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Short-term effects of increasing CO_2, nitrate and temperature on three Mediterranean macroalgae: biochemical composition

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ABSTRACT: Short-term effects of increasing pCO_2; 380 ppm (LC) vs. 700 ppm (HC); at different nitrogen levels; 5 µM nitrate (LN) vs. 50 µM (HN); on the contents of protein, mycosporine-like amino acids (MAAs), phenolic compounds and total fatty acids, antioxidant activity, calcification and C:N ratios were analyzed in 3 eulittoral Mediterranean macroalgae with different bio-optical characteristics and carbon assimilation efficiencies: *Cystoseira tamariscifolia* (Heterokontophyta), *Ulva rigida* (Chlorophyta) and *Ellisolandia elongata* (Rhodophyta). After acclimation to different pCO_2 and nitrogen conditions for 6 d, the algae were subjected to a 4°C temperature increase for 3 d. Increasing temperature and pCO_2 produced alterations in the biochemical composition of the 3 macroalgae. Short-term variations of protein levels were observed in *U. rigida*, with clearly decreased values in the HCLN treatment. In *C. tamariscifolia*, protein decreased after the temperature increase but only under LC. The interaction of temperature and N affected phenolic compounds only in *U. rigida* and the content of MAAs in *E. elongata*. The functional patterns of the 3 macroalgae in response to the pCO_2, nitrogen and temperature regimes may be explained in terms of their bio-optical characteristics and antioxidant activity. The vulnerability and acclimation of the 3 species to the expected variations of climate change factors are discussed.

KEY WORDS: Acidification · Climate change · *Cystoseira tamariscifolia* · *Ellisolandia elongata* · Nitrate · Short-term experiment · Temperature · *Ulva rigida*

INTRODUCTION

Sea surface temperature has been increasing by 0.13°C per decade over the last 50 yr, mainly due to the increase of CO_2 levels in the atmosphere (IPCC 2013). One-third of the atmospheric CO_2 produced by human activity has been absorbed by the oceans in the past 200 yr, which has led to a reduction in the pH of surface seawater by 0.1 units (Orr et al. 2005). Oceans will be further acidified by 0.3 to 0.4 units by 2100 due to the expected rise in the atmospheric CO_2 level to 800–1000 ppmv by the end of this century, according to the 'business-as-usual' CO_2 emission scenario (Brewer 1997, Caldeira & Wickett 2003). This ocean acidification process may significantly change the water carbonate chemistry (Sabine et al. 2004, Pelejero et al. 2010) and may harm marine calcifying organisms (Gao et al. 1993, Riebesell et al.

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2000). Ocean acidification has been consistently related to reduced growth rates in calcified macroalgae (Kroeker et al. 2010). Reductions in calcification rate at elevated pCO_2 have been demonstrated for crustose and articulated coralline red algae, as well for the calcified green algae of the genus *Halimeda* (Gao et al. 1993, Büdenbender et al. 2011, Price et al. 2011). However, reduced calcification at increased pCO_2 did not emerge as a general pattern in a meta-analysis of multiple seaweed studies (Kroeker et al. 2010). This may partly be because the effects of ocean acidification on calcification vary among seaweeds due the diversity of calcification strategies amongst macroalgae, e.g. many species are able to create microclimates of chemistry favorable for calcification regardless of ambient conditions (Price et al. 2011, Roleda et al. 2012a). Reduced pH may have important consequences for non-calcifying taxa as well (Roleda et al. 2012b), although the cumulative effects of climatically realistic, CO_2-driven pH change on non-calcifying seaweeds remain poorly understood (Kroeker et al. 2010). Studies on the ecological and physiological impacts of elevated CO_2 concentrations on macroalgae were initiated in the early 1990s: growth of *Pyropia* (*Porphyra*) *yezoensis* juveniles was significantly enhanced in cultivation enriched with CO_2 up to 1000 ppmv (Gao et al. 1991, 1993). The photosynthetic carbon fixation rate of some intertidal macroalgal species increased during low-tide periods when they were exposed to air containing high CO_2 concentration (Gao et al. 1991, Zou & Gao 2002). Macroalgae are a highly diverse group with complex functional morphologies and varied ecological roles. Thus, morphological plasticity in addition to physiological mechanisms may determine their capacity to acclimate to global climate changes (Falkenberg et al. 2013).

In recent years, there has been a significant effort to predict the future impact of climate change on seaweed communities (Graham et al. 2007, Halpern et al. 2008, Wernberg et al. 2010). Two main approaches have been followed: (1) experimental approaches designed as factorial experiments, incubating macroalgae for days or months at different growth temperatures according to the future predicted scenarios, and evaluating the interactive responses with other variables, such as acidification, UV radiation (UVR), and nutrient availability, amongst others (Baulch et al. 2003, Hoppe et al. 2008, Porzio et al. 2011); and (2) field studies of seaweeds growing at their temperature limit for growth and reproduction, while monitoring the temporal and spatial variation of temperature and other variables (Viejo et al. 2011, Martínez et al. 2012). Most investigations have been conducted on individual species separately, rather than communities (Olabarria et al. 2013), although it has been reported that community-level impacts might be less noticeable (Kroeker et al. 2010). Moreover, studies on the effect of global climate changes on aquatic organisms have mostly been conducted with 1 or 2 variables, and interactions between multiple factors have scarcely been studied (Franklin & Foster 1997, Gordillo et al. 2001, Bischof et al. 2006a, Häder et al. 2007). For example, the effect of increased UVR, temperature and CO_2 on photosynthetic metabolism, nutrient incorporation and growth has been studied in algae from Mediterranean and Atlantic waters off the southern Iberian Peninsula (reviewed in Häder & Figueroa 1997, Figueroa & Gómez 2001), but also in algae from the North Sea (Hanelt et al. 1997b) and the Arctic region (Hanelt et al. 1997a, Hanelt 1998, Roleda 2009). Investigations have been conducted in the field (*in situ*) and in experimentally controlled conditions under solar radiation (outdoor) and under artificial conditions (indoor). Mechanisms for acclimation to global climate changes have been evaluated as the effect on photoinhibition, photoprotection, nutrient uptake systems and patterns of growth, reproduction and morphogenesis of different developmental stages of macroalgae (Häder & Figueroa 1997, Gómez & Figueroa 1998, Figueroa et al. 2003b, Villafañe et al. 2003, De la Coba et al. 2009).

To date, few short-term (<1 yr) algal manipulation experiments have been performed. Previous studies have revealed mixed responses depending on the algal species and the culture conditions applied (Porzio et al. 2011, Zou et al. 2011, Cornwall et al. 2012). In macroalgae, doubling the CO_2 level caused an increase in photosynthetic activity of 52 to 130%, depending on the species (Gao et al. 1993, Kübler et al. 1999, Riebesell et al. 2007). Overall, the sensitivity of algae to acidification is expected to be complex, due to interactions between the effects of pH and CO_2 on the enhancement of photosynthesis. Although increasing ocean CO_2 concentration may enhance rates of photosynthesis and growth, particularly in species without carbon concentrating mechanisms (CCMs), such increases may be limited by the availability of other limiting nutrients (Raven et al. 2005). Mercado et al. (1998) reported a relationship between CCMs and light energy availability in intertidal macroalgae, but not with inorganic carbon availability: intertidal algae with emersion periods presented higher photosynthetic rates (Mercado et al. 1998) and carbon uptake (Flores-Moya et al. 1998)

due to the higher availability of CO_2 compared to submerged algae. Seasonal changes in temperature, nutrient availability and light are also likely to interact with the effect of CO_2 on metabolic processes in algae (Tyrell et al. 2008, Martin & Gattuso 2009, Mercado & Gordillo 2011, Hofmann et al. 2013). As calcification, photosynthesis, nutrient uptake, growth, and other metabolic processes are affected by temperature, light and nutrient availability, changes in these parameters are likely to have a strong influence on the enzymatic response of macroalgae to increasing CO_2. Therefore, outdoor mesocosm studies are useful for monitoring CO_2 effects over time during natural temperature, nutrient and light fluctuations.

The aim of this study was to evaluate the short-term (6 d) vulnerability and acclimation to increasing pCO_2 (low CO_2, LC: 380 ppm vs. high CO_2, HC: 700 ppm), under different nitrogen levels through nitrate pulses: high nitrate level (HN, 50 µM) vs. low nitrate level (LN, 5 µM) on 3 common eulittoral macroalgal species from the coastal area of Málaga in the Alborán Sea (Mediterranean Sea). In this region, nitrate concentrations are decreasing as a consequence of the weakening of wind-induced upwelling; thus, oligotrophication is becoming more recurrent (Mercado et al. 2012). The Mediterranean Sea is a priority area for studies on climate change due to the well-recognized increase in average seawater temperatures and the 200 to 500% increase in the number of days with heat extremes (Mieszkowska et al. 2006, Diffenbaugh et al. 2007, Louanchi et al. 2009).

The vulnerability of seaweeds to climate change factors in the short-term was evaluated by using physiological indicators including stoichiometry (C:N), quality of biomass (soluble proteins, photoprotectors and lipids) and antioxidant substances according to Figueroa & Korbee (2010) and Figueroa et al. (2014b). The experiments were conducted in the framework of the International Workshop of the Group on Aquatic Primary Productivity (GAP 9) hosted in September 2012, in Málaga. In addition to this study, the results of the experiments on these macroalgae have been presented in other reports (Stengel et al. 2014, Figueroa et al. 2014a, Parages et al. 2014; all this Theme Section).

MATERIALS AND METHODS

Algae and experimental design

Macroalgae from different morpho-functional groups defined according to Littler et al. (1983) were collected in La Araña (36° 45′ N, 4° 18′ W) on the Málaga coastline (Alborán Sea, southern Iberian Peninsula) characterized by a good ecological status of coastal waters as defined by the European Water Framework Directive (2000/60/EC) (Mercado et al. 2012, Bermejo et al. 2013).

Ulva rigida C. Agardh (Chlorophyta) is a distromatic laminar alga consisting of 2 cell layers, characterized by rapid growth and a nitrophilic pattern; the leathery *Cystoseira tamariscifolia* (Hudson) Papenfuss (Phaeophyceae) displays a complex parenchymatic morphology with a thick, non-calcareous thallus with cortical and medullar cells, and presents a low growth rate. Both *U. rigida* and *C. tamariscifolia* contain phenolic compounds. Finally, the calcareous *Ellisolandia elongata* (J. Ellis & Solander, K. R. Hind & G. W. Saunders), formerly *Corallina elongata* J. Ellis et Solander (Rhodophyta) according to Hind & Saunders (2013), presents a complex pseudoparenchymatic morphology with a thick calcareous thallus, cortical and medullar cells, slow growth rate, and contains mycosporine-like amino acids (MAAs), as do other red algae. These macroalgal species were selected since they are dominant species on the Mediterranean coast of the southern Iberian Peninsula and possess different morphologies, bio-optical properties and carbon acquisition efficiencies; in addition, their ecophysiological responses have been extensively studied in these or similar species (Mercado et al. 1998, Figueroa & Viñegla 2001, Gordillo et al. 2001, Abdala-Díaz et al. 2006, Cabello-Pasini et al. 2011, amongst others).

The collected algae were transported to the laboratory in an icebox, cleaned of epibiota and acclimated for 4 d to low C and low N conditions prior to experimental treatment conditions. The experimental design is described in detail by Stengel et al. (2014). Briefly, the 3 species were cultivated in a mesocosm system, with either high or low C and N conditions: high CO_2 (HC): 700 ppm vs. low CO_2 (LC): 380 ppm; high nitrate level (HN): 50 µM vs. low nitrate level (LN): 5 µM. The level of 380 ppm used in the experiment as ambient level (LC) of CO_2 is the range surface pCO_2 of the Alborán Sea (367 to 394 ppm of pCO_2) according to Takahashi et al. (2011) and McElhany & Busch (2013). This region can be characterized as oligotrophic (Ramírez et al. 2005, Mercado et al. 2012). The level of 5 µM nitrate is the highest range of the natural levels found during certain periods of the year (Ramírez et al. 2005). Thus, the LCLN treatment can be considered a control treatment because it was closest to natural conditions. In addition to the different C and N conditions, the algae

were subjected to a 4°C temperature increase for 3 d after a 6 d acclimation to the different carbon and nitrogen conditions. As a result, temperature fluctuated between 19°C (08:00 h GMT min.) and 24.5°C (17:00 h GMT max.) during the acclimation phase, and between 24°C (08:00 h GMT min.) and 29°C (17:00 h GMT max.) during the +4°C treatment. Detailed hourly temperature fluctuations can be found in Stengel et al. (2014). Orthophosphate at 2 µM was included in both LN and HN treatments.

Biochemical composition

Variables including accumulation of secondary metabolites, antioxidant capacity and stoichiometry were used as physiological indicators and were estimated at different time periods (morning, noon, and evening) (Figueroa & Korbee 2010, García-Sánchez et al. 2012, Figueroa et al. 2014b) as follows.

Soluble proteins (SPs). Total SPs were extracted in a mortar from 50 mg dry weight (DW) of macroalgae in a 0.1 M phosphate buffer (pH 6.5). After centrifugation at 13 000 rpm (15493 *g*), 50 µl of supernatant was mixed with 750 µl of 0.1 M phosphate buffer (pH 6.5) and 200 µl of assay mix (Bio-Rad Protein Assay). The absorbance at 595 nm was determined using a spectrophotometer (UVMini-1240 model, Shimadzu) after 15 min of incubation at room temperature (Bradford 1976). Concentrations were calculated by means of standards prepared with bovine serum albumin (Sigma).

Total Fatty Acids (Total FAs). Total FA content was determined by gas chromatography-flame ionization detector (GC/FID) after direct transmethylation of the freeze-dried biomass as described previously by Schmid et al. (2014). For quantification, pentadecanoic acid 15:0 (99%, Alfa Aesar) was added as an internal standard.

MAAs. MAAs of *E. elongata* were determined using HPLC (Waters 600) as described in Korbee-Peinado et al. (2004). Total MAAs were expressed in mg g^{-1} DW, and the different MAAs identified as shinorine, palythine, asterina-330 and palythinol as % of total MAAs.

Phenolic compounds and antioxidant activity (DPPH method). Phenolic compounds of *U. rigida* and *C. tamariscifolia* were extracted using 0.25 g fresh weight (FW) of macroalga and 2.5 ml of 80% methanol (80% MeOH:20% H_2O). After being kept overnight, the mixture was centrifuged at 4000 rpm (2253 *g*) for 15 min at 4°C and the supernatant was collected. Total phenolic compounds were determined colorimetrically using the Folin-Ciocalteu reagent (Folin & Ciocalteu 1927). Phloroglucinol (1,3,5-trihydroxybenzene, Sigma) was used as a standard. Finally, the absorbance was read at 760 nm using a spectrophotometer (UVMini-1240 model, Shimadzu). Phenolic concentrations were expressed as mg g^{-1} DW.

The antioxidant activity was measured on the phenolic compound extracts using the reduction of the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) according to Blois (1958): 150 µl of DPPH (1.27 mM) prepared in 90% methanol (90% MeOH:10% H_2O) was added to each extract. The reaction was completed after 30 min in the dark at room temperature (~20°C), and the absorbance was read at 517 nm using a spectrophotometer (UVMini-1240 model). The calibration curve made with DPPH was used to calculate the remaining concentration of DPPH in the reaction mixture after incubation. Values of DPPH concentration (mM) were plotted against algal extract concentration (mg DW ml^{-1}) in order to obtain the oxidation index EC_{50}, which represents the concentration of algal extract required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was used as positive control (Connan et al. 2006).

Calcification. Samples of *E. elongata* were kept at 40°C until desiccated. Initial dry weight (DW_I) was obtained and then 10% HCl was added in order to initiate $CaCO_3$ dissolution, maintaining the same weight/volume ratio. After complete $CaCO_3$ removal, samples were washed with distilled water, dried again at 40°C and final dry weight (DW_F) was measured. $CaCO_3$ content (%) was calculated according to the formula:

$$CaCO_3 = [(DW_I - DW_F) / DW_F] \times 100 \quad (1)$$

Carbon (C) and nitrogen (N) content. Seaweed samples were dried for 24 h at 60°C and kept desiccated until analyses. Total internal C and N contents on a DW basis were determined using a CNHS-932elemental analyzer (Leco Corporation).The molar ratio between C and N was obtained by dividing total internal C by total internal N content.

FW/DW ratio. To acquire the FW:DW ratio, 15 to 20 samples with known FW of each algal species collected at each sampling time and under each treatment were dried at 60°C for 24 h, reaching constant DW.

Statistical analyses. After visually checking for normality, data were tested for homogeneity of variances using Cochran's test. Logarithm transformations were used when the assumptions were not achieved. Multi-factorial ANOVAs were performed including temperature (ambient and high), nitrogen (low and high), CO_2 (low and high) and time of sam-

pling (morning, noon, evening), all considered fixed crossed factors, and tank as a random factor nested in the interaction of N × C and crossed with the other 2 factors. Tank (alone or in interactions) was not significant ($p > 0.25$) and thus this factor was not included in the final models (Underwood 1997). When significant differences were detected, post hoc tests were performed using a Student-Newman-Keuls (SNK) test. The significance level was set to $\alpha = 0.05$. Data sets of the different variables corresponded to 2 measurement days (n = 6). In addition, Pearson correlation coefficients were calculated and tested between all measured dependent variables.

RESULTS

SPs

In the 'initial' samples after 6 d of acclimation, SP concentrations were similarly high in *Ulva rigida* and *Cystoseira tamariscifolia*, and about 3 times higher than that of *Ellisolandia elongata* (Table 1). During the experimental period, the highest protein levels were reached in *C. tamariscifolia* (15.02 ± 1.48 mg g^{-1} DW), followed by *U. rigida* (9.27 ± 0.57 mg g^{-1} DW) and *E. elongata* (5.91 ± 0.30 mg g^{-1} DW) (Fig. 1). In *C. tamariscifolia*, the interactive effect of the 4 factors was significant (Table 2). C and N treatments also produced a significant effect as did C and time (Table 2). In general, SP values were lower at noon and in the evening under the HCHN treatment (Fig. 1a). In *U. rigida*, 2 significant interactions were observed: time and temperature, and C and N (Table 3). For both the ambient and increased temperature, we found the highest SP contents in the morning and at noon respectively (Fig. 1b). The lowest value was observed in HCLN, and it was lower at LCLN than at HCHN (Fig. 1c). Finally, in *E. elongata*, N and temperature and the interaction of time × temperature had significant effects on protein levels (Table 4). As was expected, the lowest content of SP was observed at LN and for the interactive effect, the highest value was detected at ambient temperature and during the evening (Fig. 1d).

Total FAs

The average levels of total FAs were 0.94 ± 0.01 and 1.07 ± 0.03% g^{-1} DW in *C. tamariscifolia*, 1.03 ± 0.02 and 0.90 ± 0.03% g^{-1} DW in *U. rigida* and 0.24 ± 0.01 and 0.11 ± 0.005% g^{-1} DW in *E. elongata* under ambient and ambient + 4°C temperatures respectively. Temperature produced significant effects on total FA contents in the 3 species (Tables 2–4). The increased temperature caused a decrease of total FAs in *C. tamariscifolia* and *E. elongata*, whereas in *U. rigida* it resulted in an increase in FAs (data not shown).

MAAs

E. elongata contained 0.23 mg total MAAs g^{-1} DW at the initial time period (Table 1). The interactive effects of N × temperature, time × temperature and the triple interaction of C × time × temperature were significant (Table 5); at ambient temperature, no significant differences through the day between LC and HC treatments were found except in the evening (Fig. 2). No differences were found at ambient temperature +4°C amongst treatments nor over time. Shinorine was the dominant MAA in general, with proportions higher than 40%. Palythine was also present (>25%) and palythinol (around 10 to 20%). Asterina-330 presented the lowest proportion (2 to 5%, data not shown). Asterina-330 and palythine presented a similar pattern with the most double (5 for both) and triple (4 and 3 respectively) interactions among variables, whereas in shinorine, only the double interactions C × time, N × time and time × temperature, and the triple interactions C × N × time and C × time × temperature were found (Table 5). Finally, the palythinol level was significantly affected by the double interaction N × temperature, and the triple interaction C × N × temperature (Table 5).

Phenolic compounds and antioxidant activity (DPPH scavenger capacity)

Phenolic contents in *C. tamariscifolia* at the initial time were about 4 times higher than in *U. rigida* (Table 1). For *C. tamariscifolia*, an interactive effect of C × temperature was found (Table 2), with phenolic compounds (plentics) being highest at ambient temperature and LC (Fig. 3a). EC_{50} values presented a complex pattern (Fig. 3b), with a significant interactive effect between C, N time and temperature (Table 2). At ambient temperature, the maximal values (lowest antioxidant activity) were reached at HCLN in the morning and HC at noon, whereas the maximal antioxidant activity was reached under LCLN in the morning (Fig. 3b). Under ambient +4°C, the minimal value of EC_{50} was reached at noon under the LCHN treatment (Fig. 3b).

Table 1. Means ± SD (n = 3) of different variables measured in *Cystoseira tamariscifolia*, *Ulva rigida* and *Ellisolandia elongata* after 6 d of acclimation to ambient CO_2 (LC), LN condition (5 µM nitrate) and ambient temperature (23 to 26°C) (i.e. conditions at initial time). SPs = total soluble proteins; C = total internal carbon; N = total internal nitrogen; phenolics = phenolic compounds; EC_{50} = concentration of the methanolic extract required to scavenge 50% of the DPPH. Total MAAs = total content of mycosporine-like amino acids, shinorine, palythine, asterina-330 and palythinol are expressed as of total MAAs

	SPs (mg g^{-1} DW)	C (mg g^{-1} DW)	N (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	EC_{50} (mg DW ml^{-1})	Total MAAs (mg g^{-1} DW)	Shinorine (%)	Palythine (%)	Asterina (%)	Palythinol (%)
C. tamariscifolia	8.85 ± 3.11	309.33 ± 2.21	14.16 ± 2.28	23.20 ± 1.15	0.64 ± 0.19	–	–	–	–	–
U. rigida	10.13 ± 0.62	236.93 ± 4.42	17.82 ± 0.62	6.48 ± 1.01	–	–	–	–	–	–
E. elongata	3.85 ± 0.4	153.90 ± 0.85	7.16 ± 0.49	–	–	0.23 ± 0.04	57.3 ± 3.21	24.76 ± 0.75	2.70 ± 0.10	15.25 ± 2.53

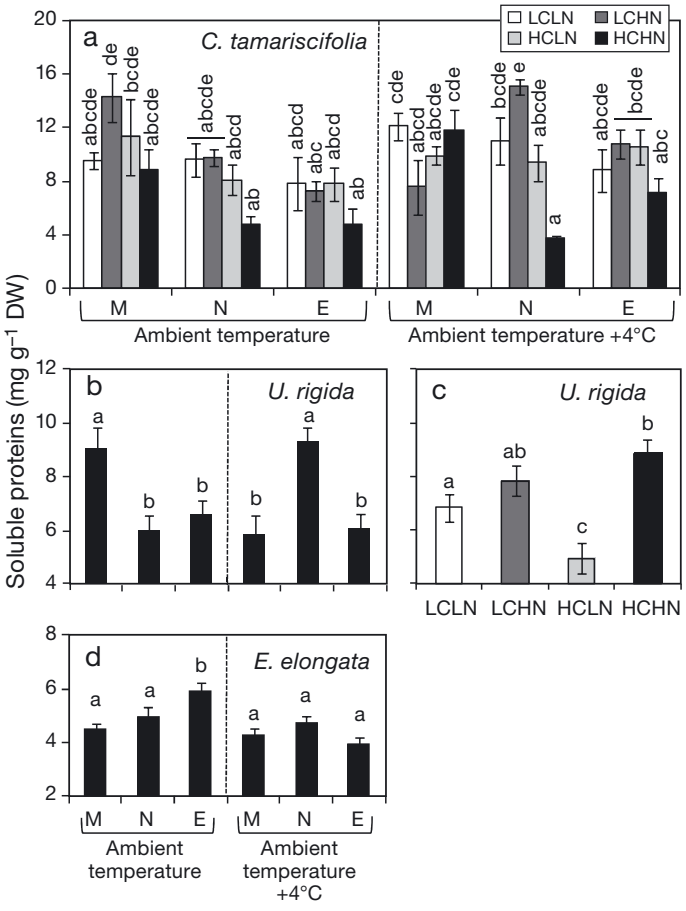

Fig. 1. Soluble protein content of (a) *Cystoseira tamariscifolia*, (b,c) *Ulva rigida*, and (d) *Ellisolandia elongata* exposed to combined conditions of C (high carbon, HC: air enriched with CO_2 at 700 ppm; low carbon, LC: air without CO_2 addition, i.e. 390 ppm), N (high nutrient enrichment, HN: 50 µM nitrate; low nutrient enrichment, LN: 5 µM), and temperature (first 6 d at ambient temperature followed by 3 d at an increase of 4°C). Measurements were conducted with samples collected in the morning (M), noon (N) and evening (E) of the experimental period. Data are pooled means ± SE, in accordance with significant effects obtained by ANOVA: *C. tamariscifolia*, interactive significant effects of C and N concentrations, time, and temperature (a, n = 6); *U. rigida*, interactive significant effects of time and temperature (b, n = 24) and interactive effects of C and N concentrations (c, n = 36); *E. elongata*, interactive effects of time and temperature (d, n = 24). Different letters above the histograms indicate significant differences ($p < 0.05$, SNK post hoc test)

In *U. rigida*, phenolic contents were significantly affected by the interactions C × time, C × temperature, N × temperature, time × temperature and the triple interaction C × time × temperature (Table 3), with the lowest values found in the LC treatments in the morning and the LN treatments at noon (Fig. 4). In this species, no antioxidant activity of methanolic extracts was found as EC_{50} values were below the detection limit of the DPPH method.

Table 2. ANOVA results for experiment with *Cystoseira tamariscifolia* after being exposed to combined conditions of C (high carbon, HC: air enriched with CO_2 at 700 ppm; low carbon, LC: air without CO_2 addition, i.e. 390 ppm), N (high nutrient enrichment, HN: 50 µM; low nutrient enrichment, LN: 5 µM), time (morning, noon and evening) and temperature (ambient temperature and ambient temperature +4°C) on the soluble proteins (SPs), internal C and N, phenolic compounds (phenolics) and antioxidant activity (EC_{50}). Results for the fatty acids (FAs) were for the effects of C, N, and temperature only. Significant differences at $p < 0.05$ are shown in **bold**

Source	df	SPs			C			N			Phenolics			EC_{50}			FAs		
		MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
C (1)	1	**159.28**	**14.58**	**<0.001**	148.93	1.32	0.25	2.99	0.91	0.34	**202.55**	**26.48**	**<0.001**	**0.30**	**12.33**	**<0.001**	0.00	0.11	0.74
N (2)	1	25.52	2.34	0.13	50.94	0.45	0.50	10.79	3.29	0.07	**90.34**	**11.81**	**<0.001**	0.01	0.42	0.52	0.03	0.87	0.35
Time (3)	2	**81.12**	**7.42**	**<0.001**	**2182.90**	**19.38**	**<0.001**	6.34	1.94	0.15	16.72	2.19	0.12	0.03	1.17	0.31			
Temperature (4)	1	**49.11**	**4.49**	**0.04**	19.19	0.17	0.68	**15.30**	**4.67**	**0.03**	**161.64**	**21.13**	**<0.001**	0.00	0.06	0.81	**0.29**	**8.13**	**0.01**
(1) × (2)	1	**117.03**	**10.71**	**<0.001**	**591.15**	**5.25**	**0.02**	5.73	1.75	0.19	5.12	0.67	0.42	0.02	0.85	0.36	0.01	0.26	0.61
(1) × (3)	2	**69.34**	**6.35**	**<0.001**	108.28	0.96	0.39	0.21	0.06	0.94	23.23	3.04	0.05	**0.36**	**14.74**	**<0.001**			
(1) × (4)	1	0.00	0.00	0.99	36.84	0.33	0.57	1.70	0.52	0.47	**68.92**	**9.01**	**<0.001**	**0.19**	**7.93**	**0.01**	0.04	1.09	0.30
(2) × (3)	2	5.24	0.48	0.62	25.21	0.22	0.80	9.94	3.03	0.05	2.88	0.38	0.69	**0.10**	**4.25**	**0.02**			
(2) × (4)	1	0.37	0.03	0.85	14.80	0.13	0.72	**22.74**	**6.94**	**0.01**	1.01	0.13	0.72	**0.15**	**6.34**	**0.01**	0.01	0.22	0.64
(3) × (4)	2	30.25	2.77	0.07	154.45	1.37	0.26	3.74	1.14	0.32	15.93	2.08	0.13	**0.29**	**11.97**	**<0.001**			
(1) × (2) × (3)	2	29.31	2.68	0.07	11.78	0.10	0.90	4.45	1.36	0.26	2.56	0.33	0.72	**0.31**	**12.75**	**<0.001**	0.13	3.76	0.06
(1) × (2) × (4)	1	5.04	0.46	0.50	3.23	0.03	0.87	0.14	0.04	0.84	6.16	0.81	0.37	0.02	0.69	0.41			
(1) × (3) × (4)	2	27.78	2.54	0.08	19.58	0.17	0.84	0.78	0.24	0.79	10.27	1.34	0.27	**0.43**	**18.01**	**<0.001**			
(2) × (3) × (4)	2	11.38	1.04	0.36	**394.65**	**3.50**	**0.03**	**18.38**	**5.61**	**<0.001**	2.88	0.38	0.69	**0.15**	**6.41**	**<0.001**			
(1) × (2) × (3) × (4)	2	**84.11**	**7.70**	**<0.001**	55.15	0.49	0.61	1.06	0.32	0.72	19.53	2.55	0.08	**0.22**	**9.12**	**<0.001**			
Residual		10.93			112.64			3.28			7.65			0.02			0.04		

Calcification

$CaCO_3$ content of *E. elongata* was greater than 70% of the total algal DW in all treatments both under ambient and increased temperature. No effect of C and N treatments were found (data not shown).

Total internal C and N

In the 'initial' samples (after 6 d acclimatisation) C content was higher in *C. tamariscifolia* than that in *U. rigida*, followed by *E. elongata* (Table 1). In *C. tamariscifolia*, C content was significantly affected by the interactions of C × N and N × time × temperature (Table 2). The internal C content was significantly higher in the LCHN treatment than in HCHN or LCLN (Fig. 5b). Among the triple interactions, significantly higher values for both temperature treatments during the evening were found for the N treatment (Fig. 5a, Table 2). In *U. rigida*, total internal C did not vary as a function of time. It was only impacted significantly by C and temperature as single factors (Table 3). Under ambient temperature, the C content was 278 mg g^{-1} DW, and 286 mg g^{-1} DW under increased temperature. C content increased under the HCHN treatment (Fig. 5c). Finally, in *E. elongata*, C content was significantly affected by time, temperature and the interaction of C × temperature (Table 4); the values were maintained close to 150 mg C g^{-1} DW (Fig. 5d,e). Under HC, the C content was higher at ambient than at increased temperature (Fig. 5d), and higher in the evening than in the morning or at noon (Fig. 5e).

In *C. tamariscifolia,* internal N content was affected by the interactive effects of N × temperature and N × time × temperature (Table 2). No significant variations in N content throughout the time and treatments were observed, except in the evening under ambient temperature (Fig. 5f). In *U. rigida*, the interaction of C × N had a significant effect on the internal level of N (Table 3). The lowest value was detected at the HCLN treatment followed by LCLN; N levels were similar under LCHN and HCHN (Fig. 5g). Finally, in *E. elongata*, although an interactive effect of C × time was detected by the ANOVA for internal N content, the SNK test did not detect

Table 3. ANOVA results for *Ulva rigida*, details as in Table 2

Source		SPs			C			N			Phenolics			FAs		
	df	MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
C (1)	1	7.44	0.84	0.36	**7194.92**	**20.77**	**<0.001**	**13.82**	**4.41**	**0.04**	**7.90**	**10.02**	**<0.001**	0.04	1.25	0.27
N (2)	1	**212.70**	**24.14**	**<0.001**	906.28	2.62	0.11	**387.66**	**123.67**	**<0.001**	0.86	1.09	0.30	0.00	0.10	0.75
Time (3)	2	24.14	2.74	0.07	51.84	0.15	0.86	2.14	0.68	0.51	**4.05**	**5.14**	**0.01**			
Temperature (4)	1	0.83	0.09	0.76	**1897.94**	**5.48**	**0.02**	6.98	2.23	0.14	**9.86**	**12.51**	**<0.001**	**0.25**	**7.02**	**0.01**
(1) × (2)	1	**74.23**	**8.42**	**<0.001**	**2235.46**	**6.45**	**0.01**	**45.24**	**14.43**	**<0.001**	0.37	0.46	0.50	0.00	0.06	0.80
(1) × (3)	2	10.38	1.18	0.31	180.87	0.52	0.59	3.74	1.19	0.31	**6.62**	**8.40**	**<0.001**			
(1) × (4)	1	9.31	1.06	0.31	13.20	0.04	0.85	0.09	0.03	0.87	**13.66**	**17.32**	**<0.001**	0.03	0.82	0.37
(2) × (3)	2	9.57	1.09	0.34	854.50	2.47	0.09	2.06	0.66	0.52	1.09	1.38	0.26			
(2) × (4)	1	3.44	0.39	0.53	47.86	0.14	0.71	3.22	1.03	0.31	**3.61**	**4.58**	**0.04**	0.01	0.16	0.69
(3) × (4)	2	**127.67**	**14.49**	**<0.001**	890.59	2.57	0.08	3.88	1.24	0.30	**29.46**	**37.36**	**<0.001**			
(1) × (2) × (3)	2	11.17	1.27	0.29	178.01	0.51	0.60	7.29	2.33	0.10	0.94	1.19	0.31			
(1) × (2) × (4)	1	20.68	2.35	0.13	6.95	0.02	0.89	1.79	0.57	0.45	0.05	0.06	0.80	0.04	1.04	0.31
(1) × (3) × (4)	2	13.04	1.48	0.23	11.85	0.03	0.97	0.33	0.11	0.90	**8.49**	**10.77**	**<0.001**			
(2) × (3) × (4)	2	5.76	0.65	0.52	411.68	1.19	0.31	1.61	0.52	0.60	0.10	0.13	0.88			
(1) × (2) × (3) × (4)	2	14.56	1.65	0.20	186.46	0.54	0.59	0.72	0.23	0.80	0.19	0.25	0.78			
Residual		8.81			346.49			3.13			0.79			0.04		

the differences among data (Table 4); all N data were around 7 mg N g^{-1} DW (Fig. 5h).

Relationships among the variables (Pearson correlation)

SP content was positively related with phenolic content in both *C. tamariscifolia* and *U. rigida*, and with antioxidant activity in *C. tamariscifolia* (Table 6) and N content in *U. rigida* (Table 7). Total C content was related to total N content in *C. tamariscifolia* (Table 6) and *U. rigida* (Table 7). In *C. tamariscifolia*, phenolic content and antioxidant activity were positively related (Table 6), whereas MAAs in *E. elongata* were negatively correlated to N content (Table 8); however when each MAA is analyzed separately, a negative correlation with N was found for palythine. A positive correlation for shinorine and asterina-330 with N was also found (Table 8). Total FA content was positively related to phenolic content only in *C. tamariscifolia* (Table 6). In *E. elongata*, total FA content was positively related to total SP and C contents (Table 8).

Table 4. ANOVA results for *Ellisolandia elongata*, details as in Table 2

Source		SPs			C			N			FAs		
	df	MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
C (1)	1	5.93	3.45	0.07	5.07	0.17	0.68	0.08	0.09	0.77	0.01	2.13	0.15
N (2)	1	**6.76**	**3.93**	**0.05**	32.18	1.08	0.30	1.89	2.11	0.15	0.00	0.35	0.56
Time (3)	2	4.34	2.53	0.08	**1785.58**	**59.70**	**<0.001**	0.67	0.75	0.47			
Temperature (4)	1	**24.03**	**13.99**	**<0.001**	**139.40**	**4.66**	**0.03**	2.12	2.37	0.13	**0.29**	**47.81**	**<0.001**
(1) × (2)	1	0.53	0.31	0.58	50.64	1.69	0.20	0.73	0.81	0.37	0.01	1.67	0.20
(1) × (3)	2	3.69	2.15	0.12	48.62	1.63	0.20	**5.00**	**5.57**	**0.01**			
(1) × (4)	1	1.23	0.72	0.40	**124.16**	**4.15**	**0.04**	1.31	1.46	0.23	0.00	0.02	0.90
(2) × (3)	2	1.25	0.73	0.49	75.38	2.52	0.09	0.59	0.66	0.52			
(2) × (4)	1	1.77	1.03	0.31	66.98	2.24	0.14	0.05	0.05	0.82	0.01	1.17	0.28
(3) × (4)	2	**12.31**	**7.17**	**<0.001**	6.68	0.22	0.80	0.56	0.62	0.54			
(1) × (2) × (3)	2	1.05	0.61	0.55	18.42	0.62	0.54	1.05	1.17	0.32			
(1) × (2) × (4)	1	1.74	1.01	0.32	3.26	0.11	0.74	0.39	0.44	0.51	0.00	0.34	0.56
(1) × (3) × (4)	2	1.67	0.97	0.38	5.98	0.20	0.82	0.74	0.83	0.44			
(2) × (3) × (4)	2	0.07	0.04	0.96	11.75	0.39	0.68	0.87	0.97	0.38			
(1) × (2) × (3) × (4)	2	1.33	0.77	0.46	43.78	1.46	0.24	1.86	2.07	0.13			
Residual		1.72			29.91			0.90			0.01		

Table 5. ANOVA results for *Ellisolandia elongata* (for details see Table 2), testing for effects on total mycosporine-like amino acids (MAAs), % shinorine, % asterina, % palythine and % palythinol

Source		Total MAAs			% Shinorine			% Asterina			% Palythine			% Palythinol		
	df	MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
C (1)	1	0.01	2.96	0.09	**1002.80**	**6.76**	**0.01**	**18.32**	**26.53**	**<0.001**	**1808.40**	**20.67**	**<0.001**	201.33	3.07	0.08
N (2)	1	0.00	0.01	0.92	133.36	0.90	0.35	0.29	0.41	0.52	75.74	0.87	0.36	1.85	0.03	0.87
Time (3)	2	0.00	0.66	0.52	**828.71**	**5.59**	**0.01**	**2.38**	**3.45**	**0.04**	**415.05**	**4.74**	**0.01**	**754.90**	**11.50**	**<0.001**
Temperature (4)	1	0.00	0.22	0.64	0.01	0.00	0.99	0.55	0.80	0.37	197.62	2.26	0.14	194.99	2.97	0.09
(1) × (2)	1	0.00	0.01	0.94	2.12	0.01	0.91	0.72	1.04	0.31	54.34	0.62	0.43	49.38	0.75	0.39
(1) × (3)	2	0.01	1.52	0.23	**653.58**	**4.41**	**0.02**	**2.88**	**4.17**	**0.02**	**324.50**	**3.71**	**0.03**	51.98	0.79	0.46
(1) × (4)	1	0.00	1.13	0.29	132.35	0.89	0.35	**9.81**	**14.20**	**<0.001**	**419.20**	**4.79**	**0.03**	124.38	1.89	0.17
(2) × (3)	2	0.00	0.74	0.48	**1511.60**	**10.19**	**<0.001**	**10.63**	**15.39**	**<0.001**	**1631.99**	**18.65**	**<0.001**	54.08	0.82	0.44
(2) × (4)	1	**0.01**	**4.09**	**0.05**	128.52	0.87	0.36	**6.35**	**9.20**	**<0.001**	**1450.05**	**16.57**	**<0.001**	**801.67**	**12.21**	**<0.001**
(3) × (4)	2	**0.02**	**5.52**	**0.01**	**683.38**	**4.61**	**0.01**	**10.36**	**15.00**	**<0.001**	**1454.15**	**16.62**	**<0.001**	202.28	3.08	0.05
(1) × (2) × (3)	2	0.00	1.06	0.35	**670.92**	**4.52**	**0.01**	**6.42**	**9.30**	**<0.001**	**1141.30**	**13.05**	**<0.001**	96.26	1.47	0.24
(1) × (2) × (4)	1	0.00	0.05	0.83	512.66	3.46	0.07	**6.46**	**9.36**	**<0.001**	**1383.90**	**15.82**	**<0.001**	**260.86**	**3.97**	**0.05**
(1) × (3) × (4)	2	**0.02**	**6.36**	**<0.001**	**586.23**	**3.95**	**0.02**	**3.45**	**5.00**	**0.01**	**984.94**	**11.26**	**<0.001**	182.48	2.78	0.07
(2) × (3) × (4)	2	0.00	1.06	0.35	210.27	1.42	0.25	**2.55**	**3.69**	**0.03**	200.89	2.30	0.11	28.78	0.44	0.65
(1) × (2) × (3) × (4)	2	0.00	0.39	0.68	358.12	2.41	0.10	1.59	2.30	0.11	265.77	3.04	0.05	36.85	0.56	0.57
Residual		0.00			148.35			0.69			87.49			65.64		

DISCUSSION

In this study, the results of short-term effects of pCO_2, nitrate and temperature increases on the physiology of 3 abundant eulittoral Mediterranean macroalgae with different morphological, bio-optical and physiological characteristics (Mercado et al. 1998, Figueroa et al. 2003a, 2014b, Abdala-Díaz et al. 2006) are presented. Combined effects of CO_2 (LC and HC) and nitrogen (LN and HN nitrate concentration through morning pulses) under 2 temperature conditions were tested (6 d at ambient temperature followed by 3 d after a 4°C increase). Most studies on the effects of global climate change on aquatic organisms have been conducted with 1 or 2 experimental variables, and studies on the interaction of multiple factors are very scarce (Gao & Zheng 2010, Cornwall et al. 2012, Koch et al. 2013, Yildiz et al. 2013).

N levels and the interaction C × N in *Ulva rigida* had significant effects on protein concentration, as previously reported (Gordillo et al. 2001, 2003). However, in *Cystoseira tamariscifolia,* N levels did not affect the protein level in contrast to C, temperature and the interaction of C × N, whereas in *Ellisolandia elongata,* only N and temperature as single factors influenced the protein level. Burkhardt & Riebesell (1997) observed a decrease in the C:N ratio at high CO_2 in different phytoplankton species. However, this ratio increased in the macroalgae *U. rigida* and *Pyropia* (*Porphyra*) *leucostica* grown under high CO_2 (Mercado et al. 1999, Gordillo et al. 2001). Algae grown at a high inorganic C concentration (5% CO_2) displayed a higher soluble carbohydrate concentration and maximum photosynthesis rate but had a lower photosynthetic affinity for inorganic C, and lower phycobiliprotein and Rubisco contents than those algae cultured at low inorganic C levels (air CO_2) (Andría et al. 1999). Inorganic C enrichment also affected N uptake and assimilation in *Gracilaria* sp., causing a decrease in N uptake rate even under N-replete conditions (Andría et al. 1999).

In *C. tamariscifolia*, the temperature increase produced a decrease in total FAs and phenolic com-

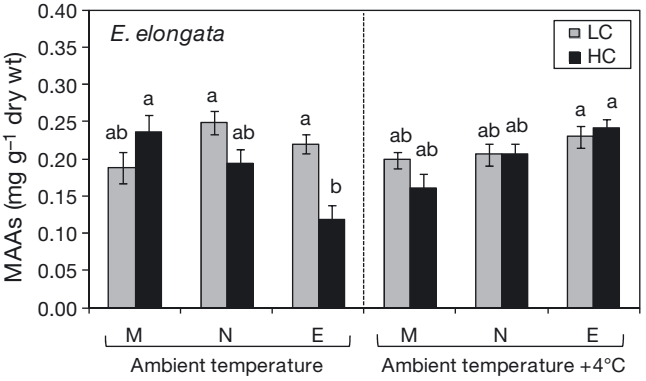

Fig. 2. Total mycosporine-like amino acid (MAA) content of *Ellisolandia elongata* exposed to combined conditions of C (HC and LC), N (HN and LN), and temperature (see Fig. 1 for description of experimental conditions). Measurements were conducted with samples collected in the morning (M), noon (N) and evening (E). Data are pooled means ± SE, in accordance with significant effects obtained by ANOVA, considering interactive significant effects of carbon, time and temperature (n = 12). Different letters above the histograms indicate significant differences ($p < 0.05$, SNK post hoc test)

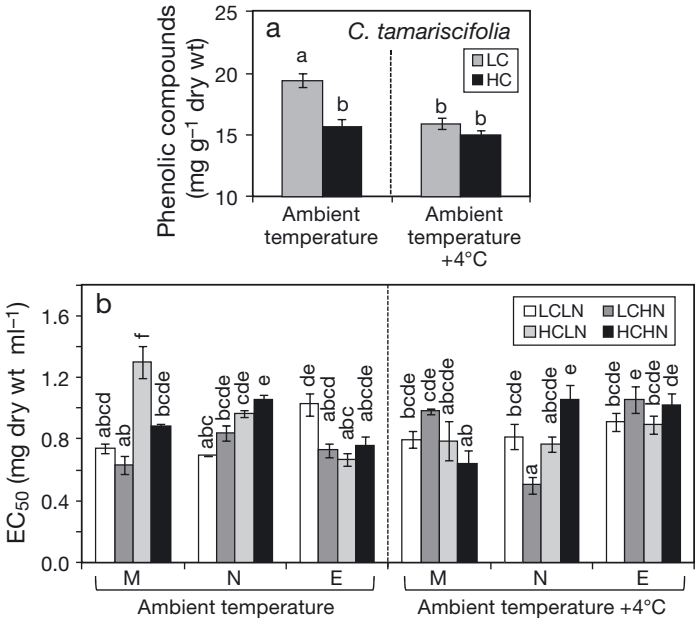

Fig. 3. (a) Total phenolic compounds and (b) oxidation index (EC_{50}) of *Cystoseira tamariscifolia* exposed to combined conditions of C (HC and LC), N (HN and LN), and temperature (see Fig. 1 for description of experimental conditions). Measurements were conducted with samples collected in the morning (M), noon (N) and evening (E) of experimental period. Data are pooled means ± SE, in accordance with significant effects obtained by ANOVA: phenolic compounds, interactive significant effects of carbon concentrations, and temperature (a, n = 36); EC_{50}, interactive significant effects of C and N concentrations, time, and temperature (b, n = 6). Different letters above the histograms indicate significant differences ($p < 0.05$, SNK post hoc test)

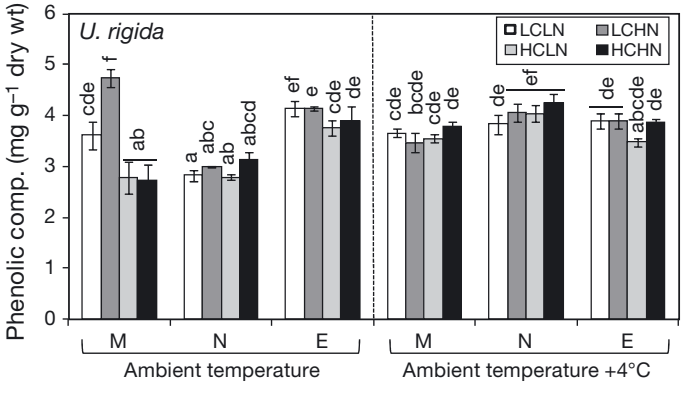

Fig. 4. Total phenolic compounds of *Ulva rigida* exposed to combined conditions of C (HC and LC), N (HN and LN), and temperature (see Fig. 1 for description of experimental conditions). Measurements were conducted with samples collected in the morning (M), noon (N) and evening (E) of experimental period. Data are pooled means ± SE, in accordance with significant effects obtained by ANOVA, with interactive significant effects of C concentrations, time and temperature (n = 3). Different letters above the histograms indicate significant differences ($p < 0.05$, SNK post hoc test)

pound levels and an increase in protein content (at noon and under HN). In *E. elongata*, a decrease in total FAs and SPs (in the evening) was observed. In contrast, in *U. rigida*, total FAs, SPs and phenolic compounds (at noon) increased under ambient + 4°C temperature. Thus, in *U. rigida* the internal biochemical compounds seem to be accumulated under stress, as has been previously shown under different stress conditions in algae (Abdala-Díaz et al. 2006, Stengel et al. 2011, Sharma et al. 2012). The effect of increased temperature in this study took into account the day/light fluctuations including the decrease in temperature during the night compared to the day period. Temperature is a major factor controlling the rate of photosynthesis in algae (Davison 1991). Temperature affects the availability of inorganic C (Surif & Raven 1989) and the rate of C fixation by Rubisco (Sukenik et al. 1987). Several primary sites of temperature sensitivity have been proposed to initiate the process of photoinhibition, including damage to PSII, in particular degradation of D1 protein (Warner et al. 1999), disintegration of the thylakoid membrane (Tchernov et al. 2004), generation of reactive oxygen species concomitant to photooxidative damage to the photosynthetic apparatus (Lesser 2006), and impairment of the Calvin-Benson cycle (Jones et al. 1998). Overall, the mechanisms of thermal tolerance are not well understood in macrophytes, but studies indicate comparable responses to heat stress amongst temperate marine macrophytes and terrestrial plants.

The different responses amongst the species to C, N and temperature treatments could be explained by the different bio-optical pattern: i.e. *C. tamariscifolia* and *E. elongata* have a very high absorptance (0.8 or 0.9) due to their thickness and complex morphology and structure, whereas the absorptance of *U. rigida* thalli, with just 2 cell layers, ranges from 0.40 to 0.65 (Salles et al. 1996). In addition, the reflectance is high in *E. elongata* (15 to 20%). In this study, the effect of increased CO_2 and temperature under different N supply was very different in thin and laminar species (*U. rigida*) compared to the shrubby macroalga *C. tamariscifolia*, or the jointed calcareous alga *E. elongata*. Additionally, different biochemical constituents were affected to different degrees by the 4 treatments. Biochemical composition of *C. tamariscifolia* responded (directly or in interaction) mainly to temperature for nearly all variables; effects of CO_2 increase were also detected, whereas composition was hardly affected by nutrient supply. In contrast, the biochemical composition of *U. rigida* was affected by increases in temperature, CO_2 and nutrient supply

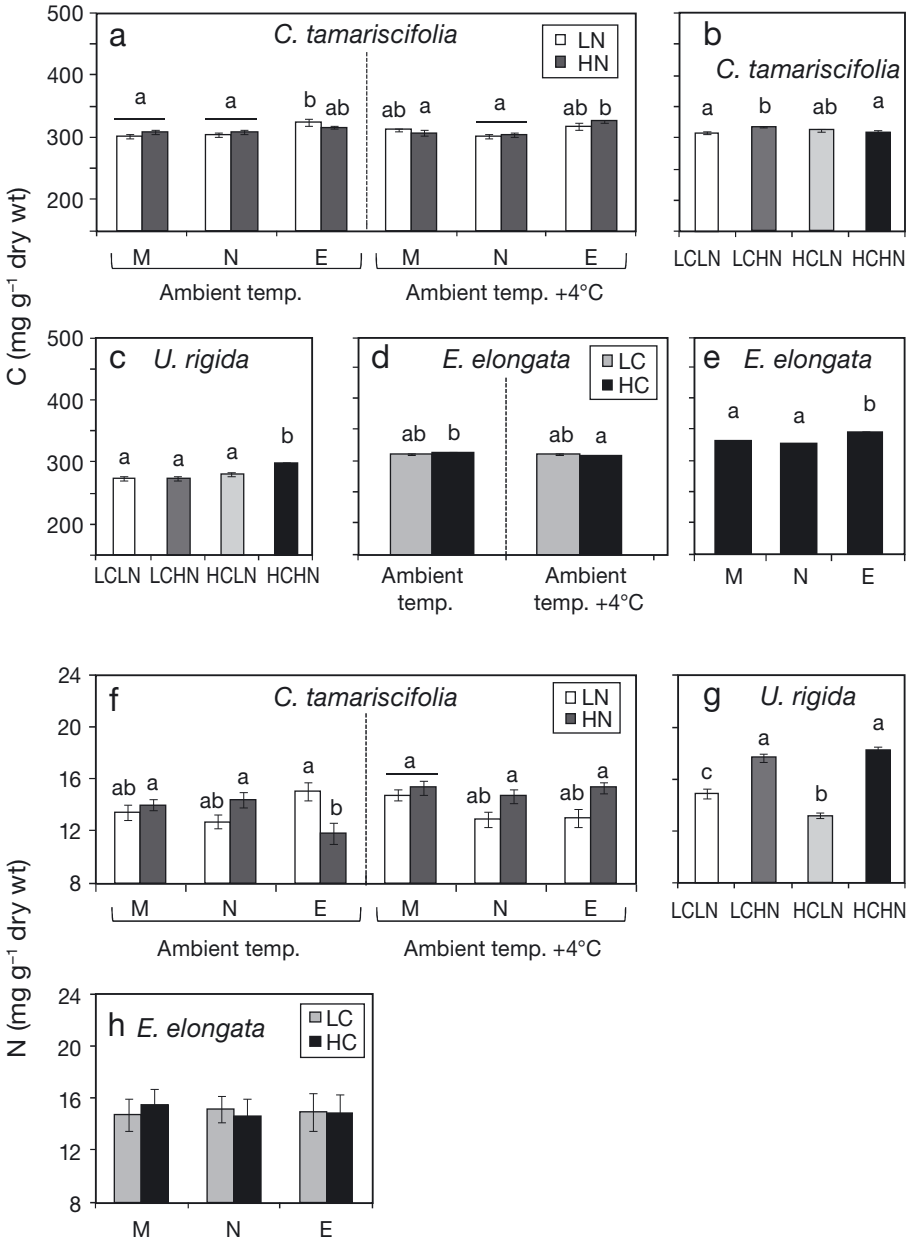

Fig. 5. Total internal (a–e) carbon content and (f–h) nitrogen content of (a,b,f) *Cystoseira tamariscifolia*, (c,g) *Ulva rigida*, and (d,e,h) *Ellisolandia elongata* exposed to combined conditions of C (HC and LC), N (HN and LN), and temperature (see Fig. 1 for description of experimental conditions). Measurements were conducted with samples collected in the morning (M), noon (N) and evening (E) of experimental period. Data are pooled means ± SE, in accordance with significant effects obtained by ANOVA. Carbon content: *C. tamariscifolia*, interactive significant effects of N concentrations, time, and temperature (a, n = 12), and interactive effects of C and N concentrations (b, n = 30); *U. rigida*, interactive significant effects of C and N concentrations (c, n = 30); *E. elongata*, interactive effects of C concentrations and temperature (d, n = 30), and isolated effect of time (e, n = 60). N content: *C. tamariscifolia*, interactive significant effects of N concentrations, time, and temperature (f, n = 12); *U. rigida*, interactive significant effects of C and N concentrations (g, n = 30); *E. elongata*, interactive effects of C concentrations and time (h, n = 24). Different letters above the histograms indicate significant differences ($p < 0.05$, SNK post hoc test)

and their interactions, although the responses were different for each variable. In addition, the light climate in the tanks depended on the positioning of the species. *U. rigida*, with its freely moving thalli in the tank's water flow, presented more variable canopy episodes than *C. tamariscifolia* (fixed position) or than *E. elongata*, which was located at the bottom (see Stengel et al. 2014). Thus, in addition to the bio-optical characteristic of the thalli, the canopy effect in the tanks must be taken into account to explain light absorption. Field studies on mats and canopies confirm this conclusion; for example, Malta et al. (2003) reported strong vertical heterogeneity in physiological characteristics of *Ulva* sp. organized in mats. Bischof et al. (2006b) also showed the photoacclimation within mats of the filamentous green macroalga *Chaetomorpha linum*, presenting much less photoinhibition than the first layers of thalli with high light exposure. Falkenberg et al. (2013) reported that turf algae responded to enrichment of CO_2 and nutrients, whereas canopy-forming species responded only to nutrient enrichment; they concluded that the identification of how these conditions modify resource availability may help predict to what extent such major ecosystem components and the communities they support may adapt to future conditions.

According to morphology, thickness and reflectance, *U. rigida* may be expected to be more vulnerable to high solar irradiance than *C. tamariscifolia* and *E. elongata*. Algal phenolic compounds are photoprotective due to their UV-screen and antioxidant capacities (Connan et al. 2006). In this study, *C. tamariscifolia* not only presented much higher levels of phenolic compounds and antioxidant capacity than *U. rigida*, but also the release of phenolic com-

Table 6. Pearson correlation values between dependent variables in *Cystoseira tamariscifolia*. Values in **bold** show significant relationships ($p < 0.05$). (*) n = 144; (**) n = 120; (***) n = 72. SPs = total soluble proteins; phenolics = phenolic compounds; FAs = fatty acids; EC_{50} = concentration of the methanolic extract required to scavenge 50% of the DPPH

	C**	N**	Phenolics*	EC_{50}*	FAs***
SPs*	–0.018 p = 0.845	0.093 p = 0.312	**0.206** **p = 0.013**	**–0.203** **p = 0.015**	–0.062 p = 0.603
C**		**0.565** **p < 0.001**	**0.185** **p = 0.043**	0.032 p = 0.725	0.188 p = 0.114
N**			–0.023 p = 0.807	–0.031 p = 0.736	0.122 p = 0.308
Phenolics*				**–0.211** **p = 0.011**	**0.244** **p = 0.039**
EC_{50}*					0.117 p = 0.330

Table 7. Pearson correlation values between dependent variables in *Ulva rigida*. Values in **bold** show significant relationships ($p < 0.05$). (*) n = 144; (**) n = 120; (***) n = 72

	C***	N***	Phenolics**	FAs***
SPs*	–0.1699 p = 0.064	**0.2517** **p = 0.006**	**0.2274** **p = 0.018**	–0.0069 p = 0.958
C**		**0.4015** **p < 0.001**	–0.0695 p = 0.530	**0.3412** **p = 0.008**
N**			–0.0648 p = 0.558	0.0692 p = 0.599
Phenolics				–0.0852 p = 0.621

pounds into the seawater in the case of *C. tamariscifolia* considerably reduced the UVR in the experimental tanks (Stengel et al. 2014). In this study, the concentration of phenolic compounds (expressed as phloroglucinol equivalent) in the seawater (experimental vessel) under ambient temperature was higher than under increased temperature. The release of phenolic compounds from *C. tamariscifolia* thalli at noon has been suggested to be a photoprotective mechanism under high irradiance conditions (Abdala-Díaz et al. 2006, Celis-Plá et al. 2014). In the green macroalga *Dasycladus vermicularis*, phenolic compounds (trihydroxycoumarins) with antioxidant capacity are released under stressful conditions, reducing photoinhibition and increasing the recovery capacity of the photosynthetic yield and productivity (Pérez-Rodríguez et al. 1998, 2001, 2003). Thus, the combination of thallus thickness, phenolic compounds (and their positive response to temperature increase), antioxidant activity and the reduction in UV penetration by the release of phenolic compounds might give *C. tamariscifolia* an advantage over *U. rigida* in high light environments that could even result in a higher production of the first species, especially with increasing temperatures. An indication of the relationship between phenolic compounds and the good physiological status of *C. tamariscifolia* and *U. rigida* is the linear and positive relationship of their phenolic content with the SP content. The increase in total FA content was also related to phenolic content only in *C. tamariscifolia*. These results indicate a link between primary metabolism, i.e. protein or lipid accumulation,

Table 8. Pearson correlation values between dependent variables in *Ellisolandia elongata*. Values in **bold** show significant relation ($p < 0.05$). (*) n = 144; (**) n = 120; (***) n = 72. SPs = total soluble proteins; FAs = fatty acids; MAAs = mycosporine-like amino acids

	C**	N**	Total MAAs***	% Palythine***	% Shinorine***	% Asterina***	% Palythinol***	% FAs****
SPs*	0.1659 p = 0.070	0.0656 p = 0.476	0.0917 p = 0.374	**–0.2452** **p = 0.016**	0.1025 p = 0.320	0.1382 p = 0.179	**0.2264** **p = 0.027**	**0.5334** **p < 0.001**
C**		0.0477 p = 0.605	–0.0795 p = 0.441	**–0.252** **p = 0.013**	**0.2383** **p = 0.019**	**0.2393** **p = 0.019**	0.0035 p = 0.973	**0.2483** **p = 0.037**
N**			**–0.2446** **p = 0.016**	**–0.3915** **p < 0.001**	**0.3883** **p < 0.001**	**0.3011** **p = 0.003**	–0.0305 p = 0.768	–0.1618 p = 0.178
Total MAAs***				**0.2826** **p = 0.005**	**–0.5196** **p < 0.001**	**–0.4311** **p < 0.001**	**0.4425** **p < 0.001**	0.1318 p = 0.273
% Palythine***					**–0.8271** **p < 0.001**	**–0.8112** **p < 0.001**	–0.1928 p = 0.060	0.0638 p = 0.597
% Shinorine***						**0.8918** **p < 0.001**	**–0.3896** **p < 0.001**	0.0141 p = 0.907
% Asterina***							**–0.2526** **p = 0.013**	0.0054 p = 0.964
% Palythinol***								–0.1515 p = 0.207

and secondary metabolism, i.e. accumulation of phenolic compounds.

E. elongata also contains UV-absorbing compounds involved in photoprotection, i.e. MAAs. C and N internal contents were positively related to some MAAs (i.e. shinorine and asterina-330) but negatively to palythine, indicating that the C and N enrichment favored the accumulation of only some MAAs. This can be advantageous under stressful conditions, since asterina-330 presented a high antioxidant activity against hydrosoluble radicals (ABTS assay) and lipid peroxidation (β-carotene oxidation assay) (De la Coba et al. 2009). N enrichment increases the accumulation of MAAs in different species of red macroalgae of the genus *Porphyra* (*Pyropia*) (Korbee-Peinado et al. 2004, Korbee et al. 2005), *Gracilaria* (Zheng & Gao 2009, Figueroa et al. 2010) and *Grateloupia* (Huovinen et al. 2006). In spite of the fact that MAAs are N-containing compounds, no significant effect of N as a single factor was found for the accumulation of MAAs, except for the double interaction of N × time for all MAAs except palythinol, and the double interaction N × temperature for all MAAs except shinorine. MAAs are synthesized from mycoporine–glycine and they are interconverted between them under the influence of light quality and N availability (Carreto & Carignan 2011). The accumulation of photoprotectors as MAAs and phenolic compounds and the high antioxidant capacity of methanolic extracts are useful indicators of the capacity of the algae to acclimate to increased UV-B radiation or other stressful conditions (Pérez-Rodríguez et al. 2003, Connan et al. 2004, Abdala-Díaz et al. 2006, Carreto & Carignan 2011, García-Sánchez et al. 2012, Figueroa et al. 2014b).

In conclusion, the 3 algal species investigated here displayed high biochemical plasticity in response to changes in nutrient concentration and temperature. Responses were species-specific and could, in part, be related to differences in their bio-optical properties and ecological strategies. Links between primary (productivity, protein production) and secondary metabolisms (antioxidant production) were established for *Ulva* and *Cystoseira*.

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