Response to oxidative stress induced by high light and carbon dioxide (CO\textsubscript{2}) in the biodiesel producer model Nannochloropsis salina (Ochrophyta, Eustigmatales)

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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\textsubscript{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 polinsaturados y alta cantidad de lípidos. Estudios previos muestran que en presencia de algunas concentraciones de CO\textsubscript{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\textsubscript{2} por medio de 3 metodologías: analizando la actividad enzimática antioxidante (catalasa, ascorbato peroxidasa y peroxirredoxina), cuantificando los compuestos fenólicos, H\textsubscript{2}O\textsubscript{2} y lipoperoxídos (i.e., marcador de daño celular) y evaluando los niveles de expresión génica. Los resultados sugieren que un aumento en la concentración de CO\textsubscript{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\textsubscript{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 microalga 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\textsubscript{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\textsubscript{2} through 3 different approaches: analysis of antioxidant enzymatic activities (catalase, ascorbate peroxidase and peroxiredoxine), analysis of gene expression and the quantification of H\textsubscript{2}O\textsubscript{2}, phenolic compounds and liperoxides (e.g., cell damage marker). The results obtained suggest that an increase in the CO\textsubscript{2} concentration in the cultures (15,000 ppm), together with high light (1,000 \textmu mol m\textsuperscript{-2} s\textsuperscript{-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

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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 microalga _Nannochloropsis salina_ D.J. Hibberd (Ochrophyta, Eustigmatophyta) 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 eicosapentaeonoic 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 microagal 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$_2$O$_2$) 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-Porcía 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, Stadtm ann & Berlett 1991, Hug & Leupin 2003) through the generation of the carbonate radical (CO$_3^{*}$). Thus, CO$_2$ enrichment increase the CO$_2^*$ 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 1/2 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 20°C, 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, Edinburg 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 2-5x10$^7$ 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 and boiled 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 Biotemns, 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., 5x10^9 cells) were directly homogenized in a mortar using pestle for 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). 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., 5x10^9 cells) were directly homogenized in a mortar using pestle for 5 min and centrifuged at 7,690 g for 15 min at 4°C.

**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 H₂O₂. After the addition of H₂O₂, its consumption was determined at 240 nm for 2 min and the activity was calculated using the extinction coefficient of H₂O₂ (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 H₂O₂. 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⁻¹cm⁻¹). Finally, the peroxiredoxine (PRX) activity, using dihiothreitol (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 H₂O₂ 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 (NH₄)₂Fe(SO₄) 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 (1x10^9 cells mL⁻¹) exposed to the different experimental conditions, were collected through centrifugation at 4°C 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 then 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 Tripian blue staining.

**Effects of intense light and high CO₂ on pH, ROS production and lipoperoxide levels**

Intense light (1,000 μmol m^2 s^-1 light) and high CO₂ (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>
<thead>
<tr>
<th>Gene Target</th>
<th>EC or KOG reference</th>
<th>Primer Name</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>EC 1.11.1.6</td>
<td>CAT-F</td>
<td>CGCACCAAGTTTCCTTTCCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAT-R</td>
<td>GATCCCCCTGGTCTGTTTGA</td>
</tr>
<tr>
<td>Peroxiredoxin</td>
<td>EC 1.11.11.15</td>
<td>PRX-F</td>
<td>TGCAGTTAUCTCCGACGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PRX-R</td>
<td>CTTCTGGTCTTCCCAATCA</td>
</tr>
<tr>
<td>Alpha tubulin</td>
<td>KOG1376</td>
<td>TUA-F</td>
<td>GCCTGCTGCCTCATGTACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TUA-R</td>
<td>GTTATGCGCCACCTTGAAG</td>
</tr>
</tbody>
</table>
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

![Graphs showing ROS formation, lipid peroxidation, phenolic compounds concentration, and specific activity of antioxidant enzymes in N. salina under different culture conditions.](image-url)
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

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**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 $P = 0.05$.
and high CO₂ 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 Tripot blue. It has been demonstrated that only dead or damaged cells are stained by Tripot blue with this method (Strober 2001). As shown in Fig. 3, *N. salina* cells were not stained by Tripot 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 CO₂ exposure.

**DISCUSSION**

The results presented in the current study suggest that high light together with high CO₂ (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 CO₂, compared with conditions of darkness (no light, ambient CO₂) and with cultures kept in high light but in ambient CO₂ (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 CO₂ 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**<sub>1/2</sub> (CO<sub>2</sub>(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:](image-url)
(2011) suggested that CO\textsubscript{2} increases death rates in a dose-specific manner due to H\textsubscript{2}O\textsubscript{2}. In the conditions of high light without an increase of CO\textsubscript{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\textsubscript{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 cooperatively protecting the cell against oxidative stress and cellular damage triggered by high CO\textsubscript{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, Ratkevičius et al. 2003, Conteras-Porcia et al. 2011b, Lovazzano et al. 2013). Expression of prx and cat genes was evaluated and they effectively responded to high CO\textsubscript{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\textsubscript{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\textsubscript{2} requires more studies to draw any conclusion. However, preliminarily this result suggest that the conditions of high light and high CO\textsubscript{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 (Suknenk et al. 1989, Fisher et al. 1996, 1998), the xanthophyll cycle (Gentile & Blanch 2001), lipid composition (Suknenk 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 25°C (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 (Roncari 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 compared 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.

**ACKNOWLEDGMENTS**

This work was supported by FONDECYT 11090234 to NE and FONDECYT 1120117 to LCP. Karen Yángüez thanks ‘Programa de becas MEF-IFARHU’ (República de Panamá). We are especially grateful to Rodrigo Ruay and Daniela Thomas for technical support and Virginia Garretón for useful discussion.

**LITERATURE CITED**


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Received 13 May 2014 and accepted 4 December 2014
Editor: Claudia Bustos D.