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An abrupt CO_2-mediated decrease in pH affects growth rates, cellular features and the interspecific interaction of *Scenedesmus (Acutodesmus) obliquus* and *Cryptomonas pyrenoidifera*

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ABSTRACT

An abrupt CO_2-mediated decrease in pH affects growth rates, cellular features and the interspecific interaction of *Scenedesmus (Acutodesmus) obliquus* and *Cryptomonas pyrenoidifera*

The technologies of anthropogenic CO_2 mitigation, such as carbon capture and sequestration, may pose an environmental threat to aquatic systems. In a scenario of CO_2 leakage from a carbon capture and sequestration process, very low-pH values might be reached and could remain over time. The main objective of this study was to detect how an abrupt lowering of pH would affect the microalgae *Scenedesmus (Acutodesmus) obliquus* and *Cryptomonas pyrenoidifera* at physiological, morphological and population levels, and also see how these effects could lead to ecological consequences. Monospecific and mixed culture experiments were run according to this purpose over 14 days and at a pH of 6.5, controlled by CO_2 injection. An increased CO_2 concentration significantly enhanced the growth rate of both species and especially affected the cell size of *C. pyrenoidifera* in the monoculture. The total biovolume of *C. pyrenoidifera* was higher than the total biovolume of *S. obliquus* in the control treatment, although neither of the two species were dominant in the culture experiments. Granularity responded in different ways for the species studied, being statistically different within subjects in monospecific and mixed culture experiments. Only chlorophyll and granularity have been significantly correlated in the low pH of *C. pyrenoidifera* monoculture. Due to its ecological relevance, the decreased colony formation ability of *S. obliquus* under a high CO_2 concentration is highlighted.

Key words: CO_2 bubbling, Colony Formation, *Cryptomonas pyrenoidifera*, Flow Cytometry, *Scenedesmus (Acutodesmus) obliquus*

RESUMEN

***Un abrupto descenso de pH mediado por inyección de CO_2 afecta a la tasa de crecimiento, a las características celulares y la interacción interespecífica de* Scenedesmus (Acutodesmus) obliquus *y* Cryptomonas pyrenoidifera**

Las tecnologías de mitigación del CO_2 antropogénico, como la captura y secuestro de carbono, pueden constituir una amenaza ambiental para los sistemas acuáticos. En un escenario de fuga de CO_2 durante el proceso de captura y secuestro de este gas, se pueden alcanzar valores muy bajos de pH. El objetivo principal de este estudio fue estudiar cómo una bajada abrupta de pH afecta a nivel fisiológico, morfológico y poblacional a las microalgas Scenedesmus (Acutodesmus) obliquus *y* Cryptomonas pyrenoidifera*, y, cómo estos efectos podrían tener consecuencias ecológicas. De acuerdo con este propósito, se llevaron a cabo experimentos con cultivos monoespecíficos y también cultivo de mezcla de ambas especies durante 14 días a un pH 6.5, mediante inyección de CO_2. La exposición a altas concentraciones de CO_2 aumentó significantemente la tasa de crecimiento en ambas especies y alteró especialmente el tamaño celular de* C. pyrenoidifera *en el monocultivo. El biovolume total fue estadísticamente diferente entre individuos de la misma especie expuestos a condiciones control y de pH 6.5. El biovolumen total de* C. pyrenoidifera *fue mayor que el de* S. obliquus *en el control, aunque ninguna de las dos especies fue dominante (en biovolumen) en el experimento de mezcla de especies. La granularidad respondió de manera distinta en las especies estudiadas, aunque en ambos casos mostraron diferencias entre tratamientos. Solamente la clorofila y la granularidad han estado significantemente correlacionadas en el tratamiento a bajo pH del monocultivo de* C. pyrenoidifera*. Debido a*

su relevancia ecológica, es necesario destacar la disminución de la capacidad de formación de colonias de S. obliquus *bajo condiciones de altas concentraciones de CO_2.*

Palabras clave: *Burbujeo CO_2, Citometría de flujo,* Cryptomonas pyrenoidifera*, Formación de colonias,* Scenedesmus (Acutodesmus) obliquus

INTRODUCTION

It is expected that atmospheric carbon dioxide (CO_2) will double over the next one hundred years (Solomon *et al.*, 2007). Carbon capture and geological storage (CCS) technologies are among the carbon mitigation strategies being currently discussed (IPCC, 2015; Szalaj *et al.*, 2017). Many studies have been carried out regarding the risk of leakage from the delivery and geological storage of large volumes of CO_2 (e.g. Basallote *et al.*, 2012), where pH might be even lower than 4 pH units (IPCC, 2005) and cause potential environmental consequences. Such acidification might affect populations that inhabit the environment around the pipe where the leakage happens. Environmental impacts on aquifers and in their microbial community have been described (Morozova *et al.*, 2010), as well as hydro-chemical changes associated with the upward migration of stored CO_2 through faults, fractures and poorly sealed or abandoned wells into shallow drinking water aquifers (Yang *et al.*, 2014). The aquifer recharge-discharge is a relevant part of the hydrology of aquatic systems and CO_2 leakages could have consequences on them.

Microalgae are responsible for most primary production in aquatic ecosystems and fuel energy transfer for the rest of the food web of these ecosystems, including the microbial loop (Cairns *et al.*, 1992;). The pH of water affects algal growth and survival (e.g. Gensemer *et al.*, 2018). The first studies carried out in the last third of the twentieth century suggested that the high CO_2 concentration in the medium would reduce the efficiency in CO_2 fixation by microalgae (Werdan *et al.*, 1975; Coleman & Colman, 1981). Primary production is expected to be stimulated in the short-term by the CO_2 increase (Hein & Sand-Jensen, 1997), so that one of the most prominent concerns about increased CO_2 is that it would induce uncontrollable primary production (see the review by Riebesell & Tortell, 2011).

Elevated CO_2 concentration will result in a decrease in pH which might affect intracellular pH, altering many physiological and structural processes (Segovia *et al.*, 2018). Moreover, it is well documented that cells grown under high CO_2 conditions have a less developed or an undetectable pyrenoid (Miyachi *et al.*, 1986). Besides, Morita *et al.* (1998) demonstrated with non-pyrenoids algae that the presence of pyrenoids or the accumulation of RuBisCO in chloroplasts is not always essential for the carbon concentration mechanism (CCM). Therefore, physiological and fine structure properties of the cells could undergo changes in high CO_2 concentration. These changes in the inner features of the cell can be detectable by flow cytometry such as granularity (Shapiro, 1995). On the other hand, the reduction of colony formation ability in *S. obliquus* has been described under acidification adjusted with HCl- (Yang *et al.*, 2016) and CO_2-enhanced concentration (Huang *et al.*, 2017). This was also reported in the species *Botryococcus braunii* when the size of the colony suffers changes under high CO_2 concentration (Ge *et al.*, 2011). This inducible defence plays an important role in preventing two important ecological challenges faced by the microalgae, sedimentation and grazing rates (Verschoor *et al.*, 2004; Stap *et al.*, 2006). Thus, studies to reveal the effect of high CO_2 concentration on the colony formation of microalgae are necessary.

The working hypothesis is that a low pH, caused by a high concentration of CO_2 in the medium will affect microalgae at three levels: physiological, morphological and population levels (e.g. colony feature changes), that could lead to ecological consequences. In order to check this hypothesis, we developed laboratory-based manipulative experiments, which aimed to disclose the effects of low pH caused by an injection of CO_2 on the growth rates and cellular features in two microalgae used as experimental models. The selected endpoints were Cell density,

Growth rate, Cell size, Granularity, Single/colony cell percentage and Biovolume. The cosmopolitan freshwater microalga, *Scenedesmus (Acutodesmus) obliquus* (Turpin) Kützing (single cells and coenobia – *S. obliquus* from here) and *Cryptomonas pyrenoidifera* Geitler (single flagellated cells – *C. pyrenoidifera* from here) were used as testing organisms due to the fact they are easy to handle and culture under experimental conditions and to the existence of previous information. Both species are widely distributed around the world (Cepák *et al.*, 2007; Guiry & Guiry, 2018) and sometimes in coexistence, e.g. in a reservoir in Turkey (Sevindik, 2010) and in a subtropical lake in China (Chen *et al.*, 2008).

For this purpose, monospecific culture experiments and a competition experiment were carried out with both species for 14 days. The competition experiment tries to clarify the interspecific interaction under CO_2-mediated low pH. Species differences in responses could lead to a shift in the community with a great ecological concern.

Besides conventional light-inverted microscopy, flow cytometry is proposed as a potential tool for phytoplankton optical properties analysis in this context of carbon capture and sequestration environmental risk assessment, which allows rapid analyses of the growth rate and cell features and had previously been used in the monitoring of the physiological state of microalgal cells (Cid *et al.*, 1996).

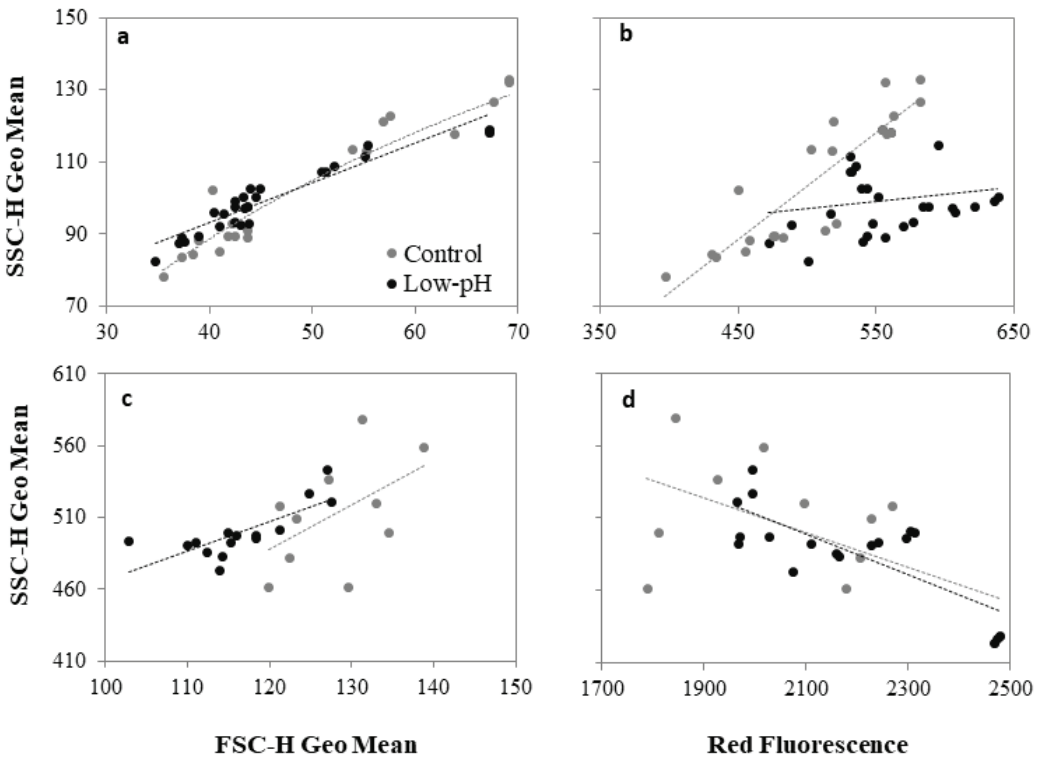

Figure 1. Correlations between: (a) FSC-H Geo Mean values (cells size) and SSC-H Geo Mean values (granularity); (b) Red fluorescence (PerCP-Cy5-5-H Geo Mean) values and SSC-H Geo Mean values (granularity) of *S. obliquus* in monoculture. (c) FSC-H Geo Mean values (cells size) and SSC-H Geo Mean values (granularity); (d) Red fluorescence (PerCP-Cy5-5-H Geo Mean) values and SSC-H Geo Mean values (granularity) of *C. pyrenoidifera* in monoculture. *Correlaciones entre: (a) Valores de FSC-H Geo Mean (tamaños celulares) y SSC-H Geo Mean (granularidad); (b) Valores de fluorescencia roja (PerCP-Cy5-5-H Geo Mean) y SSC-H Geo Mean values (granularidad) de* S. obliquus *en el monocultivo. (c) Valores de FSC-H Geo Mean (tamaños celulares) y valores de SSC-H Geo Mean (granularidad); (d) Valores de fluorescencia roja (PerCP-Cy5-5-H Geo Mean) y SSC-H Geo Mean (granularidad) de* C. pyrenoidifera *en el monocultivo.*

MATERIAL AND METHODS

Cultured species

S. obliquus (from Chemical Engineering Laboratory, University of Jaén, Spain) and *C. pyrenoidifera* (from Water Research Institute, University of Granada, Spain) were grown in 3N-BBM+V medium (recipe from CCAP, Scotland; pH corrected to 8.3-8.5 with NaOH before sterilisation by autoclaving). Cultures were maintained in a temperature-humidity-light controlled chamber at 20 ºC, supplied with a 12:12 h light cycle and there was no limitation of growth by light saturation (Sorokin & Krauss, 1958) with an irradiance of about 200 µmol photons $m^{-2} \cdot s^{-1}$ (Flöder *et al.*, 2006; Gris *et al.*, 2014).

CO_2 Laboratory-Based Manipulative Experiments

The experiments were carried out using a system of CO_2 bubbling into the water described in detail by Basallote *et al.* (2012). All the experimental vessels contained a pH sensor connected to the Aqua Medic AT Control System, which included the CO_2 bubbling pump as well as the air pump in order to decrease the rate of cell sedimentation. Before use, pH electrodes were calibrated, and the values obtained throughout the tests were regularly verified by a portable pH-meter (Crison GLP 22). CO_2 pumps were controlled by a solenoid valve that stops the CO_2 input when the system detected that the one-vessel pH had reached the established level. A computer connected to the AT control system permitted modification of the pH values (for more details, please see Fig. 1 on Bautista-Chamizo *et al.*, 2016: 957).

Three different experiments were set up, being (i) *S. obliquus* monoculture; (ii) *C. pyrenoidifera* monoculture; and (iii) *S. obliquus* plus *C. pyrenoidifera* mixed culture (competition experiment from here). All of them were carried out over a 14-day period. Samples from each vessel were monitored on Day 0 (inoculum day), 1, 2, 7, 10 and 14.

The same conditions of stock culture as mentioned above (Cultured species) were kept for the test and control vessels. Five replicated 500 ml vessels for low pH (pH 6.5) and four for control were set with an initial experimental density of approximately 2.0 $x10^5$ cells/ml *S. obliquus* and 2.0 $x10^4$ cells/ml *C. pyrenoidifera* in the monoculture, and 1.6 $x10^5$ cells/ml *S. obliquus* and 1.5 $x10^4$ cells/ml *C. pyrenoidifera* in the mixed culture. The difference between species' initial concentrations were chosen given their individual biovolumes (*C. pyrenoidifera* is bigger than *S. obliquus*), which calculations are explained below, so that the total biovolume for both species will be similar in each vessel. In addition, the initial densities are lower for each species in the mixed culture in order to prevent saturation.

The initial individual biovolume was calculated in order to find out the space occupied by each species and so to decide the initial density used for each species in the competition experiment, so saturation by one of them would be prevented. Several cells of each species were measured from photographs taken with a digital camera (Leica EC3) and then analysed using the software Image J (U. S. National Institutes of Health, Bethesda). Biovolumes were calculated following the equations proposed by Hillebrand *et al.* (1999) for a prolate spheroid shape. Individual biovolume was established according to the mean size of each species, as 104.7 μm^3 for *S. obliquus* and 1014.3 μm^3 for *C. pyrenoidifera*. Following the equation to estimate the biomass carbon content by Rocha & Duncan (1985), initially *S. obliquus* has a content carbon of 15.98 whereas *C. pyrenoidifera* has 173.82 pg C μm^{-3}.

In the competition experiment, initial densities were calculated following the total biovolume of each species (total biovolume is calculated by individual biovolume multiplied by the number of cells), so 50 % of the total biovolume contained in the vessels were occupied by each species. This calculation was also used to assess species displacement at the end of the experiments. Flow cytometric analyses were conducted immediately after sampling in order to avoid loss of fluorescence and damage in living cells. Flow cytometry settings were adapted to the sizes and fluorescence emission of *S. obliquus* and *C. pyrenoidifera*. Four different calibrating beads were used following Galotti *et al.* (2006). These beads

were Flow-Check™ and Flow-Set™, both from Beckman Coulter, Inc.; Yellow-Green Fluospheres from Molecular Probes and BD Calibrite™. The equipment utilised was a BD-LSR Fortessa, with slow flow (12 µl/minute) for 180 seconds while recording optical properties from each cell, focusing on forward scattering (FSC-channel related to cell size), side scattering (SSC-channel related to size and granularity) and red fluorescence (PerCP-Cy5-5-channel related to chlorophyll fluorescence). Cell densities were calculated from the nominal flow rate of the cytometer and the acquisition time. Data were acquired and analysed obtaining the geometric mean values (Geo-Mean; Campbell 1995) of each parameter from the different clouds. Abundance is calculated according to the number of points located on the species cloud, the time of sample acquisition and its flow velocity acquisition.

Single and colony formation has been measured using the dot clouds from flow cytometry. In addition, the approximate biovolume of *S. obliquus* events in the monoculture was calculated from the antilog transformation of the FSC-H channel. The actual value of FSC-H was multiplied by the number of events in their respective channel, and that value was designated as an approximate biovolume. In order to visualize the existence of internal biases in the dot clouds, the accumulated approximate biovolume was calculated.

Growth rate (GR) was also calculated after flow cytometric results following $GR=[lN(Nf)-lN(N_0)]/(lN_2\ (t-t_0))$, where GR is growth rate · day^{-1}, t_0 and t are the initial and final days in the study period, respectively, and N_0 and Nf are the cell density (cells/ml) at the initial and final days of study, respectively.

Samples were also taken for microscopy analysis, preserved in Lugol's solution (2 %) for the initial individual biovolume measurements (*S. obliquus* and *C. pyrenoidifera*) and the analysis of the percentage of single cells and colonies (just for *S. obliquus*). For the colony analysis, at least 200 cells were counted randomly on a Sedgwick Rafter Chamber under a Leitz DMIL inverted microscope (40x magnification). All the colonies found were counted as "one colony" independently of whether the colony (coenobia) was formed by two, four or eight cells.

Significant differences between treatments were tested by means of a repeated measures ANOVA and a DMS *post-hoc* test when suitable. Requirements for the analysis (normality and homoscedasticity) of variance were tested in all cases. Significance levels were tested at the $p = 0.05$ level. Both between-subject and within-subject were tested for significance. In addition, linear regression was tested for significance when required. Means are presented ± standard deviation (SD), where n = number of vessels for each experiment, as specified above unless otherwise stated.

RESULTS

In control treatments, the pH monitored by the system showed that the pH rose to values of 8.5-9.0 as a consequence of the algal metabolism. Treatment vessels with low-pH were maintained at pH 6.5 (±0.02) with controlled injections of CO_2 during the experimental period, and consequently no overlap occurred between treatments.

Results are organized in both tables and figures. Table S1 (*S. obliquus* monoculture), Table S2 (*C. pyrenoidifera* monoculture), Table S3 (*S. obliquus* competition experiment) and Table S4 (*C. pyrenoidifera* competition experiment) can be consulted on the supplementary information available at http://www.limnetica.net/en/limnetica. These tables present results of the proposed endpoints (cell density, growth rate, cell size, granularity, chlorophyll content, single/colony cell percentage and total biovolume) plus their SD for each day of analysis (day 0, 1, 2, 7, 10 and 14) under control and low-pH treatments.

S. obliquus monoculture

Algal cell density of *S. obliquus* increased during the experiment under low-pH and control treatment (See Table S1). Significant differences were observed between subjects (low-pH and control treatments; See Table 1). Cell density in low-pH treatment was higher than the control throughout the experimental period, starting from 22.7 % higher on day 1 up to 240.2 % on day 7, when the maximum difference was observed. The experiment finished with cell density in low-pH treatment being higher than the control at 27.6 % on day 14.

Table 1. Results of the repeated-measures ANOVA, using day in the experiment (time) as the within-subject factor and CO_2 treatment as the between-subject factor. Columns indicate the variables of interest, the degrees of freedom ($d.f._1$ and $d.f._2$), the value of the F-statistic (*F*) and the corresponding probability (*p*). *Resultados de ANOVA de medidas repetidas utilizando los días en el experimento (tiempo) como factores intra-sujetos y el tratamiento con CO_2 como factor entre-sujetos. Las columnas indican las variables de interés, grados de libertad ($d.f._1$ y $d.f._2$), el valor del estadístico F (*F*) y la probabilidad correspondiente (*p*).*

			$d.f._1$	$d.f._2$	*F*	*p*
S. obliquus	Cell Density	within-subject	1	7	73524.252	0.000
monoculture		between-subject	1	7	19.067	0.003
	Growth Rate	within-subject	1	7	132.898	0.000
		between-subject	1	7	9.642	0.017
	Cell Size	within-subject	1	7	1729693.215	0.000
		between-subject	1	7	72.979	0.000
	Granularity	within-subject	1	7	965490.399	0.000
		between-subject	1	7	3.277	0.113
	Colony formation	within-subject	1	14	17962.258	0.000
		between-subject	3	14	980.758	0.000
C. pyrenoidifera	Cell Density	within-subject	1	3	154090.764	0.000
monoculture		between-subject	1	3	2.577	0.207
	Growth Rate	within-subject	1	2	1459.294	0.001
		between-subject	1	2	10.742	0.082
	Cell Size	within-subject	1	3	1328801.449	0.000
		between-subject	1	3	41.449	0.008
	Granularity	within-subject	1	3	972096.446	0.000
		between-subject	1	3	0.930	0.406
S. obliquus	Cell Density	within-subject	1	5	176689.919	0.000
competition		between-subject	1	5	12.576	0.016
culture	Growth Rate	within-subject	1	5	625.275	0.000
		between-subject	1	5	23.128	0.005
	Cell Size	within-subject	1	5	7353.439	0.000
		between-subject	1	5	0.633	0.462
	Granularity	within-subject	1	5	536245.451	0.000
		between-subject	1	5	1.729	0.246
	Colony formation	within-subject	1	4	12697.355	0.000
		between-subject	3	4	1695.139	0.000
	Total Biovolume	within-subject	1	5	302929.250	0.000
		between-subject	1	5	12.576	0.016
C. pyrenoidifera	Cell Density	within-subject	1	5	90878.145	0.000
competition		between-subject	1	5	0.170	0.698
culture	Growth Rate	within-subject	1	5	104.789	0.000
		between-subject	1	5	3.224	0.133
	Cell Size	within-subject	1	5	143214.974	0.000
		between-subject	1	5	0.386	0.561
	Granularity	within-subject	1	5	555327.095	0.000
		between-subject	1	5	26.163	0.004
	Total Biovolume	within-subject	1	5	232660.812	0.000
		between-subject	1	5	0.221	0.658

S. obliquus growth rate (GR) in the monoculture was significantly higher in the low-pH than in the control treatment (See Table 1). The biggest difference between GR in low-pH and control treatments was seen on day 2 (See Table S1).

The biggest differences in cell size (FSC-H Geo Mean value) between treatment and control in monoculture were found on day 1 (See Table 1). However, no significant differences were found after 7 days of the experiment (See Table 1).

The oscillatory fashion of granularity was similar between the control and the low-pH treatments during the experimental period. Values of granularity were only higher in the control than in the low-pH treatment on days 1 and 2 (See Table S1).

In order to assess that granularity is related to changes in inner features rather than to cell size only, two correlations were performed. The first one between cell size (FSC-H geometric means) and granularity (SSC-H geometric means) (Fig. 1a), and the second between chlorophyll (PerCP-Cy5-5-H geometric mean = red fluorescence) and granularity (SSC-H geometric means) (Fig. 1b). The correlations between cell size and granularity in *S. obliquus* monoculture were similar for both the control and the low-pH treatments ($F_{1,19}$= 166.368; p= 0.000; r^2= 0.8986 for the control and $F_{1,25}$= 247.314; p= 0.000; r^2= 0.8947 for the low-pH treatment; Fig. 1a). There was a correlation between chlorophyll and granularity in the control treatment ($F_{1,18}$= 61.575; p= 0.000; r^2= 0.7789), while there was not under low-pH (p> 0.05; r^2= 0.0361) (Fig. 1b).

The percentage of colonies in relation to single cells of *S. obliquus* decreased in the low-pH treatments of monoculture (See Table S1) and was significantly different from control (See Table 1), indicating lower colony formation ability under 6.5 pH.

To confirm the low colony formation in low-pH treatment, flow cytometry cytograms were used. Figure 2 illustrates the response in

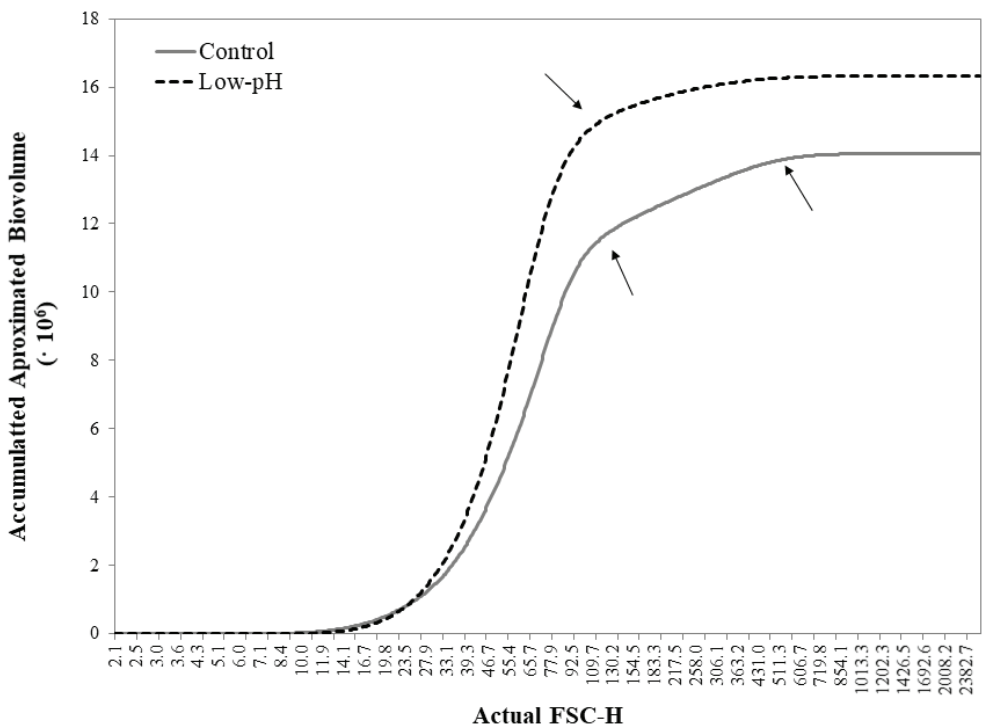

Figure 2. The accumulated approximated biovolume ($\mu m^3 \cdot 10^6$) by each FSC-H actual values (not channels from zero to 1024). Arrows highlight the inflection points on the curves. *El biovolumen aproximado acumulado ($\mu m^3 \cdot 10^6$) para cada valor real de FSC-H (no canales del cero al 1024). Las flechas apuntan los puntos de inflexión de las curvas.*

accumulated approximated biovolume (μm^3) in each treatment, considering the number of cells in each FSC channel multiplied by the calibrated size of each channel. As in the accumulative probability curves, this representation allows the detection of deviation from normal distributions in dense continuous dot clusters. The control curve showed two inflection points that corresponded to two subpopulations (singles and colony) within the cluster of this species. However, in the low-pH treatment the accumulated curve showed a single inflection point, at the level of the smaller subpopulation.

C. pyrenoidifera monoculture

C. pyrenoidifera had its growth stimulated in the low-pH. Cell density was significantly lower in control (p> 0.05; See Table 1) on day 14. By the end of the experiment, the cell density in the low-pH treatment recovery was 28.5 % higher than in the control on day 14 (Table S2).

The *C. pyrenoidifera* growth rate (d^{-1}) was not significantly different between treatments (See Table 1), and only slight differences were detected during the initial days (day 1 and 2; See Table S2).

Significant differences were seen on days 7, 10 and 14 when the cell size in the low-pH treatment was bigger than in the control (see Table S2; and Table 1 for significance levels).

C. pyrenoidifera granularity (SSC-H geometric mean) responded in a somewhat oscillatory fashion in the control and low-pH treatments, always in the contrary manner between treatments (See Table S2). Granularity was correlated with cell size and chlorophyll content (Fig. 1c and d, respectively). A significant negative correlation between granularity and red fluorescence (chlorophyll content) was only observed in the low-pH treatment ($F_{1,16}$= 22.323; p= 0.000; r^2= 0.6002).

Competition experiment

As expected, the cell density of *S. obliquus* and *C. pyrenoidifera* was significantly different between species throughout the experimental period, since the initial cell density was already different in order to accomplish similarities in total biovolume between species. However, only *S. obliquus* cell density was statistically different between treatments, low-pH and control (See Table 1). *S. obliquus* cell density was lower under the low-pH treatment of the competition experiment than in monoculture. Meanwhile, *C. pyrenoidifera* cell density under low-pH was similar in both experiments.

At the end of the competition experiment, *S. obliquus* growth rate was higher under low-pH than control but lower in both cases than those in the monoculture. Differences were significant both between and within subjects (See Table 1).

The *C. pyrenoidifera* growth rate was higher under low-pH than the control at the end of the competition experiment, being even higher than

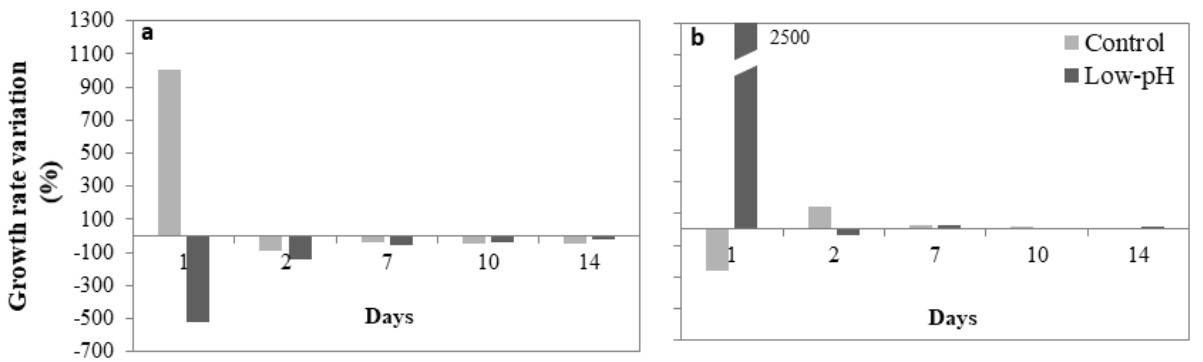

Figure 3. Growth rate variation (%) in the competition experiment in relation to the monoculture under low-pH and control treatments for *S. obliquus* (a) and *C. pyrenoidifera* (b). *Porcentaje de variación de la tasa de crecimiento en el experimento de competición en relación al monocultivo en el tratamiento con bajo pH y control para* S. obliquus *(a) y* C. pyrenoidifera *(b).*

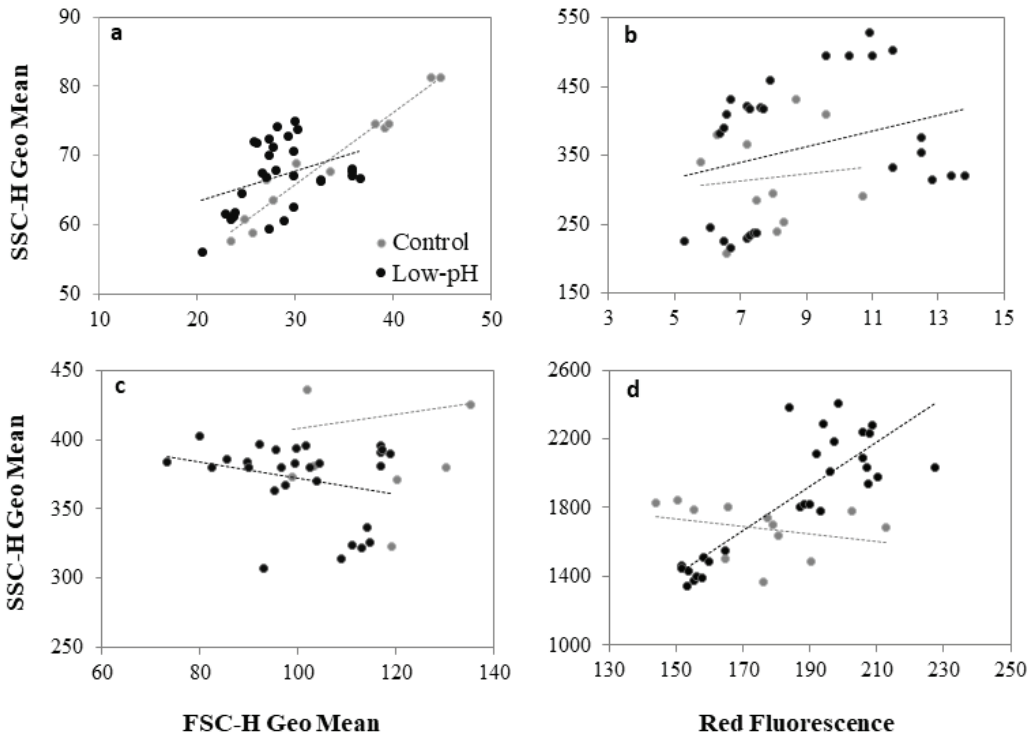

Figure 4. Correlations between: (a) FSC-H Geo Mean values (cells size) and SSC-H Geo Mean values (granularity); (b) Red fluorescence (PerCP-Cy5-5-H Geo Mean) values and SSC-H Geo Mean values (granularity) of *S. obliquus* in the competition experiment. (c) FSC-H Geo Mean values (cells size) and SSC-H Geo Mean values (granularity); (d) Red fluorescence (PerCP-Cy5-5-H Geo Mean) values and SSC-H Geo Mean values (granularity) of *C. pyrenoidifera* in the competition experiment. *Correlaciones entre: (a) Valores de FSC-H Geo Mean (tamaños celulares) y SSC-H Geo Mean (granularidad); (b) Valores de fluorescencia roja (PerCP-Cy5-5-H Geo Mean) y SSC-H Geo Mean values (granularidad) de* S. obliquus *en el experimento de competición. (c) Valores de FSC-H Geo Mean (tamaños celulares) y valores de SSC-H Geo Mean (granularidad); (d) Valores de fluorescencia roja (PerCP-Cy5-5-H Geo Mean) y SSC- H Geo Mean (granularidad) de* C. pyrenoidifera *en el experimento de competición.*

the growth rates in both treatments in the monoculture (See Table S2 and S4). Responses were significantly different among species and treatments on day 2, day 10 and day 14 ($p< 0.05$).

In order to compare the growth rates between monoculture and the competition experiment, the growth rate variation percentage was calculated (see Fig. 3). The highest percentage of variation was observed on the first day of experimentation for both species, *S. obliquus* (Fig. 3a) and *C. pyrenoidifera* (Fig. 3b). From the second day, the variation in *S. obliquus* remained negative in the control and the low-pH treatments. The variation percentage in *C. pyrenoidifera* remained positive from day 7.

The cell size of *S. obliquus* responded in an oscillatory fashion during the period of experimentation, ending larger than on the initial day. In addition, it was larger in the control than in the low-pH treatment (See Table S3 and Table 1).

In the competition experiment, the response of *C. pyrenoidifera* cell size observed between treatments was similar to monoculture, increasing both in the low-pH and control but higher in the low-pH treatment as well as in the monoculture (See Table S4).

S. obliquus granularity (SSC-H values) has shown the same pattern during the experiment under the low-pH and control treatments (See Table S3), while *C. pyrenoidifera* granularity was higher in the low-pH treatment than in the control treatment, especially on days 7 and 10 (See Table S4). The difference throughout the period of the experiment was significant for both species (See Table 1).

S. obliquus granularity was significantly correlated with cell size in control ($F_{1,11}$= 178.939; p= 0.000; r^2= 0.9471) (Fig 4a). However, there was no correlation between *S. obliquus* granularity and chlorophyll content in any of the treatments (Fig. 4b). In addition, no correlation was observed between *C. pyrenoidifera* granularity and cell size (Fig. 4c), but positive correlation was observed between *C. pyrenoidifera* granularity and chlorophyll content in the low-pH treatment ($F_{1,27}$= 64.244; p= 0.000; r^2= 0.7119) (Fig. 4d).

In the competition experiment, the percentage of colonies in relation to single cells of *S. obliquus* decreased in the low-pH treatment (See Table S3) and were statistically different than control (See Table 1). This response was similar to the monoculture experiment; however, the percentage of colonies in the control of competition experiment was much higher than in the control of the monoculture.

Tables S3 and S4 also show the total biovolume of both species in the competitive experiment. The total biovolume of both species under the low-pH treatment was significantly different on days 7 and 10 but was similar at the end of the experiment (Table 1). The total *C. pyrenoidifera* biovolume percentage in the low-pH treatment decreased throughout the last three days in comparison to its values in the control treatment (Table S4). The highest total biovolume of *S. obliquus* was observed on the last day (day 14, Table S3).

The slopes of biovolume percentage under low-pH and control were calculated in order to display the trend of *C. pyrenoidifera* displacement performed by *S. obliquus*. The slope values from day 7 to day 14 were 2.4534 (r^2 = 0.6028) and 0.7011 (r^2 = 0.083) for low-pH and control, respectively. Slopes were significantly different according to the statistical analysis (p< 0.001).

DISCUSSION

Scenedesmus obliquus grew more efficiently in the low pH treatment, while *C. pyrenoidifera* reached similar densities between treatments. Moheimani & Borowitzka (2011) found that the microalga *Pleurochrysis cearterae* seemed to act as a CO_2 user, which might be the case of *S. obliquus* in our study. On the one hand, the response of *S. obliquus* as well as *C. pyrenoidifera* under low pH was different in the monospecific and competition experiments. Specifically, in the presence of *C. pyrenoidifera*, *S. obliquus* reduced its growth rate, and it was even lower than those found by other authors (Yang & Gao, 2003) under similar CO_2 conditions. On the other hand, Low-Décarie *et al.* (2011) found that *S. obliquus* displaced the cyanobacteria under high CO_2 and within a mixed culture.

It is known that high CO_2 concentration will cause unprecedented changes, the consequences of which might be difficult to predict, since some organisms may respond positively while many others are likely to be at a disadvantage (Assunção *et al.*, 2017; Choix *et al.*, 2018). For example, the CCM differ among microalgae species (Rost *et al.*, 2003; Ainsworth & Long, 2005), especially concerning their efficacy for growing (Fu *et al.*, 2008; Kardol *et al.*, 2010), e.g. spending less energy on CCM that can be invested in growth processes (Giordano *et al.*, 2005). High CO_2 might also result in community shifts with deep ecological consequences for the whole system (Hutchins *et al.*, 2009). One of the most concerning effects in the early stages of a high CO_2 concentration in water bodies is eutrophication, being responsible for an ecological consequence over the long term. The results presented here demonstrate that *S. obliquus* reduced its growth capacities at the beginning of the competition culture under low pH; this could lead to changes in the community structure compared with the control condition. According to the resource competition theory, two species that are limited by two or more resources can coexist (Tilman, 1982); in a mixed culture at low-pH they would coexist in a somewhat oscillatory pattern (Verschoor *et al.*, 2013). Although the process of biodiversity loss under conditions of ocean acidification is well understood (i.e. Booth *et al.*, 2018) this is not the case for other water bodies.

In the present study, the two species studied changed their individual size in response to low-pH treatment. In the monoculture low-pH treatment, *S. obliquus* increased its cell size, whereas *C. pyrenoidifera* decreased its cell size under the same conditions. And in the competition

experiment, differences in cell size were also significant. The ability of *Scenedesmus* to change its cell size is well known (Trainor, 1998). Such changes in cell features could have greater consequences in higher hierarchical levels. Herbivore grazing would be promoted if cell size or induced colony formation are impaired. Besides, the ability of *S. obliquus* to form colonies has been depleted. The percentage of single-cells and colonies of *S. obliquus* were altered by low pH. *Scenedesmus* usually forms colonies of four or even eight cells (coenobia) as a strategy against grazing (see Lürling, 2003 for a review; Wu *et al.*, 2013). "This ability of a single genotype to produce one or more alternative forms of morphology in response to environmental conditions is termed *phenotypic plasticity*" (West-Eberhard, 1989) and, as aforementioned, operates such a protection against grazing for zooplankters (Wu *et al.*, 2013). Yang *et al.* (2016) found, in a study performed over 7 days, that *S. obliquus* colony formation induction is depleted under the pH from 5 to 9 adjusted with HCl. In this sense, CO_2-induced low pH likewise altered such functioning in the *S. obliquus* cells, somehow preventing colony formation and additionally, making the cells more exposed to the medium. This feature was easily detectable by flow cytometry also, and the decrease in colony formation remained throughout the 14 days of the experimental period, both in monoculture and in competition experiment. The reduction of colony formation under low-pH might be related to membrane transport processes (i.e. depolarization of the membrane potential in *S. obliquus* has been observed; unpublished data) and metabolic functions involved in algal cellular pH regulation (Jiang *et al.*, 2012). The colony formation ability was a suitable endpoint for *S. obliquus* as it shows a CO_2 exposure effect with ecological relevance. Without this defence mechanism, these microalgae might suffer a higher grazing rate, which could lead to a higher growth rate of the zooplankton, which in turn could lead to an imbalance in the trophic network. However, further research focused on how a CO_2-mediated low pH alters physiologically the colony formation mechanism should be conducted.

Granularity, measured with flow cytometry, has previously been used as an endpoint, i.e. Franqueira *et al.* (1999) found an increase in multivesicular bodies in the cytoplasm as a consequence of pesticide exposure. In this sense, Franklin *et al.* (2001) had already suggested that the light-scattering properties of two algal species (*Chlorella* sp. and *Phaeodactylum tricornutum*) could be an alternative as chronic test endpoints. The changes might be correlated with the complexity of the cytoplasm (e.g. Reader *et al.*, 1993), hence with photosynthetic structures such as chloroplasts. In this context, high levels of CO_2 enhance the production of drops of lipids (initially set to pH 6.5 in Cheng *et al.*, 2013) driven by increasing the flux of CO_2 to acetyl-CoA (acetyl coenzyme A) in microalgal chloroplasts (called "high doses" of CO_2 aeration 10 % (v/v) in Sun *et al.*, 2016). Although granularity was a suitable endpoint to *C. pyrenoidifera*, as a measurement of internal cellular changes, it was not to *S. obliquus*. The oscillatory fashion in the internal cellular granularity of *C. pyrenoidifera* might be also explained by the presence of different pigments in the Cryptomonads group, such as carotenoids (Chi c*2*) and cryptomonad phycoerythrin (Cr-PE565; Mimuro *et al.*, 1998) which could be altered by the high CO_2 concentration medium (for further information, see Rmiki *et al.*, 1999).

Apparently, following the biovolume percentage, neither of the species would displace the other. Although the results of our competition experiment have not provided sufficient evidence to state that the displacement of species will happen, if the tendency to increase the total biovolume percentage of *S. obliquus* would remain for a longer term, both eutrophication and displacement of cells might occur.

Amidst some limitations, the experiments could be used to check out the linkage between the physiological responses (endpoints) proposed and the response of microalgae species, in order to ascertain any shifts in the community dynamics that would be attributable to changes in CO_2 concentration, hence in pH. The studied endpoints: cell density, growth rate, cell size, granularity, single-cell/colonies percentage and total biovolume were suitable to show the effects of low pH on microalgae. For example, regarding cell size, although the size of *S. obliquus* decreased, its granularity in low-pH treatment

increased but not in response to an increment of chlorophyll content, contrary to what happens in *C. pyrenoidifera* under low-pH. It confirms the results taken by Yang & Gao (2003) when they found that the photosynthetic physiology of *S. obliquus* was affected by high CO_2 concentrations. *S. obliquus* physiology was not only affected in this way, because the CO_2-mediated low pH also minimizes its ability to form colonies.

Regarding flow cytometry, it responded as a potent tool capable of analysing thousands of cells in seconds. It was disclosed as a sensitive and rapid technique in the present study being determinant in providing quick and reliable results.

In conclusion, the elevated CO_2 concentration modified the growth dynamics and specific cell features in both species as well as the interaction between them.

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