Phosphorylation of MAPK-like proteins in three intertidal macroalgae under stress conditions

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ABSTRACT: The presence and activation of mitogen-activated protein kinase (MAPK)-like proteins in 3 macroalgae with different morphology and phylogeny (*Ulva rigida*, *Ellisolandia elongata* and *Cystoseira tamariscifolia*) were studied during the experimental workshop of the International Group on Aquatic Productivity. Macroalgae were exposed in the short term (days) to different carbon and nitrogen levels and raised temperature (+4°C over ambient conditions) in mesocosms under controlled conditions mimicking current and future ocean acidification. Three MAPK-like proteins of 40, 42 and 44 kDa similar to mammalian p38 kinase (p38), stress-activated protein kinase/c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) were detected, and their phosphorylation occurred both under low- and high-carbon as well as under low- and high-nitrogen conditions (LC, HC, LN, HN, respectively). Moreover, increased temperature led to differential responses of the MAPKs in these species. In *U. rigida*, p38 phosphorylation initiated the acclimation to temperature stress in LC, while this role was mainly played by JNK in HC during the first 24 h. In *E. elongata*, p38 was primarily activated in LCLN and in HC treatment. In *C. tamariscifolia*, JNK was mainly activated in LN during the first 24 h, while both p38 and JNK were activated in HC, but only after 24 h at elevated temperature. *U. rigida* was the only species in which ERK phosphorylation increased at increased temperature. These results suggest that both *E. elongata* and *C. tamariscifolia* are close to the upper limit of their temperature range at the temperatures tested in this work, and that Ulvales could have physiological advantages in Mediterranean systems in a scenario of future CO_2-enrichment with high nitrogen loads.

KEY WORDS: Macroalgae · Stress · Mitogen-activated protein kinase · MAPK · Ocean acidification · Temperature stress · Nutrient availability

INTRODUCTION

Intertidal algal growth, development, reproduction and productivity are drastically affected by changes in temperature, humidity, salinity, irradiance (both photosynthetically active radiation [PAR] and ultraviolet radiation [UVR]) and nutrient availability (Dring 1982, Lobban & Harrinson 1994, Davison & Pearson 1996, Häder & Figueroa 1997, Gévaert et al. 2003, Gao & Xu 2010, Karleskint et al. 2010). The expected increase in atmospheric CO_2 and the concomitant ocean acidification due to global climate change might increase or negate the effect of these

stressors, and the response and strategies of adaptation and acclimation to these stressors must be studied according to realistic global change scenarios.

It is widely accepted that increasing atmospheric CO_2 emissions are changing the chemistry in the surface layer of the oceans. As more CO_2 dissolves in seawater, changes in the speciation of inorganic carbon occur, resulting in more bicarbonate ions (HCO_3^-), more protons (H^+) and fewer carbonate ions (CO_3^{2-}). The consequences of these changes are a lower pH and lower saturation state of carbonate ions in the seawater (Hofmann et al. 2013). By the end of this century, the pH of surface oceans is expected to

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drop by 0.3 to 0.5 units (Caldeira & Wickett 2003, Feely et al. 2004, Orr et al. 2005) due to increasing concentrations of atmospheric CO_2 that could reach up to 970 ppm CO_2 (Houghton et al. 2001). In addition, both ocean temperature increases due to global warming and increased UVR due to the development of ozone holes may represent a real threat for all sessile marine organisms (Bischof et al. 2006).

In order to face environmental changes, plants have developed a complex metabolic network, through which they detect stress signals and acclimate to the new environmental conditions. Sensing of stress signals and their transduction into appropriate responses is thus crucial for plant survival. These response mechanisms to stress must be rapid and perfectly regulated so that plants can sense the changes. In animal cells and in yeast, these response mechanisms to stress involve the activation of a well-regulated signalling network, in which the central role is played by a specific group of cytoplasmic phosphoproteins called mitogen-activated protein kinases (MAPKs). All eukaryotic cells possess multiple MAPK pathways. External information is transferred to the cell nucleus through a series of phosphorylation/dephosphorylation reactions of the MAPKs that lead to the activation/deactivation of specific groups of genes (Kyriakis & Avruch 2001). These MAPKs, together with their activators (the so-called MAPK kinases and MAPK kinases kinases) form a cascade. The organization of this functional unit or canonic tri-module is mediated by scaffolding proteins (Pawson & Scott 1997), which preserve the efficiency and selectivity of MAPK pathways by binding pathway components, thus maintaining pathway integrity and permitting the coordinated activation of MAPK components in response to specific stimuli. To date, 6 MAPK cascades have been proposed in mammalian cells, but only for 3 of these (p38, c-Jun and extracellular signal-regulated kinase, ERK) have all the components been completely identified. When they are phosphorylated, both p38 and stress-activated protein kinase/c-Jun N-terminal kinase (JNK) trigger the response to stress (e.g. UVR, heat, osmolarity, growth factors, hormones), while ERK controls cell proliferation (cell division) and differentiation. All plants possess MAPK homologues, whose activation triggers the acclimation to stress (Ligterink 2000, Mishra et al. 2006, Sinha et al. 2011).

Despite the fact that the study of signal transduction in algae is very recent, different studies with both macro- and microalgae (Jiménez et al. 2004, 2007, García-Gómez et al. 2012, Parages et al. 2012, 2013, 2014) have clearly shown the presence of p38-like, JNK-like and ERK-like components in algae, as well as their crucial role in acclimation to stress and in cell division (Jiménez et al. 2004, 2007, Parages 2012). A procedure has been developed to detect the presence of these MAPK homologues in several species of Chlorophyta, Rhodophyta and Phaeophyta (Parages et al. 2012), revealing that phosphorylation of p38- and JNK-like MAPKs occurs in Arctic kelps in response to increasing UVR exposure and temperature (Parages et al. 2013) as well as in intertidal macroalgae at the onset of emersion and during periods of the highest irradiation (Parages et al. 2014).

Here we hypothesised that macroalgae phosphorylate and dephosphorylate MAPK-like proteins in response to environmental stress conditions such as acidification, nutrient availability and temperature increase. We investigated the presence and activation of homologues of the p38, JNK and ERK MAPK cascades in 3 different intertidal macroalgal morphotypes, i.e. *Ulva rigida*, *Ellisolandia elongata* and *Cystoseira tamariscifolia*, after exposure to different environmental stressful scenarios (increased ocean acidification, low nitrogen availability and a temperature increase).

MATERIALS AND METHODS

Algal material, location, sampling and pre-treatment

On 10 September 2012, we collected macroalgae from 3 different morpho-functional groups ('sheet-like', 'articulate-calcareous' and 'thick-leathery' according to Littler et al. 1983) from the intertidal system of La Araña (36° 45' N, 4° 18' W) on the Málaga coast (southern Spain). These macroalgae were *Ulva rigida* C. Agardh (Chlorophyta), *Ellisolandia elongata* J. Ellis & Solander (formerly *Corallina elongata* J. Ellis et Solander according to Hind & Saunders 2013) (Rhodophyta), and *Cystoseira tamariscifolia* (Hudson) Papenfuss (Heterokontophyta), respectively. Whole thalli of the 3 species were harvested and immediately transported in temperature-controlled conditions to the laboratory where epiphytic organisms were carefully removed.

Experimental design

The experiments were designed to test the interactive effects of the current pCO_2 (380 ppm, low carbon: LC) and predicted future concentration for the

year 2100 (800 ppm, high carbon: HC; Ní Longphuirt et al. 2010) in combination with 2 pulsed nitrate concentrations (5 µM, low nitrogen: LN, and 50 µM, high nitrogen: HN). The low level of nitrate represents that in coastal waters of Málaga (Ramírez et al. 2005). Thus, 4 C and N treatments were analysed: LCLN, LCHN, HCLN and HCHN. In addition, the effect of a short-term temperature increase (+4°C above ambient conditions) was also tested.

Over 4 d, thalli of the 3 study species (200 g fresh weight [FW] per replicate in the case of *U. rigida*; 400 g FW for *E. elongata* and 390 g FW for *C. tamariscifolia*) were placed in C and N conditions similar to those found in their natural habitat on the coastline of Málaga in the month of September (namely LCLN). Detailed descriptions of the experimental set up of the mesocosms, growth conditions, nutrient concentration and light regime may be found in Stengel et al. (2014, this Theme Section). In brief, the set up consisted of a mesocosm system composed of 4 groups of 9 small open tanks (0.094 m² surface area, 14 l volume). Each group of 9 tanks (3 replicates for each of the 3 species) was placed inside an open tank of 1000 l connected to a fifth 1000 l tank in which 2 water pumps (Aqua Medic OR 2500) were submersed for water circulation. Thermoregulation was achieved by using an Aqua Medic Titan at a constant flow of 0.84 ± 0.05 l min^{-1}. Temperature was established in each header tank using an AT Control System connected to 150 W submersible heaters.

Each of the four 1000 l tanks was used for a different combination of C and N (LCLN, LCHN, HCLN, HCHN). Seawater was circulated in each of the 14 l tanks by connecting groups of 3 tanks to a 60 l header tank in which pH, CO_2 and N concentration were measured and adjusted. All tanks were placed under natural solar radiation covered with a mesh neutral filter (32% of reduction of both PAR and UVR). After the 4 d acclimation period, the experimental conditions of the different treatments were established for 6 d before temperature was raised by 4°C ('temperature+4°C').

Environmental parameters: control and measurement

A pulse of nitrate was added every morning at 06:00 h in each tank using 1 M KNO_3 to obtain either a final nitrate concentration of 5 µM corresponding to the LN condition, or a concentration of 50 µM corresponding to the HN condition. The necessary volume of the 1 M KNO_3 solution was estimated from the last

sampling results of the previous day. In addition, 5 µl of 1 mM K_3PO_4 solution were also added to every tank every morning to avoid P-limitation.

Fluorometry, electron transport rate and chlorophyll *a* content

Maximal quantum yields (F_v/F_m) were determined according to Schreiber et al. (1986) using pulse amplitude modulated (PAM) fluorometers (PAM-2000, Walz) in the case of *C. tamariscifolia* and *E. elongata* (one instrument for each species) and a Mini-PAM (Walz) in the case of *U. rigida*. After 15 min of dark acclimation following an initial quasi-dark measurement (~1.5 µmol photons m^{-2} s^{-1}) to provide estimates of minimum fluorescence (F_o), a saturating flash of 0.8 s and an intensity of >4000 µmol photons m^{-2} s^{-1} was applied to obtain the maximum fluorescence level from fully reduced Photosystem II (PSII) reaction centres (F_m), and F_v/F_m was obtained as $F_v = F_m - F_o$.

Effective quantum yields ($\Delta F/F_m'$) were determined at noon *in situ*, i.e. from algae growing under solar radiation in the tanks, by using Diving PAM fluorometers (Walz) according to Genty et al. (1989). Three replicates per tank were used. $\Delta F/F_m'$ was obtained as $F_m' - F_t$, where F_m' is the maximum fluorescence yield of the illuminated sample, and F_t is the instantaneous fluorescence of illuminated algae shortly before application of a saturation pulse.

Electron transport rate (ETR, in µmol electrons m^{-2} s^{-1}) through PSII was calculated for each level of actinic light as follows:

$$ETR = \Delta F/F_m' \times E_{PAR} \times A \times F_{II} \qquad (1)$$

where E_{PAR} is the incident irradiance of PAR ($\lambda = 400$ to 700 nm). The irradiance was measured *in situ* by a spherical quantum sensor (US-SQS/L). Absorptance (A) was determined as $A = 1 - T$, where T is the transmittance as reported by Figueroa et al. (2009). F_{II} is the fraction of total absorbed quanta in PAR by cellular chlorophyll and its light-harvesting chlorophyll proteins (LHCII) associated with PSII, i.e. 0.5, 0.8 and 0.15 in green, brown and red algae, respectively, according to Grzymski et al. (1997), Figueroa et al. (2003) and G. Johnsen (pers. comm.).

Chlorophyll *a* (chl *a*) was extracted by grinding the thalli in $MgCO_3$-neutralized acetone. Concentration was calculated according to Ritchie (2006). Data presented in this work correspond to midday measurements, at the moment of highest irradiance, on Day 0 (before the temperature increase), and 24 and 72 h after the temperature increase.

Protein extraction, electrophoresis and immunoblot analysis

Samples for MAPK analysis were taken at noon on Day 0 (prior to the temperature increase) and 24 and 72 h after the temperature increase. Whole thalli were deep frozen in liquid N_2 and stored at −80°C until further processing. Frozen thalli were ground using a Retsch MM400 mixer mill. Proteins were extracted according to the method described by Parages et al. (2012), using a commercial RNA purification kit (RNeasy Plant Mini Kit, Qiagen) to yield clean protein extracts, avoiding interference from polyphenols, acidic polysaccharides and nucleic acids. Furthermore, the phosphorylation state of protein kinases is preserved with this method. Protein extracts were stored at −80°C until further analysis.

Gel electrophoresis, immunoblotting and detection of phosphorylated MAPKs were also performed according to methods described by Parages et al. (2012). Western blots were analysed using Amersham ECL Advance (GE Healthcare) as the chemoluminiscence agent and visualised on a Kodak Gel Logic 1500 Imaging System (Eastman Kodak). All antibodies used detected only the phosphorylated forms (active) of the MAPKs; they were purchased from Cell Signaling Technology and used according to the manufacturer's recommendations. Band intensity analyses were carried out using ImageJ 1.440 software (National Institutes of Health).

Statistical analysis

Four combinations of C and N treatments were considered (LCLN, LCHN, HCLN, HCHN) in the analyses. In addition, both for PAM fluorometry as well as for MAPKs, even though only 2 temperatures were tested (ambient temperature and ambient temperature+4°C), data at Time 0 (ambient temperature) were compared with data at Time 24 and 72 h after the temperature increase, and were considered as independent treatments to check acclimation to the new temperature. Results of the PAM fluorometry, ETR and chl a content were analysed by 2-way ANOVAs with 3 independent replicates. Band intensities of the blots were compared by means of 2-way ANOVAs, with 3 to 5 independent replicates for each data point. Values are expressed as means ± SD. Significant differences as well as interaction of means were compared with the post hoc Holm-Sidak test. For all analyses, p < 0.05 was considered significant. Statistical analyses were car-ried out using the SigmaPlot 11.0 statistical package (SPSS).

RESULTS

Photosynthetic responses

The physiological status of the 3 species of macroalgae under the different conditions of C, N and temperature was monitored by means of measuring their maximal quantum yield (F_v/F_m), ETR and chl a content. Fig. 1a shows that in *Ulva rigida*, F_v/F_m was initially between 0.5 and 0.7, being higher in HC than in LC, and higher in HN than in LN conditions. The 4°C temperature increase induced a significant but transitory increase in F_v/F_m in LC treatments and in HCLN after 24 h (Holm-Sidak, all p < 0.05), dropping to initial values after 72 h (Fig. 1a). In addition, F_v/F_m was similar in the LC and HCLN treatments at 24 h (Table 1). However, in HCHN the response was opposite, i.e. a drop after 24 h (Holm-Sidak, all p < 0.05), followed by an increase to initial values after 72 h. In *Ellisolandia elongata*, maximal quantum yield was initially low in the mesocosms, ranging from 0.3 to 0.5 (Fig. 1b), with lowest values in LCLN and HCHN. After 24 h at temperature+4°C, F_v/F_m did not significantly differ among treatments (Table 1), reaching values around 0.5, due to an increase in F_v/F_m values in the LCLN and HCHN treatments, and a decrease in the LCHN and HCLN treatments. However, significant differences in HCHN treatment appeared between 0 and 24 h (Holm-Sidak, p < 0.05). Final F_v/F_m was 0.40 to 0.50, with no significant differences among treatments (Table 1; Holm-Sidak, all p > 0.05). Finally, in *Cystoseira tamariscifolia*, the pattern of maximal quantum yield was not affected by the carbon concentration, nitrogen availability or temperature (Fig. 1c), since differences were not statistically different (Table 1; Holm-Sidak, all p > 0.05). In all cases, F_v/F_m values were around 0.7.

In *U. rigida*, ETR at noon under solar radiation also varied between treatments (Fig. 2a, Table 1). Initially it was highest in HN treatments, with values above 140 µmol electrons m^{-2} s^{-1} in HCHN and 120 µmol electrons m^{-2} s^{-1} in LCHN, while in LN, values were around 100 µmol electrons m^{-2} s^{-1}. In HCHN, the trend was similar to that of F_v/F_m: a drop 24 h after the increase in temperature, recovering to initial values at 72 h. In HCLN, the trend was also similar to that of F_v/F_m, with an initial increase at 24 h and a drop to initial values after 72 h. No significant differ-
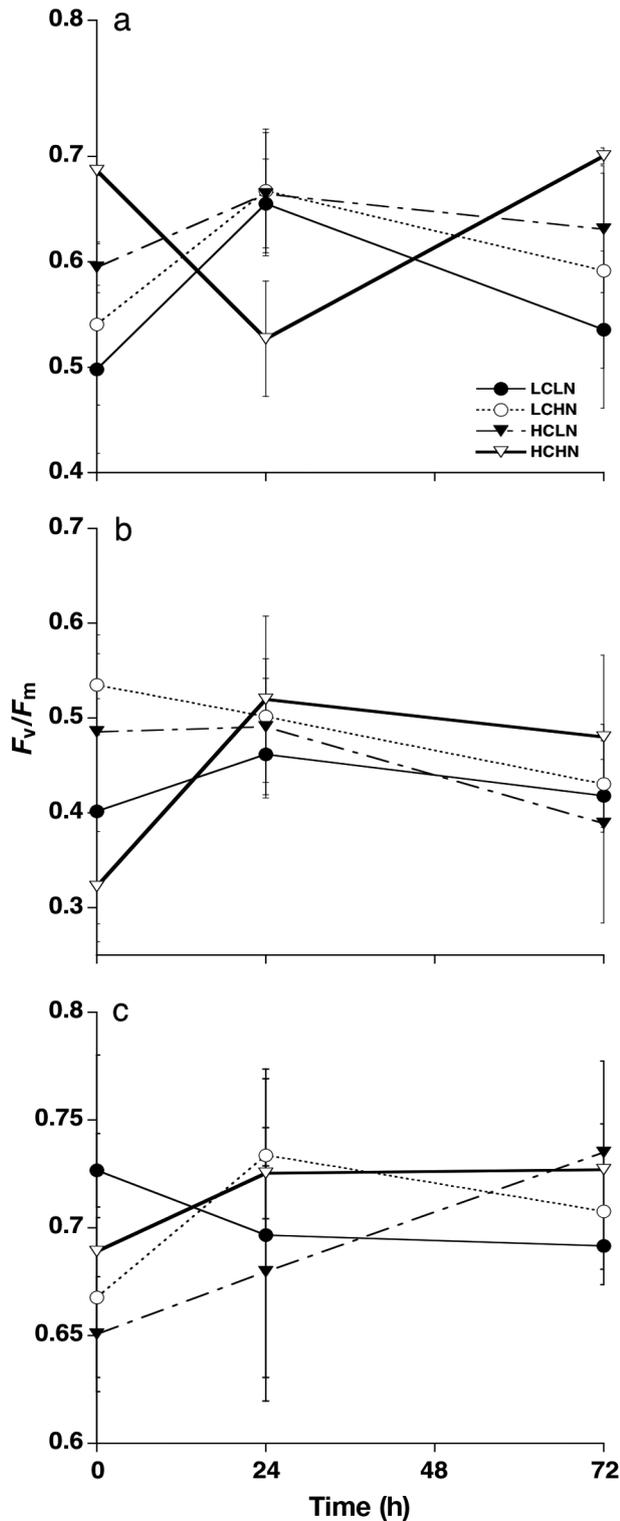
Fig. 1. Maximal quantum yield (F_v/F_m) of (a) *Ulva rigida*, (b) *Ellisolandia elongata* and (c) *Cystoseira tamariscifolia* under different conditions of carbon, nitrogen and temperature increase analysed by pulse amplitude modulated (PAM) fluorometry. Data show means ± SD. LCLN: low carbon, low nitrogen; LCHN: low carbon, high nitrogen; HCLN: high carbon, low nitrogen; HCHN: high carbon, high nitrogen

Table 1. Two-way ANOVA results for the effect of temperature and nutrient levels on maximal quantum yield (F_v/F_m), electron transport rate (ETR) and chlorophyll *a* (chl *a*) in *Ulva rigida*, *Ellisolandia elongata* and *Cystoseira tamariscifolia*. We compared data at Time 0 (= ambient temperature) with data at 24 and 72 h after a temperature increase of 4°C, and considered these as independent treatments to check acclimation to the new temperature. Algae were exposed to 4 different carbon and nitrogen treatments: low C, low N; low C, high N; high C, low N; high C, high N. *p < 0.05

Factor	df	F	p
Ulva rigida			
Maximal quantum yield			
Temperature	2, 24	2.032	0.154
C and N treatments	3, 24	2.731	0.067
Temperature × Treatments	6, 24	4.960	0.002*
ETR			
Temperature	2, 24	1.876	0.175
C and N treatments	3, 24	18.546	<0.001*
Temperature × Treatments	6, 24	1.784	0.145
Chl *a*			
Temperature	2, 24	17.228	<0.001*
C and N treatments	3, 24	16.521	<0.001*
Temperature × Treatments	6, 24	1.230	0.326
Ellisolandia elongata			
Maximal quantum yield			
Temperature	2, 24	2.699	0.088
C and N treatments	3, 24	1.172	0.342
Temperature × Treatments	6, 24	2.279	0.071
ETR			
Temperature	2, 24	1.451	0.254
C and N treatments	3, 24	2.509	0.083
Temperature × Treatments	6, 24	0.868	0.532
Chl *a*			
Temperature	2, 24	7.991	0.002*
C and N treatments	3, 24	5.038	0.008*
Temperature × Treatments	6, 24	1.728	0.158
Cystoseira tamariscifolia			
Maximal quantum yield			
Temperature	2, 24	1.587	0.226
C and N treatments	3, 24	0.454	0.717
Temperature × Treatments	6, 24	1.271	0.309
ETR			
Temperature	2, 24	18.750	<0.001*
C and N treatments	3, 24	1.252	0.313
Temperature × Treatments	6, 24	1.705	0.163
Chl *a*			
Temperature	2, 24	2.398	0.112
C and N treatments	3, 24	2.145	0.121
Temperature × Treatments	6, 24	1.226	0.328

ences appeared in LCHN during the experiments (Holm-Sidak, all p > 0.05). In general, slight or no differences appeared in ETR values after 72 h at temperature+4°C in *U. rigida*. In the case of *E. elongata* (Fig. 2b), ETR values at noon were in the range 86 to 89 µmol electrons $m^{-2} s^{-1}$ in all treatments at Time 0 (T = 0, ambient temperature), showing a small but

steady decrease to values around 80 µmol electrons m^{-2} s^{-1} after 72 h at temperature+4°C in LC and in HCHN (no significant differences between treatments were found; Table 1; Holm-Sidak, all p > 0.05). However, in HCLN, a transient increase to nearly 100 µmol electrons m^{-2} s^{-1} was detected after 24 h, after which values decreased to initial ones at 72 h. In *C. tamariscifolia*, ETR values at noon decreased slightly (but significantly; Holm-Sidak, all p < 0.05) in LN treatments after 72 h (Fig. 2c, Table 1), dropping from values around 375 µmol electrons m^{-2} s^{-1} to values around 350 µmol electrons m^{-2} s^{-1}. It is worth noting that ETR was much higher in *C. tamariscifolia* than in the other 2 species, with *E. elongata* having the lowest ETR.

Chl *a* content in *U. rigida* (Fig. 3a) was always higher in LC than in HC, and in HN than in LN, both before and after the temperature increase (Table 1). There was a transient increase in chl *a* content in HN (24 h), followed by a drop to initial values after 72 h (Holm-Sidak, all p < 0.05). In *E. elongata*, the chl *a* content (Fig. 3b) was also higher in LC than in HC after 72 h. It did not vary after the temperature increase in LC treatments; however, in HC treatments it showed a slight decrease after 72 h at increased temperature (Table 1; Holm-Sidak, all p < 0.05). Finally, the chl *a* concentration in *C. tamariscifolia* (Fig. 3c) was not affected by the transient increase in temperature, as none of the differences were significant (Table 1; Holm-Sidak, all p > 0.05).

MAPK-like proteins

Three phosphorylated MAPK-like proteins were detected in all species. In *U. rigida*, a phosphorylated 42 kDa JNK-like protein (Fig. 4a) was detected before the change in temperature (Time 0), with the highest level of this phosphorylation in LC conditions (3 times higher than in HC). However, 24 and 72 h after the increase in temperature, all growth conditions showed similar phosphorylation. The behaviour was different at LC and HC; in the first case, a continuous decrease in the degree of phosphorylation occurred after the increase in temperature, while a transient increase at 24 h was detected in HC, followed by a decrease to the initial phosphorylation state after 72 h at temperature+4°C. This transient increase in HC together with the continuous decrease in LC led to a similar degree of phosphorylation in the 4 treatments at the end of the experiments. The level of N induced significant difference in the phosphorylation of this JNK in *U. rigida* after 72 h
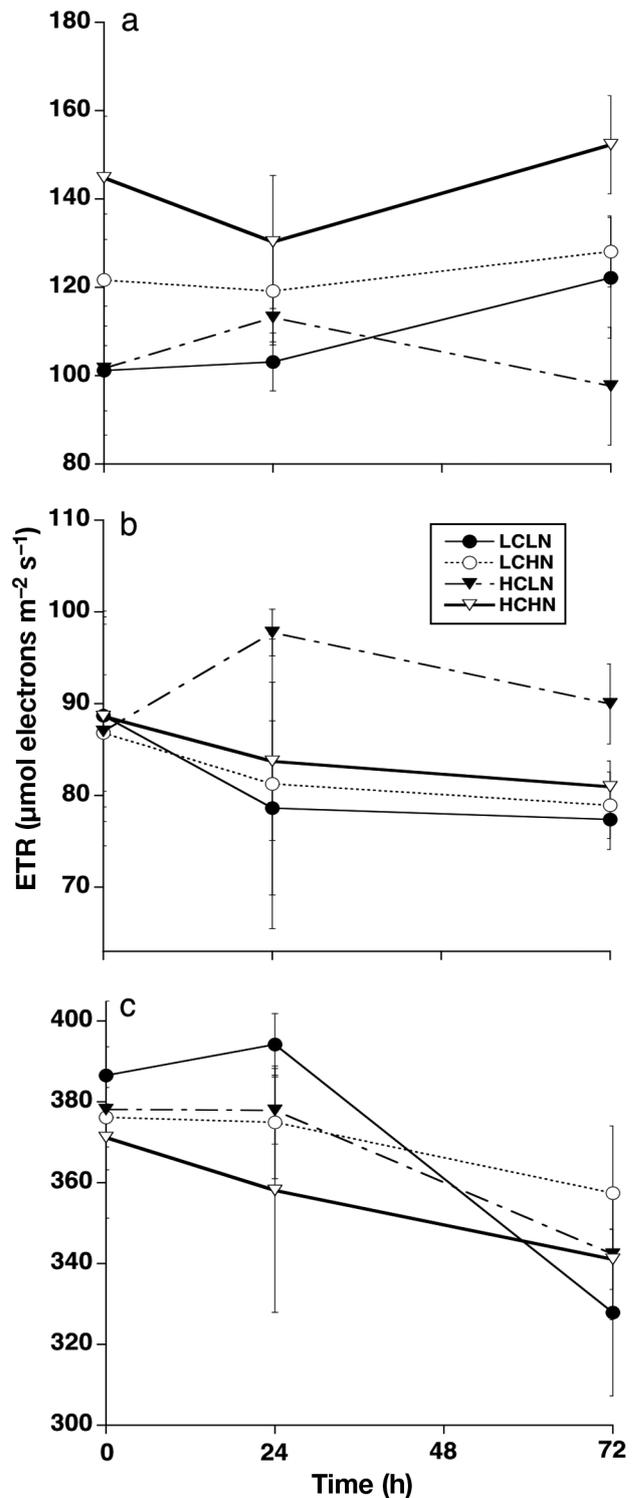

Fig. 2. Electron transport rate (ETR) through Photosystem II expressed as µmol electrons m^{-2} s^{-1} determined *in situ* (algae growing in the tanks under solar radiation) in (a) *Ulva rigida*, (b) *Ellisolandia elongata* and (c) *Cystoseira tamariscifolia* under different conditions of carbon, nitrogen and temperature increase. Data show means ± SD. LCLN: low carbon, low nitrogen; LCHN: low carbon, high nitrogen; HCLN: high carbon, low nitrogen; HCHN: high carbon, high nitrogen

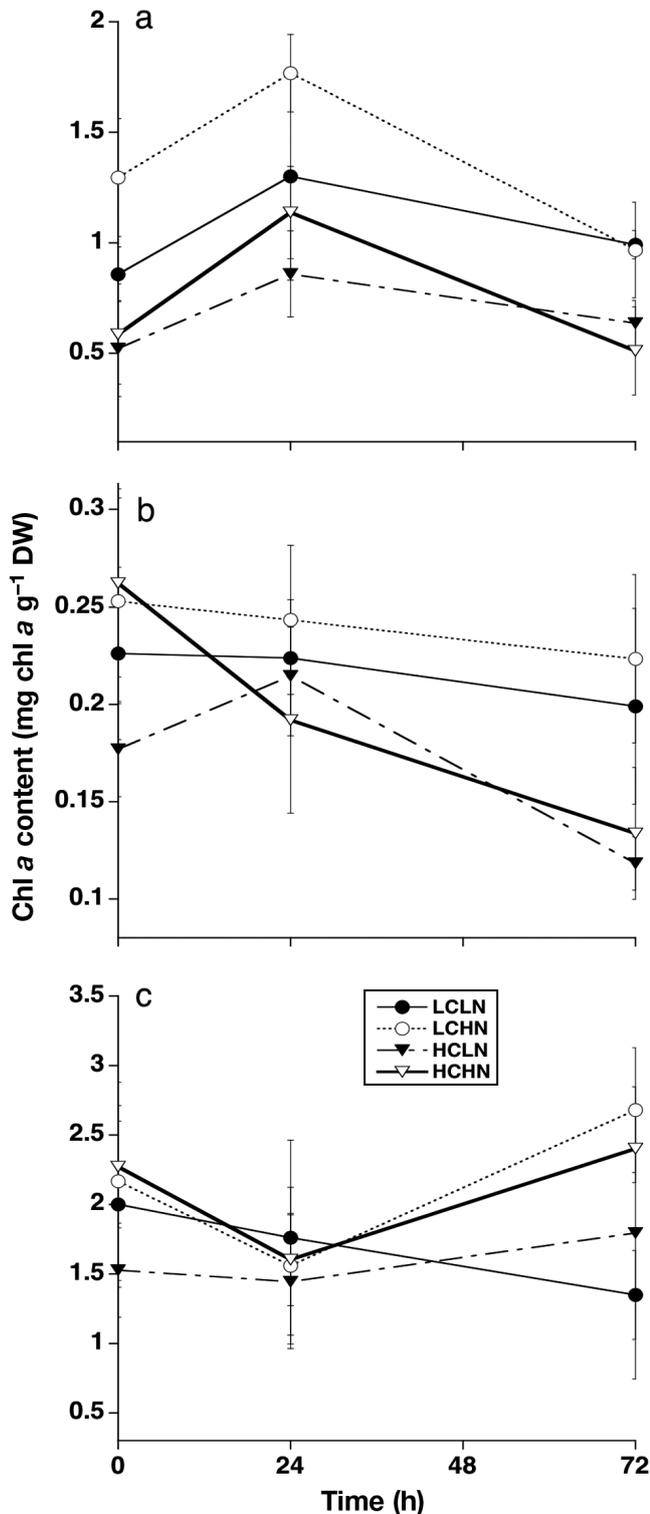Fig. 3. Chlorophyll *a* (chl *a*) content expressed as mg g^{-1} dry weight (DW) in (a) *Ulva rigida*, (b) *Ellisolandia elongata* and (c) *Cystoseira tamariscifolia* under different conditions of carbon, nitrogen and temperature increase. Data show means ± SD. LCLN: low carbon, low nitrogen; LCHN: low carbon, high nitrogen; HCLN: high carbon, low nitrogen; HCHN: high carbon, high nitrogen
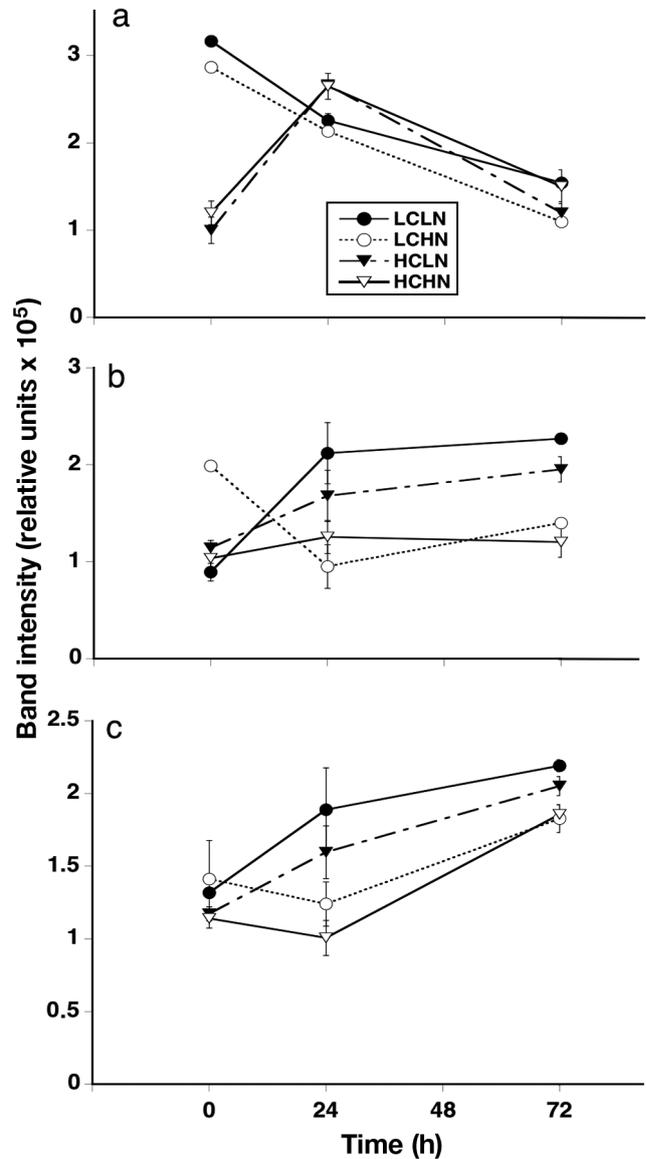

Fig. 4. Expression levels of the phosphorylated JNK-like proteins in (a) *Ulva rigida*, (b) *Ellisolandia elongata* and (c) *Cystoseira tamariscifolia* exposed to different carbon and nitrogen treatments (LCLN: low C, low N; LCHN: low C, high N; HCLN: high C, low N; HCHN: high C, high N). Algae were sampled at Time 0 (start of the experiment), and 24 and 72 h after a 4°C temperature increase. Data are mean (±SE; n = 3) band intensity measured on Western blots

(Holm-Sidak, all p < 0.05). Both temperature and treatment, as well as their interaction, induced significant differences in the level of phosphorylated JNK (Table 2). Similarly, a JNK-like protein of 42 kDa was detected in *E. elongata*, showing clear phosphorylation at Time 0, mainly in the LCHN treatment (Fig. 4b). The level of phosphorylation in this treatment dropped drastically after 24 h at tempera-

Table 2. Two-way ANOVA results for the effect of tempera-
ture and nutrient conditions on the levels (based on band
intensity in Western blots) of phosphorylated MAPK-like
proteins (Ph-JNK, Ph-p38 and Ph-ERK) in *Ulva rigida, Elli-
solandia elongata* and *Cystoseira tamariscifolia*. We com-
pared data at Time 0 (=ambient temperature) with data at 24
and 72 h after a temperature increase of 4°C, and considered
these as independent treatments to check acclimation to the
new temperature. Algae were exposed to 4 different carbon
and nitrogen treatments: low C, low N; low C, high N; high
C, low N; high C, high N). *p < 0.05

Factor	df	F	p
Ulva rigida			
Ph-JNK			
Temperature	2,12	265.337	<0.001*
C and N treatments	3,12	114.686	<0.001*
Temperature × Treatments	6,12	165.932	<0.001*
Ph-p38			
Temperature	2,12	339.931	<0.001*
C and N treatments	3,12	44.661	<0.001*
Temperature × Treatments	6,12	13.536	<0.001*
Ph-ERK			
Temperature	2,12	146.330	<0.001*
C and N treatments	3,12	96.153	<0.001*
Temperature × Treatments	6,12	40.458	<0.001*
Ellisolandia elongata			
Ph-JNK			
Temperature	2,12	14.731	<0.001*
C and N treatments	3,12	32.194	<0.001*
Temperature × Treatments	6,12	29.214	<0.001*
Ph-p38			
Temperature	2,12	151.667	<0.001*
C and N treatments	3,12	25.968	<0.001*
Temperature × Treatments	6,12	33.678	<0.001*
Ph-ERK			
Temperature	2,12	116.559	<0.001*
C and N treatments	3,12	127.744	<0.001*
Temperature × Treatments	6,12	118.957	<0.001*
Cystoseira tamariscifolia			
Ph-JNK			
Temperature	2,12	138.683	<0.001*
C and N treatments	3,12	28.267	<0.001*
Temperature × Treatments	6,12	10.027	<0.001*
Ph-p38			
Temperature	2,12	0.248	0.784
C and N treatments	3,12	4.486	0.025*
Temperature × Treatments	6,12	3.466	0.032*
Ph-ERK			
Temperature	2,12	28.419	<0.001*
C and N treatments	3,12	0.376	0.772
Temperature × Treatments	6,12	2.706	0.067

Fig. 5. Expression levels of the phosphorylated p38-like pro-
teins in (a) *Ulva rigida*, (b) *Ellisolandia elongata* and (c) *Cys-
toseira tamariscifolia* exposed to different carbon and nitro-
gen treatments (LCLN: low C, low N; LCHN: low C, high N;
HCLN: high C, low N; HCHN: high C, high N). Algae were
sampled at Time 0 (start of the experiment), and 24 and 72 h
after a 4°C temperature increase. Data are mean (±SE; n = 3)
band intensity measured on Western blots

ture+4°C (Holm-Sidak, all p < 0.05). In contrast, in
both LN treatments, irrespective of the pCO_2, a sig-
nificant increase in JNK phosphorylation occurred
after 24 h at increased temperature, which persisted
or even slightly increased at 72 h (Holm-Sidak, all p
< 0.05). In addition, a small but significant increase in
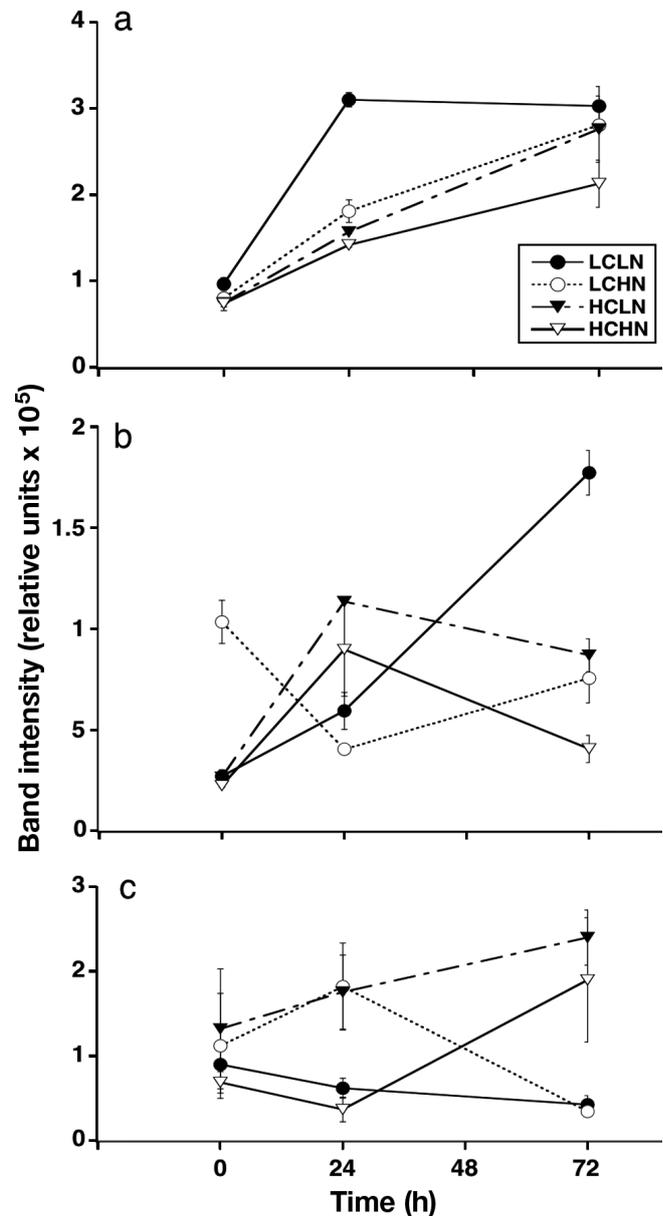JNK phosphorylation was found in HCHN after 72 h

(Holm-Sidak, all p < 0.05). In the case of *C. tamarisci-
folia*, the response to higher temperature occurred
through an increase in the phosphorylation level of
the 42 kDa JNK-like protein in all treatments (Fig. 4c,
Table 2; Holm-Sidak, all p < 0.05). As shown, the
response was faster and slightly more pronounced in
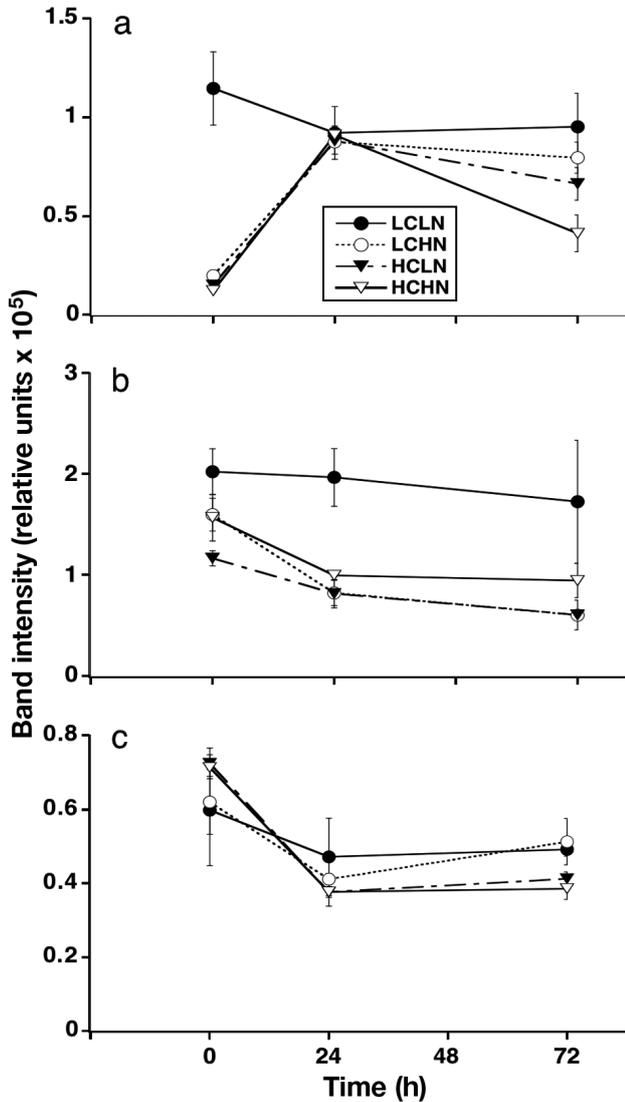LN than in HN conditions. Thus, different responses

Fig. 6. Expression levels of the phosphorylated ERK-like proteins in (a) *Ulva rigida*, (b) *Ellisolandia elongata* and (c) *Cystoseira tamariscifolia* exposed to different carbon and nitrogen treatments (LCLN: low C, low N; LCHN: low C, high N; HCLN: high C, low N; HCHN: high C, high N). Algae were sampled at Time 0 (start of the experiment), and 24 and 72 h after a 4°C temperature increase. Data are mean (±SE; n = 3) band intensity measured on Western blots

were detected for the 3 species: in *U. rigida*, new phosphorylation of JNK occurred only in HC, and this phosphorylation was transient. However, in *E. elongata*, new phosphorylation was detected in LN, while in *C. tamariscifolia*, it occurred in all treatments.

A second MAPK-like protein of 40 kDa was detected (Fig. 5) by using a phospho-p38 mammalian antibody. Phosphorylation of this kinase in *U. rigida* was very mild, and similar, in all C and N treatments in the basal state before temperature increase. A

significant increase occurred after 24 h at the new temperature in all treatments (Holm-Sidak, all p < 0.05), being more pronounced in LCLN. The intensity of phosphorylation continued to increase during the next 48 h in all treatments except in LCLN, where maximal phosphorylation was achieved after 24 h. Even though phosphorylation increased in all treatments at temperature+4°C, the final degree of phosphorylation was significantly lower in HCHN than in the rest of the treatments (Table 2; Holm-Sidak, all p < 0.05). A very different behaviour was expressed in *E. elongata* (Fig. 5b). Initial phosphorylation before the temperature increase was detected mainly in LCHN, which dropped by 50% after 24 h at temperature+4°C, and partially recovering after 72 h. In contrast, in both HC treatments there was a significant and transient rise in p38 phosphorylation at 24 h, partially dropping at 72 h (Holm-Sidak, all p < 0.05). In the case of LCLN, p38 phosphorylation rose linearly during the 72 h after temperature increase, reaching the highest level of phosphorylation detected in this seaweed. Thus, the highest activation occurred in LCLN, while the lowest was found in HCHN (Fig. 5b). In *C. tamariscifolia*, within 72 h after the rise in temperature no significant differences in the degree of phosphorylation of the 40 kDa p38-like MAPK were detected (Fig. 5c; Holm-Sidak, all p > 0.05). Phosphorylation was higher in HC than in LC after 72 h and in addition, nitrogen concentration did not introduce significant differences in phosphorylation (Table 2; Holm-Sidak, all p > 0.05). Increased temperature induced an increase in the phosphorylation in *U. rigida* in all treatments, while in *C. tamariscifolia* no significant differences occurred within 72 h; in *E. elongata*, fast activation was found in HC (24 h) but long-lasting activation occurred only in LCLN.

Finally, an ERK-like MAPK was detected in all species (Fig. 6). In *U. rigida* (Fig. 6a), this protein was mainly phosphorylated in LCLN before the temperature increase. At 24 h after the temperature increase, a sharp increase in the phosphorylation state was detected in HC and in LCHN, while a small decrease occurred in LCLN (Holm-Sidak, all p < 0.05), leading to similar phosphorylation of this 44 kDa protein in all treatments. After 72 h, phosphorylation in LCLN stabilized at values similar to 24 h; however, in the rest of the treatments, a significant drop in the phosphorylation level was found (Holm-Sidak, all p < 0.05), and was strongest in HC. Nevertheless, phosphorylation was higher after 72 h at temperature+4°C in all cases, except in LCLN, where a small decrease was found. ERK-like phosphorylation in *E.*

elongata (Fig. 6b) revealed that the highest phosphorylation in this species at Time 0 also occurred in LCLN. Results show that a partial deactivation of ERK occurred during the 72 h after the rise in temperature in HN (Fig. 6b; Holm-Sidak, all p < 0.05). Finally, ERK phosphorylation dropped in *C. tamariscifolia* in all cases during the first 24 h except in LCLN (Fig. 6c; Holm-Sidak, all p < 0.05), remaining low during the rest of the cultivation period. Thus, ERK was activated only in *U. rigida* after temperature was increased (except in LCLN), while in the rest of the species, no increase in phosphorylation was detected during the 24 to 72 h at temperature+4°C.

DISCUSSION

Macroalgal species respond to elevated CO_2 levels in different ways, and so far, no general pattern has been described. Israel et al. (1999) reported a decrease in growth rate caused by elevated CO_2 in some red algae, and similar effects as well as decreased net photosynthesis or even death of some algae have also been reported (Israel & Hophy 2002, Aline et al. 2006, Martin & Gattuso 2009). However, a more recent study reported that growth rates of 13 species of chlorophytes, rhodophytes and phaeophytes cultivated in normal seawater were comparable to their growth in CO_2-enriched seawater (Israel & Hophy 2002), most probably due to the presence of CO_2-concentrating mechanisms.

In the current study, observed response of the MAPK-like proteins varied depending on the species of macroalgae and on the growth conditions, e.g. low and high C, low and high N, and low and high temperature. Previous findings of our group have demonstrated the existence and activation of p38-like and JNK-like MAPK cascades in both microalgae (Jiménez et al. 2004, García-Gómez et al. 2012) and macroalgae (Parages et al. 2012, 2013, 2014) in response to stress. These protein kinases play a crucial role in early response and acclimation of algae to severe environments (i.e. intertidal systems, Parages et al. 2014), and a decrease in the phosphorylation of those kinases leads to reduced survival in algae (Jiménez et al. 2004, Parages 2012). In addition, presence of ERK-like MAPK was detected in all species. Active cell division occurs only when ERK is phosphorylated, and its dephosphorylation leads to cell division arrest, followed by cell death (Jiménez et al. 2007).

Intertidal communities of macroalgae are permanently exposed to a harsh and highly fluctuating environment, in which they have to be very well adapted to the severe conditions. All 3 species used in this research showed phosphorylation of MAPK-like proteins both before and after the temperature increase, and both in low and high carbon and nitrogen conditions. However, significant differences appeared between the species. No growth data are available for this set of experiments, thus no conclusions about the integrated response of these species can be drawn. *In vivo* fluorescence data obtained using PAM fluorometry as well as MAPK analysis give us a partial view of the different processes involved in the response of the different species to the stressful conditions tested in this work. According to the fluorescence data (Figs. 1 & 2), LCLN seemed to be the most stressful physiological condition for *Ulva rigida*, probably due to C limitation. Other data obtained during the GAP9 Workshop indicated that physiological fitness of *U. rigida* was highest at HC and HN before the temperature increase and that the lowest photoinhibition at elevated temperature (+4°C) occurred in the HCHN treatment (F. L. Figueroa et al. unpubl. data); thus, *U. rigida* is able to benefit from high carbon and high nitrogen availability. Those authors found that photoinhibition at midday was highest in LN, and that in parallel, ETR was lowest under N limitation (independently of the temperature). HCLN conditions seemed to be responsible for the drop in photosynthesis. In this sense, Gordillo et al. (2001) showed that growth rate increased in *U. rigida* at high CO_2, but both photosynthesis and respiration rates were decreased at high CO_2.

In the present work, we have shown that JNK-like MAPK in *U. rigida* was highly phosphorylated in LC before the temperature increase, in agreement with the house-keeping role of this MAPK (as previously shown in other algae by Jiménez et al. 2004, García-Gómez et al. 2012 and Parages et al. 2012, and in mammalian cells by Capasso et al. 2001). The rise in temperature introduced a new stress condition in the LCLN-grown thalli, which showed an immediate response driven by p38 phosphorylation (Fig. 5), while both ERK (Fig. 6) and JNK (Fig. 4) phosphorylation decreased at the elevated temperature. A clear difference appeared between HC and LC: in HC, the response to increased temperature was mostly driven by JNK phosphorylation during the first 24 h, followed by p38 phosphorylation during the next 48 h. However, increasing temperature in LC mostly induced phosphorylation of p38. In addition, activation of ERK at temperature+4°C in all cases except in LCLN suggested that cell division was enhanced by

temperature in HC and HN treatments. As mentioned, in *U. rigida* JNK was already highly activated in LC before the temperature increase, and at elevated temperature, p38 was rapidly activated in LCLN. In parallel, JNK was partially deactivated under those conditions. Jiménez et al. (2004) showed that in the unicellular microalga *Dunaliella viridis*, phosphorylation of p38-like MAPK after stress extended longer in time than that of JNK, as in the case of *U. rigida*.

It is not easy to establish a direct correlation between fluorescence data and MAPKs involved in the response to stress (i.e. JNK and p38). As shown, both F_v/F_m and ETR returned to initial values after 72 h at the elevated temperature in nearly all cases, indicating that acclimation to the new conditions was achieved. Previous data have shown that inhibition of JNK phosphorylation in macroalgae under high irradiance or UV stress led to decreased optimal quantum yield and eventually to cell death (Parages 2012, M. L. Parages & C. Jiménez unpublished data). In addition, García-Gómez et al. (2012) and Parages et al. (2013) have shown that an apparent lack of correlation between fluorescence data and MAPK phosphorylation state may temporarily occur both in microalgae and macroalgae.

The behaviour of MAPK-like proteins in *Ellisolandia elongata* was different than in *U. rigida*. A rapid and very significant increase of p38, and to a lesser extent of JNK, phosphorylation during the first 24 h at elevated temperature was found with the exception of LCHN, indicating that the stress-response mechanism had to be activated in *E. elongata* to acclimate to the new growth conditions. The finding that ERK phosphorylation decreased at elevated temperature indicates that stressful conditions may partially inhibit cell division and algal growth, in agreement with Jiménez et al. (2004). In this species, both p38 and JNK activation seemed to be responsible for triggering the stress-response system in LCLN after the temperature increase, while a transient activation (24 h) of p38 was detected in HC. Another difference compared to *U. rigida* is that activation of JNK was not transient but instead persisted in time. This behaviour in HC thalli under temperature stress was similar to that already described in *Dunaliella tertiolecta* after exposure to UVR (García-Gómez et al. 2012), where a transient increase (24 h) of p38 phosphorylation was detected. In *E. elongata*, ERK phosphorylation decreased in all C and N combinations after exposure to elevated temperature (+4°C), which could indicate that cell division and algal growth decrease compared to initial conditions at

ambient temperature (Jiménez et al. 2007, García-Gómez et al. 2012). In the case of *U. rigida*, ERK phosphorylation was higher at temperature+4°C than at ambient temperature (except in LCLN), which may indicate that this species benefits from increased temperature if C and N are not limiting. All these data indicate that *E. elongata* might be close to its upper temperature limit under the conditions tested here. Several authors (e.g. Somero 2002, Hughes et al. 2003) proposed that many marine organisms already live close to their thermal tolerances, and thus increases in temperature can negatively impact their performance and survival.

One of the effects of global change is the evolution of macroalgal communities in an acidified ocean. Calcareous species appear to be amongst the most sensitive photoautotrophs, as they have a skeletal mineralogy that dissolves easily at predicted levels of calcium carbonate saturation (Gao et al. 1993, Martin & Gattuso 2009). At a mean pH of 7.8, predictions indicate a 25% loss in the biodiversity of calcareous algae and a major reduction in cover of all erect articulated genera (the calcitic *Jania rubens*, *Corallina officinalis*, *C. elongata*, *Amphiroa rigida*, *A. criptarthrodia* and the aragonitic *Halimeda tuna*; Porzio et al. 2011). Gao & Zheng (2010) showed that growth of *Corallina sessilis* was inhibited at high CO_2 levels, decreasing the net photosynthetic rate by 29.3% and the calcification rate by 25.6% compared to a low CO_2/high pH treatment. In addition, growth, photosynthesis and calcification rates were further inhibited in the presence of UVR as compared to PAR alone. Connell & Russell (2010) reported that increased CO_2 levels (550 ppm) had no effect on the cover of turf algae at ambient temperature (17°C) but that turf cover increased considerably when increased CO_2 levels were combined with elevated temperatures. Under these conditions, corallinaceans were unable to compete for the space. Previously, Russell et al. (2009) concluded that the combination of both elevated CO_2 and nutrient concentrations had negative effects on the biomass of coralline crusts. Martin & Gattuso (2009) investigated the synergistic effects of high CO_2 and increased temperature on *Lithophyllum cabiochae*, concluding that algal necrosis appeared at elevated temperature, and initially at 700 ppm (60% of the thallus surface) and later at 400 ppm CO_2 (30%). Algal death was observed only at the highest temperature and was 2 to 3 times higher at high CO_2.

In the case of *Cystoseira tamariscifolia*, a rapid response to increasing temperature occurred in some cases without further activation of the stress-

response mechanism mediated by MAPKs. Our results demonstrated that the level of active (phosphorylated) p38- and JNK-like proteins in this species was altered when a change in temperature occurred (+4°C), but activation of MAPKs varied depending on C and N availability. Results show that JNK phosphorylation was mainly responsible for activation of the stress response in LN, with a fast and continuous increase of phosphorylation during 72 h after temperature increase. However, in HN this increase of JNK phosphorylation only started after 24 h. In general, it may be said that the response to stress in LN was principally mediated by JNK activation, and that the response in HN occurred mainly through p38 activation. In all cases, a significant drop in ERK phosphorylation occurred, which might indicate that the new temperature was above the optimal for growth of *C. tamariscifolia.* Connell et al. (2008) concluded that turf expansion at high CO_2 and elevated temperature would not just negatively affect growth of calcareous algae, but would also inhibit kelp recruitment. *C. tamariscifolia* is well acclimated to increased solar irradiance, presenting high photosynthetic yields and accumulation and release of antioxidant substances (i.e. polyphenolic compounds; Abdala-Díaz et al. 2006); however, no clear information on responses to the combination of elevated CO_2 and temperature is available.

In Arctic macroalgae, we have found differential behaviour in 2 species of phaeophytes (Parages et al. 2013). The endemic *Laminaria solidungula* presented phosphorylated JNK- and p38-like proteins at steady state conditions (PAR at 2°C), that increased after exposure to UVR and increased temperature (from 2 to 7°C). This increase in phosphorylation was more pronounced in the case of JNK than in p38 under both stressors. In turn, *Saccharina latissima*, a non-endemic species, showed a decrease in both JNK and p38 phosphorylation after temperature increase at any radiation condition, while UVR, as expected, also induced an increase in phosphorylation. In this species, p38 was more activated than JNK. *L. solidungula* is an endemic Arctic species, and increased temperature to 7°C was close to its upper tolerance limit; in the case of *S. latissima*, 2°C was close to its lower tolerance limit. This differential behaviour of p38 and JNK phosphorylation in response to environmental stress also occurs in intertidal macroalgae from southern Spain; Parages et al. (2014) described that in several species (e.g. *Chaetomorpha aerea* and *Dilophus spiralis*) JNK was primarily activated at low tide, while in *Jania rubens* p38 was the main MAPK activated during emersion. High

irradiance also induced a significant increase in MAPK phosphorylation in several species (including *U. rigida*, *Dictyota dichotoma* and *Corallina elongata*), and in all cases, p38 responded faster than JNK. In the case of the microalgae *Dunaliella viridis* (Jiménez et al. 2004) and *Dunaliella tertiolecta* (García-Gómez et al. 2012), p38 was rapidly activated after increased salinity in the former species and increased UVR in the latter species.

In conclusion, our study demonstrates that MAPK-like proteins are involved in the response of intertidal macroalgae to environmental stress imposed by ocean acidification, nitrogen limitation and elevated temperature, and that phosphorylation/dephosphorylation of several kinases (p38-like, JNK-like and ERK-like MAPKs) occurs under these conditions. The phosphorylation response of MAPKs differed between kinases and varied between macroalgal species. Activation of p38 is crucial for acclimation of *U. rigida* to the new temperature under C limitation; however, JNK played the role of activating the stress response mechanisms in HC. Similarly, p38 was activated in LCLN, but JNK phosphorylation also increased during the first 24 h. Conversely to *U. rigida*, p38 was primarily activated in *E. elongata* in HC. Finally, in *C. tamariscifolia* JNK was mainly activated in LN during the first 24 h. In addition, *U. rigida* was the only species in which ERK phosphorylation increased at temperature+4°C, indicating active cell division. As stated before, *U. rigida* probably benefitted from elevated carbon and nitrogen levels at ambient temperatures, such that increasing temperature was not as stressful as nutrient limitation. The new temperature was likely above optimal for growth of both *E. elongata* and *C. tamariscifolia*. Our data support the hypotheses of Pergent (1991) and Porzio et al. (2011) that in future climate scenarios, Ulvales might dominate Mediterranean intertidal systems in human-disturbed areas where nutrients are not limiting and in CO_2-enriched environments with no nutrient limitation.

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