



# Short-term effects of CO<sub>2</sub>, nutrients and temperature on three marine macroalgae under solar radiation

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**ABSTRACT:** Three macroalgal species belonging to Chlorophyta (*Ulva rigida*), Rhodophyta (*Ellisolandia elongata*) and Phaeophyceae (Heterokontophyta; *Cystoseira tamariscifolia*), naturally growing at the same shore level and representing 3 morpho-functional groups, were exposed to short-term changes in temperature under different carbon and nitrogen regimes. Experiments were conducted in outdoor tanks at 4 combinations of carbon and nitrogen levels under reduced solar radiation. *In vivo* chl *a* fluorescence parameters and pigment contents were monitored to assess diurnal physiological responses and potential for recovery. Strong fluctuations in chl *a* fluorescence parameters, but not in chl *a* content, were observed in response to diurnal variation in solar radiation and light climate within the tanks; sensitivity varied between algal species and, in some cases, depended on the carbon and nitrogen regime. Nitrogen uptake was similarly high in *U. rigida* and *E. elongata* and lowest in *C. tamariscifolia*. In *U. rigida* and *E. elongata*, chl *a* concentrations decreased after high-carbon treatments. Effective photosystem II quantum efficiency was reduced in all species at noon, and lowest in *C. tamariscifolia*. The results highlight the complexity of physiological short-term acclimations which were most likely linked to biochemical changes at the cellular level. Long-term experiments are required in future for more comprehensive investigation of the observed interactive effects of the different environmental parameters.

**KEY WORDS:** Chlorophyll fluorescence · Climate change · Light climate · Macroalgae · Outdoor multi-tank system · Nutrients · Ocean acidification · Temperature

## INTRODUCTION

Intertidal macroalgae inhabiting challenging environments (e.g. with periodic fluctuations in light, temperature, nutrient and osmotic conditions) typically exhibit considerable physiological plasticity. Impacts of natural environmental stressors may be exacerbated by additional pressures associated with global change, including elevated temperatures, UV radiation and eutrophication (Fabry et al. 2008, Doney et al. 2012). The degree of these effects is

likely to depend on algal group, life strategy and habitat of origin (Wahl et al. 2004, Bischof et al. 2006, Harley et al. 2012).

Anthropogenically induced increases in atmospheric CO<sub>2</sub> concentrations have led to elevated CO<sub>2</sub> concentrations in seawater, causing a reduction in seawater pH, or ocean acidification (OA) (e.g. Doney et al. 2009, Raven 2011). Despite a recent surge in research efforts, our current understanding of the impacts of OA on marine biota is still limited. However, recent studies have outlined taxa-specific

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effects on marine algae (e.g. Hurd et al. 2011, and recently reviewed by Roleda & Hurd 2012).

To date most studies have focused on the isolated impact of CO<sub>2</sub> levels (and/or reduced pH) on algal physiology such as carbon exchange (e.g. Cornwall et al. 2012, Ní Longphuirt et al. 2013), calcification (e.g. Martin & Gattuso 2009), growth (e.g. Riebesell et al. 2007), reproduction (e.g. Roleda et al. 2012), or several parameters in selected species (e.g. Bach et al. 2013). However, in natural systems, predicted and already detectable changes in seawater pH will interact with other ambient physico-chemical conditions and potential stressors such as temperature (Roleda & Hurd 2012) and solar light climate, including UV radiation (Gao & Zheng 2010, Russell et al. 2011, Yildiz et al. 2013), at species and community levels (Porzio et al. 2011, Hofmann et al. 2012). Whilst interactive impacts of coastal eutrophication and OA on water chemistry have been demonstrated (e.g. Cai et al. 2011), little is known about their combined effects on algal physiology.

Physiological characteristics of macroalgae, such as light absorption and conversion efficiency, carbon exchange and nutrient uptake rates, are impacted by thallus morphology and have been reported to differ between thin species (such as *Ulva* spp.), slow-growing calcareous species (e.g. red coralline algae) and more complex brown algae (such as *Cystoseira* spp.). Additionally, species-specific features, such as growth in dense clumps within algal turfs (Hay 1981) and physiological adaptations to facilitate protection against stressful environments (e.g. the capacity to exude phenolics during high light stress; Shibata et al. 2006), may influence measurable responses to climate change parameters of interest. Several studies have previously assessed interactive effects of nitrate and UV radiation on photosynthesis (pulse-amplitude modulated [PAM]-chlorophyll fluorescence), pigments, mycosporine-like amino acids (MAAs) and nutrient uptake characteristics of red and green macroalgae from southern Spain (e.g. Figueroa et al. 2003). Additionally, a study on nutrient uptake characteristics of algae with different life strategies suggested that nitrogen uptake characteristics were aligned to growth strategies and the adaptation of individual species to local nitrogen supply regimes (short-term pulses vs. long-term exposure) (Martínez et al. 2012).

Figueroa et al. (2009) demonstrated the effect of nutrient regime on the capability of *Ulva lactuca* to respond to temperature and light stress; how such short-term responses are influenced by additional impacts of high-CO<sub>2</sub> levels is not certain. On the

shores of southern Spain, *Ulva rigida* C. Agardh, *Ellisolandia elongata* (J. Ellis & Solander) K. R. Hind & G. W. Saunders (formerly *Corallina elongata*) and *Cystoseira tamariscifolia* (Hudson) Papenfuss inhabit exposed rocky mid- to low-intertidal shores in close vicinity (Figueroa et al. 2014a). Thus, a comparative analysis of their respective short-term responses to such stressors, most likely linked to morphological features and life strategies of these species and with potential ecological consequences, is of interest.

This paper represents the first in a series of outputs from the 2012 International Workshop of the Group on Aquatic Primary Productivity (GAP) hosted by the University of Málaga, southern Spain, and includes a description of the experimental set-up employed by the working group on macroalgae, and some primary results. Objectives of the Working Group on Macroalgae were to assess impacts of pulse-supply of nitrogen on primary productivity of selected macroalgae. Here, we describe physico-chemical parameters within tanks with different carbon and nitrogen regimes to which the 3 marine macroalgal species described above were exposed. Nitrate concentration pulses used in this experiment were in the range of those observed in the coastal water in the Bay of Málaga (Ramírez et al. 2005). Impacts of diurnal variation in light climate and ambient temperature (including an experimental 4°C temperature increase) on short-term physiological responses (PAM fluorometry: effective Photosystem II [PSII] quantum yield) and pigments are reported. The capability of different algal species to recover from mid-day exposure to high irradiances was investigated under different carbon and nitrogen regimes; this capability was further assessed after imposing additional short-term temperature stress (4°C increase for 3 days).

Results of further detailed physiological measurements (*P/E* curves measured via PAM chl *a* fluorescence under controlled conditions) and biochemical analyses, to assess impacts of the broader photosynthetic processes and overall metabolism, will be presented and discussed elsewhere (Figueroa et al. 2014b,c, this Theme Section).

## MATERIALS AND METHODS

### Macroalgal species

Thalli of 3 macroalgal species were collected from the eulittoral zone at La Araña (36°45' N, 4°18' W) on the Málaga coast (southern Iberian Peninsula) on 10 September 2012: *Ulva rigida* (Chlorophyta), *Cysto-*

*seira tamariscifolia* (Phaeophyceae) and *Ellisolandia elongata* (Rhodophyta). Algae were transported in temperature-controlled tanks to the laboratory where epiphytic organisms were carefully removed before experimentation.

### Experimental design

The experiment was designed to examine interactive effects of the current  $p\text{CO}_2$  (380 ppm, pH target above 8.0) and predicted future concentration for the year 2100 (700 ppm, pH target 7.92; Orr et al. 2005, Meehl et al. 2007) in a crossed combination with 2 pulsed nitrate concentrations (5 and 50  $\mu\text{M}$ ) according to nitrate data reported for the Alborán Sea, southern Spain (Ramírez et al. 2005). The 4 treatments were designated as low-carbon/low-nitrogen (LCLN), low-carbon/high-nitrogen (LCHN), high-carbon/low-nitrogen (HCLN) and high-carbon/high-nitrogen (HCHN) (see next section). In addition, the effect of a short-term temperature increase was tested in the second part of the experiment.

The experiments were conducted in a multi-tank system placed under semi-natural solar conditions, i.e. photosynthetically active radiation ( $E_{\text{PAR}}$ ; 400–700 nm) reduced by 35%, and UVA (320–400 nm) and UVB (280–320 nm) reduced by 39%, using a green mesh, in the Unit for Microbiology, Ecophysiology and Aquatic Organisms of Málaga University (UMEGOA), located in the Grice-Hutchinson Experimental Centre. The experimental system was composed of 3 open vessels (0.094 m<sup>2</sup> surface area, 14 l volume) per treatment and species ( $n = 3$ ) connected in parallel to a separate tank of 60 l capacity (1 tank per treatment and species, 12 tanks in total, Fig. 1a). The water flow between each vessel and its header tank was  $0.84 \pm 0.05 \text{ l min}^{-1}$ , representing a turnover rate of  $26 \pm 1 \% \text{ h}^{-1}$ . In addition to the series connection circuit causing permanent seawater supply and movement and mixing within vessels, a second closed circuit re-circulated the water along a 3 m pipe within each header tank. This secondary re-circulated system facilitated water mixing in the header tank. Aeration with ambient air caused further mixing within vessels. The seawater used originated from the Bay of Málaga and was stored in two 12 000 l tanks on site. The entire system was placed within 8 tanks of 1000 l with circulating freshwater which were permanently cooled using 2 cooling units (Titan; Aqua Medic).

The collected algal material (per replicate: 200 g fresh weight [FW] of *U. rigida*; 390 g FW for *C.*

*tamariscifolia* and 400 g FW for *E. elongata*) were randomly assigned to the different replicates of the treatments and maintained under the same conditions (LCLN and ambient temperature) for 4 d. Subsequently, the experimental conditions of the different treatments (i.e. LCLN, HCLN, LCHN, HCHN) were established and algae acclimated at ambient temperature for 6 d before the temperature was raised by 4°C (on average) for 3 further experimental days. Data presented here are based on physiological measurements conducted immediately before the temperature increase ('ambient') and 3 d after the temperature increase ('+4°C').

### Environmental parameters: control and measurement

**Carbon treatments.** In order to achieve an HC treatment, a computer-operated pH control system (AT control System, Aqua Medic) was used, with pH sensors (Aqua Medic T2001HC, Aqua Medic) located inside each of the twelve 60 l header tanks (Fig. 1b). The system automatically recorded one measurement every 15 min and was programmed to initiate the supply of pure CO<sub>2</sub> via a solenoid valve as soon as the pH exceeded a threshold of 7.92 in the header tanks (corresponding to 700 ppm of CO<sub>2</sub>, HC treatment). When the pH returned to this value, CO<sub>2</sub> injection stopped. CO<sub>2</sub> was supplied to the secondary re-circulating system of each header tank. This water circuit performed the role of a CO<sub>2</sub> reactor, facilitating rapid CO<sub>2</sub> dissolution and avoiding a sharp drop in pH. No upper pH limit was set for the LC treatment.

**Nitrogen treatments.** Nitrate was added every morning at 06:00 h GMT to each header tank using a 1 M KNO<sub>3</sub> pulse to achieve either a final nitrate concentration of 5  $\mu\text{M}$  corresponding to LN conditions, or a concentration of 50  $\mu\text{M}$  corresponding to HN conditions. The volumes of the 1 M KNO<sub>3</sub> solution required to reach each experimental concentration were estimated from the last sampling result of the previous day. In addition, 1  $\mu\text{M}$  K<sub>3</sub>PO<sub>4</sub> was added in the experimental tanks to avoid phosphate limitation. For nutrient analysis, water samples (50 ml) were collected every day just before nutrient addition, after 1 h, at noon, and again in the evening. Water samples were kept in a freezer at  $-20 \pm 1^\circ\text{C}$  until applying segmented flow analysis using a Bran-Luebbe AA3 Autoanalyzer following the methods described by Grasshoff et al. (1999). The detection limit was 0.05  $\mu\text{M}$ .

**Temperature control.** Water temperature was monitored using the same control system as for pH, with

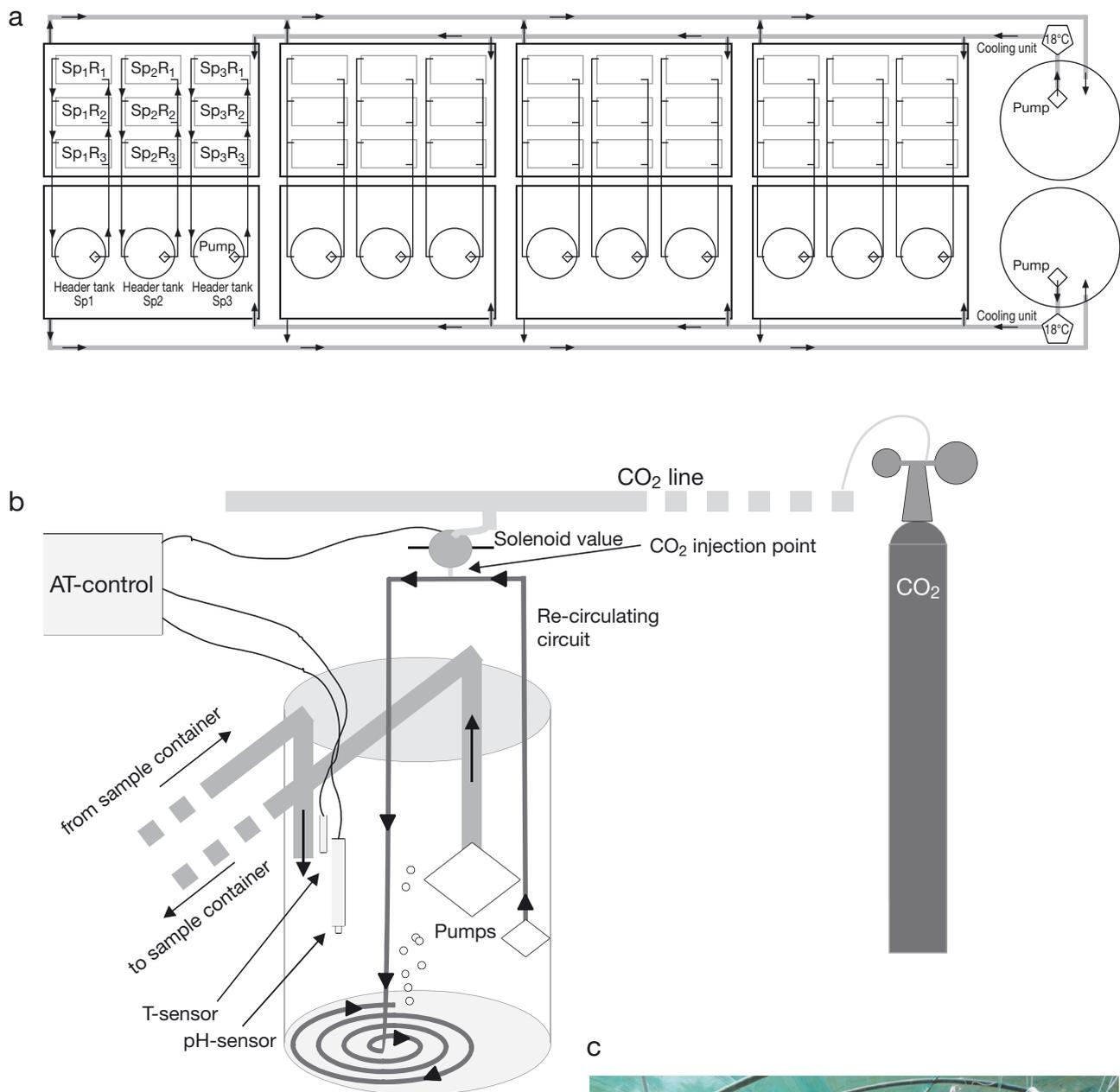


Fig. 1. Experimental set-up of header tanks and replicate vessels. For more detailed explanation and implementation of environmental controls refer to 'Experimental design'. (a) Four experimental treatments (LCLN, LCHN, HCLN, HCHN) with separate header tanks for each species (*Ulva rigida*, *Cystoseira tamariscifolia* and *Ellisolandia elongata*) and 3 replicate ('R') vessels for each species ('Sp') within in each treatment, e.g. Sp<sub>1</sub>R<sub>2</sub>, Sp<sub>2</sub>R<sub>1</sub> etc. (b) Detail of CO<sub>2</sub> adjustment using a computer-operated pH control system (AT control) with pH sensors located inside each of the twelve 60 l header tanks. HC = pCO<sub>2</sub> of 700 ppm, LC = pCO<sub>2</sub> of 380 ppm, HN = 50 μM nitrate and LN = 5 μM nitrate. (c) Photo of outdoor experimental multi-tank set-up with 3 replicated vessels for each species and treatment located under mesh, reducing solar radiation



temperature sensors also located in the header tanks. In the second part of the experiment, temperatures were raised to simulate a daily temperature curve of 4°C above the ambient temperatures experienced during the previous days. Temperature was controlled in the freshwater circulating system in the eight 1000 l tanks. In addition, a diurnal temperature curve was established in each header tank using an AT control System connected to 150 W submersible heaters (Fig. 1b).

**Monitoring conditions in vessels.** Temperature, pH and conductivity were measured in each of the 48 vessels and header tanks 3 times per day using a multi-parameter probe (HI 982X; Hanna). No significant differences (ANOVA,  $p > 0.05$ ) were found between the values measured in the vessels and their corresponding header tank (data not shown). Water loss via evaporation was monitored, and distilled water was added every day after sunset to the header tanks if salinity was higher than 38.

**Solar radiation.** Ambient photosynthetically active radiation ( $E_{\text{PAR}}$ ) at the UMEGOA experimental site beside the tanks (underneath the mesh) was measured in air at 5 min intervals from sunrise to sunset using a spherical quantum sensor (Li 193 SA, Licor) connected to a Data Logger (Li-1400). These data are presented together with effective PSII quantum yield ( $\Delta F/F_m'$ ). In addition, incident solar radiation was measured continuously in air using a UV-PAR Multi-filter radiometer NILU-6 (Geminali). Levels of UVA (320–400 nm) and UVB (280–320 nm) radiation were calculated from the data of the different UV filters according to Høiskar et al. (2003). The integrated daily light fluence expressed as  $\text{kJ m}^{-2}$  was calculated for the duration of the experiment. The NILU-6 was located on the roof of the building of the Central Services Research of Málaga University (3.7 km from the UMEGOA experimental site).

In order to characterize the underwater light field within vessels, solar radiation ( $E_{\text{PAR}}$  and UVR) was measured in the morning of the first day of the experiment just below (1 cm) the water surface and at the bottom of each vessel using Zippo-Hobo®-U12-UV sensors (PAR: SQ-212, UVA: LPUVA01; Onset Computer).

### Biological parameters

**Chl *a* fluorescence of algae in tanks.** The effective quantum yield of PSII ( $\Delta F/F_m'$ ) of algae subjected to the conditions above was determined in 15 samples from every species and treatment every 2 h from

sunrise to sunset using Diving-PAM Fluorometers (Walz).

**Pigment contents.** For pigment analysis (chlorophyll and phycobiliproteins), 1 algal sample per vessel was frozen in liquid nitrogen until extraction. For chlorophyll determination, samples were then ground with a mortar and pestle for 20 min in 1 ml of 90 % (v/v) acetone neutralized with magnesium carbonate hydroxide and sea sand. After centrifugation at 1500  $g$  for 10 min, each supernatant was used to determine chlorophyll concentrations spectrophotometrically (UV mini-1240 Spectrophotometer; Shimadzu) following Ritchie's equations (Ritchie 2008); chlorophyll contents are presented in  $\text{mg g}^{-1}$  dry weight (DW).

Phycobiliproteins (i.e. phycoerythrin and phycocyanin) were extracted from 0.1 to 0.2 g FW of *E. elongata* ground in phosphate buffer at pH 7.0. Pigments were quantified spectrophotometrically (UV mini-1240 spectrophotometer; Shimadzu) according to Beer & Eshel (1985) and are expressed in  $\text{mg g}^{-1}$  DW of seaweed material.

To acquire the ratio of FW to DW, 15 to 20 samples of known FW for every species were dried in an oven at 60°C for 24 h, followed by determination of final DW.

### Statistical analyses

For each macroalgal species, experiments investigating impacts of OA and eutrophication were carried out in triplicate ( $n = 3$ ), with 3 independent vessels used at each of the defined conditions of LCLN, LCHN, HCLN and HCHN. Chlorophyll *a* fluorescence (i.e.  $\Delta F/F_m'$ ) was determined for 15 different specimens per vessel, with the average of 15 values reflecting the overall photosynthetic performance of algae within 1 tank, i.e. the average was considered as 1 replicate measurement and used for further statistical analyses ( $n = 3$ ). Pigment concentrations were measured in 1 specimen per species per tank. All data are presented as mean  $\pm$  SD.

Main effects of the fixed factors 'time of day' (TD, i.e. morning, noon and evening), 'carbon regime' (CR, i.e. low and high carbon) and 'nitrogen regime' (NR, i.e. low and high nitrogen) as well as their respective interactions on the dependent variables 'pigment content' of each species were analysed using 3-way model I ANOVAs. All data were normally distributed (Kolmogorov-Smirnov test), variances were homogeneous (Levene's test) and mean values were generally considered as significantly dif-

ferent at  $p < 0.05$ . Where the Levene's test indicated heterogeneity of variances, even after transformation (this was the case for some pigment data), a more conservative  $\alpha$  of 0.01 was applied to minimise the probability of a Type I error (Krassoi et al. 2008). The least significant difference (LSD) test was applied to differentiate between means when significance was found.

## RESULTS

### Light climate

The solar radiation measured at the University of Málaga campus (3.7 km from the experimental site) was stable throughout the acclimation period but declined on September 18 due to clouds and associated rainfall; the daily integrated light fluence dropped from nearly 10 000 to 4000  $\text{kJ m}^{-2}$  and UVA+UVB from 1600 to 600  $\text{kJ m}^{-2}$  (Fig. 2a). For the remainder of the experimental period, solar conditions were stable again and similar to the initial period.

Despite similar light conditions measured on tank surfaces, the 3 algal species experienced different light

climates within their respective vessels (Fig. 2b–d). Even measurements of irradiance within the surface layer of *Ulva rigida* vessels showed some variation compared to the 2 other species due to *U. rigida* thalli intermittently floating above the Hobo® sensor. Both PAR and UV radiation at the bottom of the *U. rigida* tanks were considerably reduced due to heavy shading by moving thalli (Fig. 2b); by contrast, there was only a small light reduction in *Ellisolandia elongata* tanks, as algal thalli were resting at the bottom of the tanks (Fig. 2d). Regarding *Cystoseira tamariscifolia*, both PAR and UVR were considerably lower at the bottom compared to surface solar radiation (Fig. 2c), and nearly no UV radiation was available at depth due to absorption by exuded phenolic compounds (Figuroa et al. 2014c).

### Temporal variation in pH and temperature in tanks

In the HC treatments (HCLN and HCHN), average pH values were maintained below 8 at most times, with some variability observed during the daytime in all species (Fig. 3a–c), and slightly higher average values for the HCHN treatment for *U. rigida* (Fig. 3a).

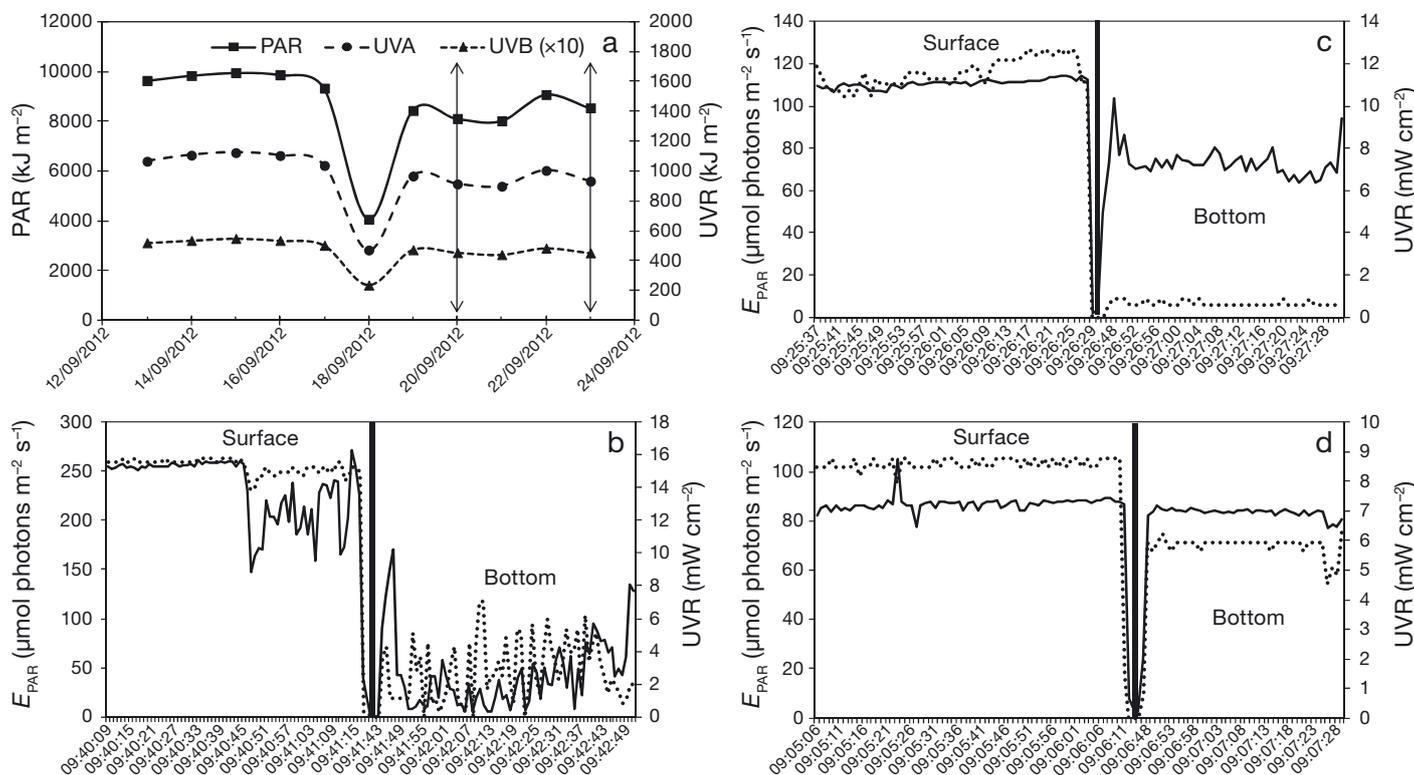


Fig. 2. Light climate: (a) Daily fluence values of photosynthetically active radiation (PAR; 400–700 nm), UVA (320–400 nm) and UVB (280–320 nm) in the air during the experiment; the arrows show the sampling dates. Light climate (relative to surface) at different depths (1 cm below surface and at bottom) within vessels at the beginning of the experiment (19 Sep 2012) with UV radiation (dotted lines) and PAR (solid line) for (b) *Ulva rigida*, (c) *Cystoseira tamariscifolia* and (d) *Ellisolandia elongata*. Note the variability in light climate at both surface and bottom of the *U. rigida* tanks due to floating thalli causing shading. There was little movement of other species within the other tanks

Considerable diurnal variation was observed for all species during daytime (from about 10:00 h GMT onwards), with most drastic increases observed in tanks containing *U. rigida*, where ambient (not controlled) pH ranged between 9 and 9.5 during hours of darkness, and reached up to 10 during daytime (Fig. 3a). In tanks containing *C. tamariscifolia* and *E. elongata*, pH decreased during the night to levels of about 7.7 before increasing again around 09:00 h GMT and reaching values of above 8.7 during periods of photosynthetic activity. In general, daily fluctuations of pH were wider in LC than in HC treatments because pH in HC was buffered by the CO<sub>2</sub> enrichment.

Temperature within the header tanks showed considerable diurnal variation, but, in contrast to pH, the pattern was consistent for all species and treatments (Fig. 4). During the ambient temperature experimental period, nighttime temperatures decreased from about 23 to 19°C until sunrise, and increased to maxima of about 24.5°C during mid-afternoon (17:00 h GMT) in all species and treatments. Lowest temperatures were observed in all tanks around 08:00 h GMT. Following the 4°C temperature increase, nighttime temperatures ranged from 24 to 25.5°C, and increased sharply after sunrise at 08:00 h GMT to reach maxima of 29°C at around 17:00 h GMT. Although all tanks were cooled permanently, heating by ambient daytime air temperatures resulted in a diurnal increase in water temperature in all tanks; temperatures in '+4°C' treatments were higher both during night- and daytime ( $p < 0.00001$ ; 4-way ANOVA) but despite variability did not vary significantly between species ( $p = 0.6945$ ).

Temperatures were similar in all tanks containing the 3 different species and for all carbon and nitrogen treatments (Fig. 4).

### Nitrate uptake

The nitrate provided in the morning was more rapidly taken up by *U. rigida* and *E. elongata* than by *C. tamariscifolia* (Fig. 5). One hour after the addition of nitrate, nearly 50% was taken up by *U. rigida* and 20% by the other 2 species; by evening, seawater in almost all vessels contained nitrate levels close to zero. When nitrate was added, uptake by *C. tamariscifolia* was faster under LC than HC conditions, and HCHN was the only treatment where some nitrate remained in the tanks by evening (Fig. 5b).

### Temporal variation in the effective PSII quantum yield

Results of 2 d of fluorescence measurements are presented in Fig. 6: 1 d at ambient temperature (after 6 d under the different treatments) and 1 d at ambient temperature '+4°C' (after 3 d at the increased

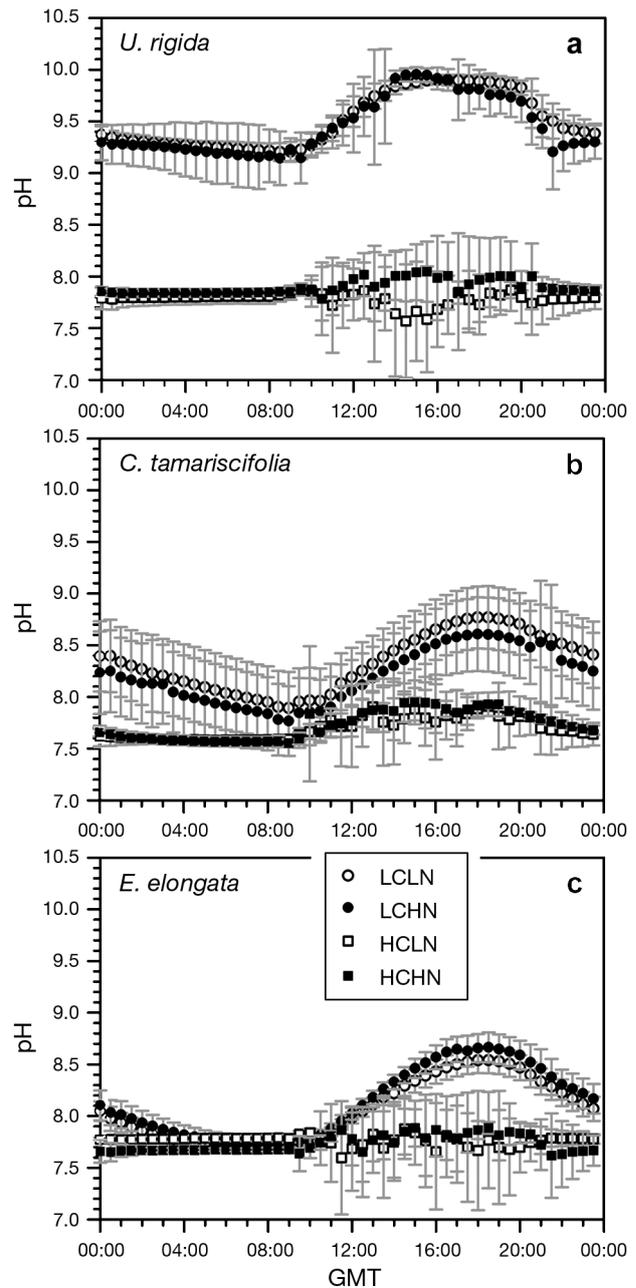


Fig. 3. Daily variation of pH in the different carbon and nutrient treatments (C: carbon; N: nitrogen; L: low; H: high) during day and night periods on (a) *Ulva rigida*, (b) *Cystoseira tamariscifolia* and (c) *Ellisolandia elongata*. Data are means  $\pm$  SD of the values measured during the 4 days of the experiment at different times of day

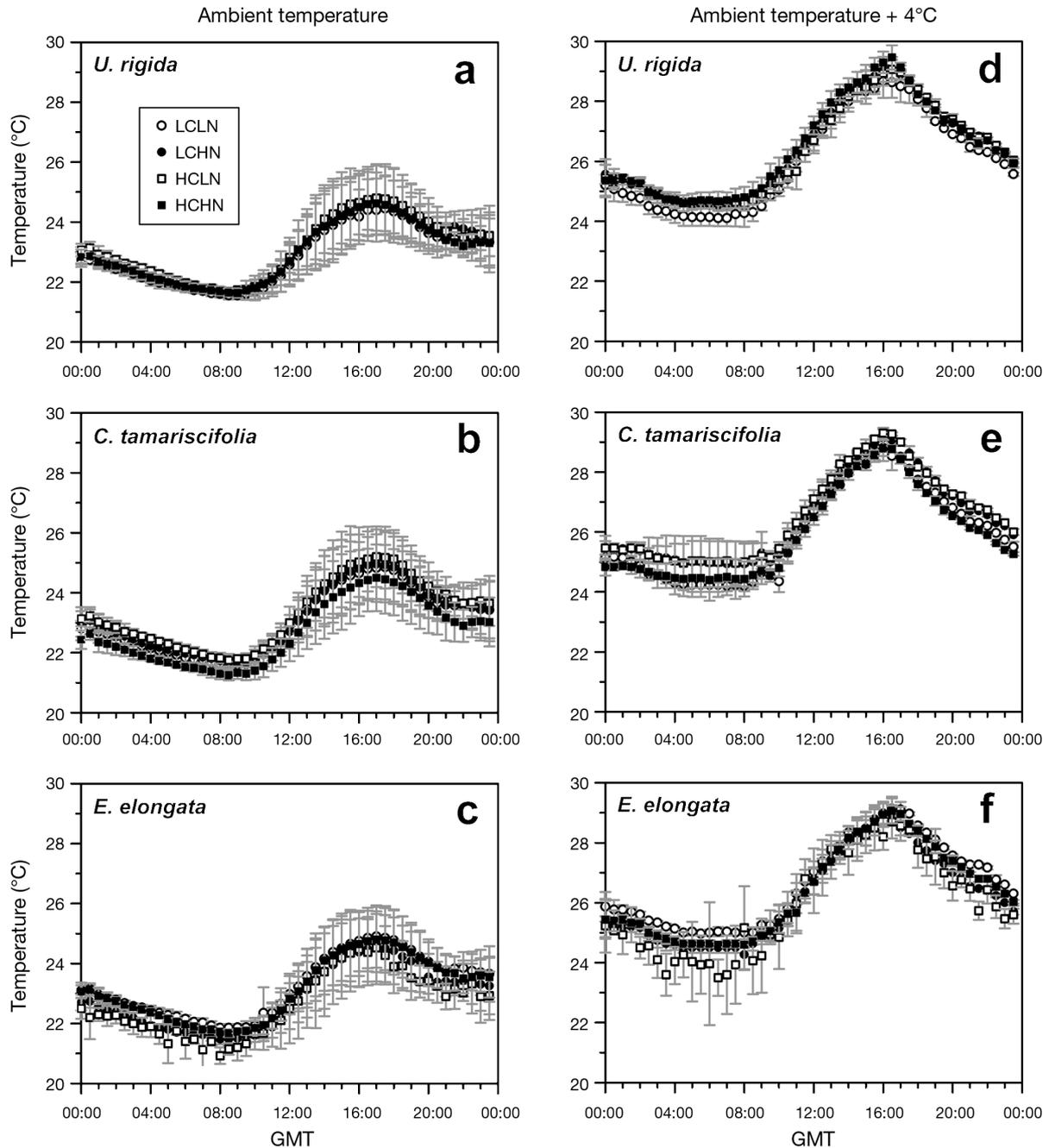


Fig. 4. Daily variation in temperature in tanks during the different carbon and nutrient treatments on (a) *Ulva rigida*, (b) *Cystoseira tamariscifolia* and (c) *Ellisolandia elongata* before increasing temperature (left) and after increasing temperature by 4°C (right); see Fig. 3 for treatment abbreviations. Data are means  $\pm$  SD of values averaged over 4 and 3 d for the first and second part of the experiment, respectively

temperature).  $\Delta F/F_m'$  exhibited a diurnal pattern regardless of treatment and temperature regime in all species, but such variations were generally more pronounced in *U. rigida* than in *C. tamariscifolia* or *E. elongata* (Fig. 6). During the early part of the day,  $\Delta F/F_m'$  decreased with increasing solar radiation (simultaneously measured beside the experimental

tanks underneath the mesh) and represented by  $E_{PAR}$  in air (Fig. 6); the reduction in  $\Delta F/F_m'$  was maximal at noon when the radiation was highest, and this was followed by an increase in  $\Delta F/F_m'$  towards the evening.

In *U. rigida*, the reduction in  $\Delta F/F_m'$  at noon appeared to be greater for LCLN than for the other car-

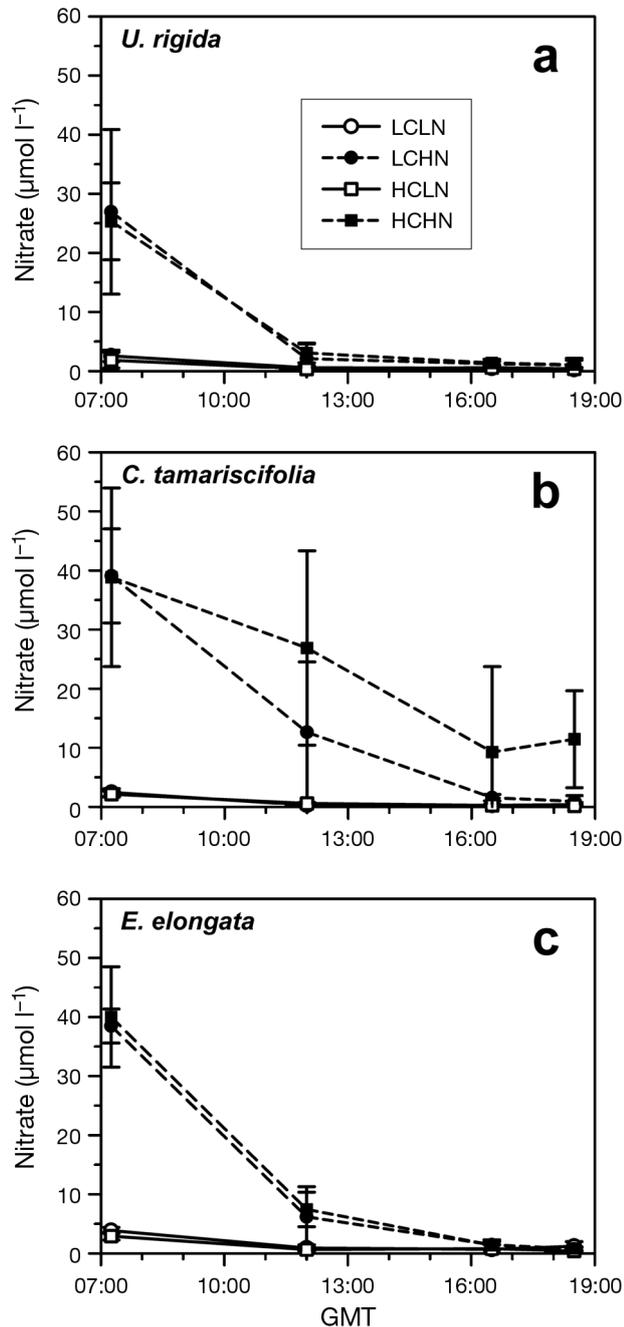


Fig. 5. Daily variation of water nitrate concentration in tanks during the different carbon and nutrient treatments on (a) *Ulva rigida*, (b) *Cystoseira tamariscifolia* and (c) *Ellisolandia elongata*; see Fig. 3 for treatment abbreviations. Data are means  $\pm$  SD of the values measured during the 7 d of the experiment. The water nitrate concentrations of the different treatments were adjusted every morning at 06:00 GMT

bon-nitrogen regimes applied, i.e. *U. rigida* probably benefitted from elevated carbon and nitrogen levels at ambient temperatures (Fig. 6a). At increased temperature a more pronounced reduction in  $\Delta F/F_m'$  at noon was again observed in both LCLN and HCLN,

indicating that the low nitrogen may have caused the observed decrease in  $\Delta F/F_m'$  (Fig. 6d).

In contrast to *U. rigida*, different carbon-nitrogen regimes had only marginal impacts on  $\Delta F/F_m'$  and its diurnal pattern in *C. tamariscifolia* and *E. elongata* at either temperature (Fig. 6b,c,e,f).  $\Delta F/F_m'$  of *E. elongata* appeared to be slightly lower for LCLN than for the other treatments in the '+4°C' temperature regime (Fig. 6f).

### Chl a concentrations

Chl a levels differed considerably between the 3 algal species, with concentrations ranging from 1 to 3 mg g<sup>-1</sup> DW for *C. tamariscifolia*, 0.5 to 1.5 mg g<sup>-1</sup> DW for *U. rigida*, and 0.1 to 0.3 mg g<sup>-1</sup> DW for *E. elongata* (Fig. 7). After the increase in temperature, there was no consistent diurnal pattern, and lower chl a content was measured in the morning in *C. tamariscifolia* but higher levels occurred in *E. elongata* (Table 1). Nitrogen and/or carbon significantly impacted the different algal species: higher chl a content was observed under higher nitrogen regimes in *U. rigida* at ambient temperature and in *C. tamariscifolia* at both temperatures. The HC treatment resulted in lower chl a levels in *U. rigida* and *E. elongata* at both temperatures, and the interaction time of day  $\times$  carbon was significant for *U. rigida* at both temperatures (Table 1).

### Accessory pigment concentrations

Chl b content of *U. rigida* ranged from 0.4 to 1.0 mg g<sup>-1</sup> DW (Fig. 8a). At ambient temperature, carbon and nitrogen had significant impacts on chl b content with higher levels observed in LC than in HC, and in HN than in LN treatments (Table 2). *U. rigida* grown in the LCHN treatment had the highest chl b level; also, the interactions carbon  $\times$  nitrogen and time of day  $\times$  nitrogen were significant. At increased temperature, only effects of carbon and the interaction time of day  $\times$  carbon were significant.

In *C. tamariscifolia*, 0.2 to 0.6 mg g<sup>-1</sup> DW of chl c were found, with no variation amongst carbon and nitrogen treatments at ambient or increased temperature (Fig. 8b; Table 2). At ambient temperature, a slight, but not significant, increase in chl c occurred; following the temperature increase, chl c values at noon were significantly higher than those measured in the morning or evening.

*E. elongata* contained between 1.5 and 3.0 mg phycoerythrin g<sup>-1</sup> DW and between 0.30 and 0.65 mg

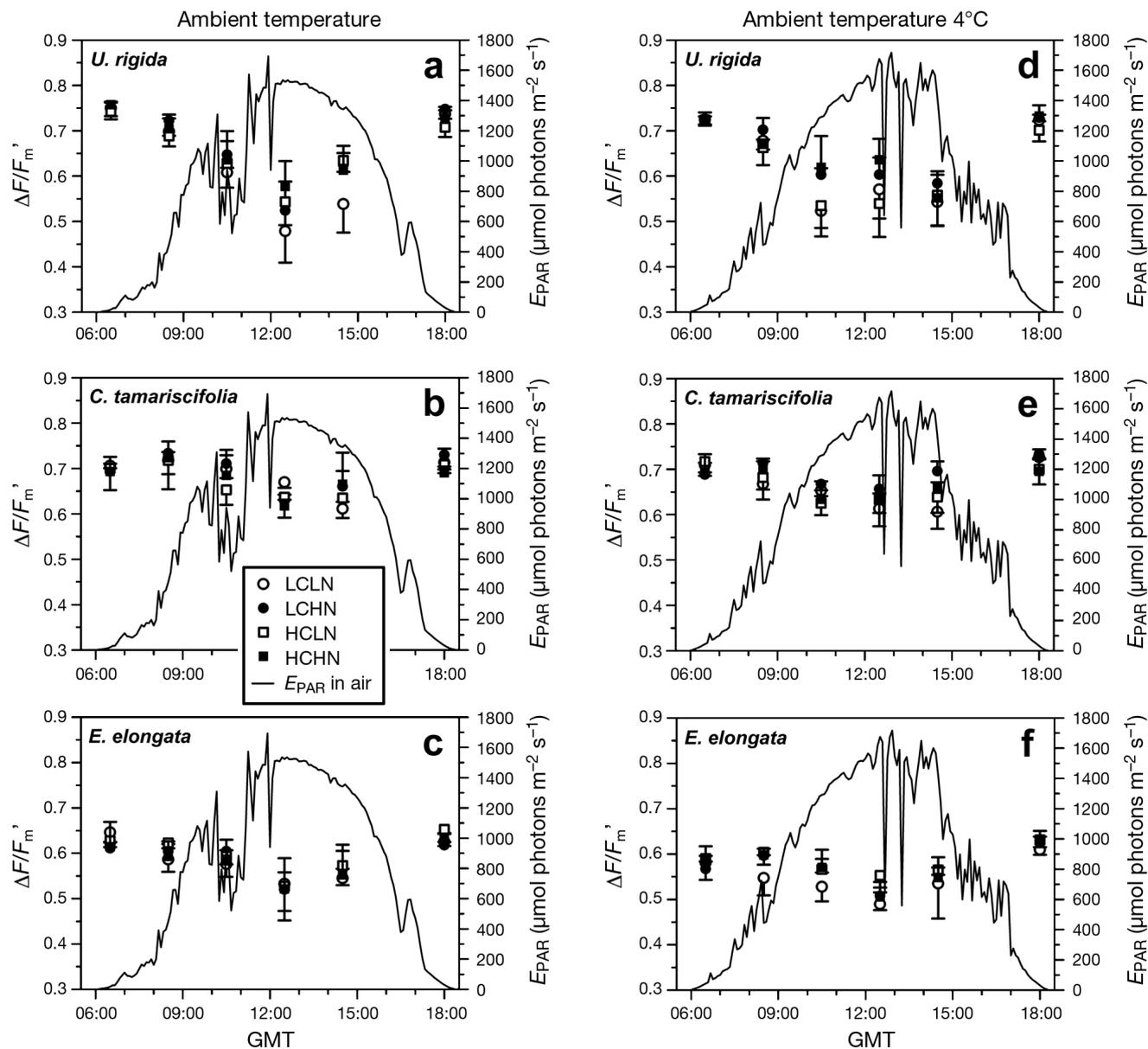


Fig. 6. Daily evolution of the effective PSII quantum yield ( $\Delta F/F_m'$ ) in 3 macroalgae exposed to different carbon and nitrogen regimes and reduced solar radiation at an ambient (a–c) and 4°C-increased ambient temperature (d–f). *Ulva rigida* (a,d), *Cystoseira tamariscifolia* (b,e) and *Ellisolandia elongata* (c,f) were subjected to either low or high carbon (LC, HC respectively) at low and high nitrogen (LN, HN respectively) levels.  $\Delta F/F_m'$  was determined on algae in tanks; data are means  $\pm$  SD (–SD for LN, +SD for HN; n = 3).  $E_{\text{PAR}}$  was measured in air immediately beside the experimental tanks using a spherical quantum sensor connected to a data logger

phycocyanin  $\text{g}^{-1}$  DW (Fig. 8c,d). Neither carbon nor nitrogen treatments, nor the increase in temperature had an impact on phycobiliprotein contents (Table 2). Phycoerythrin contents were slightly, but not significantly, higher in the evening at ambient temperature. On the other hand, time of day affected phycocyanin levels of *E. elongata* at ambient temperature, with the highest levels observed in the evening, and similar levels in the morning and noon samples.

## DISCUSSION

### Experimental setup

This paper describes the conditions in the experimental tanks used during the GAP workshop at the University of Málaga in September 2012, and the short-term photosynthetic responses of *Ulva rigida*, *Cystoseira tamariscifolia* and *Ellisolandia elongata*

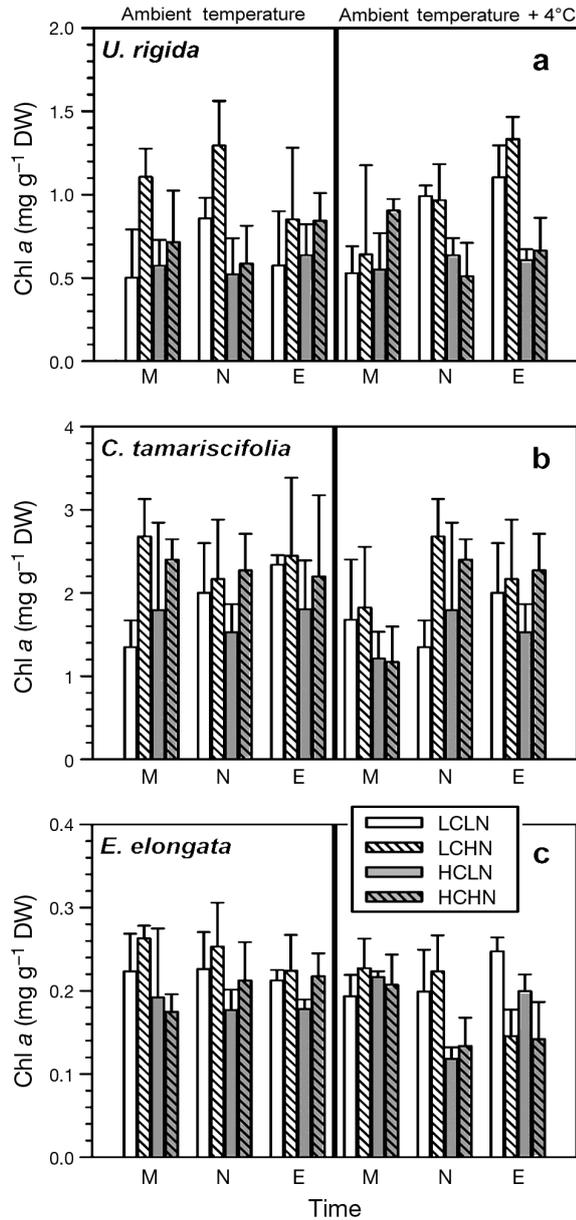


Fig. 7. Morning (M), noon (N) and evening (E) chl a contents in 3 macroalgae exposed to different carbon and nitrogen regimes and natural solar radiation at ambient (left-hand side) and 4°C-increased ambient temperature (right-hand side). *Ulva rigida* (a), *Cystoseira tamariscifolia* (b) and *Ellisolandia elongata* (c) were subjected to either low or high carbon (LC, HC respectively) at low and high nitrogen levels (LN, HN respectively). Data are means + SD

measured under ambient conditions within tanks. Results of more detailed measurements of physiological parameters are reported in Figueroa et al. (2014b,c). Thus, we emphasize the description of physico-chemical conditions within experimental tanks which are critical to the interpretation of data presented in subsequent publications.

The experimental set-up exposed 3 macroalgal species to 4 combinations of nitrogen and carbon treatments in replicated outdoor tanks; natural solar irradiance was reduced by about 35 and 39% ( $E_{PAR}$  and UVR, respectively). Temperature and pH (by CO<sub>2</sub>-enrichments) in tanks were automatically adjusted and resulted in 4 distinct nutrient and carbon conditions for each species. Additionally, pH exhibited considerable diurnal variability in the uncontrolled LC treatments, probably caused by a high metabolic activity, especially in the tanks containing *U. rigida*. This is consistent with previous studies on *Ulva* (formerly *Enteromorpha*) *intestinalis* from rock-pools where photosynthetic activities significantly increase pH in small volumes of water (pH > 9.4, Larsson et al. 1997), though no negative impacts on photosynthesis were observed here. Previous studies have reported an insensitivity of *Ulva* spp. to high pH due to its capacity to utilize bicarbonate (Axelsson et al. 1995, Frost-Christensen & Sand-Jensen 1990). Averaged pH in LC and HC in *U. rigida* tanks differed considerably throughout the duration of the experiment, whereas for *C. tamariscifolia* and *E. elongata* the differences were less pronounced. The observed species-specific differences in pH profiles for the different treatments suggest that interpretation of physiological responses should be restricted to a comparison of responses to HC/LC treatments within species, and be interpreted only with caution to explain differences amongst species. On the other hand, daily fluctuations in temperatures in tanks were more consistent for all species (and treatments); the observed diurnal increases were consistently impacting all species and treatments, and the required increase in temperature by 4°C was successfully achieved, with only small, negligible variability between species and treatments. Any changes in physiological parameters observed in response to increases in temperature should thus be comparable amongst species.

### Light climate

Spectral irradiances measured at the University of Málaga campus (3.7 km from the experimental site) were representative of natural conditions before the 35% reduction in solar irradiance caused by the mesh; some small shading effects were likely to occur due to different sun angles, but it can probably be assumed that these were, on average, similar for all tanks throughout a daily cycle and thus did not have any impacts on particular treatments, species or

Table 1. Statistical summary of the effects of time and carbon-nitrogen treatment on chl a content in 3 different macroalgae (*Ulva rigida*, *Cystoseira tamariscifolia* and *Ellisolandia elongata*) kept at ambient or +4°C temperature. Main effects of time of day (TD; M: morning, N: noon, E: evening), carbon regime (CR; LC: low carbon, HC: high carbon), and nitrogen regime (NR; LN: low nitrogen, HN: high nitrogen) as well as interactions of each of these fixed factors were computed for each species by a 3-way ANOVA; **bold** = significant p-values ( $\alpha = 0.05$  was used if variances were homogenous; if they were heterogeneous, a more conservative  $\alpha = 0.01$  was employed, indicated by *italic* font). Where a significant difference was found by ANOVA, means were compared using the least significant difference (LSD) procedur

Chl a		Ambient temperature				Ambient temperature + 4°C			
		df	F	p	LSD	df	F	p	LSD
<i>U. rigida</i>	TD	2,24	0.504	0.610		2,24	4.684	<i>0.019</i>	
	CR	1,24	6.639	<b>0.017</b>	HC < LC	1,24	15.174	<b>0.001</b>	HC < LC
	NR	1,24	11.673	<b>0.002</b>	LN < HN	1,24	1.930	0.178	
	TD × CR	2,24	3.646	<b>0.041</b>		2,24	9.103	<b>0.001</b>	
	TD × NR	2,24	0.251	0.780		2,24	1.599	0.223	
	CR × NR	1,24	3.184	0.087		1,24	0.004	0.949	
	TD × CR × NR	2,24	0.498	0.614		2,24	0.774	0.472	
<i>C. tamariscifolia</i>	TD	2,24	0.329	0.723		2,24	3.719	<b>0.039</b>	M < N = E
	CR	1,24	0.600	0.446		1,24	1.316	0.263	
	NR	1,24	6.975	<b>0.014</b>	LN < HN	1,24	6.571	<b>0.017</b>	LN < HN
	TD × CR	2,24	0.424	0.659		2,24	0.950	0.401	
	TD × NR	2,24	1.018	0.377		2,24	1.905	0.171	
	CR × NR	1,24	0.012	0.912		1,24	0.083	0.776	
	TD × CR × NR	2,24	0.871	0.432		2,24	0.969	0.394	
<i>E. elongata</i>	TD	2,24	0.150	0.862		2,24	5.251	<b>0.013</b>	M > N = E
	CR	1,24	9.395	<b>0.005</b>	HC < LC	1,24	11.300	<b>0.003</b>	HC < LC
	NR	1,24	2.771	0.109		1,24	2.147	0.156	
	TD × CR	2,24	0.718	0.498		2,24	5.558	<b>0.010</b>	
	TD × NR	2,24	0.193	0.826		2,24	8.679	<b>0.001</b>	
	CR × NR	1,24	0.066	0.799		1,24	0.014	0.906	
	TD × CR × NR	2,24	0.888	0.425		2,24	1.365	0.275	

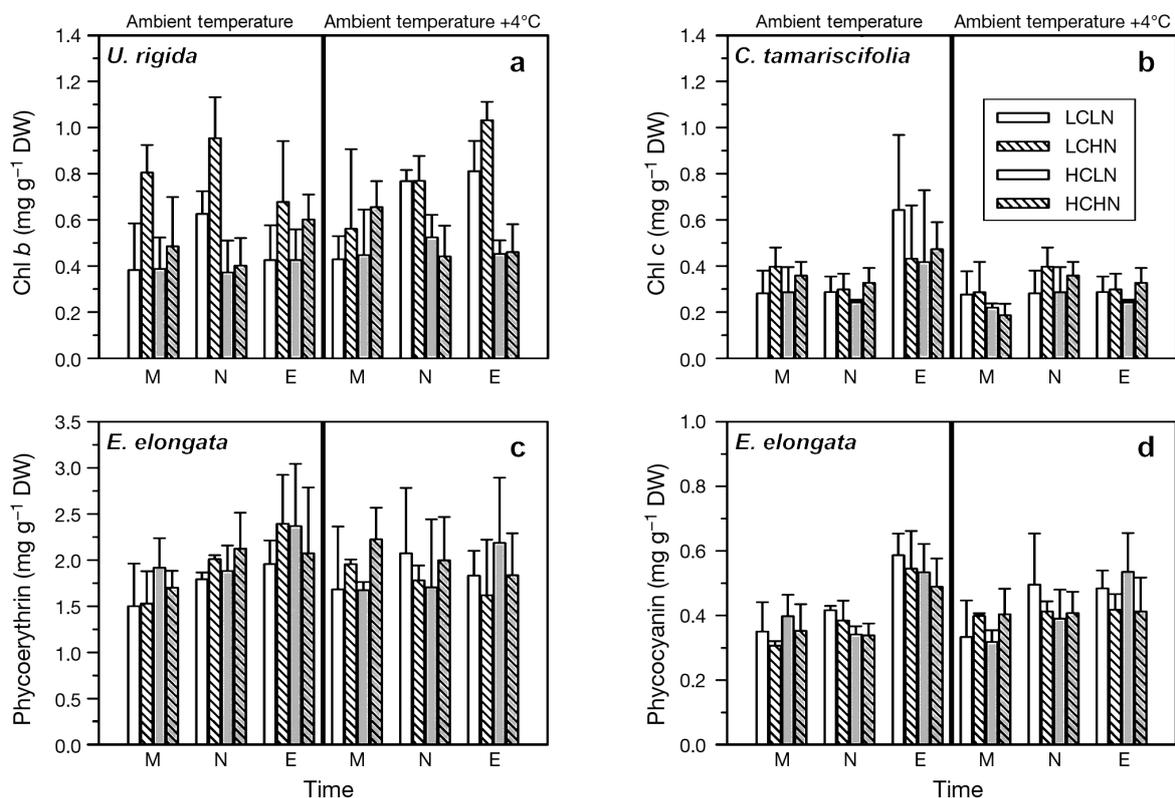


Fig. 8. As in Fig. 7 but for accessory pigments: (a) chl b in *Ulva rigida*, (b) chl c in *Cystoseira tamariscifolia*, (c) phycoerythrin and (d) phycocyanin in *Ellisolandia elongata*

Table 2. Statistical summary of the effects of time and carbon-nitrogen treatment on accessory pigments (chl *b* in *Ulva rigida*, chl *c* in *Cystoseira tamariscifolia*, and phycobiliproteins in *Ellisolandia elongata*) kept at ambient or +4°C temperature. Main effects of time of day (TD; M: morning, N: noon, E: evening), carbon regime (CR; LC: low carbon, HC: high carbon), and nitrogen regime (NR; LN: low nitrogen, HN: high nitrogen) as well as interactions of each of these fixed factors were computed for each species by a 3-way ANOVA; bold = significant p-values ( $\alpha = 0.05$  was used if variances were homogenous; if they were heterogeneous, a more conservative  $\alpha = 0.01$  was employed, indicated by *italic* font). Where a significant difference was found by ANOVA, means were compared using the least significant difference (LSD) procedure

Accessory pigments		Ambient temperature				Ambient temperature + 4°C			
		df	<i>F</i>	<i>p</i>	LSD	df	<i>F</i>	<i>p</i>	LSD
<i>U. rigida</i> : chl <i>b</i>	TD	2	0.667	0.523		2	3.824	<i>0.036</i>	
	CR	1	13.659	<b>0.001</b>	LC > HC	1	21.904	<b>&lt;0.001</b>	LC > HC
	NR	1	16.234	<b>&lt;0.001</b>	LN < HN	1	2.725	0.112	
	TD × CR	2	3.944	<b>0.033</b>		2	9.563	<b>0.001</b>	
	TD × NR	2	0.190	0.828		2	1.634	0.216	
	CR × NR	1	4.671	<b>0.041</b>		1	0.564	0.460	
	TD × CR × NR	2	0.538	0.591		2	0.716	0.499	
<i>C. tamariscifolia</i> : chl <i>c</i>	TD	2	5.188	<i>0.013</i>		2	3.742	<b>0.038</b>	N > M = E
	CR	1	0.529	0.474		1	1.687	0.206	
	NR	1	0.160	0.693		1	2.697	0.114	
	TD × CR	2	0.243	0.786		2	0.677	0.518	
	TD × NR	2	0.900	0.420		2	1.330	0.283	
	CR × NR	1	0.840	0.369		1	0.007	0.934	
	TD × CR × NR	2	0.705	0.504		2	0.537	0.591	
<i>E. elongata</i> : phycoerythrin	TD	2	5.141	<i>0.014</i>		2	0.005	0.995	
	CR	1	1.157	0.293		1	0.457	0.506	
	NR	1	0.256	0.618		1	0.068	0.796	
	TD × CR	2	0.305	0.740		2	0.391	0.681	
	TD × NR	2	0.465	0.634		2	1.448	0.255	
	CR × NR	1	1.351	0.257		1	0.530	0.474	
	TD × CR × NR	2	0.652	0.530		2	0.394	0.678	
<i>E. elongata</i> : phycocyanin	TD	2	25.856	<b>&lt;0.001</b>	M = N < E	2	4.002	<i>0.032</i>	
	CR	1	0.955	0.338		1	0.177	0.677	
	NR	1	2.238	0.148		1	0.363	0.553	
	TD × CR	2	2.220	0.130		2	0.627	0.543	
	TD × NR	2	0.140	0.870		2	2.963	0.071	
	CR × NR	1	0.029	0.866		1	0.141	0.710	
	TD × CR × NR	2	0.051	0.950		2	0.617	0.548	

replicate. In addition to the expected diurnal natural variation in light climate, daily radiation differed between experimental days due to partial cloud cover, but again differences were similar for all species and treatments. Changes in natural irradiance may potentially have impacted on observed differences between measurements taken on different days which also coincided with the increase in temperature by 4°C; on the other hand, most significant changes in light climate, expectedly, occurred over the daily cycle and caused the diurnal variation in e.g. photosynthetic parameters such as  $\Delta F/F_m'$ .

#### Light climate within vessels

Whilst every attempt was made to provide different species and vessels with the same light climate,

species-specific effects were observed which may have resulted in different micro-climates within vessels, and these were likely to be similar for all treatments within species. Thus differences in physiological parameters measured were most likely due to different treatments, but a comparison between such effects in different species needs to be interpreted in view of the different light climates experienced by different species within vessels. The resultant light micro-climates were caused by the different morphologies of the different species, e.g. severe shading was observed within *U. rigida* vessels due to movement of thalli caused by water motion, which produced efficient mixing of algal fronds which were then used for the assessment of physiological parameters. In *U. rigida* vessels, the light climate fluctuated greatly and may also have resulted in light flecks (Kübler & Raven 1996). Sam-

ples of *U. rigida* would thus have experienced a mixed and rapidly changing light climate, but it is also possible that some pieces were based at the bottom of the tank for extended periods and thus became shade-acclimated. For physiological analyses, samples were consistently taken from the surface, but it is possible that individual algae had only just reached the surface from lower layers following water movement within the vessels that allowed mixing within tanks. By contrast, due to their weight, all *C. tamariscifolia* and *E. elongata* specimens were permanently placed at the bottom of the vessels, even though the same vigorous water movement was applied, and it is likely that these 2 species experienced a more constant light climate. In the case of *C. tamariscifolia*, water colour changed over time, which explained the differences in light levels between surface and bottom measurements; most likely this change in water colour and light penetration was related to the exudation of phenolics released by *C. tamariscifolia* (Figueroa et al. 2014c); such exudation of screening compounds has already been observed under high light for the brown macroalga *Macrocystis integrifolia* (Swanson & Druehl 2002). In the confined environment of the experimental vessels, this exudation resulted in reduced light, particularly UV, availability. It is also likely that samples removed from *C. tamariscifolia* vessels on different days had experienced slightly different light climates due to the summative release (and subsequent reduction/change in light) over time. By contrast, the removal of biomass over time (for different measurements and analyses) may have reduced the degree of shading and thus increased the availability of light within *U. rigida* vessels.

The most consistent light climate was probably experienced by *E. elongata*, with only small differences measured between surface and bottom light within tanks, and again the use of samples from the surface was always attempted, although some movement of samples may have occurred that caused some variability between replicate samples.

### Nitrogen uptake

Despite spiking all HN tanks equally every morning, resultant N levels in tanks varied between species, although in each case nitrogen-enrichment (HN) resulted in concentrations about 4 times those of unenriched (LN) treatments. This suggests that significantly different N-regimes were achieved. As

might be expected due to its high surface-to-volume ratio, *U. rigida* rapidly took up nitrogen provided to HN treatments within about 5 h, with concentrations measured during mid- and late afternoon reaching those of unenriched vessels. An assessment of possible effects attributed to N-enrichment (or the lack thereof) should consider that elevated N conditions were still present in *C. tamariscifolia* vessels, whereas in the case of *U. rigida* and *E. elongata*, such effects may be caused by nutrients previously absorbed and thus by associated internal N-storage; it is likely that N-depletion occurred from noon onwards. With the exception of N levels in *C. tamariscifolia* exhibiting some variability, there were no differences between HCHN and LCHN treatments.

The conditions provided in the tanks therefore represented realistic nutrient regimes experienced by algae in their natural habitats on the southern coast of Spain, where nutrient pulsing is a well-documented phenomenon (e.g. Ramírez et al. 2005).

### Pigments

Nitrogen enrichment is likely to affect pigment contents including chlorophyll (Figueroa et al. 2009), protein (Ribeiro et al. 2013) and MAA (Figueroa et al. 2010) concentrations. Previous studies have shown a direct relationship between N-uptake and chl *a* and *b* levels in *Ulva* sp. (Altamirano et al. 2000) and chl *a* in *Gracilaria conferta* (Figueroa et al. 2010). Indeed, in our study, all specimens of *U. rigida* grown under HN had higher chl *a* and chl *b* concentrations. The relationship between seawater N concentrations and pigment contents in brown algae is less well documented, although under controlled conditions, pigment levels increased e.g. in *Ascophyllum nodosum* (Stengel & Dring 1998) and several kelp species (Korb & Gerard 2000); also several observations from field populations report on pigment changes in response to *in situ* nutrient levels (Henley & Dunton 1995). In line with its coarsely branched morphology and thus lower surface-to-volume ratio compared to the other species, N uptake by *C. tamariscifolia* (as indicated by disappearance from the media) was less pronounced than that by the other species, although both LCHN and HCHN treatments resulted in elevated chl *a* levels.

Chl *a* levels generally exhibited some variability. The most striking effects were observed in response to N-enrichment and these were most pronounced in *U. rigida* under LC conditions before the temperature rise, but relatively consistently higher levels also oc-

curred in HN treatments of *C. tamariscifolia*. Differences observed in *E. elongata* were more likely due to HC treatments with no consistent trends observed for HN impacts, even though N was taken up. At elevated temperatures, observed changes in chl *a* levels were caused by interactions between light, nutrients and carbon treatments. In *U. rigida* and *E. elongata* in particular, HC appeared to reduce chl *a* concentrations.

In this study, phycobiliprotein (i.e. phycoerythrin and phycocyanin) contents of *E. elongata* were not influenced by carbon and nitrogen treatment, contrary to previous studies: in *Gracilaria* spp. phycoerythrin increased with seawater N concentration and internal N content (Rosenberg & Ramus 1982, Figueroa et al. 2010). On the other hand, Gao & Zheng (2010) observed a decrease in the phycoerythrin content of *Corallina sessilis* when exposed to high CO<sub>2</sub> (1000 ppmv) for 30 d. The short experimental duration of the present study may explain the lack of change in phycobiliprotein levels. However, some diurnal variation was observed, with the highest phycocyanin levels measured in *E. elongata* in the evening.

To assist with the interpretation of the impacts of the different treatments described here, further biochemical analyses were conducted (e.g. soluble proteins, fatty acids, MAAs, phenolics; Figueroa et al. 2014c).

### Effective PSII quantum yield

In all species, considerable diurnal variation was observed on the 2 experimental days, with lower yields during periods of high solar radiation in the afternoon; greatest impacts on yield under different carbon and nitrogen treatments were observed for *U. rigida* where LN caused a slow recovery at ambient temperature and a fast decline under the 'ambient +4°C' treatment. Recovery of yields occurred in all species and under both temperature regimes, but, typically, yields of *U. rigida* were higher than those of *C. tamariscifolia* and *E. elongata*, corresponding to values previously reported for these species and other Chlorophyta, Phaeophyceae and Rhodophyta (e.g. Büchel & Wilhelm 1993).

In *U. rigida*, yields decreased more rapidly at elevated temperatures but on this day, natural solar radiation was also higher. Under these more extreme conditions, recovery under LN conditions was slower and suggests a N requirement for photoprotective mechanisms in *U. rigida* (Henley et al. 1991).

### Conclusions

The established multi-tank system allowed the successful conditioning of 3 macroalgal species under semi-natural light conditions and modified carbon, nitrogen and temperature regimes. Different micro-scale light environments were observed within tanks which potentially influenced physiological responses, but most of the significant physiological changes occurred in response to diurnal variation in light climate. However, responses were species-specific and may have further been affected by *in vitro* experimental conditions (self-shading, release of phenolics). Although water temperatures were highly variable diurnally, the experimental approach succeeded in increasing the average temperature by 4°C; however this was accompanied by a larger temperature variation within tanks. This short-term temperature increase did not appear to impact the ability of any species to recover to morning levels in any of the treatments. The most significant impacts of the carbon and nitrogen treatments were observed in *U. rigida*, probably due to its sheet-like morphological structure, high N dependency and generally high metabolic rates; the observed physiological responses were impacted by interactions of the environmental factors carbon and nitrogen, whereas temperature effects were less pronounced for all species. Physiological responses of *U. rigida* appeared to be most sensitive to rapid changes in irradiances and both carbon and nutrient treatment, whilst *C. tamariscifolia* was impacted the least by any environmental factor.

The 3 species grow in close proximity on exposed rocky outcrops in the low intertidal in southern Spain; however, the differences in their physiological responses were likely due to a combination of morphological structures (e.g. shrubby nature of *C. tamariscifolia*), low productivity of the calcified *E. elongata* and photoprotective mechanisms in *C. tamariscifolia* and *E. elongata*. In their natural habitat all species occur predominantly in dense clumps, with extreme self-shading providing at least partial protection from certain stressors, including solar radiation and desiccation, at least for *E. elongata*.

Analysis of further biochemical parameters and more detailed PAM fluorescence measurements conducted under controlled conditions are presented in other papers (Figueroa et al. 2014b,c) and will help further elucidate the protective mechanisms employed by the 3 species. Further, longer-term studies should

be conducted which incorporate different temporal scales and assess the impacts at multiple, physiological, biochemical and genetic levels.

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