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ARTICLE

Response to oxidative stress induced by high light and carbon dioxide (CO_2) in the biodiesel producer model *Nannochloropsis salina* (Ochrophyta, Eustigmatales)

Respuesta a estrés oxidativo generado por alta luz y dióxido de carbono (CO_2) en el modelo productor de biodiesel *Nannochloropsis salina* (Ochrophyta, Eustigmatales)

Karen Yangüez[1], Carlos Lovazzano[2], Loretto Contreras-Porcia[2,3] and Nicole Ehrenfeld[1,4]

[1]Universidad Santo Tomás, Escuela de Biotecnología. Ejército 146, Santiago, Chile
[2]Departamento de Ecología y Biodiversidad, Facultad de Ecología y Recursos Naturales, Universidad Andrés Bello, República 440, Santiago, Chile
[3]Center of Applied Ecology & Sustainability (CAPES), Pontificia Universidad Católica de Chile, Santiago, Chile
[4]Austral Biotech S.A., Ejército 146, 1er subterráneo Ed. C, Santiago, Chile. nicole.ehrenfeld@australbiotech.cl

Resumen.- Producto del agotamiento de los recursos mundiales de combustibles fósiles, las microalgas han tomado fuerza como alternativa de biocombustible. Buscando hacer sustentable el proceso, en general se propone realizar los cultivos acoplados a fuentes de emisión de CO_2, logrando con ello mayores rendimientos en biomasa y mitigando la huella de carbono de los procesos de combustión. *Nannochloropsis salina* es una microalga de la familia Monodopsidaceae de fácil crecimiento y que produce compuestos de valor comercial, tales como pigmentos esenciales, ácidos grasos poliinsaturados y alta cantidad de lípidos. Estudios previos muestran que en presencia de algunas concentraciones de CO_2 (hasta 2%) se produce un aumento de la biomasa y de la producción de ácidos grasos. Sin embargo, estas condiciones traen consigo una acidificación del medio, condición que afecta la eficiencia del proceso de fotosíntesis y promueve la generación de especies reactivas de oxígeno. En este trabajo, se estudió la respuesta antioxidante de cultivos de *Nannochloropsis salina* suplementados con CO_2, por medio de 3 metodologías: analizando la actividad enzimática antioxidante (catalasa, ascorbato peroxidasa y peroxirredoxina), cuantificando los compuestos fenólicos, H_2O_2 y lipoperóxidos (*i.e.,* marcador de daño celular) y evaluando los niveles de expresión génica. Los resultados sugieren que un aumento en la concentración de CO_2 en el cultivo, junto con alta luz, induce una condición de estrés oxidativo en *Nannochloropsis salina*. Sin embargo, la respuesta celular observada en esta microalga logra atenuar este estrés, sin afectar su rendimiento global.

Palabras clave: *Nannochloropsis salina*, especies reactivas de oxígeno, enzimas antioxidantes, dióxido de carbono, qPCR

Abstract.- Due to overconsumption of fossil fuels, microalgae have arrived as an alternative source of biofuel. Looking forward to generate a sustainable process, it is proposed to couple the cultures to CO_2 emission sources, reaching in this way higher biomass performance and helping in the way with the capture of carbon released by the combustion processes. *Nannochloropsis salina* is a microalgae from the Monodopsidaceae family, which is easy to grow and produces high value compounds like essential pigments, polyunsaturated fatty acids and high amounts of lipids. Previous studies showed that adding CO_2 to cultures (until 2%) generated an increment in biomass and in the production of fatty acids. However, these conditions also induce acidification of the media, a condition that may promote the generation of oxygen reactive species. In this work, the antioxidant performance of *N. salina* was studied under different culture conditions involving CO_2 through 3 different approaches: analysis of antioxidant enzymatic activities (catalase, ascorbate peroxidase and peroxiredoxine), analysis of gene expression and the quantification of H_2O_2, phenolic compounds and lipoperoxides (*e.g.*, cell damage marker). The results obtained suggest that an increase in the CO_2 concentration in the cultures (15,000 ppm), together with high light (1,000 μmol m^{-1} s^{-1}) induces an oxidative stress condition in *N. salina* cells. However, the antioxidant response observed in the microalgae manages to soften this stress, adapting themselves to these conditions without affecting their global performance.

Key words: *Nannochloropsis salina*, reactive oxygen species, antioxidant enzymes, carbon dioxide, qPCR

INTRODUCTION

Overconsumption of fossils fuels due to industrial activities has prompted the search for renewable and environmentally friendly energy sources. One promising alternative under consideration is microalgae-based biodiesel, since many algae species can produce large amounts of lipidic-storage products that can be easily

converted into biodiesel through chemical methods similar to those used with vegetable oil (Sheehan 2009). A potential oil production of over 100 tons/ha per year was initially predicted, but no large scale, long-term experiments have ever reached this high projection (Chisti 2007). When searching for alternatives to obtain greater biomass production together with environmental benefits, many models apply another advantage of microalgae – their ability to capture CO_2 from power or industrial plants (Chisti 2007). This ability has generated worldwide hope that microalgae cultivation for energy generation could contribute to a sustainable energy supply in the future helping to reduce CO_2 emissions.

The microalgae *Nannochloropsis salina* D.J. Hibberd (Ochrophyta, Eustigmatales) is one of the species that has been studied as a potential candidate for biodiesel production, and have been proposed as alternative sources for commercial production of eicosapentaenoic acid production (EPA, C20:5), a high-value omega-3 polyunsaturated fatty acid (PUFA) (Sukenik *et al.* 1989). Rodolfi *et al*. (2009) reported that several *Nannochloropsis* strains have a dry weight lipid content of 30% or higher and lipid productivity ranging from 55 to 61 mg L^{-1} day^{-1}. These factors make these strains some of the best lipid producers among 30 marine and freshwater microalgae in terms of both lipid content and lipid productivity (Rodolfi *et al*. 2009). At present, *Nannochloropsis* have been successfully grown in indoor and outdoor systems for biodiesel or biomass production, as feeding source for rotifers and for creating a 'green-water effect' in fish larvae tanks (Rodolfi *et al.* 2009, Moazami *et al.* 2012, Quinn *et al.* 2012).

During excess light conditions, increased production of damaging reactive oxygen species as byproducts of photosynthesis has been described for algae and plants (Anderson *et al*. 1995). For *Nannochloropsis*, various aspects of the high light response have been investigated so far, including changes in pigmentation and ultrastructure (Sukenik *et al*. 1989, Fisher *et al*. 1996, Fisher *et al*. 1998), the xanthophyll cycle (Gentile & Blanch 2001), lipid composition (Sukenik *et al*. 1993), non-photochemical quenching system (NPQ; Cao *et al*. 2013) and the photosynthetic response (Fisher *et al*. 1996, Tamburic *et al*. 2014). Evidences suggests that all these responses together give to *Nannochloropsis* a high capacity for handling conditions of excess light.

In general for autotroph algae like *Nannochloropsis*, increasing CO_2 inputs contribute to higher biomass productivity (Chisti 2007). However, it has been seen that the growth of most microalgae is generally inhibited under high concentrations of CO_2 (> 5%; Solovchenko & Kozhin-Goldberg 2013). Optimal CO_2 supply for saturation of microalgal growth has been proposed as approximately 5% in the unicellular green alga *Chlorella* (Nielsen 1955) and 2% for *Nannochloropsis oculata* (Chiu *et al*. 2009). It was described that high-CO_2 conditions produces inhibition of photosynthesis, which could be a consequence of inactivation of the key enzymes of the Calvin cycle due to acidification of the stromal compartment of the chloroplast (Krause & Weis 1991). *Chlorococcum littorale* cells growing in a range of CO_2 concentrations from 1 to 40% CO_2 showed a drop in intracellular pH within 1 h at 40% CO_2 (Satoh *et al*. 2002). Also other studies have suggested that the transference of microalgae culture to a higher CO_2 condition affects photosynthetic apparatus (Sergeenko *et al*. 2000), increasing cyclic electron transport over photosystem I, to facilitate generation of ATP necessary for pH homeostasis in the algal cell (Miyachi *et al.* 2003). In addition, the effect of CO_2 is species dependent, since elevated CO_2 levels accompanied with high light induced photo-inhibition in sensitive species like *Chlamydomonas reinhardtii*, but not in CO_2 tolerant species like *Chlorella pyrenoidosa* (Yang & Gao 2003). Therefore, current evidence strongly suggests that high CO_2 adaptation is a complex process involving adjustment of numerous functions of microalgal cells and with several mechanisms specific for each microalgae.

For other types of cells, for example for human neutrophils, increases in CO_2 concentration (from 1 to 10%) are known to affect several cellular reactions, leading, for example, to increased intracellular oxidative stress by means of reactive oxygen species (ROS) (Coakley *et al.* 2002, Abolhassani *et al.* 2009, Schwartz *et al.* 2010, Visca *et al.* 2002, Karsten *et al.* 2009). For microalgae, only a recent publication has evaluated ROS production related to an increase in CO_2 concentration. In this report, studies in *Dunaliella tertiolecta* suggest that intermediate concentration of CO_2 (0.1% CO_2) would protect this unicellular chlorophyte from high light and UV stress, increasing productivity, diminishing ROS accumulation and DNA damage (García-Gómez *et al*. 2014). However, in the same work the authors discussed the specificity of this response, which depends on the specie and the CO_2 concentration, since for diatoms CO_2 addition lead to a decrease in productivity (Gao *et al.* 2012).

High levels of ROS are responsible for abnormal physiological reactions, consequentially leading to a condition of oxidative stress (Mittler 2002). This physiological state occurs when the levels of ROS exceed the buffering capabilities of cells, causing the oxidation of important macromolecules (Foyer & Noctor 2009). The most common ROS are superoxide anion (O_2*), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO*) (Imlay 2008). Several studies, both in macro- and microalgae have demonstrated the importance of antioxidant enzymes, including ascorbate peroxidase (AP) and catalase (CAT), to cope with the effects of ROS (*e.g.*, Randhawa *et al.* 2001, Contreras *et al.* 2007, Contreras-Porcia *et al.* 2011a, b). Besides, water soluble phenolic compounds, like flavonoids, phenylpropanoids, tannins and other substances containing aromatic rings and hydroxyl groups, have been reported as scavengers of ROS in plants (Michalak 2006).

External inputs of CO_2 affects the equilibrium between CO_2 concentration dissolved in aqueous solution (dCO_2) and the partial pressure of atmospheric CO_2 (pCO_2). The dCO_2 dissociates into bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) and these three species of dissolved inorganic carbon (DIC) attain equilibrium at a fix ratio depending on pH, ion concentrations and salinity (Falkowski & Raven 1997). Interestingly, under CO_2 exposure it has been shown that the oxidation of biomolecules such as proteins and lipids is dependent on the presence of the bicarbonate ion (HCO_3^-) (Berlett *et al.* 1990, Stadtman & Berlett 1991, Hug & Leupin 2003) through the generation of the carbonate radical (CO_3*$^-$). Thus, CO_2 enrichment increase the CO_3*$^-$ concentration, which is a potentially toxic radical generated by the reaction between HCO_3^- or CO_3^{2-} and HO* (Augusto *et al.* 2002, Medinas *et al.* 2007).

It has been proposed that *Nannochloropsis* fix carbon principally as bicarbonate ion (HCO_3^-) (Sukenik *et al.* 1997, Huertas *et al.* 2000a). Also it has been shown that *N. oculata* is affected by concentrations of CO_2 higher that 2% (Chiu *et al*. 2009). Thus, based on these previous observations, we wanted to evaluate whether supplementary CO_2 and high light would have a direct effect on the physiological antioxidant mechanisms of *N. salina* and its performance for biodiesel production.

Materials and methods

Culture conditions

Nannochloropsis salina was obtained from the library of algae CSIRO (Australian Scientific and Industrial Research Organization), Australia. *N. salina* cells were grown photoautotrophically in artificial seawater (Goldman & McCarthy 1978) supplemented with f/2 medium (Guillard & Ryther 1962) under 20°C, 90 rpm agitation and illuminated continuously with 60-80 μmol m^{-2} s^{-1} blue light.

Four treatments, involving *N. salina* cultures with $1x10^6$ to $1x10^7$ cells mL^{-1} grown at 20ºC, were started in the same moment. The cultures were first placed 4 h in Dark, then 2 h in High Light with atmospheric CO_2 and finally 2 more hours in High Light with: atmospheric or High CO_2. Samples were taken after: i) 4 h in dark (DARK) (cultures without light and flasks covered with aluminum foil), ii) 4 h in dark plus 2 hours in high light and atmospheric CO_2 (HL; 1,000 μmol m^{-2} s^{-1} light intensity), iii) 4 h in dark plus 4 h in high light and 4 h in low CO_2 (HLLC, HL low CO_2; 300 ppm; 0.03%) and iv) 4 h in dark plus 4 h in high light and 2 h in high CO_2 (HLHC, HL High carbon dioxide, HC; 15,000 ppm, 1.5%). All experiments were performed in triplicate (n= 3) and CO_2 was added through bubbling at a rate of 1 v/v and its concentration in the line was confirmed through an infrared sensor (CO_2 Sensor OEM Gascard NG, Edimburg Instruments). This pre-acclimation allowed us to compare dark *versus* high light and atmospheric CO_2 *versus* high CO_2 under high light.

pH measurements

pH was measured at room temperature using a HI8424 microcomputer pHmeter (Hanna Instruments, Woonsocket, RI, USA) in 10 mL of culture medium after each experimental treatment.

Quantification of reactive oxygen species (ROS)

ROS were determined by incubating $2\text{-}5x10^7$ cells of *N. salina* in each condition in 30 mL culture medium supplemented with 5 μM, 2', 7'-dichlorodihydrofluorescein diacetate (DCHF-DA, Calbiochem, San Diego, USA) for 1 h at room temperature. Algal cells were centrifuged at 5,000 *g* for 5 min, the supernatant discarded and the pellet rinsed in seawater mixed with PBS (50:50 v/v). The rinsed pellet was resuspended in 1 mL of 40 mM Tris-HCl pH 7.0 and frozen in liquid nitrogen. The cells were homogenized boiling them for 10 min, with repeatedly vortexing. The homogenate was centrifuged at 16,100 *g* for 15 min and the supernatant was recovered. Fluorescence of the clear extract was determined in a Modulus Single Tube spectrofluorometer (Turner Biosystems, Inc., Sunnyvale, CA, USA) using Blue module (excitation wavelength of 460 nm and emission wavelength in range of 515-570 nm).

Detection of lipoperoxides

Lipoperoxide levels were determined as thio-barbituric acid reactive species (T-BARS) according to Ratkevicius *et al.* (2003) with the following modifications: pellets cells (*ca.*, $5x10^8$ cells) were directly homogenized in a mortar using pestle during 5 min and centrifuged at 7,690 *g* for 15 min at 4°C.

Detection of total Phenolic compounds

Microalgae samples were homogenized in mortar using a pestle. A total of 3 mL of 0.1 M phosphate buffer pH 7.0 was added during the homogenization. The homogenate was centrifuged at 12,800 *g* for 15 min. Aliquots of 100 µL were added to a reaction mixture containing 20% of sodium carbonate and 0.3 M Folin-Ciocalteau reagent in a final volume of 1 mL. This reaction was incubated for 2 h at room temperature, and the absorbance was determined at 765 nm (Contreras *et al.* 2005).

Protein extraction

Protein extracts were obtained as described by Contreras *et al.* (2005) with the following modifications: pellets cells (*ca.*, $5x10^8$ cells) were directly homogenized in a mortar using pestle during 5 min and centrifuged at 7,690 *g* for 30 min at 4°C. Proteins were precipitated by addition of 0.5 g of ammonium sulfate per mL and centrifuged at 7,690 *g* for 1 h at 4°C. The supernatant was discarded and was added 300 µL of 0.1 M phosphate buffer pH 7.0, containing 5 mM 2-mercaptoethanol and centrifuge for 1 h. The protein pellet was dissolved in 150 µL of 0.1 M phosphate buffer pH 7.0, containing 2 mM 2-mercaptoethanol. Protein concentration was obtained using the bicinchoninic acid assay (Smith *et al.* 1985).

Detection of antioxidant enzymes activities

The catalase (CAT) activity was determined as described by Contreras *et al.* (2005). The reaction contained 0.1 M phosphate buffer pH 7.0 and 14 mM H_2O_2. After the addition of H_2O_2, its consumption was determines at 240 nm for 2 min and the activity was calculated using the extinction coefficient of H_2O_2 (ε= 39.4 mM^{-1} cm^{-1}). For AP determination, the reaction mixture contained 0.1 M phosphate buffer pH 7.0, 800 µM ascorbate (ASC) and 16 mM H_2O_2. After the addition of ASC, its consumption was determined at 290 nm for 1 min and the activity was calculated using the extinction coefficient of ASC (ε= 2.8 $mM^{-1}cm^{-1}$). Finally, the peroxiredoxine (PRX) activity was measured as described by Lovazzano *et al.* (2013). Briefly, PRX activity, using dithiotreitol (DTT) as reducing agent (TDP/DTT), was determined preincubating 50-100 µg of protein extract with DTT 0.2 mM in phosphate buffer 0.1 M pH 7.0 for 30 min at 37°C. Reaction was initiated by adding 50 µM H_2O_2 to the protein extract, and incubated for 30 min at 37°C. Reaction was stopped by adding trichloroacetic acid (10% final concentration) and centrifuged at 18,700 *g* for 10 min to precipitate the proteins. 700 µL aliquot of the supernatant with the remaining peroxide was mixed with 200 µL of $(NH_4)_2$ $Fe(SO_4)$ 10 mM and 100 µL of KSCN 2.5 M. Peroxide concentrations were spectrophotometrically determined at 480 nm, using a spectrophotometer UV/Visible SmartSpec 3000 (BioRad, Laboratories, Inc., USA).

RNA extraction and cDNA synthesis

Cells in early exponential phase ($1x10^7$ cells mL^{-1}) exposed to the different experimental conditions, were collected through centrifugation at 4ºC and the pellet was immediately frozen in liquid nitrogen until RNA extraction. RNA extraction was performed using NAES buffer (50 mM NaOAc, 10 mM EDTA, 1% SDS) and acid phenol in 50:50 vol/vol. Samples were homogenized using a Bead Bug Microtube homogenizer (Benchmark Scientific, Inc.) at 4,000 *g* for 60 s. The plastic tubes were than incubated for 5 min at 65°C and placed back in the Bead Bug for 30 s at 4,000 *g* and incubated at 65°C for 5 min. After this time, the tubes were incubated on ice for 5 min and centrifuged at 16,000 *g* for 7 min. An equal volume of phenol acid was incorporated and centrifuged at 16,000 *g* for 7 min at 4°C. The supernatant was recovered, and equal volume of phenol:chloroform was added to the tube and centrifuged at 4°C for 10 min at 16,000 *g*. Posteriorly, 0.1 volume of 8 M LiCl and 2.5 volume of absolute ethanol was added and the tube was incubated for 30 min at -80°C and centrifuged at 4°C for 15 min at 16,000 *g*. A last 70% ethanol wash was used. The pellet was resuspended in 50 µL of water treated with diethylpyrocarbonate (DEPC). For cDNA synthesis, Reverse Transcripts ImProm-II System (Promega) was used following manufacturer's instructions.

Quantitative real-time PCR (qPCR)

PCR products were generated for the genes of interest: catalase, *cat* and peroxiredoxin, *prx* by amplifying cDNA prepared as described above. Primers were design using Primer3 free software (primer3.ut.ee) using as gene reference *N. salina* EST's obtained in a previous

transcriptomic study (Loira *et al*., in progress). All quantitative PCR reactions were carried out on the Roche LightCycler 1.5 instrument. qPCR was conducted using SYBR® Green fluorescence Master mix Brilliant II (Agilent Technologies, Inc.) according to the manufacturer's instructions, in 10 μL reactions containing 2.5 μL of diluted cDNA and 0.5 μL each of forward and reverse primer (Table 1) using the following cycling program: 95°C for 10 min followed by 40 cycles of 94°C for 10 s, 60 and 58°C for 20 s and 72°C for 10s. To correct for differences in RNA starting material and variations in cDNA synthesis efficiency, the abundance of each transcript was normalized to the abundance of the transcripts of gene α-tubulin (copies transcripts of interest/copy α-tubulin) used as housekeeping (Cao *et al*. 2012). Amount of transcripts were estimated using previously quantified copies of PCR amplicon diluted in cDNA mix.

Cellular viability effects

To evaluate the effects on cell viability under different culture conditions, microalgae samples were stained with Trypan blue 0.4% (LifeTechnologies, Thermo Fisher Scientific, Inc.) and incubated at room temperature for 3 min after the culture time exposure (Strober 2001). Images were visualized in an optical microscope coupled to a Moticam camera, and analyzed using Motic Images Plus Version 2.0© software (Motic China Group, Ltd. Hong Kong, China), where dead cells are shown with a distinctive blue colour, checking around 40 cells in each of the 5 replicates for each treatment.

Statistical analysis

Data were analyzed using MINITAB software (Minitab Inc., State College, PA). Cell densities were expressed as the mean value ± standard deviation (n= 3, different cultures). Comparisons between treatments were determined by analysis of variance (ANOVA), followed by Tukey's multiple comparisons test (*T*) for all parameters measured. Prior to the statistical analysis, data were checked for variance homogeneity using Levene's test and for normal distribution using Kolmogorov-Smirnov test (Zar 2010).

Results

The functional responses to oxidative stress induces by different culture conditions in *Nannochloropsis salina* was studied by monitoring and recording: (i) pH; hydrogen peroxide accumulation and oxidation of biomolecules by means of lipoperoxide quantification; (ii) attenuation of ROS over-production by the quantification of antioxidant enzymes activity and phenolic compounds; (iii) transcript accumulation of antioxidant enzymes; and (iv) cellular alteration through cell viability using Tripan blue staining.

Effects of intense light and high CO_2 on pH, ROS production and lipoperoxide levels

Intense light (1,000 μmol m^{-2} s^{-1} light) and high CO_2 (1.5% or 15,000 ppm) both affected the pH of *N. salina* culture. After 4 h cultures in the dark had values of pH 8.2 ± 0.02, while cultures kept in HL condition had pH 9.2 ± 0.05.

Table 1. Sequences of primers used for qPCR analysis in *Nannochloropsis salina* under different culture conditions / Secuencia de los partidores usados para los análisis de qPCR en *Nannochloropsis salina* bajo distintas condiciones de cultivo

Gene Target	EC or KOG reference	Primer Name	Sequence 5'-3'
Catalase	EC 1.11.1.6	CAT-F	CGCACCAAGTTTCCTTTCTC
		CAT-R	GATCCCCTGGTCTGTCTTGA
Peroxiredoxin	EC 1.11.1.15	PRX-F	TGCAGTTACTCTCCGACGTG
		PRX-R	CTTCTGGTCCTTCCCAATCA
Alpha tubulin	KOG1376	TUA-F	GCCTGCTGCCTCATGTACC
		TUA-R	GTTGATGCCGCACTTGAAG

Also, we determined that cultures in HLLC had pH 9.4 ± 0.06 and cultures in HLHC had pH 6.5 ± 0.004.

ROS production found maximum levels under intense light and high CO_2 exposure (HLHC, Fig. 1A), reaching twice times values than those recorded during Dark exposure (T= 8.80, P < 0.001). After 2 h high light conditions (HL), the levels of ROS in *N. salina* cells decreased, however after 4 h of high light (HLLC), ROS production was similar to cultures kept in Dark (T= 0.71, P= 0.996) (Fig. 1A).

Intense light and high CO_2 (HLHC) also had significant effects on membrane oxidation in *N. salina*, as determined by the quantification of lipoperoxides (LPX). Although membrane oxidation increased under intense light as compared to Dark, it was not statistically significant (Fig. 1B). Instead, under intense light and high CO_2, LPX levels

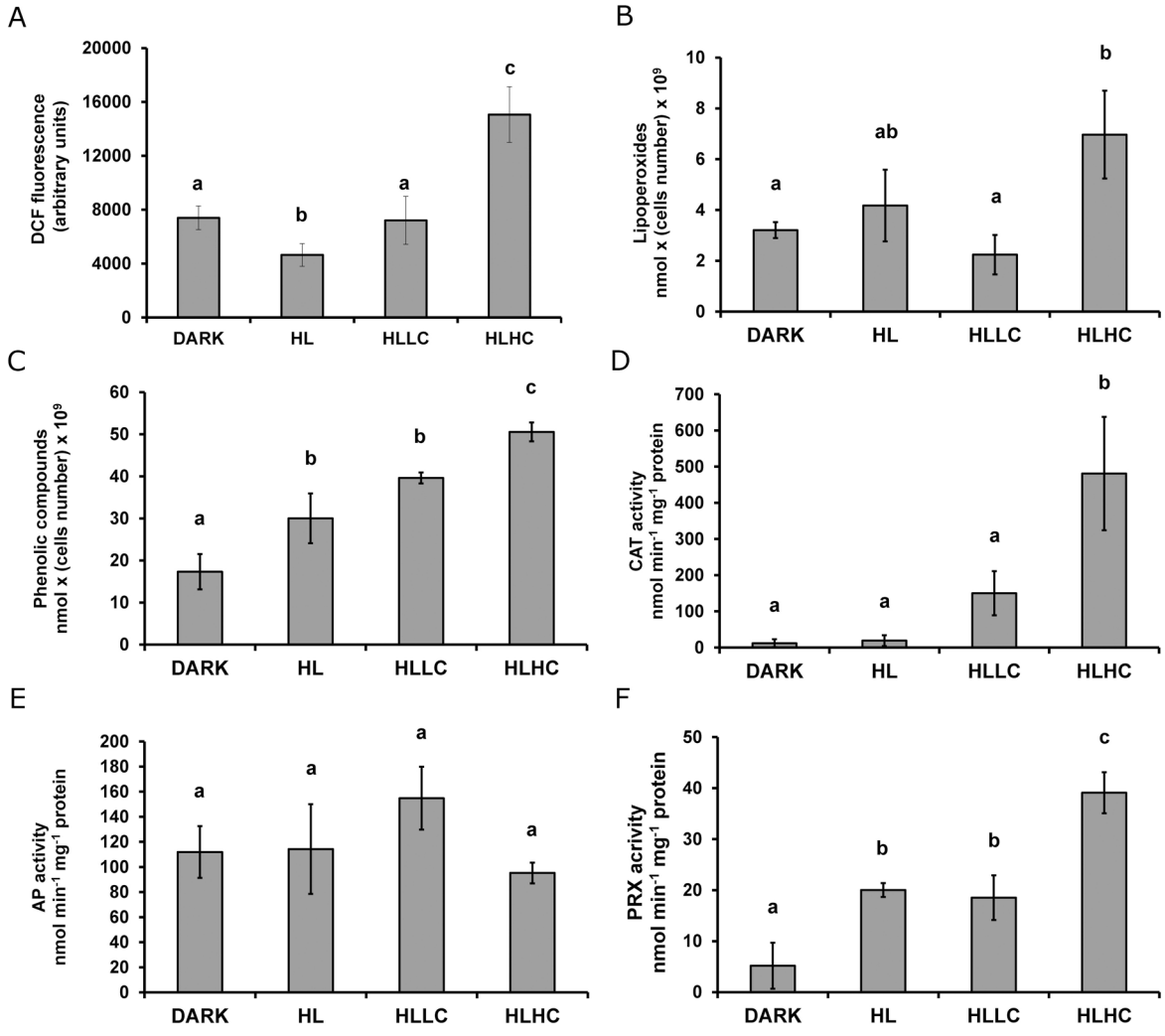

Figure 1. Oxidative stress and antioxidant response in *Nannochloropsis salina* under diverse culture conditions: DARK; high light (HL); high light and low CO_2 (0.03%) (HLLC) and high light and high CO_2 (1.5%) (HLHC). (A) ROS formation, (B) lipid peroxidation, (C) phenolic compounds concentration, and specific activity of the antioxidant enzymes catalase (CAT, D), ascorbate peroxidase (AP, E) and peroxiredoxine (PRX, F). Values are mean ± SD of 3 replicates. Letters above histograms indicate results of Tukey tests; means with the same letter are not significantly different at P= 0.05 / Estrés oxidativo y respuesta antioxidante en *Nannochloropsis salina* bajo diversas condiciones de cultivo: Oscuridad (DARK); alta luz (HL); alta luz y bajo CO_2 (0,03%) (HLLC) y alta luz y alto CO_2 (1,5%) (HLHC). (A) formación de EROs, (B) peroxidación lipídica, (C) concentración de compuestos fenólicos, y actividad específica de las enzimas antioxidantes catalasa (CAT, D), ascorbato peroxidasa (AP, E) y peroxiredoxina (PRX, F). Valores promedios ± DE de 3 réplicas. Las letras sobre los histogramas indican resultados de análisis de Tukey; promedios con la misma letra no son diferentes significativamente a P= 0,05

were more than 3 times higher than the basal levels (T= 3.86, P= 0.020). Thus, a condition of stress was principally triggered under high light and high CO_2 in *N. salina*, however we currently cannot separate the effect of HL from the effect of HC.

Antioxidant responses under intense light and CO_2

Subsequently, the accumulation of phenolic compounds in *N. salina* cultures was evaluated. Intense light and high CO_2 (HLHC) exposures induced 2.8 times higher levels of phenolic compounds than those observed in the basal condition (T= 10.58, P < 0.001) (Fig. 1C). HL condition also increased accumulation of phenolic compounds (T= 7.10, P < 0.001), but in lesser extent than the condition with high CO_2 (Fig. 1C).

In regards to the antioxidant enzymes activity, CAT was significantly induced under conditions of maximum stress: intense light and high CO_2 (HLHC) (T= 6.79, P < 0.001), going from 20 nmol min^{-1} mg^{-1} of protein in darkness to 500 nmol min^{-1} mg^{-1} of protein under intense light and high CO_2 (Fig. 1D). AP activity was not modified in any of the cultures evaluated (in all cases P > 0.05), with results ranging between 100-150 nmol min^{-1} mg^{-1} of protein (Fig. 1E). Finally, PRX activity increased for all culture conditions in comparison with the basal condition in darkness (in all cases P < 0.05). A maximum PRX activity of 40 nmol min^{-1} mg^{-1} of protein was detected under intense light and high CO_2 exposure (Fig. 1F). Therefore, the accumulation of water- and lipid-soluble compounds together with an increase in the activity of enzymes with antioxidant functions revealed an active antioxidant response to high CO_2 and high light in *N. salina*. Interestingly, the increase in the antioxidant response was specific, because apparently the activity of AP was not affected by the culture conditions evaluated.

Effects of CO_2 on the expression profile of genes coding for stress related enzymes

The expressions of genes coding for catalase (*cat*) and peroxiredoxin (*prx*) in *N. salina* were analyzed using qPCR in samples of cells grown in different CO_2 and light conditions and normalized against the housekeeping gene α-tubulin (*tubA*, Cao *et al*. 2012). The results showed that the expression of *cat* was significantly induced only under intense light and high CO_2 (T= 12.06, P < 0.010), with a slight increase of *cat* mRNA under intense light (Fig. 2A), which was not significant (T= 1.57, P= 0.443). On the other hand, *prx* gene showed low levels of absolute expression (data not shown) and also low levels of relative expression under all conditions evaluated, with a major expression when extra CO_2 was included in the system (T= 20.935, P < 0.001) (Fig. 2B). These results reveal that higher levels of CO_2 trigger an active transcriptional response in *N. salina* cells.

Cellular viability under oxidative stress conditions

Our results regarding ROS production, lipoperoxide and antioxidant levels in *N. salina* suggest that intense light

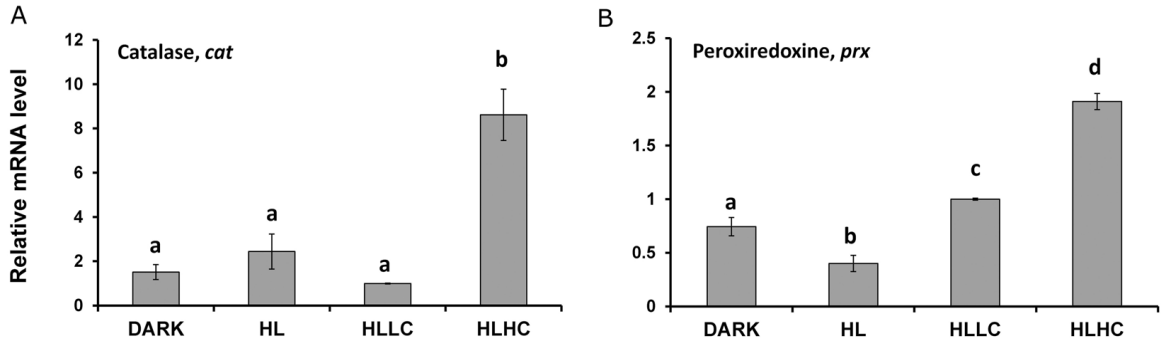

Figure 2. Gene expression changes in *Nannochloropsis salina* measured using qPCR under diverse stress conditions: Dark; high light (HL); high light and low CO_2 (0.03%) (HLLC) and high light and high CO_2 (1.5%) (HLHC). (A) catalase, *cat* and (B) peroxiredoxine, *prx*. Values are mean ± SD of 3 replicates. Letters above histograms indicate results of Tukey tests; means with the same letter are not significantly different at P= 0.05
I Cambio en la expresión génica de *Nannochloropsis salina* a través de qPCR bajo diversas condiciones de estrés: Oscuridad (DARK); alta luz (HL); alta luz y bajo CO_2 (0,03%) (HLLC) y alta luz y alto CO_2 (1,5%) (HLHC). (A) catalasa, *cat* y (B) peroxiredoxina, *prx*. Valores promedios ± DE de 3 réplicas. Las letras sobre los histogramas indican resultados de análisis de Tukey; promedios con la misma letra no son diferentes significativamente a P= 0,05

and high CO_2 induced an oxidative stress condition, which could affect the cellular viability of this specie. With the purpose of testing the viability of *N. salina*, cells grown under the same culture conditions used previously were stained with Tripan blue. It has been demonstrated that only dead or damaged cells are stained by Tripan blue with this method (Strober 2001). As shown in Fig. 3, *N. salina* cells were not stained by Tripan blue for any of the evaluated conditions as compared to the positive control, where cells were exposed to a high temperature (*ca.*, 100°C) (Fig. 3E). These results provide evidence that *N. salina* cells keep their integrity and viability under intense light and high CO_2 exposure.

Discussion

The results presented in the current study suggest that high light together with high CO_2 (HLHC) bring about a stressed state in *Nannochloropsis salina* cells. However, the stress condition is not sufficiently damaging to affect cell viability and biomass accumulation. ROS production and antioxidant response stimulation was significantly evidenced in high light and high CO_2, compared with conditions of darkness (no light, ambient CO_2) and with cultures kept in high light but in ambient CO_2 (HL and HLLC). Our results also demonstrate that *N. salina* was able to acclimatize to the conditions evaluated, since no cell death was observed in any tested condition, which agrees with the fact that all cultures continue growing after finishing the tests (data not shown).

The addition of CO_2 in the *N. salina* culture medium induced an acidification of the medium reaching a pH 6.5 ± 0.004. Under this condition, a reduced efficiency of bicarbonate uptake in *N. salina* cultures may happen. Sukenik *et al.* (1997) reported for *Nannochloropsis* sp. cultures that under regular light conditions, the maximal photosynthetic rate was hardly affected by raising the pH from 5.0 to 9.0, and that $K_{1/2}$ ($CO_{2(aq)}$) was not significantly affected by raising the alkalinity in the interval of pH 6.5-9.0. It can therefore be inferred that the differences in pH may not cause a reduction in the efficiency of bicarbonate uptake in *N. salina* cultures. Nonetheless, Gentile & Blanch (2001) reported that pH reduction might affect xanthophyll cycle activity and, as a consequence, an increase redox pressure could be caused by high light. On the other hand, Ezraty *et al.*

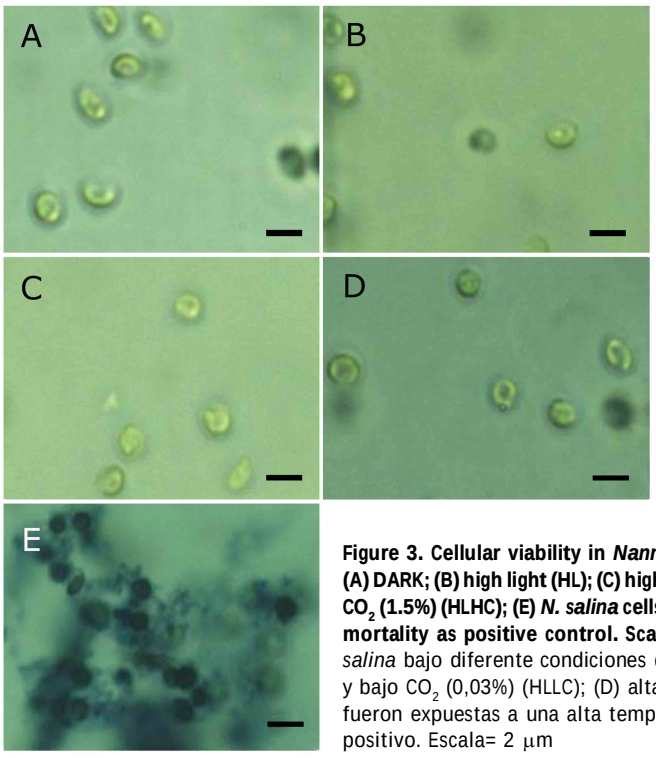

Figure 3. Cellular viability in *Nannochloropsis salina* under different culture conditions: (A) DARK; (B) high light (HL); (C) high light and low CO_2 (0.03%) (HLLC); (D) high light and high CO_2 (1.5%) (HLHC); (E) *N. salina* cells were exposed to high temperature in order to induced mortality as positive control. Scale bar= 2 μm / Viabilidad celular en *Nannochloropsis salina* bajo diferente condiciones de cultivo: (A) Oscuridad; (B) alta luz (HL); (C) alta luz y bajo CO_2 (0,03%) (HLLC); (D) alta luz y alto CO_2 (1,5%) (HLHC); (E) Células de *N. salina* fueron expuestas a una alta temperatura con el fin de inducir mortalidad como control positivo. Escala= 2 μm

(2011) suggested that CO_2 increases death rates in a dose-specific manner due to H_2O_2. In the conditions of high light without an increase of CO_2 (HL and HLLC) the pH raised significantly compared to darkness (pH 8.2 to pH 9.4). Similar changes in pH were described between day and night for *N.oculata* cultures (Tamburic *et al*. 2014) and may be because of photosynthetic carbon uptake during the day.

As suggest by Ezraty *et al*. (2011) our data indicate that during HLHC condition, the higher CO_2 concentration increases ROS production in *N. salina* cultures. At the same time, because of decrease in the pH, the xanthophyll cycle activity in *N. salina* cells might be diminished, affecting one of the basal barriers in antioxidant response, such as was demonstrated by Gentile & Blanch (2001). In this work, we established an increase in active oxygen species inside the cell, which triggered an effective antioxidant response, confirmed by the production of phenolic compounds and activation of the antioxidant enzymes catalase (CAT) and peroxiredoxin (PRX). We suggest that these antioxidant components work co-operatively protecting the cell against oxidative stress and cellular damage triggered by high CO_2 exposure which was confirmed with the Tripan blue staining. In fact, this coordination has been described in other tolerant algae species in front several abiotic stressors (*e.g.*, Randhawa *et al*. 2001, Ratkevicius *et al*. 2003, Contreras-Porcia *et al*. 2011b, Lovazzano *et al.* 2013). Expression of *prx* and *cat* genes was evaluated and they effectively responded to high CO_2 in *N. salina*, demonstrating the active participation of gene regulation in the oxidative stress tolerance.

The *prx* sequences used in the qPCR gene expression analyses of this work corresponded to two copies of a peroxiredoxin Q (Dietz 2011). In the *N. salina* transcriptome, 9 coding sequences were found for peroxiredoxin activity (EC. 1.11.1.15): 4 genes coding for the chloroplastic PRX type II, 2 genes coding for the chloroplastic PRX Q, and 3 genes coding for the cytoplasmic A-type PRX (Dietz 2011). In general, PRX Q is coded in the nuclear genome, but its protein is transported to the chloroplast and is located in the thylakoids (Lamkemeyer *et al*. 2006, Pitsch *et al*. 2010). PrxQ has an important role in antioxidant response, since *A. thaliana* knock-out for *prxQ* showed increased ROS and altered transcript levels of proteins involved in maintaining the redox homeostasis and antioxidant defense (Lamkemeyer *et al.* 2006). On the other hand, primers used for *cat* gene matched one sequence in *N. gaditana* genome (Radakovits *et al.* 2012) and also on one sequence present in the transcriptome of *N. salina* used in the present study. This gene codes for clade 3 of the heme-binding enzyme CAT, which is a ubiquitous enzyme found in both prokaryotes and eukaryotes (Chelikani *et al.* 2004). Thus, both genes used in this study were related to the antioxidant response in microalgae.

Although we only quantified gene expression of one out of 9 PRX genes present in *N. salina*, a strong correlation was found between activity and mRNA levels for this enzyme (Figs. 1F and 2B). The enzyme activity evaluated in the current study was performed with whole cell macerate, and should therefore reflect the activity of all PRXs present in the different compartments of *N. salina* cells. Even so, enzyme activity and gene expression demonstrated a coordinated response, thus highlighting the synchronization of the antioxidant response. In the case of PRX, our results showed that either enzyme activity as well as gene expression had low levels, but these levels were significantly incremented under HL and higher in HLHC. For CAT, enzyme activity and gene expression were induced only in the condition of HLHC, again suggesting that the addition of CO_2 to the system was detrimental for the redox balance in *N. salina* cells (Figs. 1D and 2A). The fact that no differences were observed for AP after high light and high CO_2 requires more studies to draw any conclusion. However, preliminarily this result suggest that the conditions of high light and high CO_2 used in this study are able to affect certain ROS and antioxidants pathways, but not all.

The behavior observed in this work suggests that *N. salina* cells have an exceptional capacity for handling conditions of excess light. Various aspects of the photo-acclimation response of *Nannochloropsis* have been investigated so far, including changes in pigmentation and ultrastructure (Sukenik *et al.* 1989, Fisher *et al.* 1996, 1998), the xanthophyll cycle (Gentile & Blanch 2001), lipid composition (Sukenik *et al.* 1993) and the photosynthetic response (Fisher *et al.* 1996). However, most of these studies compared the changes caused by going from a steady state of high light to one of low light, and none of them review antioxidant response. Nonetheless, previous results obtained for *Nannochloropsis* under HL conditions evidenced an efficient NPQ system (Cao *et al.* 2013) together with controlled activity of xanthophyll cycle (Gentile & Blanch 2001). Down-regulation of photosynthesis with a reduction in cellular chlorophyll a (Chl *a*) and in photosynthetic units has been also reported (Fisher *et al.* 1998, Tamburic *et al.* 2014). However, this

reduction in photosynthetic activity was not detrimental, since the cell recovered after the light excess corresponding to midday was left behind, finding that the cell size remained constant but filled with accumulation bodies (Fisher *et al*. 1996, 1998; Van Wagenen *et al*. 2012). Similarly, intact cells were also observed in our study after HL conditions, but we did not analyzed accumulation bodies presence.

At equilibrium with atmospheric CO_2, seawater can be found between pH 7.8 and pH 8.4; where the main dissolved inorganic carbon (DIC) is HCO_3^- (Soli & Byrne 2002). The concentration of HCO_3^- is approximately 2 mM, whereas the level of dissolved CO_2 (dCO_2), the inorganic carbon species utilized by the carboxylating enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO), is ca. 12-15 mM, at 25ºC (Round 1981). This limited CO_2 availability may restrict its supply to marine microalgae for photosynthesis. However, most of the microalgae examined so far have an efficient dissolved inorganic carbon concentrating mechanism (CCM), which permits them to use either CO_2 or HCO_3^- as external sources of DIC (see Falkowski & Raven 1997). For *Nannochloropsis*, investigations have notably indicated the presence of a light dependent bicarbonate transport system (Huertas & Lubian 1998, Huertas *et al.* 2000b). Although an increase in the carbon dioxide concentration is presumed to rise the rate at which carbon is incorporated into carbohydrate in the light-independent reaction, previous work has shown that high CO_2 aeration (5-15%) may have a harmful effect on the growth of microalgal cells (Chiu *et al.* 2009). Several authors have stated that 2% CO_2 is maximum for *Nannochloropsis* growth (Roncarti *et al.* 2004, Hu & Gao 2003, Chiu *et al.* 2009). In the present work 1.5% CO_2 aeration (15,000 ppm) was used in *N. salina* cultures and only a slightly higher growth rate was obtained compare to cultures with ambient levels of CO_2 (data not shown). Huertas *et al.* (2000a) reported similar results in *N. gaditana*, where the culture with high CO_2 aeration (1% v/v) reached maximum cell density only somewhat faster than the control culture. These results suggest that the CO_2 or C are not the limiting factor for biomass accumulation, therefore if better biomass production or better CO_2 fixation rates are aimed, as previously described, the microalgae should be supplemented with other nutrients besides (Flynn *et al*. 1993).

Thus, our results suggested that CO_2 supply is a stressing agent for *N. salina* cultures. However, the cellular antioxidant response observed in *N. salina* manages to soften this stress condition, without affecting its global performance. Higher CO_2 concentrations may overpass antioxidant cellular capacity and may cause cellular damage, affecting biomass generation and biodiesel production as seen in conditions over 2% CO_2. Further experiments with higher CO_2 concentrations controlling pH and light intensity should be made to prove this hypothesis.

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Literature cited

Abolhassani M, M Guais, P Chaumet-Riffaud, AJ Sasco & L Schwartz. 2009. Carbon dioxide inhalation causes pulmonary inflammation. American Journal of Physiology, Lung Cellular and Molecular Physiology 296(4): L657-L665.

Anderson JM, WS Chow & YI Park. 1995. The grand design of photosynthesis: acclimation of the photosynthetic apparatus to environmental cues. Photosynthesis Research 46: 129-139.

Augusto O, MG Bonini, AM Amanso, E Linares, CCX Santos & SL De Menezes. 2002. Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. Free Radical Biology and Medicine 32(9): 841-859.

Berlett BS, PB Chock, MB Yim & ER Stadtman. 1990. Manganese (II) catalyzes the bicarbonate-dependent oxidation of amino acids by hydrogen peroxide and the amino acid-facilitated dismutation of hydrogen peroxide. Proceedings of the National Academy of Sciences of the United States of America 87(1): 389-393.

Cao S, X Zhang, N Ye, X Fan, S Mou, D Xu, C Liang, Y Wang & W Wang. 2012. Evaluation of putative internal reference genes for gene expression normalization in *Nannochloropsis* sp. by quantitative real-time RT-PCR. Biochemical and Biophysical Research Communications 424(1): 118-123.

Cao S, X Zhang, D Xu, X Fan, S Mou, Y Wang, N Ye & W Wang. 2013. A transthylakoid proton gradient and inhibitors induce a non-photochemical fluorescence quenching in unicellular algae *Nannochloropsis* sp. FEBS Letters 587(9): 1310-1315.

Chelikani P, I Fita & PC Loewen. 2004. Diversity of structures and properties among catalases. Cellular and Molecular Life Sciences 61: 192-208.

Chisti Y. 2007. Biodiesel from microalgae. Biotechnology Advances 25(3): 294-306.

Chiu S-Y, C-Y Kao, M-T Tsai, S-C Ong, C-H Chen & C-S Lin. 2009. Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. Bioresource Technology 100: 833-838.

Coakley RJ, C Taggart, C Greene, NG McElvaney & SJ O'Neill. 2002. Ambient pCO_2 modulates intracellular pH, intracellular oxidant generation, and interleukin-8 secretion in human neutrophils. Journal of Leukocyte Biology 71(4): 603-610.

Contreras L, A Moenne & JA Correa. 2005. Antioxidant responses in *Scytosiphon lomentaria* (Phaeophyceae) inhabiting copper-enriched coastal environments. Journal of Phycology 41: 1184-1195.

Contreras L, G Dennett, A Moenne, E Palma & JA Correa. 2007. Molecular and morphologically distinct *Scytosiphon* species (Scytosiphonales, Phaeophyceae) display similar antioxidant capacities. Journal of Phycology 43(6): 1320-1328.

Contreras-Porcia L, G Dennett, A González, E Vergara, C Medina, JA Correa & A Moenne. 2011a. Identification of copper-induces genes in the marine alga *Ulva compressa* (Chlorophyta). Marine Biotechnology 13(3): 544-556.

Contreras-Porcia L, D Thomas, V Flores & JA Correa. 2011b. Tolerance to oxidative stress induced by desiccation in *Porphyra columbina* (Bangiales, Rhodophyta). Journal of Experimental Botany 62: 1815-1829.

Dietz KJ. 2011. Peroxiredoxins in plants and cyanobacteria. Antioxidants & Redox Signaling 15(4): 1129-1159.

Ezraty B, M Chabalier, A Ducret, E Maisonneuve & S Dukan. 2011. CO_2 exacerbates oxygen toxicity. EMBO Reports 12(4): 321-326.

Falkowski PG & JA Raven. 1997. Carbon acquisition and assimilation. In: Falkowski PG & JA Raven (eds). Aquatic photosynthesis, pp. 128-163. Blackwell Science, Capital City Press, Massachusetts.

Fisher T, J Minnaard & Z Dubinsky. 1996. Photoacclimation in the marine alga *Nannochloropsis* sp. (Eustigmatophyte): a kinetic study. Journal of Plankton Research 18: 1797-1818.

Fisher T, T Berner, D Iluz & Z Dubinsky. 1998. The kinetics of the photoacclimation response of *Nannochloropsis* sp. (Eustigmatophyceae): a study of changes in ultrastructure and PSU density. Journal of Phycology 34: 818-824.

Flynn KJ, K Davidson & A Cunningham. 1993. Relations between carbon and nitrogen during growth of *Nannochloropsis oculata* (Droop) Hibberd under continuous illumination. New Phycologist 125: 717-722.

Foyer CH & G Noctor. 2009. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxidants & Redox Signaling 11(4): 861-905.

Gao K, J Xu, G Gao, Y Li, DA Hutchins, B Huang, L Wang, Y Zheng, P Jin, X Cai, D-P Häder, W Li, K Xu, N Liu & U Riebesell. 2012. Rising CO_2 and increased light exposure synergistically reduce marine primary productivity. Nature Climate Change 2: 519-523.

García-Gómez C, FJL Gordillo, A Palma, MR Lorenzo & M Segovia. 2014. Elevated CO_2 alleviates high PAR and UV stress in the unicellular chlorophyte *Dunaliella tertiolecta*. Photochemical Photobiological Sciences 13: 1347-1358.

Gentile M-P & HW Blanch. 2001. Physiology and xanthophyll cycle activity of *Nannochloropsis gaditana*. Biotechnology and Bioengineering 75(1): 1-12.

Goldman JC & JJ McCarthy. 1978. Steady-state growth and ammonium uptake of a fast-growing marine diatom. Limnology & Oceanography 23(4): 695-703.

Guillard RR & JH Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (cleve) Gran. Canadian Journal of Microbiology 8: 229-239.

Hu H & K Gao. 2003. Optimization of growth and fatty acid composition of an unicellular marine picoplankton, *Nannochloropsis* sp., with enriched carbon sources. Biotechnology Letters 25: 421-425.

Huertas IE & LM Lubian. 1998. Comparative study of dissolved inorganic carbon utilization and photosynthetic responses in *Nannochloris* (Chlorophyceae) and *Nannochloropsis* (Eustigmatophyceae) species. Canadian Journal of Botany 76: 1104-1108.

Huertas IE, O Montero & LM Lubián. 2000a. Effects of dissolved inorganic carbon availability on growth, nutrient uptake and chlorophyll fluorescence of two species of marine microalgae. Aquacultural Engineering 22: 181-197.

Huertas IE, GS Espie, B Coman & LM Lubian. 2000b. Light-dependent bicarbonate uptake and CO_2 efflux in the marine microalga *Nannochloropsis gaditana*. Planta 211: 43-49.

Hug SJ & O Leupin. 2003. Iron-catalyzed oxidation of arsenic(III) by oxygen and by hydrogen peroxide: pH-dependent formation of oxidants in the Fenton reaction. Environmental Science & Technology 37(12): 2734-2742.

Imlay JA. 2008. Cellular defenses against superoxide and hydrogen peroxide. Annual Review of Biochemistry 77: 755-776.

Karsten V, SR Murray, J Pike, K Troy, M Ittensohn, M Kondradzhyan, KB Low & D Bermudes. 2009. *msb*B deletion confers acute sensitivity to CO_2 in *Salmonella enterica* serovar Typhimurium that can be suppressed by a loss-of-function mutation in *zwf*. BMC Microbiology 9: 170.

Krause G & E Weis. 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annual Review in Plant Biology 42: 313-349.

Lamkemeyer P, M Laxa, V Collin, W Li, I Finkemeier, MA Schöttler, V Holtkamp, VB Tognetti, E Issakidis-Bourguet, A Kandlbinder, E Weis, M Iginiac-Maslow & KJ Dietz. 2006. Peroxiredoxin Q of *Arabidopsis thaliana* is attached to the thylakoids and functions in context of photosynthesis. The Plant Journal 45: 968-998.

Lovazzano C, C Serrano, JA Correa & L Contreras-Porcia. 2013. Comparative analysis of the peroxiredoxin activation in the brown macroalgae *Scytosiphon gracilis* and *Lessonia nigrescens* (Phaeophyceae) under copper stress. Physiologia Plantarum 149(3): 378-388.

Medinas DB, G Cerchiaro, DF Trindade & O Augusto. 2007. The carbonate radical and related oxidants derived from bicarbonate buffer. IUBMB Life 59(4-5): 255-262.

Michalak A. 2006. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Environmental Studies 15(4): 523-530.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7(9): 405-410.

Miyachi S, I Iwasaki & Y Shiraiwa. 2003. Historical perspective on microalgal and cyanobacterial acclimation to low- and extremely high-CO_2 conditions. Photosynthesis Research 77: 139-153.

Moazami N, A Ashori, R Ranjbar, M Tangestani, R Eghtesadi & AS Nejad. 2012. Large-scale biodiesel production using microalgae biomass of *Nannochloropsis*. Biomass and Bioenergy 39: 449-453.

Nielsen ES. 1955. Carbon dioxide as carbon source and narcotic in photosynthesis and growth of *Chlorella pyrenoidosa*. Physiologia Plantrum 8(2): 317-335.

Pitsch NT, B Witsch & M Baier. 2010. Comparison of the chloroplast peroxidase system in the chlorophyte *Chlamydomonas reinhardtii*, the bryophyte *Physcomitrella patens*, the lycophyte *Selaginella moellendorffii* and the seed plant *Arabidopsis thaliana*. BMC Plant Biology 10: 133.

Quinn JC, T Yates, N Douglas, K Weyer, J Butler, TH Bradley & PJ Lammers. 2012. *Nannochloropsis* production metrics in a scalable outdoor photobioreactor for commercial applications. Bioresource Technology 117: 164-171.

Radakovits R, RE Jinkerson, SI Fuerstenberg, H Tae, RE Settlage, JL Boore & MC Posewitz. 2012. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. Nature Communications 3: 686.

Randhawa VK, FZ Zhou, XL Jin, C Nalewajko & DJ Kushner. 2001. Role of oxidative stress and thiol antioxidant enzymes in nickel toxicity and resistance in strains of the green alga (*Scenedesmus acutus* f. alternans). Canadian Journal of Microbiology 47: 987-993.

Ratkevicius N, JA Correa & A Moenne. 2003. Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev. (Chlorophyta) from heavy-metal enriched environments in northern Chile. Plant, Cell & Environment 26(10): 1599-1608.

Rodolfi L, G Chini-Zittelli, N Bassi, G Padovani, N Biondi, G Bonini & MR Tredici. 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 102: 100-112.

Roncarti A, A Meluzzi, S Acciarri, N Tallarico & P Melotti. 2004. Fatty acid composition of different microalgae strains (*Nannochloropsis* sp., *Nannochloropsis oculata* (Droop) Hibberd, *Nannochloris atomus* Butcher and *Isochrysis* sp.) according to the culture phase and the carbon dioxide concentration. Journal of the World Aquaculture Society 35(3): 401-411.

Round FE. 1981. The physical and chemical characteristics of the environment. In: Round FE (ed). The ecology of algae, pp. 7-26. Cambridge University Press, Cambridge.

Satoh A, N Kurano, H Senger & S Miyachi. 2002. Regulation of energy balance in photosystems in response to changes in CO_2 concentrations and light intensities during growth in extremely-high-CO_2-tolerant green microalgae. Plant and Cell Physiology 43: 440-451.

Schwartz L, A Guais, P Chaumet-Riffaud, G Grévillot, AJ Sasco, TJ Molina & A Mohammad. 2010. Carbon dioxide is largely responsible for the acute inflammatory effects of tobacco smoke. Inhalation Toxicology 22(7): 543-551.

Sergeenko T, E Muradyan, N Pronina, G Klyachko-Gurvich, I Mishina & L Tsoglin. 2000. The effect of extremely high CO_2 concentration on the growth and biochemical composition of microalgae. Russian Journal of Plant Physiology 47: 632-638.

Sheehan J. 2009. Engineering direct conversion of CO_2 to biofuel. Nature Biotechnology 27(12): 1128-1129.

Smith PK, RI Krohn, GT Hermanson, AK Mallia, FH Gartner, MD Provenzano, EK Fujimoto, NM Goeke, BJ Olson & DC Klenk. 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150(1): 76-85. [Erratum in: Analytical Biochemistry (1987) 163(1): 279]

Soli AL & RH Byrne. 2002. CO_2 system hydration and dehydration kinetics and the equilibrium CO_2/H_2CO_3 ratio in aqueous NaCl solution. Marine chemistry 78(2-3): 65-73.

Solovchenko A & I Khozin-Goldberg. 2013. High-CO_2 tolerance in microalgae: possible mechanisms and implications for biotechnology and bioremediation. Biotechnology Letters 35: 1745-1752.

Stadtman ER & BS Berlett. 1991. Fenton chemistry. Amino acid oxidation. The Journal of Biological Chemistry 266(26): 17201-17211.

Strober W. 2001. Trypan blue exclusion test of cell viability. Current Protocols in Immunology: Appendix 3: Appendix 3B. < doi: 10.1002/0471142735.ima03bs21>

Sukenik A, Y Carmeli & T Berner. 1989. Regulation of fatty acid composition by irradiance level in the Eustigmatophyte *Nannochloropsis* sp. Journal of Phycology 25: 686-692.

Sukenik A, Y Yamaguchi & A Livne. 1993. Alterations in lipid molecular species of the marine Eustigmatophyte *Nannochloropsis* sp. Journal of Phycology 29: 620-626.

Sukenik A, A Tchernov, A Kaplan, E Huertas, LM Lubian & A Livne. 1997. Uptake, efflux, and photosynthetic utilization of inorganic carbon by the marine Eustigmatophyite *Nannochloropsis* sp. Journal of Phycology 33: 969-974.

Tamburic B, S Guruprasad, DT Radford, M Szabó, RM Lilley, AWD Larkum, JB Franklin, DM Kramer, SI Blackburn, JA Raven, M Schliep & PJ Ralph. 2014. The effect of diel temperature and light cycles on the growth of *Nannochloropsis oculata* in a photobioreactor matrix. PLoS ONE 9(1): e86047 <doi: 10.1371/journal.pone.0086047>

Van Wagenen J, TW Miller, S Hobs, P Hook, B Crowe & M Huesemann. 2012. Effects of light and temperature on fatty acid production in *Nannochloropsis salina*. Energies 5: 731-740.

Visca P, G Fabozzi, M Milani & P Ascenzi. 2002. Nitric oxide and *Mycobacterium leprae* pathogenicity. International Union of Biochemistry and Molecular Biology Life 54: 95-99.

Yang Y & K Gao. 2003. Effects of CO_2 concentrations on the freshwater microalgae, *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Chlorophyta). Journal of Applied Phycology 15: 379-389.

Zar JH. 2010. Biostatistical analysis, 944 pp. Prentice Hall, Englewood Cliffs.

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