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Artículo Original

Effects of CO_2 concentration on nutrient uptake and starch accumulation by duckweed used for wastewater treatment and bioethanol production

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Abstract

The aquatic macrophytes commonly known as duckweed has been successfully used in wastewater treatment plants during decades. Besides the efficiency of these plants to remove nutrient from wastewater, duckweed has drawn increasing attention for bioethanol production due to its high biomass and starch production. Recently several studies have been evaluating techniques to promote starch accumulation in duckweed biomass and thus improve ethanol yield. Therefore, the present study aimed to evaluate the effect of CO_2 concentration ($[CO_2]$) and availability in nutrient removal and starch accumulation by duckweed grown in photobioreactors (PBRs). Thus, duckweed was grown in hermetic PBRs (24 L) exposed to three different CO_2 concentrations (C1-1,500; C2-6,000 and C3-100,000 ppm), as well as a control group (CC-380 ppm), without CO_2 replacement for a seven-day test period. The decay of NO_3^- and PO_4^- was monitored along the test, as well the $[CO_2]$ and biomass growth rates. The results showed that in C1 and C2, duckweed quickly consumed the CO_2 in the gas phase, causing a reduction of nutrient removal efficiency and the consumption of storage starch. By contrast, the higher $[CO_2]$ improved the starch content by approximately 150%, from 9.6 to 24.7%, and presented the best results for nitrate and phosphate removal (82 and 79% from 308 $mgNO_3\ L^{-1}$ and 28 $mgPO_4\ L^{-1}$, respectively).The findings pointed that $[CO_2]$ is an important parameter to be monitored in closed duckweed systems, and CO_2 supply could improve the starch content and nutrient removal rates.

Keywords: *Duckweed, starch content, nutrient removal, CO_2 enrichment*

Resumen

Las plantas acuáticas conocidas comúnmente como lenteja de agua, o lemnas, se han utilizado con éxito en sistemas de tratamiento de aguas residuales durante décadas. Además de la eficiencia de estas plantas para la remoción de nutrientes de efluentes, las lentejas de agua han atraído una creciente atención para la producción de bioetanol debido a la alta tasa de crecimiento de su biomasa y el contenido de almidón. Recientemente, muchos estudios han evaluado técnicas para promover la acumulación de almidón en la biomasa de lemnas y así aumentar la producción de etanol. Es por ello

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que el presente estudio tuvo como objetivo evaluar el efecto de la concentración de CO_2 ($[CO_2]$) en la eficiencia de remoción de nutrientes por lemnas y en el contenido de almidón en la biomasa, cultivadas en fotobiorreactores (FBR). Las lemnas fueron producidas en un medio Stenberg modificado dentro de los FBR, con 24L y se expusieron a tres diferentes concentraciones de CO_2 (C1-1,500; C2-6,000 y C3-100,000 ppm), además del grupo de control (380ppm), sin reposición de CO_2 por una semana. Durante las pruebas, el decaimiento de NO_3^- y PO_4^- en el medio fueron evaluados, así como la $[CO_2]$ en la composición del gas, además de la tasa de crecimiento y la composición de la biomasa. Los resultados mostraron que para las concentraciones C1 y C2, el CO_2 se consumió en pocas horas reduciendo la tasa de crecimiento y consecuentemente la eficiencia de eliminación de nutrientes, además de causar el consumo de almidón. Por otro lado, la biomasa producida en la concentración de CO_2 superior (C3) mostró un aumento en la concentración de almidón de aproximadamente 150% (de 9,6 a 24,7%). Asimismo, la eficiencia de eliminación de nutrientes fue mayor en C3 alcanzando 82 y 79% (a partir de 308 $mgNO_3.L^{-1}$ y $28mgPO_4.L^{-1}$, respectivamente). Los resultados muestran que la concentración de CO_2 es un parámetro importante para ser monitoreado en sistemas cerrados para el cultivo de lemnas y el suministro de este gas en altas concentraciones puede mejorar la eficiencia de eliminación de nutrientes y la acumulación de almidón para la producción de bioetanol.

Palabras clave: *Lenteja de agua, contenido de almidón, remoción de nutrientes, enriquecimiento de CO_2*

1. Introduction

The group of aquatic plants commonly named duckweed has been successfully used in effluent treatment systems, mainly for agricultural and municipal wastewater (Skillicorn et al., 1993; Körner and Vermaat,1998, Mohedano et al., 2012a, Zhao et al., 2015). In addition to the high nutrient removal rate, the conditions created by duckweed mats provide a suitable microenvironment for microorganism growth, thus improving nitrification and organic matter degradation (Sims et al, 2013; Mohedano et al., 2014., Zhao et al., 2014).

Under ideal conditions duckweeds presents the higher growth rate between vascular plants and consequently high amounts of surplus biomass should be removed for efficient wastewater treatment (Iqbal, 1999). However, the great advantage of this plant group over other macrophytes used in effluent treatment (such as water hyacinth) is the production of high nutritional-value biomass containing more than 40% of crude protein (Landesman et al., 2002, Zhao et al., 2015).

In addition to its nutritional value, other use for duckweed biomass has arisen in recent years: the bioethanol production. Since 2009, pioneer studies developed in North Carolina (USA) have been demonstrating the great potential of duckweeds for ethanol production. This process is based on the hydrolysis of starch and other carbohydrates present in duckweed biomass (leafs and roots) for subsequent alcoholic fermentation, similar to the process used for corn-based ethanol. According to these studies, the annual duckweed bioethanol yield could reach 6,420 $L.ha^{-1}$, about 50% over than obtained for corn-based ethanol (Cheng & Stomp, 2009; Xu et al., 2011).

To this end, researchers have been attempting to develop techniques for

starch accumulation in duckweed biomass and thus improve ethanol yield. The findings to date show that lower temperatures, nutrient starvation, and higher DLIs (daily light integrals) favor starch accumulation (Xu et al., 2011; Xiao et al., 2013; Cui and Cheng, 2015). In a recent review concerning biofuel production using duckweed, Cui and Cheng (2015) cite that to increase starch content, enrichment of CO_2 in growth media could stimulate photosynthesis and carbohydrate production. However, none results regarding the CO_2 enrichment on duckweed systems were cited in reviewed recent literature.

All photosynthetic organisms, from the most primitive algae to more complex angiosperm, reduce CO_2 to carbohydrates by the same basic mechanism, that is, Calvin's Cycle or the Reductive Pentose Phosphate Cycle [RPP]. In general, the enzyme (Ribulose-1,5-bisphosphate carboxylase oxygenase - RuBisCo) that catalyzes carbon fixation for sugar production also promotes photorespiration, causing carbon loss. However, due to competition for the active site on RuBisCo, this carbon loss could be reduced by elevating atmospheric CO_2 concentration and improving carbon fixation rates. Consequently the carbon enrichment may improve sugar production and storage by plant tissues. Despite of the scarcity of studies focused on duckweed growth under CO_2 enrichment, this technique is widely used in algae-based photobioreactors as well for food production in greenhouses and through FACE (free-air carbon enrichment) in agricultural crops (Langley, et al., 2012; Bind et al., 2001).

Duckweeds species growing under high [CO_2] could present some advantages comparing to others plants group. First, some duckweed species have nonfunctional stomata (facing up) that never close, benefiting gas exchange with the atmosphere. Additionally, the photosynthetic activity in plants with C3 metabolism (such as duckweeds) may be improved by increasing CO_2 and light, which not happen in C4 metabolism plants. In this sense, Andersen et al. (1984) demonstrated a growth rate improvement of 46% for *Lemna giba* exposed to 6,000 ppm of CO_2 compared with exposure to normal atmospheric concentration (350 ppm of CO_2).

Taking into account their application in full-scale wastewater treatment plants, the biogas produced in anaerobic digesters such as UASBs should be a potential source of CO_2 (before or after burning) to be used in duckweed pond enrichment (Byrns, et al. 2012). Moreover, duckweed ponds supplied by CO_2 enrichment may contribute to the reduction of GHG (greenhouse gas) emissions due to their high growth rate, which improves carbon fixation.

Thus, the aim of this study was to evaluate a new technique to improve starch accumulation and nutrient uptake using carbon dioxide (CO_2) enrichment. The CO_2 enrichment technique is commonly used for biomass gain in greenhouses for vegetable growth; however, their effect on duckweed intended for biofuel production and nutrient recovery has not been assessed or reported in the scientific literature.

2. MATERIALS AND METHODS

2.1 Experimental design

To evaluate the effect of CO_2 on duckweed starch content and nutrient uptake, a pilot system was designed and operated. Duckweeds from the species *Landoltia punctata* were grown in 12 hermetic chambers or photobioreactors

(PBRs) made of transparent acrylic with dimensions of 16.5 x 30 x 50 cm and a volume of 24.75 L (Figure 1). The surface area of each PBR to support duckweed growth was 833cm^2 (0,083m^2). An illumination structure was constructed using bulb lamps to provide PAR (photosynthetic active radiation) of approximately 125 µmol.m^{-2}.s^{-1}, measured with a Quantum Radiometer Photometer (Li-COR, model Li-250 Light Meter); the photoperiod was controlled using a D/L (dark/light) ratio of 12/12. The experiment was carried out in a climate-controlled room to maintain a constant temperature of 24±2 ºC. These conditions were based on ISO/DIS 20079 standardization (ISO/DIS, 2003).

All chambers were filled with 4 L of nutritive medium (modified Stenberg Medium), resulting in a 5 cm depth of the water column (Figure 1). This medium was modified by improving nitrate and phosphate concentration in order to avoid nutrient starvation considering that the medium was not replaced during the test period (seven days). Then, 30 g of fresh duckweed (*Landoltia punctata*) from an axenic culture was placed in each chamber and allowed to sit for 24 h for the adaptation. CO_2 gas from commercial bottles was introduced into the chambers through specific holes at three different concentrations: 1,500 ppm or 0.15%, 6,000 or 0.6% and 100,000 ppm or 10% identified as C1, C2 and C3, respectively. A control treatment was maintained with a normal atmospheric concentration of approximately 380 ppm or 0.038%, which was denoted CC (control concentration).

To measure the [CO_2] in gas phase inside the photobioreactor, two devices were used: an NDIR analyzer (Non Dispersive Infrared/ Instrultherm® / C-02) for concentrations below 6,000 ppm and GEM-2000 by Landtec® for higher concentrations. Each treatment was performed in triplicate, meaning three chambers for each concentration (C1, C2, C3 and CC), which amounted to 12 photobioreactors (Figure 1) where each triplicate was randomly located. Inside each PBR was placed one little fan (computer cooler) to homogenize the gas before taking samples.

Duckweeds were exposed to high [CO_2] for seven-day test periods, and after closing the PBRs, the CO_2 was not replaced with the intent of observing the natural decay caused by carbon fixation in plants, as well as the effects of CO_2 starvation.

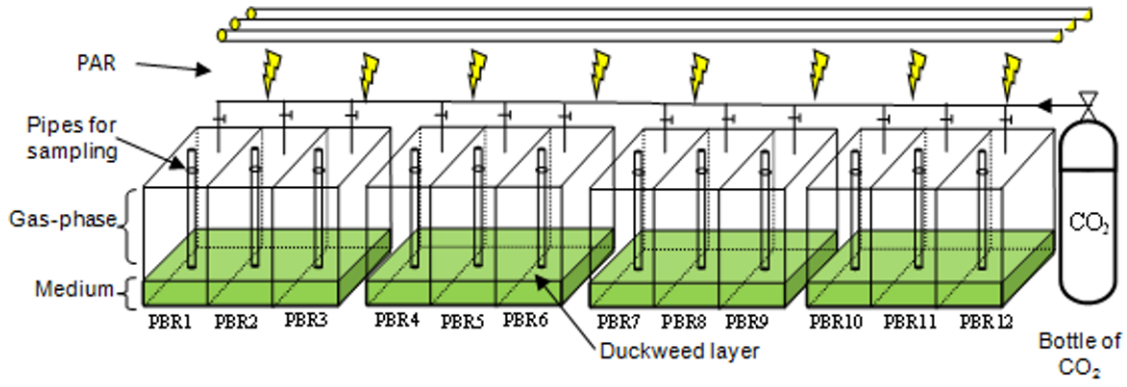

Figure 1. Scheme of experimental system, with 12 photobioreactors (PBR).

2.2 Monitoring

To assess the effects of CO_2 supply on nutrient uptake and starch accumulation by duckweeds, a number of parameters were monitored, including variables of water quality, gas phase CO_2 concentration, biomass growth and composition (starch content and TOC - Total organic carbon).

To quantify the nutrient removal from water (culture medium), samples were collected from each photobioreactor twice per day for seven days and analyzed by ion chromatography (Dionex®) to detect nitrate (NO_3^-) and phosphate (PO_4^-) concentration. Temperature and pH measurement were made *in loco* by portable probes and dissolved CO_2 concentration was determined by titration method.

To evaluate the carbon dioxide concentration in the gas phase, the probes cited before were connected to hermetic apertures to taking samples from each PBR. This procedure was repeated twice a day, more specifically, soon after light period (12h) and soon after dark period (12h) to comprise the both phases of photosynthesis. To estimate carbon mass was used stoichiometric calculations under STP (standard temperature and pressure) conditions where 1 mol of CO_2 have 44g and occupies 22.4L. The amount of carbon fixed was estimated by subtracting the final mass from initial mass. Also the TOC content in biomass was measured to assist the quantifying of carbon fixed by duckweed considering that all the carbon present in the biomass came from fixation.

The biomass growth rate was obtained based on fresh and dry weight according to equations 1, 2 and 3.

$$SGR = \frac{ln\,(p1) - ln\,(p0)}{\Delta t} \quad (1)$$

$$TG = p1 - p0 \quad (2)$$

$$RGR = (TG * A)/\Delta t \quad (3)$$

Where: SGR = Specific growth rate ($g.g^{-1}.d^{-1}$); TG =Total growth (g); RGR Relative growth rate ($g.m^{-2}.d^{-1}$); p0 = initial weight (g); p1 = final weight (g); Δt = t1- t0 = time range in days; A = surface area (m^2).

The biomass was collected only once at the end of testing to assess all exposed duckweeds. For fresh weight evaluation, the biomass was carefully dried with paper towels and immediately weighed; for dry weight, the biomass was then oven dried at 55 °C for 24 h. After weighing, the dry biomass was submitted to starch content analysis using the Amyloglucosidase/α-amylase method (AOAC - Official Method 996.11). To determine the carbon fixed and stored by plant tissue, the biomass collected soon after PBR opening was submitted TOC analyze by using oxidation catalytic combustion method, performed by TOC -L analyzer Shimadzu with accessory for solid samples SSM- 5000A. The obtained values were subjected to statistical tests (ANOVA) carried through the software STATISTICA® 7.1.

3. RESULTS

3.1 Nutrient and Carbon dioxide uptake

Regarding the nutrient uptake by duckweed grown in experimental conditions, the efficiency of nitrate removal was 6, 9, 16 and 82% for CC, C1, C2, and C3, respectively, from initial concentration of 308 $mg.L^{-1}$ in all reactors. Notwithstanding, the surface nitrogen load rate removal in C3 conditions was 3.8 $g.m^{-2}.d^{-1}$. Additionally, the same pattern was observed for phosphate reduction, where the efficiency was 10, 11, 24 and 79% from an initial concentration of 28 mg $PO_4.L^{-1}$. In Table 1 and Figure 2 are shown the data of nutrient concentration

(NO_3 and PO_4) along the experiment, removal rates and efficiencies. Non statistic difference were observed between CC and C1 considering nutrient uptake. The pH values remained near to neutrality (between 6.8 and 7.1) presenting no expressive variation along the period. Also, the dissolved CO_2 values remained low with a maximum concentration of 35 $mg.L^{-1}$ at the start and followed by a slight decreasing in all treatments.

Table 1. Nitrate and phosphate removal in different CO_2 concentrations (CC- 380; C1-1,500; C2-6,000; C3-100,000 ppm).

	Nitrate (NO_3^-)				Phosphate (PO_4^-)			
	CC	**C1**	**C2**	**C3**	**CC**	**C1**	**C2**	**C3**
Initial conc.($mg\ L^{-1}$)	308	308	308	308	28	28	28	28
Final conc.($mg\ L^{-1}$)	288^a	281^a	257^b	53^c	25^a	25^a	21^b	6^c
Removed ($mg\ L^{-1}$)	20	27	51	255	3	3	7	23
Efficiency (%)	6	9	16	82	10	11	24	79
RR ($mg.L^{-1}\ d^{-1}$)	0.13	0.17	0.33	1.57	0.02	0.02	0.04	0.15
SRR ($mg\ m^{-2}\ d^{-1}$)	296	400	760	3,786	40	48	96	344

RR- removal rate; SRR - surface removal rate;
a, b, c, d - different letters means different statistical significance.

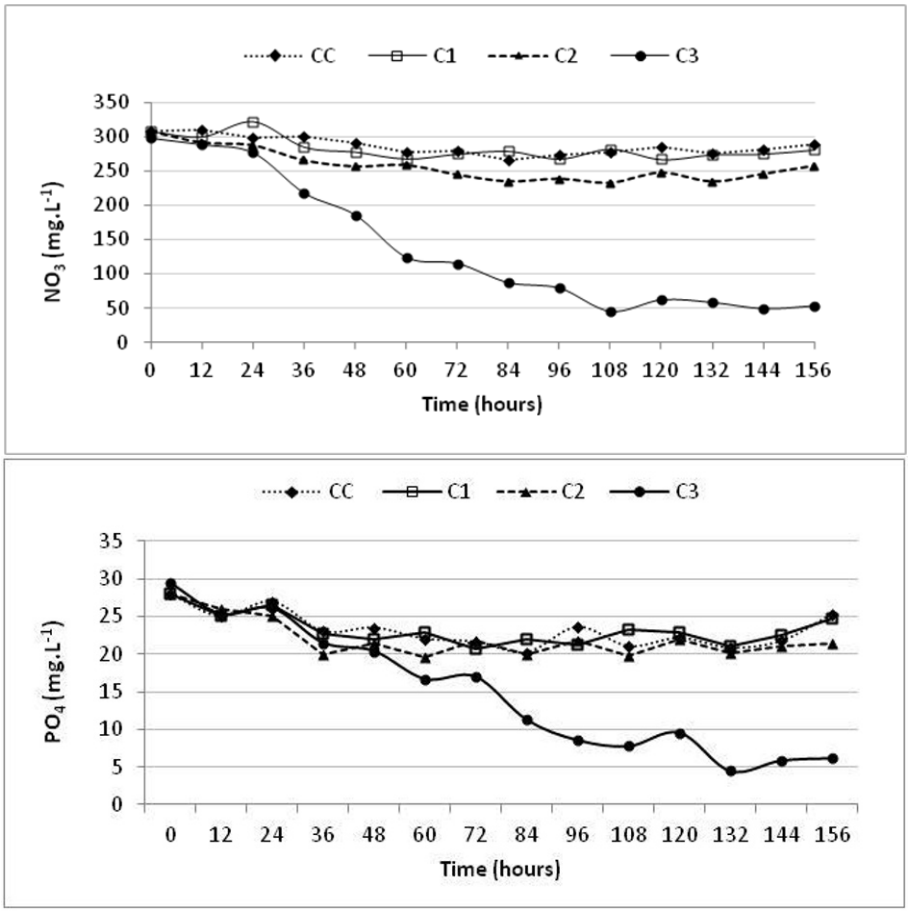

Figure 2. Nitrate and phosphate reduction in different CO_2 concentrations (CC- 380; C1-1,500; C2-6,000; C3-100,000 ppm)

Dissolved CO_2 decay is shown in Figure 3. Concerning the CO_2 concentration in gas phase the Table 2 presents the mass balance considering the amount (mg) of carbon introduced and fixed by plants, as well the fixation rates. Also in Figure 4 it is shown the carbon mass (mg) available for fixation in each treatment during start conditions.

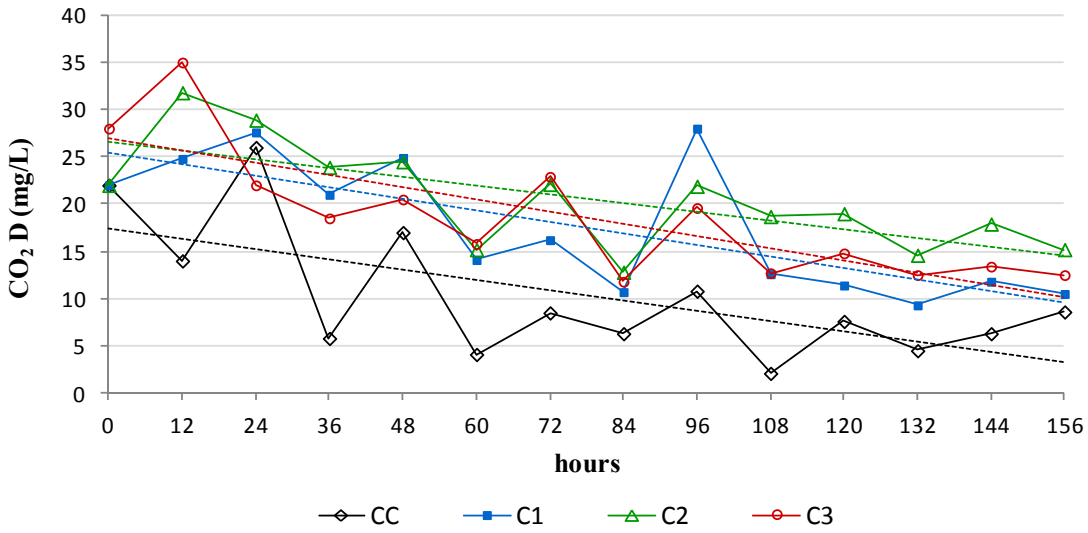

Figure 3. Dissolved carbon dioxide concentration in nutritive medium along the experimental period (CC- 380; C1-1,500; C2-6,000; C3-100,000 ppm).

Table 2. Biomass growth and carbon fixation rates in different CO_2 concentrations (CC- 380; C1-1,500; C2-6,000; C3-100,000 ppm).

	CC	C1	C2	C3
Initial fresh weight (g)	30	30	30	30
Final fresh weight (g)	30.6±0.5	31.1±0.8	33.15±1	61.2±4.2
Total weight gain (g)	0.6	1.1	3.15	31.23
Biomass moisture (%)	94	93.8	92	90.5
Dry weight gain (mg)	36 a	79.2 b	252 c	2.966 d
Relative Growth Rate ($g.g^{-1}.d^{-1}$)	0.003	0.005	0.014	0.102
Specific Growth Rate ($g.m^{-2}.d^{-1}$)	1,0	1,9	5,4	53,5
***TOC** (%)	26a	33b	32b	41c
C fixed in each PBR (mg)	9.36	26.14	80.64	1127.40
C fixation rate ($mg.m^{-2}.h^{-1}$)	0.06	0.17	0.52	7.23
CO_2 removed in each PBR (mg)	34.29	95.74	295.38	4129.68
CO_2 removal rate ($mg.m^{-2}.h^{-1}$)	0.22	0.62	1.9	26.5

(a, b, c, d) different letters means different statistical significance
*TOC - Total Organic Carbon

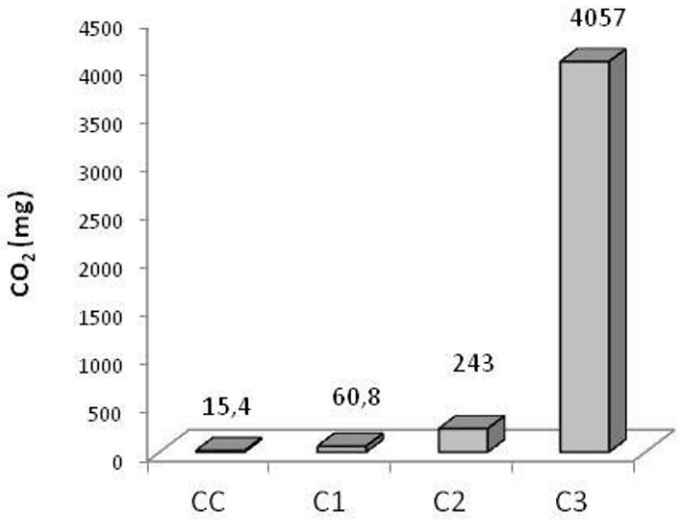

Figure 4. Starch content (%) in duckweed biomass exposed to different CO_2 concentrations (CC- 380; C1-1,500; C2-6,000; C3-100,000 ppm)

3.2 Biomass evaluation (growth and starch content)

The relative growth rates found to the end of the experiment were 1.0, 1.9, 5.4 and 53.5 $g.m^{-2}.d^{-1}$, in CC, C1, C2, C3 respectively. Therefore, the total weight gain in C3 was 31g (from 30 to 61g) and for CC, C1 and C2 the growth was almost negligible (0.6 g, 1.0 g and 3.15 g). The behaviors of biomass growth during experimental period for the different treatments are shown in Table 2 and Figure 5.

While in C3 the starch content rose from 9.6 to 24.7% (150% increase), in other treatments, this carbohydrate dropped from 9.6 to 1.4, 3.1 and 3.4% (CC, C1 and C2, respectively). The starch content in duckweed biomass growing in all the treatments is shown in Figure 6.

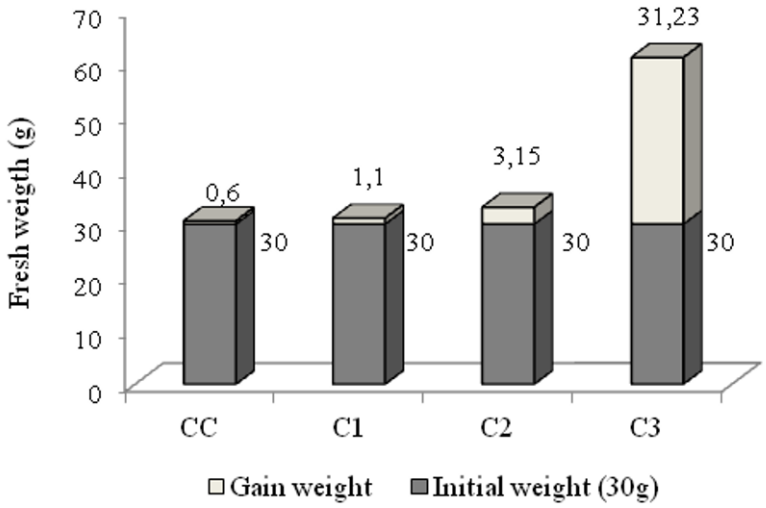

Figure 5. Amount of CO_2 available in each treatment (CC- 380; C1-1,500; C2-6,000; C3-100,000 ppm).

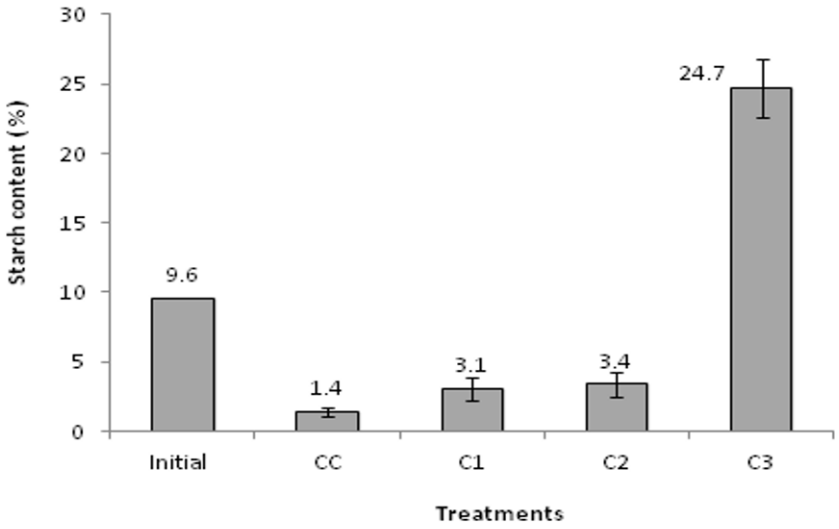

Figure 6. Weight gain of duckweed biomass under different CO2 concentration (CC- 380; C1-1,500; C2-6,000; C3-100,000 ppm).

4. DISCUSSION

4.1 Nutrient and Carbon dioxide uptake

According to results presented in table 1 and figure 2, was clearly demonstrated the effect of CO_2 availability on nutrient removal from the nutritive medium. Due to the non replacement of CO_2 along a week, the lack of carbon prevented the duckweed growth causing a reduction on nutrient uptake rates. Thus, considering the amount of CO_2 available in C3 PBRs the plants were able to grow vigorously, although in the others treatments (CC, C1 and C2) the biomass growth was almost negligible (Figures 4 and 5) . Differently of full scale treatment ponds where nitrogen could be removed by many ways, in PBR the nitrate uptake by duckweeds is the only way for nitrogen removal, thus the growth rate is directly related to nutrient removal.

When compared with other studies, the initial nitrate concentration (308 mg $NO_3.L^{-1}$) was intentionally high because the modified medium could not be replaced or recharged during the tests. Notwithstanding, the nitrogen load removal in C3 conditions was one of the highest reported (3.8 $g.m^{-2}.d^{-1}$) compared with the nitrogen removal rates in previous studies, such as 0.4 $g.m^{-2}.d^{-1}$ (Zhao et al., 2014), 0.54 $g.m^{-2}.d^{-1}$ (Körner and Vermaat,1998), 1.2 $g.m^{-2}.d^{-1}$ (Benjawan and Koottatep, 2007) and 3.4 $g.m^{-2}.d^{-1}$ (Cheng et al., 2002). Additionally, the same pattern was observed in phosphate reduction, where the higher efficiency was in C3 treatment (Table 1 and figure 2).

It seems evident that a lack of CO_2 causes growth inhibition in lower [CO_2] treatments and the high [CO_2] in C3 does not cause inhibitory effects. This means that carbon dioxide is consumed until the compensation point (limit required for fixation processes by RuBisCo), as described by Muller et al. (1977), which states that carbon fixation in the duckweed *Lemna minor* ceases under [CO_2] values lower than 40 ppm.

Due to the carbon consumed by plants, the [CO_2] in the gas phase decreased until the limit level within the first few hours (approximately 6 hours) in C1 (380 ppm), causing a low growth rate and, consequently, low nutrient uptake. Some authors have cited problems

resulting from CO_2 limitation in overgrown duckweed populations, where the lower layer suffered metabolic stress due to low light and CO_2 availability (Driever et al., 2005). However, were not found in recent literature studies with focus on the effects of low CO_2 in closed systems to grow duckweed.

Despite the high carbon dioxide concentration in the gaseous phase, the pH values did not suffered acidification as expected due to H_2CO_3 (carbonic acid) formation. The duckweed layer most likely provides a barrier to CO_2 diffusion into the aqueous phase, and the medium has a suitable buffer capacity. This hypothesis is supported by the dissolved CO_2 values, which were observed to reach a maximum at 35 $mg.L^{-1}$. However, in Figure 3 is possible to note that all treatments presents a zigzagging line and this behavior could be explained by the photoperiod changes with a slight CO_2 reduction during the light phase and increasing during dark period.

4.2 Biomass evaluation (growth and starch content)

The effect of low carbon available for duckweed growth was evident (Table 2), while in CC, C1 and C2, the growth rate was almost negligible, in C3 ($[CO_2] = 10^5$ ppm), the biomass doubled, from 30 to 61 g (fresh weight). Similarly, Andersen et al. (1984) demonstrated a growth rate increase of 46% for *Lemna giba* exposed to 6,000 ppm of CO_2 compared to the growth rate under exposure to normal atmospheric concentrations (350 ppm of CO_2). Thus, in photobioreactors that use duckweeds for nutrient removal and biofuel production, the CO_2 supply is an important factor for both processes.

The starch produced and stored by plants increased sharply under high $[CO_2]$, as did the TOC (Figure 3 and Table 2). While in C3, the starch content rose from 9.6 to 24.7% (150% increase), in other treatments, this carbohydrate dropped to 1.4, 3.1 and 3.4% (CC, C1 and C2, respectively). In addition, Guy et al. (1990) find 38% of starch content increase in *Lemna gibba* growing under 6,000 ppm of $[CO_2]$. Indeed, under low carbon availability, duckweeds have to consume the stored starch for basic metabolic maintenance; by contrast, under high CO_2 conditions, the nonstructural carbohydrates produced were stored. An increase in sugar content under high $[CO_2]$ has also been demonstrated in other important species such as grapevine and soybean (Bindi et al., 2001; Sicher, 2013).

To advance the research beyond the findings presented is recommended to carrying out tests with the maintenance of CO_2 concentration for extended periods.

5. CONCLUSION

Regarding the use of duckweeds for nutrient uptake and bioethanol production in photobioreactors, the effect of CO_2 concentration demonstrated great importance. Without CO_2 replacement, the duckweed exposed to normal atmospheric $[CO_2]$ (380 ppm) consumed the gas quickly (6 hours) until reaching the limit (47 ppm). By contrast, the PBR with higher initial $[CO_2]$ (C3 - 100,000 ppm) presented a higher growth rate and, consequently, a higher nutrient removal rate. Additionally, starch accumulation was enhanced by approximately 150% under higher CO_2 concentrations, from 9.6 to 24.7%. These results might contribute to the understanding and improvement of photobioreactors designed to grow duckweed intending the wastewater treatment and bioethanol production.

6. ACKNOWLEDGEMENTS

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