cm^{-2} s^{-1} light intensity under 14/10 light/dark cycle.

2.2. Culture media and experimental procedure

Two different culture media have been tested. The first was a treated urban wastewater (WW), samples were taken from the effluent of the WWTP of Arcos de la Frontera (Spain), where the WW is submitted to pretreatment, primary settling, activated sludge and secondary settling. Nutrient concentration in WW was: $N{-}NH_4$ = 21.0 mg L^{-1}, $N{-}NO_3$ = 1.6 mg L^{-1}, $P{-}PO_4$ = 5.6 mg L^1 and COD: 100 mg O_2 L^{-1}

The second culture medium was a synthetic wastewater (SW), consisting of autoclaved bi-distilled water (Milli-Q quality) enriched with a modified Combo medium to contain 5.6 mg L^{-1} $P{-}PO_4$ as K_2HPO_4 and 21.0 mg L^{-1} $N{-}NH_4$ as NH_4Cl and 1.6 mg L^{-1} $N{-}NO_3$ as $NaNO_3$. These concentrations of nitrogen and phosphorus in the SW were added to match the nutrient composition of the WW.

The experiments were conducted in 2000 mL Pyrex flasks. The fluid was mixed and aerated with 0.2 μm prefiltered air using a membrane air pump. The air stream was bubbled into the reactors from the bottom at a flow rate of 1 vvm (L_{air} $L_{reactor}^{-1}$ min^{-1}). The air bubbled into the cultivation was enriched with 5% CO_2 (Abello Linde S.A). The enriched aeration kept the reactor under completely mixed conditions, preventing cell sedimentation, and also provided input of inorganic carbon. A set of 6 fluorescent lamps (3 Sylvania Gro-Lux F57W and 3 Philips TLD 58W) were used as the light source, providing 250 μmol cm^{-2} s^{-1}, measured with a Hansatech QRT1 Quantitherm light meter. The assays were conducted inside a climate chamber at a controlled temperature of 20 ± 1 °C and a 14/10 light/dark photoperiod. At the beginning of the experiment all reactors were inoculated with a calculated volume of microalgae in order to obtain a similar initial concentration of biomass in all assays (initial optical density at 680 nm between 0.15 and 0.20). For each test media, the assay was run in a control reactor containing the culturing media but without microalgae, in order to study physical-chemicals changes during the assay.

2.3. Analytical methods

Microalgae biomass was measured daily by optical density at 680 nm (OD_{680}). Samples with high biomass were diluted by appropriate ratios to ensure that absorbance values were in the range of 0.1–1.0. To convert the OD_{680} values to biomass as dry weight, a calibration curve was obtained for each strain and culture medium tested (Table 1). Biomass dry weight as suspended solids was determined gravimetrically according to Standard Methods (APHA-AWWA-WPCF, 1992). Microalgae biomass is the portion of solids retained by filtration through a pre-dried and pre-weighed glass fiber filter of 0.45 μm (pore diameter) (PALL Corporation, Type A/E).

Samples from each flask were taken daily to determine phosphorus and nitrogen concentration after filtration. Phosphorus was measured as orthophosphate ($P-PO_4^{3-}$) and nitrogen as the sum of nitrate and ammonium (ΣN: $N-NO_3^-$ + $N-NH_4^+$). Analyses of nitrate, ammonium and phosphate were performed colorimetrically according to Standard Methods (APHA-AWWA-WPCF, 1992) with a Spectroquant® NOVA 60 spectrophotometer (Merck Chemicals). COD was determined according to Standard Methods 5220-D (APHA-AWWA-WPCF, 1992).

Once the cultures reached the stationary growth stage, biomass was harvested by means of centrifugation at 6000 rpm for 15 min (Centrifuge Mixtasel-BL Selecta®). The same procedure was repeated three times with the addition of distilled water. The pellet obtained was dried in a vacuum freeze-dryer (LABCOCNO®) during 72 h and the percentages of C, N, H and S were determined by elementary analysis of the biomass (Leco® CHNS 932).

The crude protein concentration of microalgae was estimated by Equation (1), where the nitrogen content (% dw) was multiplied by 6.25 (Becker et al., 1994; Fuentes et al., 2000; Ho et al., 2010).

$$\text{Crude protein}(\%) = 6.25\%\,N \quad (1)$$

Lipid content of the biomass was determined in duplicate. Lipids were extracted according to a modified method reported by Takagi et al. (2006) and Wiltshire et al. (2000). To 90 mg of lyophilized pellets, 12 ml of 2:1 trichloromethane:methanol mixture and 0.6 g of analytical grade quartz were added, and the mixture was sonicated in a bath (60 kHz; 360 W) for 90 min. The extraction was performed twice and the two extracts were mixed, centrifuged and filtered to ensure quartz separation. The filtrate was evaporated under reduced pressure (2.93 psi) in a rotary evaporator. The remainder was dried at 100–105 °C for 12 h and weighed, as the total lipids.

Table 1 – Calibration curve OD680 to biomass as dry weight.

Strain & culture medium	SS (gr L^{-1}) = A OD_{680} + B	r^2
CvSW	y = 0.310x + 0.003	0.994
CvWW	y = 0.404x + 0.026	0.993
CkSW	y = 0.602x − 0.047	0.997
CkWW	y = 0.547x − 0.013	0.998
ScSW	y = 0.691x − 0.031	0.995
ScWW	y = 0.959x − 0.075	0.991
BlSW	y = 1.008x − 0.055	0.999
BlWW	y = 1.266x − 0.158	0.995

2.4. Determination of growth kinetic parameters

The Verhulst logistic kinetic model (Verhulst, 1838) was used to model the evolution of the experimental biomass concentration in the reactors. The model is a substrate-independent equation and can accurately describe biomass growth in the different culture conditions which occur in many batch bioreactors (Gong and Lun, 1996). According to the model, the microbial growth can be expressed as a sinusoidal curve, as described by Equation (2).

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}}\right) \quad (2)$$

Integrating this equation, we get Equation (3), where, μ_{max} is the maximum specific growth rate (d^{-1}), X_{max}, X_0 and X are the concentration of biomass (g L^{-1}) at an operating time equal to infinity, zero and t respectively.

$$X = \frac{X_0 X_{max} e^{\mu} max^t}{X_{max} - X_0 + X_0 e^{\mu} max^t} \quad (3)$$

Productivity is an important parameter to consider in the technology for cultivating microalgae, as it shows the capacity of a reactor to produce biomass under specific operating conditions. Firstly the overall productivity ($P_{overall}$) calculated according to the Equation (4),

$$P_{overall} = \frac{\Delta X}{\Delta X} = \frac{(X_{max} - X_0)}{(t_m - t_o)} \quad (4)$$

where X_{max} is the maximum final concentration achieved at the end of the experiment, and is the highest asymptotic value of the sinusoidal growth curve; X_0 is the initial biomass concentration in mg L^{-1}; t_m is the time required to reach X_{max}; and t_0 is equal to zero. For many authors this is the equation most frequently applied (Ho et al., 2010; Ho et al., 2011; Tang et al., 2011), and it takes into account only the experimental data.

To estimate productivity with a new methodology, the lag phase should be minimized or not be considered, because this is an extremely variable phase of growth that depends not only on the experimental conditions, but also on the experimental methodology, such as the initial inoculum concentration used and how this inoculum was obtained. Therefore the inclusion of the lag phase in productivity calculations leads to a wide and unnecessary dispersion of productivity data between authors. As stated in many guidelines for testing biodegradability (e.g. OECD, 2004), the lag phase is considered as the time elapsed until 10% of the total biodegradation has been reached. In this work, that consideration could be comparable to an increase of 10% in the initial biomass concentration in the reactor ($X_{10} = 1.1 \cdot X_0$). Therefore t_{10} is the time required to reach X_{10}, and t_{90} is the time required to reach X_{90} ($X_{90} = 0.9 \cdot X_{max}$) (Table 2). From Equation (4) an expression is obtained for the time as a function of biomass concentrations (Equation (5)).

$$t = \frac{1}{\mu} \mathrm{Ln}\left(\frac{X \cdot (X_{max} - X_0)}{X_0 \cdot (X_{max} - X)}\right) \quad (5)$$

With this equation t_{90} and t_{10} can be obtained (Equations (6) and (7)).

$$t_{90} = \frac{1}{\mu} \mathrm{Ln}\left(\frac{9 \cdot (X_{max} - X_0)}{X_0}\right) \quad (6)$$

$$t_{10} = \frac{1}{\mu} \mathrm{Ln}\left(\frac{1.1 \cdot (X_{max} - X_0)}{(X_{max} - 1.1 \cdot X_0)}\right) \quad (7)$$

Considering that productivity in the exponential growth period in the batch photobioreactor could be approximated to Equation (8).

$$P_B = \frac{(X_{90} - X_{10})}{(t_{90} - t_{10})} = \frac{(0.9 \cdot X_{max} - 1.1 \cdot X_0)}{(t_{90} - t_{10})} \quad (8)$$

by the combination of Equations (6)–(8), an expression is obtained that relates productivity with the kinetic parameters (μ, t_{10}, t_{90}) (Equation (9)).

$$P_B = \frac{\mu \cdot (0.90 \cdot X_{max} - 1.10 X_0)}{\mathrm{Ln}\left(\frac{9 \cdot (X_{max} - 1.1 \cdot X_0)}{1.1 \cdot X_0}\right)} \quad (9)$$

2.5. Kinetic parameters for nutrient removal

To compare the kinetics for nitrogen and phosphorus removal, a calculation has been made of the time required to reach 10 mg L^{-1} (ΣN) and 1 mg L^{-1} ($P-PO_4^{3-}$) (the most restrictive concentrations in European Union Directive 98/15/CE concerning requirements of N and P in the effluents permitted from urban wastewater treatment). In order to obtain these times, phosphorous and nitrogen consumption has been modeled according to the Quiroga-Sales kinetic model for substrate consumption by microorganisms in batch reactors (Equation (10)) (Quiroga et al., 1999).

$$v = K_2 \cdot S_2 + K_1 + K_0 \quad (10)$$

In this equation, S is the substrate concentration in the medium (nitrogen or phosphorous) (mg L^{-1}) and K_2, K_1, and K_0 are kinetic constants.

2.6. Determination of carbon dioxide biofixation rate

In accordance with the method described by De Morais and Costa (2007a,b), the carbon dioxide biofixation rate (P_{CO_2}) was calculated using Equation (11),

$$P_{CO_2} = \%C \; P_b \left(\frac{MW_{CO_2}}{MW_c}\right) \quad (11)$$

where %C is the carbon content in dried biomass obtained by elementary analysis; P_b is the batch productivity; and MW_{CO_2} and MW_C are the molecular weight of CO_2 and C respectively.

2.7. Statistical analysis

Non-linear regression for data kinetic modeling was performed using the software STATISTICA 6.0 (StatSoft Company). The Quasi-Newton method of estimation was used, with a convergence criterion of 10^{-4}. A confidence interval of $p \leq 0.05$ was calculated using Equation (12), where x is the mean, δ is the standard deviation, n is the sample size and $t_{(\alpha/2)}$ is the t critical value.

$$CI = X \pm t_{(\alpha/2)} \cdot \left(\frac{S}{\sqrt{n}}\right) \quad (12)$$

3. Results and discussion

3.1. Biomass growth rate

Fig. 1 shows biomass evolution in all the assays conducted. As can be observed, it was confirmed that all the strains grow in both culture media with the addition of 5% CO_2 in air. The typical evolution in four phases of a batch culture was observed in all the assays: (1) there was an initial period of physiological adjustment (lag phase) due to changes in nutrient or culture conditions (this phase in all the tests was less than 25 h, except for *C. vulgaris* in synthetic wastewater (*Cv*SW) for which the lag phase was longer (50 h); (2) an accelerating growth phase where the cells begin to grow and multiply once they were adapted to the new environment; (3) an exponential growth phase characterized by cell doubling and biomass growth at a constant rate; and finally, (4) a stationary phase where biomass growth rate was practically zero as a result of nutrient depletion in the culture medium. All the

Table 2 – Maximum final biomass concentration (X_{max}, mg L^{-1}), maximum specific growth rate (μ_{max}, d^{-1}), Overall productivity ($P_{overall}$, mg L^{-1} d^{-1}), batch productivity (P_b, mg L^{-1} d^{-1}), time to reach 90% of Xmax (t_{90}, h), and time to reach 110% of Xo (t_{10}, h) of *S. obliquus* (Sc), *C. Vulgaris* (Cv), *C. Kessleri* (Ck) and *Natural Bloom* (BL) cultivated in wastewater (WW) and synthetic wastewater (SW) with addition of 5% CO_2 in air, obtained by the Verhulst logistic model (1838).

Strains	X_{max} (mg L^{-1})	μ_{max} (d^{-1})	r^2	t_{90} (h)	t_{10} (h)	$P_{overall}$ (mg L^{-1} d^{-1})	P_b (mg L^{-1} d^{-1})
*Cv*SW	821 ± 88	0.648 ± 0.216	0.979	177	3.81	73.5	94.1
*Cv*WW	1303 ± 270	0.480 ± 0.144	0.979	217	5.35	112.5	116.0
*Ck*SW	1260 ± 91	0.624 ± 0.144	0.991	170	4.00	114	140.2
*Ck*WW	1172 ± 110	0.624 ± 0.168	0.983	176	4.02	111.3	132.3
*Sc*SW	1810 ± 194	0.600 ± 0.168	0.984	188	4.14	163.5	193.2
*Sc*WW	1684 ± 105	0.672 ± 0.168	0.984	168	3.70	152.3	201.4
*BL*SW	1950 ± 243	0.600 ± 0.192	0.982	198	4.10	171.3	201.3
*BL*WW	1884 ± 161	0.600 ± 0.144	0.998	189	4.13	167.3	200.4

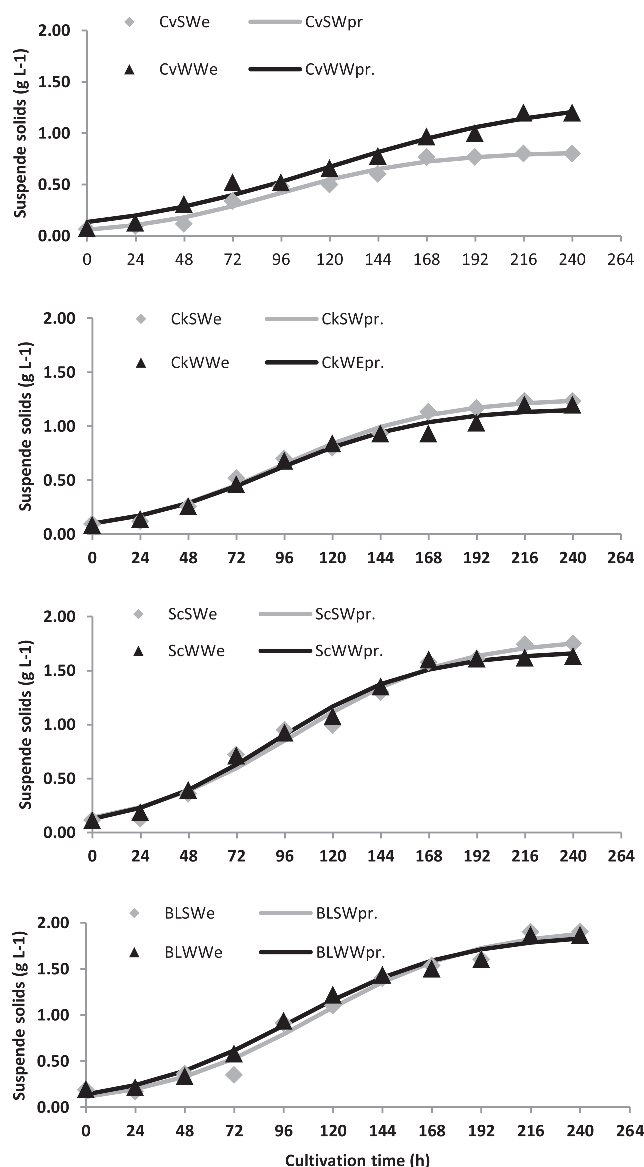

Fig. 1 – Modeled biomass evolution using the Verhulst logistic model (1938) for *Chlorella vulgaris* (Cv), *Chorella kessleri* (Ck), *Scenedemus obliquus* (Sc) and *natural Bloom* (BL) in wastewater (WW) and synthetic wastewater (SW) Solid lines indicate the predicted data (pr), and the experimental data is shown by the symbols (e).

strains cultivated show a similar biomass concentration in WW and SW, except for *Cv* which grew better in WW than in SW.

At the beginning of the experiments all the reactors shows a drop in the pH (data not presented) associated with the CO_2 injection; then as the trial progresses an increase of pH occurs, except in the control reactors *CtrSW* and *CtrWW*. In the control reactors, pH suffers an initial drop from 6.25 to 5.78 for *CtrSW*, and from 7.15 to 6.73 for *CtrWW* but it does not rise subsequently, as in the inoculated assays. In both cases this pH behavior is because of the formation of carbonic acid in water as the result of the CO_2 injection. Once the culture begins to grow, the CO_2 consumption rate increases, as well as the inorganic carbon consumption kinetic; so in those reactors with biomass growth the pH increases.

Modeled data (Fig. 1) showed a good fit between experimental data (symbols) and the predicted values obtained with the Verhulst model (lines). In all the experiments the regression coefficient (r^2) was higher than 0.978 (Table 2). Regarding the final biomass concentration achieved (X_{max}) (Table 2) three different groups can clearly be distinguished. The first one was composed of the *CvSW* where X_{max} achieved at the end of the assay was 821 ± 88 mg L^{-1}, the lowest value obtained in all the trials. Chiu et al. (2008) reported similar results (X_{max}: 899 ± 30 mg L^{-1}) cultivating *Chlorella* sp. inoculated at high cell density ($8 \cdot 10^6$ cell ml^{-1}) in a modified f/2 medium in artificial seawater with addition of 5% CO_2. Sydney et al. (2010) reported an X_{max} of 1940 mg L^{-1}, twice as high as the value obtained in our work, when they cultured *C. vulgaris* Leb-104 in Modified Bristol medium and with addition of 5% CO_2.

The second group was composed of *CvWW*, *CkWW* and *CkSW*. There were no significant differences between these microalgae ($p < 0.05$), and the final concentration achieved was 1303 ± 270, 1260 ± 91 and 1172 ± 110 mg L^{-1} for *CvWW*, *CkWW* and *CkSW* respectively. De Morais and Costa (2007b) reported similar results for *C. Kessleri* when cultivated in Bristol medium, 1450 ± 10 and 980 ± 10 mg L^{-1} with addition of 0.0389% and 6% CO_2 (v/v) respectively.

The third group consisted of *S. obliquus* and *Bl* in both culture media (SW and WW). No significant differences can be seen between strains or between culture media. The X_{max} obtained in this group was 32% higher than the group composed of *CvWW*, *CkWW* and *CkSW*, and 55.18% higher than *CvSW*. The X_{max} obtained was 1810 ± 194, 1684 ± 105, 1950 ± 243 1884 ± 161 mg L^{-1} for *ScSW*, *ScWW*, *BlSW* and *BlWW* respectively. These results are similar to those obtained by Tang et al. (2011) for *Scenedesmus obliquus* cultivated in modified BG11 medium with 5% CO_2 (X_{max}: 1800 ± 20 mg L^{-1}). De Morais and Costa (2007a) also obtained similar X_{max} for *Scenedesmus obliquus* when cultivated in vertical tubular reactors enriched with 6% CO_2 in MC medium (X_{max}: 1560 mg L^{-1}).

The maximum specific growth rates (μ_{max}; d^{-1}) obtained do not show significant differences between any of the strains tested (Table 2), nor between culture media, since there were no differences between the trials in light intensity, nutrient concentrations or CO_2 addition. However the μ_{max} values in this study were slightly higher than those obtained by other authors: for *Scenesdemus obliquus* and *Chlorella kessleri* cultivated with 6% CO_2, De Morais and Costa (2007a) obtained 0.261 ± 0.020 d^{-1} and 0.267 ± 0.013 d^{-1} respectively (Table 3); this difference could be attributed to the very low light intensity employed by De Morais and Costa (2007b) in the experiment (88 μmol cm^{-2} s^{-1}), indicating that in the case of De Morais and Costa (2007a,b) the strains suffered lack of light. On the other hand, for *Scenesdemus obliquus* with 5% CO_2, Tang et al. (2011) reported a slightly higher μ_{max} (0.943 ± 0.021 d^{-1}) (Table 3), in this case this difference can attributed not to the light intensity used (180 μmol cm^{-2} s^{-1}) but to a temperature effect: Tang et al. (2011) operated at 25 ± 1 °C while in our work the temperature was 20 ± 1 °C, this indicate a great effect of the temperature in the growth rate.

Table 3 – Comparison of cell growth rate and carbon dioxide biofixation capability of *S. obliquus*, *C. vulgaris* and *C. Kessleri* reported in the literature by different authors.

Species	% CO_2	X_{max} (mg L^{-1})	P (mg SS L^{-1} d^{-1})	P_{CO_2} (mg $CO_2L^{-1}day^{-1}$)	μ_{max} (d^{-1})	Culture médium	Reference
C. vulgaris	Air	nd	40	75	nd	Watanabe	Scragg et al., 2002
	Unknown	nd	100	188	nd		Yoo et al., 2010
	Unknown	nd	10	188	nd		Liang et al., 2009
	Air	nd	180	338	nd		Gouveia and Oliveira, 2009
	Unknown	nd	30–40	0.056–0.0752	nd		Illman et al., 2000
	Unknown	nd	170	319	nd		Rodolfi et al., 2009
	Unknown	nd	200	376	nd		Rodolfi et al., 2009
C. kessleri	Air	1450 ± 10	90 ± 1	169	0.257 ± 0.02	Bristol	De Morais and Costa, 2007a,b
	6	980 ± 10	87 ± 1	163	0.267 ± 0.01		
	12	80 ± 10	86 ± 1	161	0.267 ± 0.01		
	18	88 ± 10	61 ± 1	114	0.199 ± 0.01		
Sc. obliquus	Air	nd	90	16	nd	Wastewater	Gomez villa et al., 2005
	Air	nd	16	31	nd	Wastewater	Gomez villa et al., 2005
	Air	nd	190	357	nd	Nd	Mandal and Mallik, 2009
Sc. obliquus	Unknown	nd	220	413	nd	Nd	Yoo et al., 2010
	Nd	nd	260	488	nd	Nd	Rodolfi et al., 2009
	Nd	nd	266	500	nd	Nd	Rodolfi et al., 2009
	Air	1050 ± 20	83 ± 2	150 ± 7	0.507 ± 0.01	Modified BG11	
	5	1800 ± 20	158 ± 6	286 ± 7	0.943 ± 0.02		
	10	1840 ± 10	155 ± 4	288 ± 4	0.887 ± 0.01		
	20	1650 ± 10	134 ± 1	246 ± 2	0.780 ± 0.09		
	30	1030 ± 60	81 ± 2	151 ± 2	0.544 ± 0.09		
	50	820 ± 60	56 ± 4	105 ± 6	0.299 ± 0.01		
	Air	1110 ± 10	64 ± 1	0.12032	0.216 ± 0.03	Bristol	De Morais and Costa, 2007a
	6	1100 ± 10	85 ± 2	0.1598	0.261 ± 0.02		
	12	1140 ± 10	76 ± 1	0.14288	0.249 ± 0.02		
	18	1120 ± 10	74 ± 1	0.13912	0.260 ± 0.02		
	Air	310	40	0.0752	0.15	MC médium	De Morais and Costa, 2007b
	6	1560	100	0.188	0.22		

3.1.1. *Productivity*

Table 2 present productivities obtained from experimental data in the assays, using the two calculation procedures compared in Section 2.4. Results indicate that there were significant differences between $P_{overall}$ and P_B; in fact, P_B was between 24 and 38% higher than $P_{overall}$ in all the assays. The smallest difference was found in *CvWW* since that assay recorded the lowest specific growth rate measured (0.480 ± 0.144 d^{-1}). It is important to note that almost all the strains (except *CvWW*) showed a similar difference (29–35%) between P_b and $P_{overall}$ in the two culture media tested. This was probably because no significant differences in growth rate were observed between strains or between culture media.

The new method proposed for calculating productivity, where the lag and stationary phases are minimized or even excluded, gives more homogeneous results, since the wide dispersion of data in respect of these two phases is prevented. For this reason, by excluding the unnecessary data from the calculation, we obtained higher values of productivity in comparison with the classic method of calculation ($P_{overall}$); this shows the real potential of the microalgae for biomass generation. In fact, when microalgae productivities values obtained in batch reactors by different authors are consulted, a great dispersion can be appreciated. This dispersion of productivities between different authors are due to the wide variety of experimental conditions used: light intensity, light dark cycles, culture medium, species of microalgae, temperature, aeration, source and proportion of carbon dioxide, type of photobiorector, etc, but one of the most crucial source of variation lies in the way of calculating the productivity, which is strongly related with the variety of criteria used to stop the experiments. The use of a homogeneous, easy and realistic procedure for productivities calculation, as the proposed one, could contribute to the reduction of productivities data dispersion and facilitate comparison of data from different authors (Arbib et al., 2013).

3.2. *Nutrient removal*

Figs. 2 and 3 represents the evolution of total nitrogen and total phosphorus concentration during the experiments.

3.2.1. *Nitrogen removal*

Regarding removal of total dissolved nitrogen (ΣN: $N{-}NO_3^{+} + N{-}NH_4^{+}$), Fig. 2 shows that at the end of the experiments, in all the reactors the removal was higher than 90% except for *BlSW* (Fig. 2D) where it was slightly lower, at 79%. This behavior is to be expected since Bl was isolated from

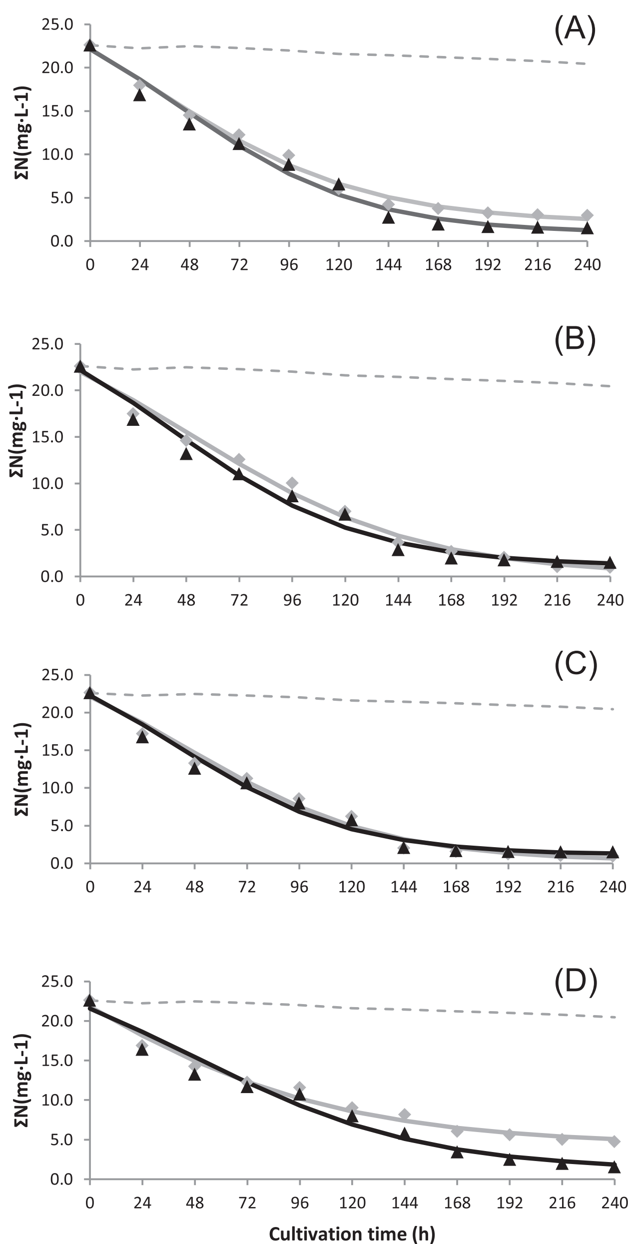

Fig. 2 – Evolution of total nitrogen as ΣN: $N-NH_4^+ + N-NO_3^-$ concentration in test media. (A): *Chlorella vulgaris*; (B) *Chlorella Kessleri*; (C) *Scenedemus obliquus* and (D) *Bloom*. Triangle and rhombus represents wastewater and Synthetic water respectively. Black line and gray line represents predicted data for synthetic water and wastewater respectively. Discontinuous line represent control reactor.

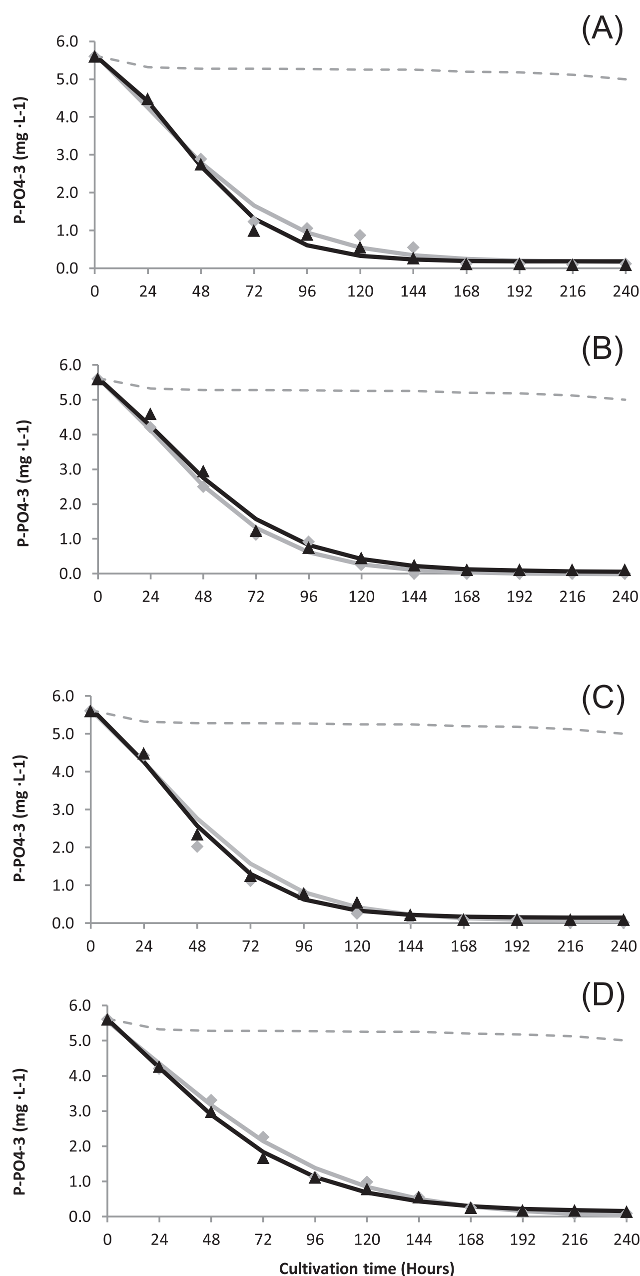

Fig. 3 – Evolution of $P-PO_4^{3-}$ concentration in test media. (A): *Chlorella vulgaris*; (B) *Chlorella Kessleri*; (C) *Scenedemus obliquus* and (D) *Bloom*. Triangle and rhombus represents wastewater and Synthetic water respectively. Black line and gray line represents predicted data for synthetic water and wastewater respectively. Discontinuous line represent control reactor.

downstream of the WWTP and therefore it was more acclimated to WW than to SW.

All the strains studied were able to remove the total nitrogen from the wastewater, and no significant differences were observed between the two different culture media. In control reactors, no removal was appreciated; therefore total nitrogen depletion observed in bioreactors was due to biological processes (Arbib et al., 2012). It Is noteworthy to note that as the pH was maintained at low levels by the addition of CO_2, no NH_3 inhibition took place during the tests, NH_3 inhibition has been described as one of the most important selection criteria for microalgae in WW field due to the low tolerance of some species and the relative high concentration of this compound in some kind of wastewaters (de Godos et al., 2010).

During the course of the trial, the experimental data (symbols) corresponded well to the model applied for nutrient

Table 4 – Time needed to reach 10 mgN L^{-1} (t_{10N}) and 1 mg P L^{-1} (t_{1P}) in filtered test media (most restrictive concentrations value in Directive 98/15/CE), obtained by Quiroga et al. (1999) model.

Strain & culture	Total nitrogen		Phosphorus	
	r^2	t_{10N} (h)	r^2	t_{1P} (h)
CvSW	0.992	85	0.989	92
CvWWW	0.984	79	0.989	80
CkSW	0.99	87.5	0.996	80.5
CkWW	0.983	78	0.994	88.7
ScSW	0.985	77.5	0.984	89.1
ScWW	0.983	72.5	0.995	80.5
BLSW	0.982	101.6	0.996	112
BLWW	0.969	90.0	0.998	101.4

removal. In all the reactors R^2 was higher than 0.969 (Table 4). It can be appreciated in Table 4 that, for the four species tested, the time required to reach 10 mg l^{-1}N (t_{10N}) was higher in synthetic than in wastewater (Ruiz et al., 2011; Arbib et al., 2012), which demonstrate again the great capability of microalgae in growing in WW. Between species, t_{10N} was very similar except for *BlSW* with the slower nitrogen uptake kinetic (101.6 h).

3.2.2. *Phosphorus removal*

For phosphorus (Fig. 3), the behavior observed is similar to that for total nitrogen removal. At the end of the experiments phosphorous removal in all the assays was higher than 98%. As in the case of nitrogen, in the control reactors no phosphorous removal was observed, so only biological processes are involved in the bioreactors (Arbib et al., 2012).

It is noteworthy that after the first 25 h of the assays the phosphorous concentration suffers a sudden drop. This initial drop implies the removal of up to 20% of the total. For *CkSW* and *ScSW* this initial removal was slightly higher, at 25% for both. According to several authors (Boyd and Musig, 1981; Okada et al, 2004; Khummongkol et al., 1982; El Yousri, 1995; Arbib et al., 2012) this drop can be attributed to two different processes: (1) Precipitation and deposition at the bottom of the reactor due to a high pH (above 9); and/or (2) adsorption to the microalgae cell wall and subsequent assimilation. However because this initial drop was not observed in the control reactors (Fig. 3) and the pH during the entire assay was below 9 in all the reactors (data not presented), it can be concluded there is no removal due to a precipitation process; therefore the initial large percentage drop in phosphorous concentration was attributed to the adsorption to the microalgae cell wall.

As for total nitrogen, the experimental data of phosphorus consumption (Fig. 3) showed a good fit to the kinetic model applied for nutrient uptake ($R^2 > 0.911$) (Table 4). The time needed to reach the discharge limit for P ($t_{1P} = 1$ mg L^{-1}) is presented in Table 4. In *WW* the t_{1P} was lower than in *SW*, except for *C. Kessleri* where the t_{1P} was 80.5 and 88.7 for *SW* and *WW* respectively, this results indicate again the great capability of different microalgae strain in growing in WW.

An important point to note regarding the nutrient removal capability, is that the time needed to reach the two discharge limits (t_{1P} and t_{10N}) was comparable in all the reactors (Table 4). Therefore our study shows that the simultaneous removal of nitrogen and phosphorus to below the most restrictive limits for effluent discharge can be achieved. Hence, in the operating conditions employed in these assays, neither nitrogen nor phosphorus assimilation kinetics limit the overall process.

3.3. *Biomass composition*

Table 5 presents the biomass composition for the four microalgae studied. Regarding the percentage of carbon in the biomass, it can be seen that there were significant differences between strains. The maximum was obtained in *CvSW* (56.6 ± 2.5% C) and the minimum in *BLWW* (46.5 ± 0.7% C). But no significant differences were observed between the two media tested for each strain. For example, for *ScWW* and *ScSW* the values obtained were 47.8 ± 0.2%C and 48.8 ± 0.9% C respectively.

The total lipids content (also in Table 5) from the microalgae cultivated in this study ranged from 18.50 ± 2.22% to 29.55 ± 2.55% of the dry weight. It can be appreciated that there were no significant differences in lipid content between the two culture media tested for each strain, as the nutrient concentrations (nitrogen and phosphorous) were similar in WW and SW, and the culture conditions were also the same. Lipid content of biomass from *Bl* was the highest in both

Table 5 – Elementary analysis of dried biomass obtained by Leco CHNS 932, Crude protein content and Lipid percentage in biomass. Kinetic parameters of carbon dioxide biofixation.

Strain and culture médium	C (% dw)	N (%dw)	Crude protein (% dw)	Lipids (% dw)	$P^*_{CO_2}$ (mg CO_2 L^{-1} d^{-1})	P_{CO_2} (mg CO_2 L^{-1} d^{-1})	$Y_{CO_2/SS}$ (mg CO_2 L^{-1})
CvWW	54.8 ± 2.6	3.5 ± 0.2	21.85 ± 1.25	22.02 ± 2.20	214.12	236.42	2.076
CvSW	56.6 ± 2.5	3.4 ± 0.3	21.25 ± 1.87	21.75 ± 2.75	277.34	296.75	2.012
CkWW	50.4 ± 0.8	3.3 ± 0.3	20.62 ± 1.87	20.55 ± 3.00	323.70	319.99	1.858
CkSW	50.7 ± 1.2	3.5 ± 0.1	21.87 ± 0.62	19.25 ± 2.25	307.19	302.17	1.849
ScWW	47.8 ± 0.2	2.6 ± 0.5	16.25 ± 3.12	19.38 ± 1.75	444.99	424.40	1.793
ScSW	48.8 ± 0.9	2.5 ± 0.3	15.62 ± 1.87	18.50 ± 2.22	461.63	436.67	1.778
BLWW	46.5 ± 0.7	3.8 ± 0.8	23.75 ± 5.0	26.82 ± 2.75	453.80	413.77	1.714
BLSW	46.7 ± 1.1	3.9 ± 0.7	24.37 ± 4.37	29.55 ± 2.55	460.37	417.52	1.705

$P^*_{CO_2}$: 1.88 × P_B, which is derived from the typical molecular formula of microalgal biomass, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Chisti, 2007).
P_{CO_2}: $P_{Batch2} \times (\%C) \times (MW_{CO_2}/MW_C)$
$Y_{CO_2/SS}$: P_{CO_2}/P_B.

culture media (26.82 ± 2.75 and 29.55 ± 2.55% for *BlWW* and *BlSW* respectively).

The lowest crude protein content (Table 5) was observed for *S. obliquus* in both cultures (16.25 ± 3.12% and 15.62 ± 1.87% for ScWW and ScSW respectively), and in *Chlorella* strains there were no differences between the two strains tested in the two culture media.

3.3.1. *Carbon dioxide biofixation capacity*

Batch productivity (P_B) (Table 2) and carbon content in biomass (Table 5) were used to calculate the kinetic parameters related to the carbon dioxide fixation (De Morais and Costa, 2007b; Tang et al., 2011). Those parameters were: P_{CO_2} (g CO_2 L^{-1} d^{-1}) and Y_{CO_2} (g CO_2 g SS^{-1}) (Table 5). P_{CO_2} was also calculated by the typical molecular formula of microalgal biomass $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$, according to Equation (11) (De Morais and Costa, 2007a,b).

The yield coefficient (Y_{CO_2}) obtained ranged between 1.705 and 2.010 g CO_2 g SS^{-1} (Table 5). Values were similar to those obtained by other authors for different microalgae species. Scragg et al. (2002) have reported that *C. vulgaris* cultivated in Watanabe's medium and low nitrogen medium gave a yield coefficient of 1.875 g CO_2 g SS^{-1} for both cultures. For *Chlorella kessleri*, De Morais and Costa (2007b) have reported a yield coefficient of 1.875 g CO_2 g SS^{-1}. The yield coefficient for *Scenedesmus obliquus* was 1.931 and 1.771 g CO_2 g SS^{-1} respectively when it was cultivated in wastewater in summer and winter (Gomez-Villa et al., 2005). It is noteworthy that the Y_{CO2} obtained was close to 1.88, the constant used in the equation of Chisti (See Table 5). This value is directly related to the carbon percentage in the biomass dry weight.

With respect to the P_{CO_2}, it can be appreciated that differences between the P_{CO_2} obtained through kinetic parameters and the equation of Chisti ($P^*_{CO_2}$) are very small, between (10.4% and −1.1%)

4. Conclusions

All the strains tested were able to grow in wastewater media. No significant differences in the maximum biomass concentration were found when *Scenedesmus obliquus, Chlorella kessleri* and the natural bloom were cultured in either synthetic or actual wastewater media. A new procedure (P_B) for calculating the productivity of a batch reactor has been proposed, in order to avoid data variations, although an established procedure ($P_{overall}$) was also used in these experiments, In all the assays P_B was between 24 and 38% higher than $P_{overall}$.

All four species tested have high potential for removing nitrogen and phosphorous from urban wastewater, to levels even lower than the most restrictive currently imposed (Directive 98/15/CE). Results indicate that the time needed to reach the stipulated limits for removal of both nutrients (t_{1P} and t_{10N}) were comparable in all the reactors. This means that the simultaneous photobiological removal of Nitrogen and Phosphorus from wastewater is technically feasible.

No influence of the culture media was observed in relation to the carbon dioxide biofixation capacity. This parameter (P_{CO_2}) was related more closely with the strain tested. Those strains with higher biomass productivities (*Scenedesmus obliquus* and the *bloom*) were also the ones with higher CO_2 biofixation kinetics, as biomass carbon content was very similar between experiments.

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