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A novel *in situ* system to evaluate the effect of high CO_2 on photosynthesis and biochemistry of seaweeds

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ABSTRACT: Previous studies of the impact of increased CO_2 on macroalgae have mainly been done in laboratories or mesocosm systems, placing organisms under both artificial light and seawater conditions. In this study, macroalgae were incubated *in situ* in UV-transparent cylinders under conditions similar to the external environment. This system was tested in a short-term study (5.5 h incubation) on the effect of 2 partial pressures of CO_2 (pCO_2): air (ambient CO_2) and the pCO_2 predicted by the end of the 21st century (700 µatm, high CO_2), on photosynthesis, photosynthetic pigments and photoprotection in calcifying (*Ellisolandia elongata* and *Padina pavonica*) and non-calcifying (*Cystoseira tamariscifolia*) macroalgae. The calcifying *P. pavonica* showed higher net photosynthesis under high CO_2 than under ambient CO_2 conditions, whereas the opposite occurred in *C. tamariscifolia*. Both brown algae (*P. pavonica* and *C. tamariscifolia*) showed activation of non-photochemical quenching mechanisms under high CO_2 conditions. However, in *P. pavonica* the phenol content was reduced after CO_2 enrichment. In contrast to phenols, in *E. elongata* other photoprotectors such as zeaxanthin and palythine (mycosporine-like amino acid) tended to increase in the high CO_2 treatment. The different responses of these species to elevated pCO_2 may be due to anatomical and physiological differences and could represent a shift in their relative dominance as key species in the face of ocean acidification (OA). More *in situ* studies could be carried out to evaluate how macroalgae will respond to increases in pCO_2 in a future OA scenario. The *in situ* incubator system proposed in this work may contribute towards increasing this knowledge.

KEY WORDS: Macroalgae · Non-photochemical quenching · Ocean acidification · Photoprotection · Photosynthesis

INTRODUCTION

The ocean absorbs about 30% of the emitted anthropogenic CO_2, causing significant changes in the marine carbon cycle and carbonate system. These changes include an increase in the concentration of dissolved CO_2, a smaller proportional increase of bicarbonate ions (HCO_3^-), a decrease of carbonate

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ions (CO_3^{2-}) and changes in the saturation state of calcium carbonate ($CaCO_3$) (Caldeira & Wickett 2005, Orr et al. 2005). The predicted changes in dissolved inorganic carbon distribution and abundance will result in an increase in hydrogen ion (H^+) concentration and, consequently, a decrease in seawater pH. These interrelated chemical changes in the inorganic carbon system are referred as 'ocean acidification (OA)' (Zeebe et al. 2008, Shi et al. 2009). The pH of ocean surface waters has decreased by 0.1 units since the beginning of the industrial era to its current mean value of 8.2 (Caldeira & Wickett 2003), corresponding to a 26% increase in H^+ concentration. The most recent models project a global increase in OA, with a corresponding decrease in surface ocean pH by the end of 21st century in the range of 0.06–0.07, up to 0.30–0.32, depending on the case scenarios of CO_2 atmospheric concentration (Representative Concentration Pathways, RCP) (IPCC 2013).

While the chemistry of carbonate systems has been well studied (Zeebe 2012), the impacts of OA on marine organisms and ecosystems remain poorly understood (Gattuso et al. 2010). While many studies have pointed out that OA may have negative effects on macroalgae (Mercado et al. 1999, Riebesell et al. 2000, Zondervan et al. 2001, Hall-Spencer et al. 2008, Kuffner et al. 2008, Gao & Zheng 2010, Sinutok et al. 2011, Johnson et al. 2012), a few found no effects (Israel & Hophy 2002, Egilsdottir et al. 2013) and others even found increases in photosynthesis, growth and calcification rates (Gao et al. 1993, Kübler et al. 1999, Zou 2005, Iglesias-Rodríguez et al. 2008, Zou & Gao 2009, Roleda et al. 2012). Many factors may be involved in the discrepancies among results, such as morpho-functional traits, the form of C acquisition, species adaptation/acclimation to environmental conditions, pre-experimental conditions and other methodological aspects (Ries et al. 2009, Olabarria et al. 2013). In addition, most of these studies have been carried out under laboratory conditions and in different temporal scales (see Hurd et al. 2009 and Raven 2011 for a review), impeding an appropriate comparison among the results. Although mesocosm experiments have improved our understanding of how submerged macrophytes will respond to future OA, information from *in situ* experiments is scarce. To date, *in situ* responses of marine organism to dissolved CO_2 increase are based on observations made near volcanic vents (Hall-Spencer et al. 2008, Martin et al. 2008, Porzio et al. 2011, Johnson et al. 2012). Recently, different techniques of *in situ* CO_2 manipulation, such as the Coral–Proto Free Ocean Carbon Enrichment System (Kline et al. 2012), the Free Ocean Carbon Enrichment System (Arnold et al. 2012), and the Carbon-Enriched Open Chamber System (Campbell & Fourqurean 2011, 2013) have been proposed. The development of new CO_2 *in situ* experiments can provide new insights and give straightforward answers or at least provide a piece of the puzzle about the effect of OA on macroalgae.

The objective of this study was to present a novel and simple experimental design to incubate macroalgae *in situ* under different partial pressures of CO_2 (pCO_2). Our design was tested in the lower intertidal environment of Cabo de Gata National Park (Spain), by comparing the responses of non-calcifying (*Cystoseira tamariscifolia*) vs. calcifying (*Padina pavonica* and *Ellisolandia elongata*) macroalgal species to changes in pCO_2. The studied calcifying species are lightly calcified with aragonite (*P. pavonica*) and heavily calcified with magnesium calcite (*E. elongata*). We analyzed the short-term effects of increased pCO_2 in photosynthetic parameters (both O_2 evolution- and chlorophyll fluorescence-based parameters), as well as pigment and photoprotector concentrations (mycosporine-like aminoacids [MAAs] and phenolic compounds); all of which are good indicators of physiological status (Figueroa & Korbee 2010).

MATERIALS AND METHODS

Studied site and species

The macroalgae were collected from the intertidal rocky shores of the Cabo de Gata-Níjar Natural Park (Southern Iberian Peninsula, 36° 52′ N, 2° 12′ W). *Cystoseira tamariscifolia* (Hudson) Papenfuss (Phaeophyceae), *Padina pavonica* (Linnaeus) Thivy (Phaeophyceae) and *Ellisolandia elongata* (J Ellis & Solander) KR Hind & GW Saunders (Florideophyceae, Corallinales) were selected for study in the present work. The species were chosen on the basis of their key ecological role in rocky photophilous habitats.

The brown macroalgae *C. tamariscifolia* is an Atlantic species that occurs from Scotland and Ireland to Mauritania and Cape Verde Islands. The species is present in Mediterranean waters of Atlantic influence, occurring across Iberian Coast as far as the province of Almería (Gómez-Garreta et al. 1994). It presents blue-green iridescence, can reach up to 1 m height and is fixed to the substrate by a thick disk (Gómez-Garreta et al. 2001). The brown macroalgae *P. pavonica* is a highly spread tropical/subtropical species, common in the Mediterranean coastal waters (www.algaebase.org). The thallus is brown to tan

in colour, forming fan-shaped clusters. The blades are calcifying, heavier above and lighter below, and curl inward near the edges. Both the upper and lower blade surfaces bear minute surface hairs arranged in a series of bands approximately 1.5 to 6 mm apart (Taylor 1979, Littler & Littler 2000, Littler et al. 2008). The blades attach to the substratum via a holdfast, which is often matted. The red *E. elongata* is an articulated calcareous species, whitish-pink to reddish-lilac, calcified, up to 50 mm high. The species is present in Mediterranean and Eastern Atlantic waters (www.algaebase.org).

Incubation and experimental design

The studied macroalgae were collected at 0.5 m depth and immediately incubated in 6 transparent UV cylinders (0.8 l; 33 × 7 cm) in a sheltered bay. The cylinders were fixed perpendicularly to the sun and not deeper than 0.5 m using a wooden frame and several buoys and weights (Fig. 1). Two pCO_2 conditions were applied in triplicate cylinders: (1) bubbling air (control, ambient CO_2) and (2) a commercial mix (Praxair España) of CO_2 and air with a final concentration of 700 ppm (high CO_2). The air pump and the bottle with 700 ppm CO_2 were maintained on the beach edge. Aeration was provided from the bottom of the cylinders at a rate of 0.5 l min^{-1}. Before algal incubation, the water was aerated for 30 min in both pCO_2 treatments. In a previous experiment it was determined that this time was enough to equilibrate the carbonate system inside the cylinders. Each cylinder received 20 g fresh weight (FW) of algae. The incubation experiments were performed over 3 d in September 2012, with 1 d for each species.

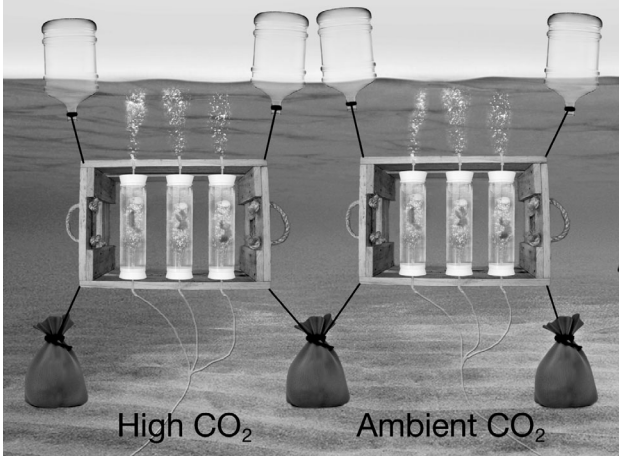

Fig. 1. Experimental design: cylinders were fixed using a wooden frame

After bubbling for 30 min, aeration was stopped in the 6 experimental cylinders in order to avoid extra oxygenation, and 1 h incubation allowed for net photosynthesis determination. This time is optimal for the incubations in function of the high volume: biomass incubation ratio inside the cylinders. At the end of this time, water samples were collected for the final oxygen concentration. After that, the cylinders were closed and were continuously aerated for 5.5 h.

Photosynthetic parameters including maximal quantum yield (F_v/F_m), electron transport rate (ETR) and non-photochemical quenching (NPQ) were measured by *in vivo* chlorophyll *a* (chl *a*) fluorescence associated to Photosystem II at the beginning and at the end of the incubation period of 5.5 h. Samples for absorptance, chlorophylls, carotenoids, photoprotective compounds (phenols and mycosporine-like amino acids) as well as antioxidant capacity were also determined at both periods.

To analyse phenolic compounds, DPPH and photosynthetic pigments, samples were collected, immediately frozen in liquid nitrogen and stored at –80°C until analyses. Samples for MAAs were kept desiccated until analysis. Water samples for pH measurement and alkalinity analysis were obtained at the end of the 5.5 h of incubation; the samples for alkalinity were poisoned with a small amount of saturated mercury chloride solution until analysis. The water chemistry for incubation without algae was also determined.

Measurement of solar radiation, temperature and nutrients

The irradiance of solar radiation was determined at 3 wavelength bands (PAR = 400 to 700 nm, UVA = 315 to 400 nm and UVB = 280 to 315 nm) using 2 Hyperspectral Irradiance Sensors for UV and PAR (Ramses, TrioS). Due to the sensor size, irradiances were measured outside the cylinders.

Temperature was measured during the incubation periods inside the cylinders. The concentration of nitrate and phosphate was measured in the seawater, and after the incubation it was also determined inside of the cylinders.

Measurement of pH and salinity

The pH within the incubation cylinders was measured using a pH meter (Crison Basic 20, Crison Instruments). The pH electrode was calibrated regu-

larly with standard National Bureau of Standards (NBS) buffer solutions (Oakton) to ensure a stable response. Salinity was estimated using a conductivity meter (Crison CM35, Crison Instruments).

Total alkalinity

Total alkalinity was measured by titrating (stepwise addition of reagent) the water sample with HCl to a final pH_{NBS} of 3. Once the water sample reached a pH of 3, all the bicarbonate, carbonate and hydroxide were neutralized. An automated titration system (877 Titrino plus, Metrohm) was used selecting the option of monotonic titrations with automatic equivalence point finding (MET).

The HCl solution nominal molarity used was 0.97 M at 20°C. This molarity was verified titrating 80 ml of $NaHCO_3$. The total inorganic carbon was determined using continuous and *in situ* measurements or pH and temperature and determinations of total alkalinity and salinity. The total alkalinity was determined by the Gran (1952) titration method. Total inorganic carbon concentration was calculated using the program CO_2sys (v.2.1, Pierrot et al. 2006) using dissociation constants for carbonic and boric acids determined on the NBS scale. To calculate the speciation of total inorganic carbon into carbonate, bicarbonate and dissolved CO_2 forms, the CO_2 seawater solubility coefficient proposed by Weiss (1974) was used. The first and second dissociation constants of carbonic acid in seawater by Mehrbach et al. (1973), refit by Dickson & Millero (1987), and the first dissociation constant of boric acid in seawater by Lyman (1956) were used.

Photosynthetic measurements as oxygen evolution

Net photosynthesis was estimated as photosynthetic oxygen evolution under *in situ* incubation by the difference between the oxygen concentrations after (final) and before (initial) 1 h incubation under the 2 pCO_2 treatments. The Spectrophotometric Winkler method was used to estimate the concentration of dissolved oxygen (Labasque et al. 2004). In each case, after fixing the soluble oxygen with R1 and R2 Winkler reagents, samples were kept in darkness and at 4°C. Within 24 h of collection, R3 was added and absorbance was measured at 466 nm, using a Genesis 10S Vis Thermo Scientific (Thermo Fisher Scientific). Standardization relied on the preparation of I2+I3 solutions by oxidation of iodide with iodate. A standard solution KIO_3 (0.01M) was used to obtain the standard curve.

In vivo chl *a* fluorescence

In vivo chl *a* fluorescence associated to Photosystem II was determined by using a portable pulse modulated fluorometer (Diving-PAM, Walz). Algal samples were collected from each treatment at the initial time, and after 5.5 h incubation were put into 10 ml incubation chambers to conduct rapid light curves (RLCs). For these incubations, the medium was taken directly from each of the cylinders. Minimum (F_o), maximum (F_m) and maximum variable fluorescence ($F_v = F_m - F_o$) were determined after 15 min in darkness to obtain F_v/F_m (Schreiber et al. 1995, Figueroa et al. 2003).

RLCs were obtained in order to determine the ETRs. After 15 min in the darkness for F_v/F_m determination, the algae were exposed for 20 s to 8 incremental irradiances (9.3, 33.8, 76, 145, 217, 301, 452, 629 and 947 µmol photons m^2 s^{-1}) of white light (internal Diving-PAM halogen lamp).

The ETR (µmol electrons m^{-2} s^{-1}) was calculated according to Schreiber et al. (1995) as follows:

$$ETR = \Delta F / F_m' \times E \times A \times F_{II} \quad (1)$$

where E is the incident irradiance. $\Delta F = F_m' - F_t$ and is the variable fluorescence in light, F_m' is the maximum fluorescence in light and F_t is the intrinsic fluorescence under a specific irradiance. Absorptance, $A = 1 - (E_t/E_o)$, was calculated from the light transmitted through a piece of each species (E_t) placed on a cosine-corrected PAR sensor (Licor 192 SB, Li-Cor) connected to a data-logger (Licor-1000), and E_o is the incident irradiance in the absence of the algal piece. The measured absorptances were 0.91 ± 0.02 for *C. tamariscifolia*, 0.79 ± 0.03 for *P. pavonica* and 0.69 ± 0.04 for *E. elongata*. F_{II} is the fraction of chlorophyll related to PSII (400 to 700 nm), being 0.8 in the brown macroalgae and 0.15 in the red algae (Grzymski et al. 1997). A tangential function was applied to the ETR versus irradiance curves (Eilers & Peeters 1988), and the photosynthetic parameters maximum ETR (ETR_{max}) and the initial slope of the curve (α_{ETR}, as estimator of photosynthetic efficiency) were determined. The irradiance of ETR saturation (Ek_{ETR}) was calculated from the intercept between ETR_{max} and α_{ETR}.

NPQ was calculated according to Schreiber et al. (1995) as:

$$NPQ = (F_m - F_m') / F_m' \quad (2)$$

Maximal NPQ (NPQ_{max}) and the initial slope of NPQ versus light curve (α_{NPQ}) were obtained from the tangential function according to Eilers & Peeters (1988). Finally, the saturation light for NPQ (Ek_{NPQ}) was calculated from the intercept between NPQ_{max} and α_{NPQ}.

Photosynthetic pigments

Photosynthetic pigments were measured for each species and cylinder in duplicate. Results were expressed as mg g^{-1} DW (dry weight). The FW:DW ratios were calculated from 10 thalli of each species. FW was determined after blotting off surface water with absorbent paper. Afterwards, the thalli were oven-dried for 2 d at 60°C to obtain the DW. The ratios were 4.3 ± 0.08 for *C. tamariscifolia*, 3.1 ± 0.04 for *P. pavonica* and 1.5 ± 0.01 for *E. elongata*.

Chl *a* content was determined spectrophotometrically (Shimadzu UVmini 1240, Shimadzu Scientific Instruments), while chl *c* and carotenoids were identified and quantified by high-performance liquid chromatography (HPLC, Waters 600 HPLC system, Waters Cromatografía). Both analyses were made from the same extract using 15 mg FW in 1 ml of N,N-dimethylformamide (DMF) and maintained in darkness at 4°C for 12 h. The chl *a* concentration was calculated using Wellburn (1994) equations. The carotenoid composition and concentration were determined by HPLC according to García-Sánchez et al. (2012). Chl *c*, fucoxanthin, violaxanthin, antheraxanthin, zeaxanthin and β-carotene were identified using commercial standards (DHI LAB Products).

Phycobiliproteins for *E. elongata* were extracted in 0.1 M phosphate buffer (pH 6.5), centrifuged at 2253 × *g* for 30 min. Phycoerythrin and phycocyanin concentrations were calculated following Sampath-Wiley & Neefus (2007) equations.

Phenolic compounds

The phenol concentration was determined using 0.25 g FW. Samples were pulverized in a mortar and pestle with sea-sand using 2.5 ml of 80% methanol. After being maintained overnight, the mixture was centrifuged at 2253 × *g* for 15 min at 4°C and the supernatant was collected. These supernatants were used for phenol determination and to determine the antioxidant activity. Total phenolic compounds were estimated colourimetrically using Folin-Ciocalteu assay (Folin & Ciocalteu 1927). Phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) was used as standard. Finally, the absorbance of 760 nm was determined in a UVmini-1240 spectrophotometer (Shimadzu Scientific Instruments) (Abdala-Díaz et al. 2006).

Antioxidant activity

Antioxidant activity of the algal extracts of *C. tamariscifolia* and *P. pavonica* was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical assay (Blois 1958). A volume of 150 µl of DPPH solution (diluted in 90% methanol) was added to each algal extract, obtained as previously explained above for phenols. The samples were reacted with the stable DPPH solution during 30 min in the dark at room temperature (~20°C). The absorbance of the solutions was read at 517 nm in a UVmini-1240 spectrophotometer. A calibration curve made for a set of DPPH concentrations was used to calculate the remaining concentration of DPPH in the reaction mixture after incubation. Concentrations of DPPH (mM) were plotted against plant extract concentration (mg ml^{-1} DW) in order to obtain the EC_{50} value (oxidation index), which represents the concentration of the extract (mg ml^{-1}) required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was used as positive control (Connan et al. 2006).

MAAs

Samples (10 to 20 mg DW) of *E. elongata* were extracted for 2 h in screw-capped centrifuge vials filled with 1 ml 20% aqueous methanol (v/v) at 45°C. The concentration and composition of different MAAs were analysed by HPLC (Waters 600 HPLC system, Waters Cromatografía) according to Korbee-Peinado et al. (2004).

Statistical analysis

The effects of the treatments (ambient CO_2 and high CO_2) on the photosynthetic parameters were analysed using ANOVAs (α = 0.05) (Underwood 1997). One test was performed including CO_2 treatment as fixed factor with 2 levels, and cylinder as a random factor nested within CO_2 treatment. Homogeneity of variance was tested using Cochran's tests and by visual inspection of the residuals (Underwood 1997). Photosynthetic parameters, phenolic

compounds, antioxidant capacity and photosynthetic pigments were measured from 2 replicates in each cylinder (n = 12). For oxygen evolution, 4 measurements were done in each cylinder (n = 24); for the other variables (alkalinity, pH, temperature and carbonate chemistry) 1 measurement was done per cylinder (n = 6). Therefore, this source of variation (i.e. cylinder), is not included in the latest analysis. Analyses were done with SPSS v.21 (IBM). All results are expressed as mean ± SE.

RESULTS

Physical and chemical variables

Irradiance and temperature within the cylinders showed little variation during each species' incubation period, but changed along the 3 experimental days on which each species was incubated (Table 1). Daily integrated irradiance was higher during the *Cystoseira tamariscifolia* experiment, compared to *Padina pavonica* and *Ellisolandia elongata*. Water temperature was lower during the *E. elongata* experiment than on the other 2 experimental days (Table 1).

After 30 min of pumping high (700 ppm) and ambient CO_2 inside the cylinders without algae, values of DIC were significantly higher in high CO_2 (2.2 ± 0.01 mM) compared to ambient CO_2 (2.0 ± 0.003 mM) (F = 135.42, df = 1, n = 6, p = 0.00031). Values of pH were lower in high CO_2 (8.0 ± 0.01) compared to ambient CO_2 (8.3 ± 0.01) (F = 539.35, df = 1, n = 6, p = 0.00002), while the total alkalinity did not show differences between treatments (2424 ± 10 and 2450 ± 10 µM, for high and ambient CO_2, respectively; F = 4.49, df = 1, n = 6, p = 0.101). After algae incubation, the carbonate chemistry changed with respect to the above-reported values (Table 1). No differences in pH were found anymore between ambient and high CO_2 treatments in *E. elongata* and *P. pavonica* whereas the difference in pH in *C. tamariscifolia* was still evident after incubation (Tables 1 & 2). The pH increased with respect to the value found without algae, except for *E. elongata* at ambient CO_2 (Table 1).

During incubation, nutrient concentrations in the seawater were 1.4 µM nitrate and 0.1 µM phosphate. After incubation, the concentration of phosphate slightly decreased inside the cylinders, while nitrate decreased to values in the range of 0.2 to 0.4 µM in all species and both pCO_2 treatments.

Photosynthetic parameters

The results of net photosynthesis (determined by oxygen evolution) showed that, under ambient CO_2 conditions, net productivity rates of *C. tamariscifolia* (0.54 ± 0.02 mg O_2 g^{-1} DW h^{-1}) were twice as high as values found for *P. pavonica* (0.23 ± 0.01 mg O_2 g^{-1} DW h^{-1}), and 10 times higher than values for *E. elongata* (0.02 ± 0.003 mg O_2 g^{-1} DW h^{-1}). When the different species were incubated in high CO_2 conditions, *C. tamariscifolia* showed a significant reduction in net photosynthesis, while *P. pavonica* showed a significant increase in this parameter (Fig. 2, Table 3). *E. elongata* showed a slight increase in photosynthetic rates under high CO_2, but differences between the treatments were not significant (Fig. 2, Table 3).

Table 1. Daily integrated irradiance PAR (400 to 700 nm), UVA (315 to 400 nm) and UVB (280 to 315 nm) radiation, determined by integrating the instantaneous irradiances from dawn to dusk, temperature, pH, salinity, total alkalinity and seawater (SW) carbonate chemistry in cylinders with *Cystoseira tamariscifolia, Padina pavonica* and *Ellisolandia elongata* after 5.5 h *in situ* incubation under high CO_2 and ambient CO_2 conditions. Values reported are mean ± SE (n = 6). TA: total alkalinity; pCO_2: partial pressure of CO_2; DIC: dissolved inorganic carbon. Lower-case letters denote significant differences (ANOVA, p < 0.05)

	Cystoseira tamariscifolia		*Padina pavonica*		*Ellisolandia elongata*	
	High CO_2	Ambient CO_2	High CO_2	Ambient CO_2	High CO_2	Ambient CO_2
PAR (KJ m^{-2})	7662		4790		4416	
UVA (KJ m^{-2})	900.2		585.7		542.3	
UVB (KJ m^{-2})	37.0		24.2		21.9	
Temperature (°C)	26.1 ± 0.2	26.3 ± 0.2	25.8 ± 0.1	25.5 ± 0.2	22.5 ± 0.2	22.6 ± 0.2
pH	8.53 ± 0.03[b]	8.70 ± 0.02[a]	8.66 ± 0.02	8.57 ± 0.07	8.28 ± 0.04	8.27 ± 0.03
Salinity	37.0 ± 0.06	37.2 ± 0.06	37.0 ± 0.05	37.0 ± 0.02	37.0 ± 0.04	37.1 ± 0.08
TA (µmol kg^{-1} SW)	2289 ± 28	2323 ± 36	2300 ± 38	2344 ± 26	2497 ± 65	2394 ± 30
pCO_2 (µatm)	142 ± 13	79 ± 67	92 ± 34	132 ± 35	327 ± 29	319 ± 27
HCO_3^- (µmol kg^{-1}SW)	1360 ± 16	1120 ± 43	1202 ± 7	1317 ± 105	1847 ±36	1751 ±46
CO_3^{-2} (µmol kg^{-1} SW)	389 ± 22	471.8 ± 6.5	458.1 ± 18	401.9 ± 34	265.9 ± 21	245.0 ± 10
DIC (µmol kg^{-1} SW)	1753 ± 6	1594 ± 38	1663 ± 24	1723 ± 71	2123 ± 45	2006 ± 39

Table 2. ANOVAs of the effect of CO_2 treatments on alkalinity, temperature, and pH for *Cystoseira tamariscifolia*, *Padina pavonica* and *Ellisolandia elongata*. Significant results (ANOVA, $p < 0.05$) indicated in **bold**

			Cystoseira tamariscifolia			*Padina pavonica*			*Ellisolandia elongata*		
		df	MS	*F*	p	MS	*F*	p	MS	*F*	p
Alkalinity	Treatment	1	1734	0.55	0.499	643	0.173	0.698	29260	3.880	0.120
	Residual	4	3143			3706			7542		
Temperature	Treatment	1	0.060	0.95	0.386	0.107	1.60	0.275	0.007	0.077	0.795
	Residual	4	0.063			0.067			0.087		
pH	Treatment	1	**0.043**	**19.4**	**0.012**	0.013	1.57	0.278	0.000	0.071	0.803
	Residual	4	0.002			0.008			0.003		

No significant differences were found between field samples (initial values) and those for the control treatment (ambient CO_2) in any of the photosynthetic or biochemical variables, so initial values are not shown. In the ambient CO_2 treatment, values of F_v/F_m registered for *C. tamariscifolia*, *P. pavonica* and *E. elongata* were 0.64 ± 0.02, 0.61 ± 0.03 and 0.58 ± 0.02, respectively (Table 4). The analysis of the RLCs showed that, under ambient CO_2 conditions, *C. tamariscifolia* and *P. pavonica* had higher values of ETR_{max}, α_{ETR} and Ek_{ETR} compared to the calcareous red alga *E. elongata*. Values of ETR_{max} for *C. tamariscifolia* and *P. pavonica* were 18 and 16 times higher than for *E. elongata*. The latter also showed a fall in photosynthetic rates under irradiances above 500 µmol m^{-2} s^{-1} (Fig. 3). NPQ parameters were also higher for the brown algae than for the red calcareous alga. An exception was found for α_{NPQ}, where in *E. elongata* it showed higher values than that in the other 2 species. When the seaweeds were incubated in high CO_2, increases of F_v/F_m and NPQ_{max} in *C. tamariscifolia* and of α_{NPQ} in *P. pavonica* were observed compared to the control (Fig. 3, Table 4). Nevertheless, the tendency towards higher values for NPQ_{max} and/or α_{NPQ} under high CO_2 was found for all 3 species.

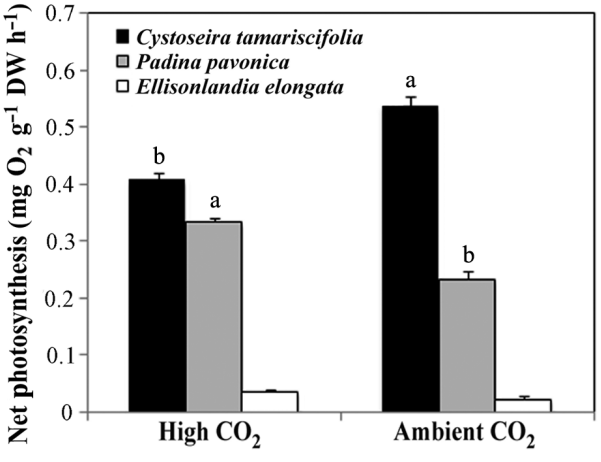

Fig. 2. Net photosynthesis in *Cystoseira tamariscifolia, Padina pavonica* and *Ellisolandia elongata* after 1.5 h *in situ* incubation in high CO_2 and ambient CO_2. Data are expressed as mean values ± SE (n = 24). Lowercase letters denote significant differences (ANOVA, $p < 0.05$)

Biochemical analysis

The analysis of photosynthetic and photoprotective pigments showed that *C. tamariscifolia* presented the highest concentrations of analysed pigments (chlorophylls, fucoxanthin, violaxanthin, antheraxanthin and β-carotene) (Table 5). Among the brown algae, concentrations of chl *a*, chl *c* and fucoxanthin were about twice as high in *C. tamariscifolia* compared to *P. pavonica*. The red alga *E. elongata* showed the lowest concentration of chlorophylls and carotenoids among the 3 species, particularly for fucoxanthin and violaxanthin (Table 5). In addition, a higher proportion of antheraxanthin+zeaxanthin to violaxanthin content were found in *E. elongata* compared to the 2 brown algae (Table 5). After the incubation period, the concentration of some photosynthetic pigments (chl *a*, chl *c* and/or phycobiliproteins) did not change significantly between ambient and high CO_2 treatments (Table 3). A significant difference between treatments was found for *E. elongata*, in which the zeaxanthin content was significantly higher under high CO_2 than under ambient CO_2 conditions (Tables 3 & 5).

Among the brown algae, *C. tamariscifolia* showed higher phenol content (25 mg g^{-1} DW) and antioxidant capacity than *P. pavonica* (20 mg g^{-1} DW) (Fig. 4). Significant differences in phenols and antioxidant capacity between treatments were only found for *P. pavonica*, in which a decrease in phenolic compounds was observed under high CO_2 (Fig. 4, Table 3). A negative correlation was found between

Table 3. ANOVAs of the effect of CO_2 treatments on the photosynthetic parameters, photoprotective compounds, antioxidant capacity, photosynthetic pigments and net photosynthesis for *Cystoseira tamariscifolia, Padina pavonica and Ellisolandia elongata.* df for *E. elongata* in net photosynthesis were 1, 2 and 12 for treatment, cylinder(treatment) and residual, respectively, and in the carotenoids (fucoxanthin, violaxanthin, antheraxanthin, zeaxanthin, β-carotene) 1, 2 and 4, respectively. EC50: oxidation index; see Table 4 for other abbreviations. For treatment only, significant results (ANOVA, $p < 0.05$) are indicated in **bold**; nd: no data

			C. tamariscifolia			*P. pavonica*			*E. elongata*		
		df	MS	*F*	p	MS	*F*	p	MS	*F*	p
Net photosynth.	**Treatment**	**1**	**0.1**	**9.518**	**0.037**	**0.061**	**10.172**	**0.033**	0.001	3.475	0.203
	Cylinder(treatment)	4	0.011	32.712	<0.001	0.006	52.048	<0.001	0.000	9.51	0.003
	Residual	18	0.000			0.000			0.000		
F_v/F_m	**Treatment**	**1**	**0.011**	**25.061**	**0.007**	0.014	1.391	0.304	0.001	0.444	0.542
	Cylinder(treatment)	4	0.000	0.169	0.947	0.01	3.026	0.109	0.002	0.839	0.547
	Residual	6	0.003			0.003			0.003		
ETR_{max}	Treatment	1	2.54	0.09	0.779	6.721	0.297	0.615	0.394	1.609	0.273
	Cylinder(treatment)	4	28.156	0.579	0.689	22.643	0.632	0.658	0.245	1.098	0.436
	Residual	6	48.604			35.837			0.223		
α_{ETR}	Treatment	1	0.005	2.691	0.176	0.02	2.509	0.188	0.000	0.009	0.927
	Cylinder(treatment)	4	0.002	0.16	0.951	0.008	0.683	0.629	0.000	3.716	0.075
	Residual	6	0.011			0.011			0.000		
Ek_{ETR}	Treatment	1	241	1.772	0.254	1234	1.264	0.324	116	1.609	0.273
	Cylinder(treatment)	4	136	0.152	0.955	977	1.359	0.35	72	1.194	0.402
	Residual	6	894			719			60		
NPQ_{max}	**Treatment**	**1**	**0.318**	**36.083**	**0.004**	1.155	2.323	0.202	0.009	0.881	0.401
	Cylinder(treatment)	4	0.009	0.067	0.99	0.497	14.306	0.003	0.011	0.654	0.646
	Residual	6	0.132			0.035			0.016		
α_{NPQ}	**Treatment**	**1**	0.000	3.371	0.14	**0.000**	**9.883**	**0.035**	0.000	3.604	0.13
	Cylinder(treatment)	4	0.000	0.371	0.822	0.000	1.668	0.274	0.000	0.493	0.742
	Residual	6	0.000			0.000			0.000		
Ek_{NPQ}	Treatment	1	4368	0.701	0.45	49009	4.299	0.107	1036	5.214	0.084
	Cylinder(treatment)	4	6234	1.438	0.329	11400	1.443	0.327	199	0.434	0.781
	Residual	6	4336			7903			458		
Chl *a*	Treatment	1	0.227	0.602	0.481	0.011	0.564	0.494	0.005	0.114	0.753
	Cylinder(treatment)	4	0.378	1.792	0.249	0.019	2.168	0.19	0.048	4.411	0.053
	Residual	6	0.211			0.009			0.011		
Chl *c*	Treatment	1	0.0024	1.729	0.259	0.0002	0.981	0.378			
	Cylinder(treatment)	4	0.0014	0.979	0.484	0.0002	1.663	0.275		nd	
	Residual	6	0.0014			0.0001					
Fucoxanthin	Treatment	1	17085	0.664	0.461	2648	0.896	0.397	12.18	5.442	0.145
	Cylinder(treatment)	4	102886	3.328	0.092	2956	8.447	0.012	2.238	0.498	0.641
	Residual	6	46372			350			4.493		
Violaxanthin	Treatment	1	21.35	0.041	0.849	474	1.634	0.27	0.018	0.507	0.55
	Cylinder(treatment)	4	2067	7.04	0.019	290	2.844	0.122	0.035	2.545	0.194
	Residual	6	440.44			102			0.014		
Antheraxanthin	Treatment	1	79.24	0.511	0.514	2.177	0.031	0.87	16.545	1.501	0.345
	Cylinder(treatment)	4	620.02	2.223	0.182	71.06	3.183	0.1	11.022	1.332	0.36
	Residual	6	418.27			22.328			8.277		
Zeaxanthin	**Treatment**	**1**	12.77	0.061	0.817	1.395	0.02	0.896	**5.011**	**186.2**	**0.005**
	Cylinder(treatment)	4	834.27	6.368	0.024	71.333	1.661	0.275	0.027	0.122	0.888
	Residual	6	196.51			42.933			0.221		
β-carotene	Treatment	1	27.94	0.086	0.784	122.119	2.094	0.221	22.144	1.469	0.349
	Cylinder(treatment)	4	1304.07	3.523	0.083	58.308	1.882	0.233	15.073	4.441	0.096
	Residual	6	555.31			30.978			3.394		
Phenols	**Treatment**	**1**	1.6	0.068	0.808	**45.827**	**9.967**	**0.034**			
	Cylinder(treatment)	4	23.69	0.882	0.527	4.598	0.516	0.728		nd	
	Residual	6	26.85			8.909					
EC_{50}	Treatment	1	0.002	0.042	0.847	0.017	0.779	0.427			
	Cylinder(treatment)	4	0.056	4.638	0.048	0.022	2.302	0.173		nd	
	Residual	6	0.012			0.009					
Phycoerythrin	Treatment	1							0.079	1.13	0.348
	Cylinder(treatment)	4		nd			nd		0.07	0.918	0.511
	Residual	6							0.076		
Phycocyanin	Treatment	1							0.001	0.133	0.734
	Cylinder(treatment)	4		nd			nd		0.005	1.677	0.272
	Residual	6							0.003		
Total MAAs	Treatment	1							0.006	0.216	0.666
	Cylinder(treatment)	4		nd			nd		0.027	1.199	0.401
	Residual	6							0.022		
Shinorine	Treatment	1							351	1.202	0.334
	Cylinder(treatment)	4		nd			nd		292	12.085	0.005
	Residual	6							24.181		
Palythine	Treatment	1							224	0.922	0.391
	Cylinder(treatment)	4		nd			nd		243	13.601	0.004
	Residual	6							17.888		

Table 4. Maximal quantum yield (F_v/F_m), maximal electron transport rate (ETR_{max}), photosynthetic efficiency (α_{ETR}), saturation irradiance for ETR (Ek_{ETR}), maximal non-photochemical quenching (NPQ_{max}), the slope of the NPQ versus irradiance (α_{NPQ}) and the saturation irradiance for NPQ (Ek_{NPQ}) of *Cystoseira tamariscifolia, Padina pavonica* and *Ellisolandia elongata* after 5.5 h *in situ* incubation in high CO_2 and ambient CO_2 conditions. Values are mean ± SE (n = 12). Lowercase letters denote significant differences (ANOVA, $p < 0.05$)

	Cystoseira tamariscifolia		*Padina pavonica*		*Ellisolandia elongata*	
	High CO_2	Ambient CO_2	High CO_2	Ambient CO_2	High CO_2	Ambient CO_2
F_v/F_m	0.70 ± 0.02[a]	0.64 ± 0.02[b]	0.68 ± 0.03	0.61 ± 0.03	0.60 ± 0.02	0.58 ± 0.02
ETR_{max}	47.23 ± 2.82	48.15 ± 2.35	39.43 ± 2.85	40.94 ± 1.45	2.20 ± 0.19	2.56 ± 0.20
α_{ETR}	0.55 ± 0.04	0.51 ± 0.04	0.55 ± 0.04	0.47 ± 0.04	0.07 ± 0.01	0.06 ± 0.01
Ek_{ETR}	88.48 ± 9.82	97.45 ± 10.03	73.81 ± 6.94	94.09 ± 15.03	34.08 ± 3.03	40.29 ± 3.53
NPQ_{max}	1.09 ± 0.10[a]	0.76 ± 0.13[b]	1.39 ± 0.22	0.77 ± 0.16	0.68 ± 0.04	0.63 ± 0.06
α_{NPQ}	0.003 ± 0.0005	0.002 ± 0.0003	0.004 ± 0.0006[a]	0.002 ± 0.0004[b]	0.021 ± 0.0036	0.014 ± 0.0029
Ek_{NPQ}	394.6 ± 33.1	356.4 ± 24.6	323.7 ± 45.4	451.5 ± 32.2	36.6 ± 4.6	55.1 ± 9.8

phenols and EC_{50}, for both species (Figs. 4 & 5; R^2 = 0.674, n = 24, $p < 0.05$).

The content of total MAAs found in the red alga *E. elongata* was not significantly different between either CO_2 treatment (0.62 ± 0.06 mg g^{-1} DW for high CO_2 and 0.66 ± 0.07 mg g^{-1} DW for ambient CO_2, n = 12) (Table 3). The MAA composition was also similar between treatments as follows: 50 to 60% shinorine, 40% palythine and 5 to 10% asterina-330 (Fig. 6, Table 3).

DISCUSSION

Most studies on the effects of OA on marine macrophytes have been conducted *ex situ* in laboratories or mesocosms under controlled conditions. While those studies are useful to better understand some isolated effects of increasing dissolved CO_2 on algal photosynthesis and biochemistry, the results do not reflect the response of the natural populations. *In situ* experimental approaches can operate under more realistic

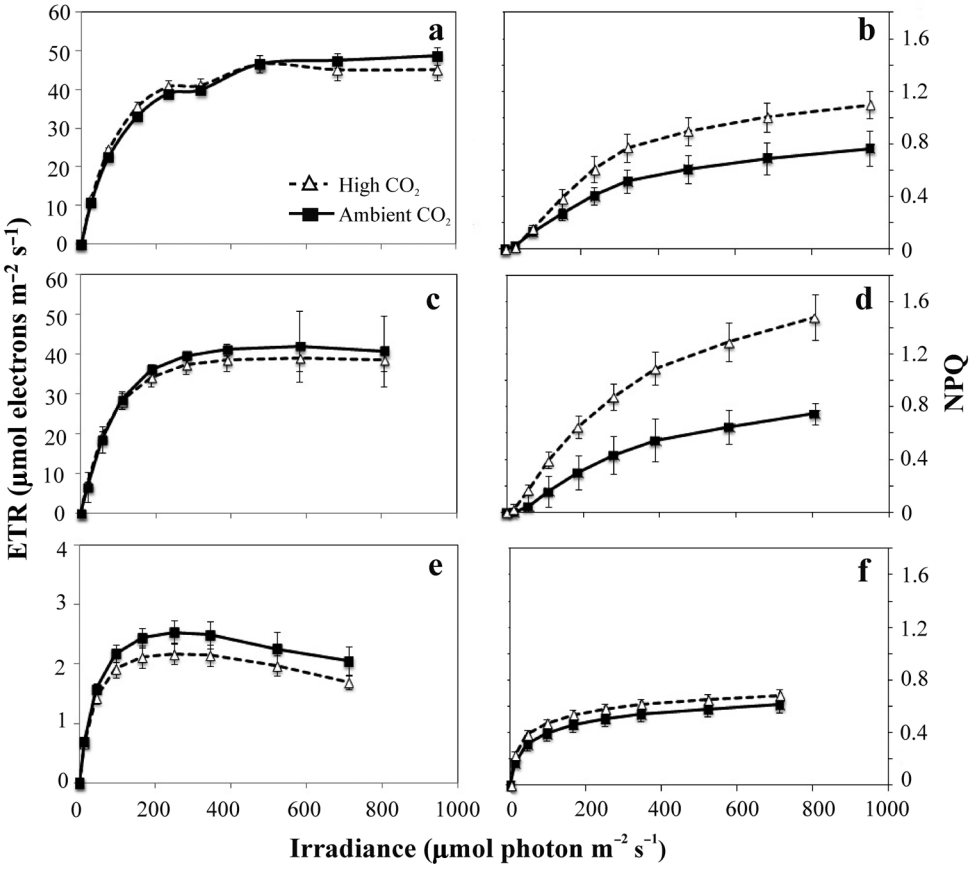

Fig. 3. Electron transport rate (ETR) and non-photochemical quenching (NPQ) versus irradiance curves for (a,b) *Cystoseira tamariscifolia*, (c,d) *Padina pavonica* and (e,f) *Ellisolandia elongata* after 5.5 h *in situ* incubation in high CO_2 and ambient CO_2 conditions. Data are mean ± SE (n = 12)

Table 5. Pigment concentration of *Cystoseira tamariscifolia, Padina pavoniva* and *Ellisolandia elongata* after 5.5 h *in situ* incubation in high CO_2 and ambient CO_2 conditions. Values are mean ± SE (n = 12). Chl *a*, chl *c*, phycoerythrin and phycocyanin are in mg g^{-1} DW. The other pigments are expressed as µg g^{-1} DW. Lowercase letters denote significant differences (ANOVA, $p < 0.05$)

	Cystoseira tamariscifolia		*Padina pavonica*		*Ellisolandia elongata*	
	High CO_2	Ambient CO_2	High CO_2	Ambient CO_2	High CO_2	Ambient CO_2
Chl *a*	1.97 ± 0.21	2.25 ± 0.23	0.81 ± 0.05	0.75 ± 0.05	0.61 ± 0.07	0.65 ± 0.06
Chl *c*	0.17 ± 0.014	0.20 ± 0.017	0.07 ± 0.005	0.06 ± 0.005	–	–
Phycoerythrin	–	–	–	–	2.55 ± 0.08	2.72 ± 0.14
Phycocyanin	–	–	–	–	0.49 ± 0.02	0.47 ± 0.03
Fucoxanthin	550.1 ± 50.5	625.6 ± 49.2	308.9 ± 14.3	279.2 ± 16.1	9.74 ± 0.63	7.27 ± 1.22
Violaxanthin	68.3 ± 8.1	65.6 ± 4.2	53.5 ± 3.6	40.9 ± 6.8	0.44 ± 0.04	0.53 ± 0.09
Antheraxanthin	46.7 ± 3.5	51.9 ± 4.8	25.6 ± 1.7	24.7 ± 3.3	19.6 ± 1.5	22.5 ± 1.5
Zeaxanthin	16.7 ± 2.9	18.8 ± 5.1	16.8 ± 2.2	17.51 ± 3.6	3.48 ± 0.25[a]	1.90 ± 0.12[b]
β-carotene	61.3 ± 5.0	64.4 ± 6.1	45.9 ± 3.4	39.6 ± 1.5	18.6 ± 1.5	21.9 ± 1.2

environmental conditions and deal with the variation of the natural populations (Wernberg et al. 2012). In this study, macroalgae were incubated *in situ* at 2 different pCO_2 levels, while other parameters remain relatively unchanged. The experiments were performed in a pristine marine environment, by evaluating the short-term responses of *Cystoseira tamariscifolia, Padina pavonica* and *Ellisolandia elongata*, which are dominant macroalgae on the Mediterranean shores.

The results showed that the short-term responses of the seaweeds to seawater CO_2 enrichment varied according to species and their functional traits. While the brown algae *C. tamariscifolia* showed a reduction in photosynthetic rate (based on O_2 evolution), the calcified brown algae *P. pavonica* showed an increase under high CO_2 conditions. Both algae enhanced the NPQ mechanisms, but no changes in pigment composition or concentration were found. The enhanced production by *P. pavonica* under high CO_2 came with reductions in phenol. The calcareous red algae *E. elongata* was not significantly affected by CO_2 enrichment in most of the photosynthetic and biochemical parameters, but it showed increases in the concentration of its photoprotective pigment, zeaxanthin. The contrasting results found for the 3 studied species in response to pCO_2 enrichment may be related to the striking differences in the photosynthetic apparatus, including pigment composition and concentration, and their highly distinct morphological traits and growth strategies. Among the brown algae, *C. tamariscifolia* has thick blades, with highly corticated and complex thallus, compared to the simpler sheet-like, thinner *P. pavonica*. The articulated calcareous *E. elongata* has the lowest ratio of photosynthetic to non-photosynthetic (calcified) tissue.

At the end of the incubation period, the effect of pCO_2 on seawater carbonate chemistry and pH was different depending on the incubated species. These may be related to differences in metabolism, since seawater carbonate chemistry is strongly affected by biological activity (Feely et al. 2004, Raven 2011). Although the pH values were significantly lowered (by 0.3 units) in the cylinders treated with high CO_2

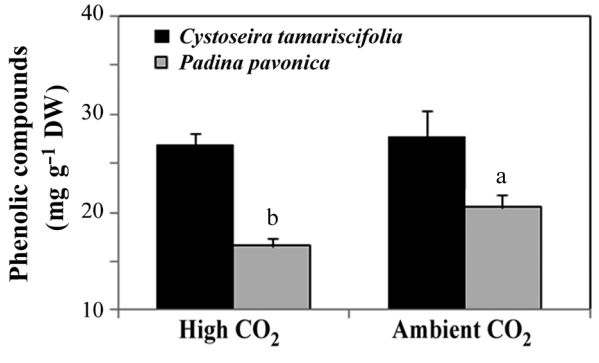

Fig. 4. Phenolic compound tissue concentration in *Cystoseira tamariscifolia* and *Padina pavonica* after 5.5 h *in situ* incubation in high CO_2 and ambient CO_2. Data are expressed as mean + SE (n = 12). Lowercase letters denote significant differences

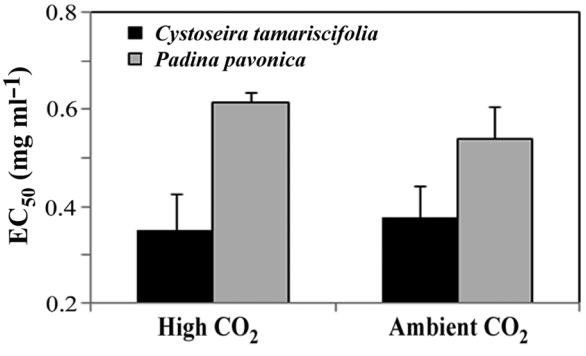

Fig. 5. Antioxidant capacity of *Cystoseira tamariscifolia* and *Padina pavonica* after 5.5 h *in situ* incubation in high CO_2 and ambient CO_2 conditions. Data are mean + SE (n = 12)

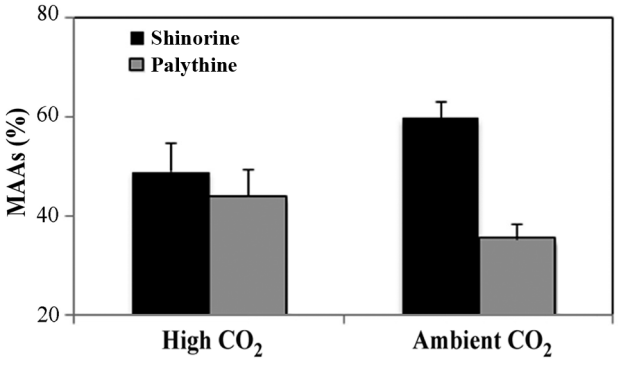

Fig. 6. Percentage of shinorine and palythine in *Ellisolandia elongata* after 5.5 h *in situ* incubation in high CO_2 and ambient CO_2 conditions. Data are mean + SE (n = 12)

before algae incubation, the pH tended to increase, reaching similar values in both pCO_2 treatments during the experiment. The initial difference after the incubation was only maintained in *C. tamariscifolia*. In general, respiration and dissolution of CO_2 into seawater lowers the pH, while photosynthetic carbon removal from the medium increases it. Additionally, calcium carbonate dissolution of macroalgae skeletons also affects the seawater carbonate chemistry speciation and seawater pH (Feely et al. 2004). Therefore, the pH within incubations mainly depends on the balance among those processes (Gao et al. 1991). In our study, *E. elongata* only raised the pH of the surrounding seawater at high pCO_2, and showed the lowest increase among species. Cornwall et al. (2012) also found the lowest increase in pH in a coralline alga, *Corallina officinalis*. On the other hand, the absence of calcium carbonate in the thallus of *C. tamariscifolia* could in part explain the maintenance of the difference between treatments after the incubation (Caldeira & Wickett 2003, Cornwall et al. 2012). These results can have at least 2 implications: (1) methodological: the results of CO_2 experiments are dependent on container volume, amount of algal material and air flux rate in each incubation chamber; or (2) ecophysiological: the results draw attention to the lower capacity of *C. tamariscifolia* to buffer its surrounding seawater pH, compared to calcified algae.

While calcareous algae are known to be vulnerable to OA, by decreasing their calcium carbonate fixation and increasing dissolution (Feely et al. 2004), the non-calcified species are likely to show positive responses to elevated pCO_2, since many macroalgal species have been shown to be carbon-limited in nature (Mercado et al. 1999, Koch et al. 2013), in part due to the inefficacies of Rubisco and inorganic carbon uptake mechanisms, as well as boundary layer-related problems (Raven et al. 2012). However, the degree of macroalgae photosynthetic responses to elevated pCO_2 is uncertain and variable (Koch et al. 2013). Mercado & Gordillo (2011) reported that changes in CO_2 concentration by natural processes or climate change could have a limited impact on primary production in a variety of aquatic ecosystems due to the effective acclimation processes (Beardall & Raven 2004).

In the present study, a reduction in photosynthetic capacity under the short-term incubation at elevated pCO_2 was observed for *C. tamariscifolia*, while *P. pavonica* increased its net photosynthesis. These observed changes in net photosynthesis were not followed by changes in ETR_{max}, nor in other chl *a* fluorescence parameters. In fact, an increase in F_v/F_m was detected under high pCO_2 in *C. tamariscifolia*. By comparing the results of several studies, one can say that the effects of OA on photosynthesis of macroalgae varies according to species and functional traits, as well as incubation characteristics (time exposure, light quality and quantity, type and size of incubator, etc.) (Martin & Gattuso 2009, Semesi et al. 2009, Gao & Zheng 2010, Sinutok et al. 2011, Zou et al. 2011). Important physiological aspects to be considered are the presence of an HCO_3^- transport system, the type of carbon-concentrating mechanisms (CCM) (Mercado et al. 1998, Wu et al. 2008) and the possible inhibitory effect of CO_2 on respiration, among others. CCMs involve the enzyme external carbonic anhydrase (exCA). A previous study showed that exCA was not present in *P. pavonica*, but it was detected in *C. tamariscifolia*, indicating a low affinity of this latter species for inorganic carbon compared to other macroalgae (Mercado et al. 1998). An exCA in *P. pavonica* could be present, but Mercado et al. (1998) did not detect it as consequence of the limitation in the methodology used to detect this enzyme. For another *Padina* species, *P. sanctae-crucis*, Enríquez & Rodríguez-Román (2006) suggested the presence of an efficient CCM, probably related to HCO_3^- uptake. A CCM requires an energetic investment for expression and operation (Raven et al. 2012). A down-regulation of the CCM activity in response to enriched inorganic carbon has been proposed; therefore, the alga has more energy to invest in other processes such as growth (Giordano et al. 2005). In spite of these mechanisms, we did not find any positive effect of elevated pCO_2 on photosynthesis in the non-calcifying macroalgae *C. tamariscifolia*.

In this study, *E. elongata* showed the lowest pigment concentration among the 3 species (especially fucoxanthin and violaxanthin contents which were half

those in the brown algae), although antheraxanthin was maintained in the same order of magnitude. To our knowledge, no short-term study (hours) on the effect of high CO_2 on pigment content exists. In this study, no significant effect of pCO_2 treatments in pigment content was found in the non-calcifying *C. tamariscifolia* or in the calcifying *P. pavonica*. Nevertheless, *P. pavonica* showed a tendency to have higher pigment contents under high CO_2, especially fucoxanthin, which is an accessory photosynthetic pigment with strong antioxidant properties (Mori et al. 2004, Fung et al. 2013) and its presence strongly enhanced cell viability against H_2O_2 induced oxidative damage (Heo et al. 2008). Additionally, both brown algae species showed increases in NPQ parameters (NPQ_{max} and α_{NPQ}, for *C. tamariscifolia* and *P. pavonica*, respectively) and decreased Ek_{ETR} under high CO_2. The lower Ek_{ETR} indicates a higher rate of reactive oxygen species (ROS) production at incubation irradiances (solar radiation) (Lesser 2006). It is possible that the short-term exposure to high CO_2 activated their heat dissipation mechanisms; hence, these species became protected against the increased photooxidative damage (Demmig-Adams & Adams 2006).

In response to intense solar radiation, algae have evolved certain photoprotective mechanisms by accumulating a series of photoprotective compounds, such as carotenoids, phenolic compounds (brown algae) and MAAs (red algae). Regarding phenolic compounds, a higher content was observed in *C. tamariscifolia* than in *P. pavonica*. It is possible that the acidification of the medium drove the phenolic losses in *C. tamariscifolia* and *P. pavonica* by photodegradation and release, as has been observed in marine angiosperms along a natural gradient of CO_2 (Arnold et al. 2012). A decrease of phenolic compounds and the antioxidant activity during a submarine volcanic eruption, which produced a decrease in seawater pH level, was also observed (Betancor et al. 2014). The higher concentration of zeaxanthin observed in *E. elongata* under high CO_2 treatment could indicate a higher photoprotection potential under a future scenario of high pCO_2. Hence, a photoprotective role could be argued for zeaxanthin, since it has been described as a zeaxanthin-dependent amplification of NPQ exclusively found in thylakoids containing zeaxanthin (Goss et al. 2006). To our knowledge, this is the first time that the effect of different pCO_2 on MAAs has been studied. Although no effect on total MAA content was found in *E. elongata*, a tendency to accumulate relatively more palythine under high CO_2 was observed. It is known that palythine possesses a higher antioxidant capacity than the other MAAs (De la Coba et al. 2009). Thus, even though this species may show a loss of carbonate skeleton under OA, it could still maintain high photoprotective and antioxidant capacities.

The ecophysiological responses of macroalgae to high CO_2 concentrations are variable and complex, and for calcareous macroalgae, the responses may be even more complex due to the calcification process. Some evidences show that certain calcifying Phaeophyceae could be amongst the ecological winners under OA scenarios (Kübler et al. 1999, Porzio et al. 2011, Raven 2011, Johnson et al. 2012), and that the function and structure of future ecosystems could be drifting towards these species. However, the ecological impacts on these particular species and further consequences for the surrounding macroalgal community are unknown and difficult to predict.

CONCLUSIONS

In this study, we present a novel experimental design to incubate macroalgae *in situ* at different pCO_2. Our design tested non-calcifying (*Cystoseira tamariscifolia*) versus calcifying (*Padina pavonica* and *Ellisolandia elongata*) marine macroalgae in a short-term incubation at 2 different pCO_2: air (ambient CO_2) and the pCO_2 predicted by the end of the 21st century (700 µatm, high CO_2). Slight differences were detected in the 3 studied species after the short-term incubations. Although one would expect an increase in net photosynthesis under enriched CO_2 as the algae would have more substrate for Rubisco, slight differences were found between CO_2 levels within *in vivo* chlorophyll fluorescence parameters and a positive effect of increasing pCO_2 on net photosynthesis was only observed in *P. pavonica*. On the other hand, although the activation of the NPQ dissipation mechanism occurred under high CO_2 in the brown algae *P. pavonica* (indicating a high protection mechanism), this capacity was counteracted by a loss of phenols. In *E. elongata*, the higher zeaxanthin and palythine contents under high CO_2 could indicate a higher photoprotection capacity. This is one of the few studies in which the effect of CO_2 on macroalgal photoprotective compounds has been evaluated (but see Betancor et al. 2014).

Despite the increasing number of studies on the effect of changes in pCO_2 on macroalgae, the ecophysiological responses of these species to a future scenario of OA are still unknown. Together with the other systems proposed for different habitats and macrophyte species (Campbell & Fourqurean 2011,

2013, Kline et al. 2012, Arnold et al. 2012), the *in situ* incubation system proposed here may contribute to this knowledge and has the advantage of being simple, reproducible and cheap. Nevertheless, for the near future, parameters such as different pCO_2 and gas flux rates, carbonate system parameters, chamber volume and algal density or biomass (among others) should be exhaustively controlled. The development of long-term experiments including day–night cycles and enabling acclimation responses should be studied for a better understanding of how macroalgae will respond to a future OA scenario.

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