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Photosynthetic performance and photoprotection of *Cystoseira humilis* (Phaeophyceae) and *Digenea simplex* (Rhodophyceae) in an intertidal rock pool

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ABSTRACT

Rock pools are dynamic and intermittently isolated habitats in the rocky intertidal. In this study, we assessed if the photosynthesis and physiological activity of the brown macroalga *Cystoseira humilis* and the red macroalga *Digenea simplex* in a rock pool at Lanzarote Island (eastern Atlantic) was affected due by their vertical distribution. Photosynthetic responses were measured at three depth levels (0.05–1 m, 0.4–2.5 and 3.5 m) through in vivo chlorophyll a fluorescence, in particular the maximal quantum yield (F_v/F_m) as an estimator of the physiological status and photoinhibition, and the electron transport rate (ETR) as an estimator of the photosynthetic capacity. Algal photoprotection and photodamage processes were related to algal zonation; shallow-water thalli had active mechanisms of dynamic photoinhibition compared to algae from the deeper level. The progressive increase of solar radiation during the day caused different responses for each macroalga, where *C. humilis* showed lower photoinhibition, higher ETR values than *D. simplex*. Algae from the shallow level had lower pigment content and higher resistance to high solar radiation through the accumulation of photoprotective compounds and higher antioxidant activity (DPPH) compared to thalli from the deeper level. In summary, this study corroborates that algae are vertically distributed inside rock pools according to their adaptive responses to light-induced stress conditions and that photoacclimation occur in a short-term period.

1. Introduction

The intertidal is a harsh environment routinely subjected to extreme conditions that influence the colonization of species and their subsequent spatial distribution across the horizontal and vertical axis (Benedetti-Cecchi et al., 2000). In a few meters along the vertical axis, environmental conditions such as temperature, salinity, wave action and irradiation vary from a completely aquatic medium to a completely terrestrial environment, generating steep gradients in a range of ecological processes (Harley and Helmuth, 2003). Physical factors interact with biological factors to determine the distribution of species (Benedetti-Cecchi et al., 2000; Martins et al., 2007); zonation patterns typically reflect different vertical patterns of species' adaptation (Davison and Pearson, 1996). In the intertidal, rock pools are singular microcosms of varying ecological structure and composition, as a result of differences in their size, depth and position in the intertidal, what affects, for example, patterns in temperature, pH and salinity fluctuations (Martins et al., 2007). In general, the size of pools and the water renovation rate largely determine the community structure inside pools, where deeper pools and those situated at exposed areas often contain a greater biodiversity (Martins et al., 2007). Because of these peculiarities, rock pools are adequate systems to study the adaptation of macroalgae to fluctuating environmental stress (Davison and Pearson, 1996).

Macroalgal zonation patterns are often related to their ability to resist high radiation stress (Hanelt, 1998), where upper-shore species are more resistant to elevated solar UVR (Bischof et al., 1998). In aquatic ecosystems, the increase in UVB by ozone depletion, altogether with a high exposure to UVA according to the latitude and altitude, has been related to the damage over DNA, RNA, proteins and lipids in a variety of aquatic organisms (Bischof et al., 1998; Roleda et al., 2004). Algal species may present different sensitivity to UVB according to their morphology and life cycle

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(Roleda et al., 2004, 2006), and it is related to the action of photoprotection mechanisms (Mitchell and Karentz, 1993), accumulation of lipidic and water-soluble antioxidants, and the activation of antioxidant enzymes (Cockell and Knowland, 1999), as well as the accumulation of UV-screen photoprotectors as mycosporine-like amino acids (MAAs) in red macroalgae (Korbee-Peinado et al., 2004) and phenolic compounds in brown algae (Abdala-Díaz et al., 2006).

Plant facilitation through the alleviation of physical stress is an important driver of community structure across many ecosystem types, particularly the intertidal (Harley and Helmuth, 2003). Zonation of macroalgae in the intertidal can be, from a physiological perspective, characterized through light absorption, pigment contents, photosynthetic parameters and photoinhibition (Gómez and Huovinen, 2011). Most comparative investigations on photoinhibition in relation to preadaptation status (e.g. depth) have been carried out on algae of different species (Hanelt et al., 1992); only a few studies, however, selected the same species across different depths (Sagert et al., 1997; Bischof et al., 1998; Borum et al., 2002; Hanelt and Roleda, 2009). Additionally, the potential for acclimation and recovery at different stress conditions is an important pre-requisite for the recruitment and ecological success of algae growing in the intertidal (Roleda et al., 2006).

In the intertidal of the Canary Islands (eastern Atlantic), intertidal rock pools support stands of the brown macroalga *Cystoseira humilis* (Fucales, Phaeophyceae) and the red macroalga *Digenea simplex* (Ceramiales, Rhodophyceae). Normally, these algae cover the walls of rock pools, from the surface to −4 m of depth. In this study, we took advantage of the presence of these algae inside a large rock pool to in situ evaluate the physiological status and adaptive responses of both *C. humilis* and *D. simplex* with depth. We hypothesized that shallow-water algae have photoprotective mechanisms more efficient relative to deep-water algae.

2. Material and methods

2.1. Description of the species

C. humilis Kützing (Fucales, Phaeophyceae) and *D. simplex* (Wulfen) C. Agardh (Ceramiales, Rhodophyceae) are algal species that grow in the intertidal rocky shore of Canary Islands (Espino et al., 2006). Across its distribution range, *C. humilis* is often found covering the bottom and walls of protected and semi-protected rock pools; their thallus may reach about 15 cm in total length (Haroun et al., 2003). *D. simplex* is found on intertidal rocky platforms and often inside rock pools; this alga has a cartilaginous erect thallus, typically thicker than *C. humilis*, with a total length up to 10 cm (Haroun et al., 2003).

2.2. Study area and experimental design

This study was carried out during September 2010 in an intertidal rock pool situated at Lanzarote Island (28°54′52.1″N; 13°50′50.4″W) (Fig. A1). The pool has a surface area of approximately 60 m^2 and a maximum depth of 5 m, and remains isolated during low tides (Fig. A1). Thalli of both macroalgal species (*C. humilis* and *D. simplex*) were located at different depths in the rock pool (Fig. A1). We selected 3 depths for each seaweed; in the case of *C. humilis*: −0.05, −0.4 and −3.5 m, and for *D. simplex*: −1, −2.5 and −3.5 m, corresponding to a shallow, middle and deep level. In the morning (11 h) and the afternoon (13 h), several environmental parameters and algal collections took place according to the following routines. In all cases, we selected the apical part of thalli of different specimens of each species for each specific measurement.

2.3. Light and temperature measurements

Underwater (0.01 to 5 m depth) and air photosynthetic active radiation (PAR) expressed as μmol photons m^{-2} s^{-1} were measured by a PAR spherical quantum sensor (Li 193 SB) connected to a radiometer (Li-1000). The irradiance of ultraviolet-A (UVA, λ = 320–400 nm) and ultraviolet B (UVB, λ = 280–320 nm) radiation, expressed as W m^{-2}, were determined through a multidiode spectroradiometer (Ramses ACC-UV, TrioS GmbH).

The vertical attenuation coefficient of the downward radiation (K_d) was calculated, in the PAR region and in the UVA and UVB bands, by linear regression between the surface irradiance (0.1 m depth) and the irradiance at the different depths, according to the following equation:

$$K_d = \frac{(\ln E_0 - \ln E_z)}{z}$$

where E_0 is the irradiance at the surface (0.1 m depth) and E_z the irradiance at the depth. K_d was determined at 11:00 and 12:30 and the average and standard deviation calculated.

The temperature was measured continuously every 5 min through submersed thermometers (Hobos U22, Onset computer corporation) at each depth.

2.4. Photosynthetic activity as in vivo chlorophyll fluorescence

In vivo chlorophyll *a* fluorescence of photosystem II (PSII) was assessed through a portable pulse amplitude modulation fluorometer (Diving-PAM, Waltz). On each depth, thalli of both seaweeds were collected at 11:00 and 13:00 h (Betancor et al., 2014). After 15 min of dark adaptation, the minimum (basal) fluorescence was measured (F_o) and the maximum fluorescence (F_m) obtained immediately after applying a saturated pulse of actinic light (>4000 μmol photons m^{-2} s^{-1}, 0.8 s); the maximal quantum yield was therefore calculated as: $F_v/F_m = F_m - F_o/F_m$ ($n = 8$), which is an indicator of physiological stress (Maxwell and Johnson, 2000). For seaweeds directly exposed to solar light, the minimum and maximum fluorescence were similarly calculated (F and F'_m, respectively) after applying a saturated pulse of actinic light; the effective quantum yield was then calculated as: $\Delta F/F'_m = (F'_m - F)/F'_m (n = 8)$. After 15 min of dark adaptation, a rapid light curve (RLC) ($n = 3$) was initiated, involving 20 s of exposure to 9 successive irradiances, from 85 to 1748 μmol photons m^{-2} s^{-1}. RLCs were then obtained by calculating the electron transport rate (ETR) through the PSII for each level of actinic light:

$$\mathrm{ETR}\left(\mu\mathrm{mol\ \ electrons\ \ m^{-2}\ \ s^{-1}}\right) = \left(\Delta F/F'_m\right) \times E \times A \times \mathrm{FII}$$

where '*E*' is the irradiance, '*A*' is the absorptance of each seaweed (0.85 ± 0.02 for *C. humilis* and 0.72 ± 0.05 for *D. simplex* (Figueroa et al., 2009), and 'FII' is the fraction cellular chlorophyll a associated to PSII, being 0.8 and 0.15 in brown and red algae, respectively, according to Grzymski et al. (1997). RLCs were fitted through the model provided by Jassby and Platt (1976) to obtain the initial slope of the curve (α_{ETR}, i.e. the photosynthetic efficiency), the saturation irradiance (E_k) and maximal ETR (ETR_{max}); the model of Platt and Gallegos (1980) was applied when photo-inhibition was detected.

2.5. Photosynthetic pigments

The content of chlorophyll-*a* (chl-*a*), chlorophyll-*c* (chl-*c*) in *C. humilis*, chlorophyll-*d* (Chl-*d*) in *D. simplex*, and carotenoids in both macroalgae was determined spectro-photometrically. The analyses were carried out by extracting pigments from plants (ca. 20 mg FW, $n = 3$) using 1 ml of saturation solution of acetone 90% + $C_4Mg_4O_{12}$ and maintained in darkness at 4 °C for 12 h.

After centrifugation at 4000 rpm for 20 min, each supernatant was used to measure pigments in a spectrophotometer, using absorption spectra between 480 and 750 nm. The pigment concentration, expressed as mg g^{-1} DW, were calculated using equations provided by Ritchie (2008).

2.6. Photoprotective compounds

The phenolic compound of *C. humilis* was obtained by grounding tissue (ca. 0.25 g FW, $n = 3$) with a mortar and a pestle in sand at 4 °C, and extracted overnight in centrifuge tubes with 2.5 ml of 80% (v/v) methanol. The mixture was centrifuged at 4000 rpm for 30 min and the supernatants were collected (Sigma 2-16PK). Total phenolic compounds, expressed as mg g^{-1} DW, were determined using phloroglucinol as a standard (Folin and Ciocalteu, 1927). The reaction was complete after 120 min in darkness at 4 °C, and the absorbance was measured at 760 nm in a spectrophotometer (Thermo Scientific Evolution 201).

MAAs determination in *D. simplex* was assayed according to Korbee-Peinado et al. (2004). Dried algal samples (10–20 mg DW, $n = 3$) were extracted for 2 h in screw-capped centrifuge vials filled with 1 ml 20% aqueous methanol (v/v) in a water bath at 45 °C. After centrifugation at 10,000 rpm for 5 min, 600 μl of the supernatant was evaporated to dryness under a vacuum at 45 °C (Jouan evaporator centrifuge). Dried extracts were redissolved in 600 μl of 100% methanol and mixed for 30 s. After passing through a 0.2-μm membrane filter, samples were analyzed with a waters HPLC system (Waters 600). The mobile phase was 2.5% aqueous methanol (v/v) plus 0.1% acetic acid (v/v) in water, and run isocratically at 0.5 ml min^{-1}. Sample volumes of 10 μl were injected into the C8 chromatographic column (5-μm pore size, 250 × 4 mm; Sphereclone; Phenomenex) with a guard column (C8, Octyl, MOS; Phenomenex). MAAs were detected online with a Waters Photodiode Array Detector 996 at 330 nm, and absorption spectra (290–400 nm) were recorded each second directly on the HPLC-separated peaks. Identification of MAAs was performed by comparison of the absorption spectra and retention times with various well-characterized standards (*Mastocarpus stellatus*, *Bostrychia scorpioides*, *Porphyra yezoensis* and fish lenses of the coral trout *Plectropomus leopardus*). Quantification was carried out by using published extinction coefficients (Takano et al., 1978; Tsujino et al., 1980; Dunlap et al., 1986).

2.7. Antioxidant activity

The DPPH (2,2-diphenyl-1-picrylhydrasyl) free-radical scavenging assay was carried out according to the method of Blois (1958) for *C. humilis* ($n = 3$). Briefly, 150 μl of each methanolic extract were mixed with 1.5 ml of a 90% methanol and 150 μl of DPPH solution prepared daily at 1.27 mM. The reaction was complete after 30 min in darkness at room temperature, and the absorbance was registered at 517 nm. The calibration curve made with DPPH was used to calculate the remaining concentration of DPPH in the reaction mixture after incubations. Values of DPPH concentration (μM) were plotted against plant extract concentration (mg DW ml^{-1}) in order to obtain the EC_{50} value (oxidation index), which represents the concentration of the extract (mg/ml) required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was used as a positive control.

2.8. Statistical analysis

Differences in photosynthetic activity and biochemical responses with depth was tested through 2-way factorial ANOVAs, separately for each species, including 'depth' (fixed factor with 3 levels: shallow, middle and deep) and 'hours' (random factor with 2 levels: 11 h and 13 h). The Cochran's test checked for homogeneity of variances (Underwood, 1997); data were transformed when necessary to achieve homogeneous variances. In case of no homogeneous variances despite data transformations, we adjusted the alpha value to 0.01, instead of the conventional 0.05 level, to decrease a type I error (Underwood, 1997). Where appropriate, pair-wise tests were run (*a posteriori* comparisons).

3. Results

3.1. Abiotic parameters

Higher temperatures were detected in the shallower level, followed by the middle and the deeper level. Maximum temperature variation was registered in the shallow level (3.9 ± 0.06 °C), being lower in the middle (0.6 ± 0.05 °C) and the deep level (0.3 ± 0.04 °C). Water temperature increase about 14.7% from 11:00 to 13:00 in shallow waters, whereas the increase in middle and deep waters was 2.5% and 1.3%, respectively. Incidence PAR increased from 1600 at 11:00 to 2142 μmol photons m^{-2} s^{-1} at 13:00 (33.8% increase), whereas UVA increased from 38.2 to 51.2 W m^{-2} (34.0% increase) and UVB from 1.84 to 2.48 W m^{-2} (34.7% increase). Penetration of both PAR and UVR was high during the study, as expected for very clear coastal waters (Type I according to the Jerlov's classification). The attenuation coefficient decreased from shorter (UVB) to longer wavebands (UVA and PAR). The K_d of PAR was 0.14 ± 0.008 m^{-1}, 2.2 times lower that UV-A (0.325 ± 0.005 m^{-1}) and ~3.5 times lower than that UVB (0.5 ± 0.03 m^{-1}).

3.2. Photosynthetic activity

The maximal and effective quantum yield of both *C. humilis* and *D. simplex* varied between depths according to the time of day ('De' × 'Ho', $p < 0.001$, Table 1). In the morning (11 h) and the afternoon (13 h), the maximal quantum yield (F_v/F_m) of *C. humilis* showed the highest values at the deep level, followed by the shallow level; the lowest values were detected in the middle level (Fig. 1a, pairwise tests, $p < 0.05$). In the morning, *D. simplex* had larger values for the maximal quantum yield at the shallow and deep levels (Fig. 1b, pairwise tests, $p > 0.05$) compared to the middle level. In contrast, in the afternoon, there was no difference between depths (Fig. 1b, pairwise tests, $p > 0.05$). In general, both species showed higher values of F_v/F_m at 11 h than that 13 h (Fig. 1a and b, 'Ho', $p < 0.001$, Table 1). Deep thalli of *C. humilis* showed high photoinhibition (6.8%), while photoinhibition was not observed for shallow-water thalli (increment of 2.7%). In contrast, shallow- and deep-water thalli of *D. simplex* presented high photoinhibition (20.9 and 13.6%, respectively), which was otherwise absent in specimens from the middle level (1.5%).

The electron transport rate (ETR) of both species varied between depths differentially in the morning (11 h) and the afternoon (13 h) ('De × Ho', $p < 0.001$, Table 1). In the morning, *C. humilis* did not show differences in ETR values between the shallow and middle level, although individuals from the deep level had a lower ETR (Fig. 1c, pairwise tests, $p < 0.01$). In the afternoon (13 h), the ETR was significantly higher in the shallow level, decreasing with depth (Fig. 1c, pairwise tests, $p < 0.01$). In the morning, *D. simplex* did not show significant differences in ETR with depth, while in the afternoon (13 h), ETR values were significant higher in the shallow and middle level than at the deep level (Fig. 1d, pairwise tests, $p < 0.01$).

The photosynthetic parameters (derived from RLCs) of both macroalgae did not vary between depths from the morning to the afternoon ('De × Ho', $p > 0.05$, Table 1), except the photosynthetic efficiency (α_{ETR}) ('De × Ho', $p < 0.05$, Table 1). For *C. humilis*, specimens from the shallow level had a larger ETR_{max} at 13 h relative

Table 1
Results of two-way ANOVAs testing the effect of 'Depth' and 'Hours' on the effective ($\Delta F/F'_m$) and maximal quantum yield (F_v/F_m), the ETR_{max}, the photosynthetic efficiency (α_{ETR}), and the saturation irradiance (E_k) of *C. humilis* and *D. simplex*. Significant values are highlighted in bold.

	C. humilis			*D. simplex*		
	MS	*F*	*p*	MS	*F*	*p*
$\Delta F/F'_m$						
De	0.1965	3.843	0.0668	0.0185	0.909	0.6464
Ho	0.2023	61.375	0.0002	0.0108	5.054	0.0284
De × Ho	0.0511	15.519	**0.0002**	0.0203	9.480	**0.0002**
Residual	0.0033			0.0021		
F_v/F_m						
De	0.1154	11.761	0.1212	0.0021	0.3835	0.7032
Ho	0.0008	2.643	0.1076	0.032	39.348	0.0002
De × Ho	0.0098	32.059	**0.0002**	0.0055	6.726	**0.0052**
Residual	0.0003			0.0008		
ETR_{max}						
De	2027.17	13.370	0.0935	7.910	4.117	0.2625
Ho	124.71	1.076	0.3215	0.6923	0.1562	0.682
De × Ho	151.62	1.308	0.297	1.921	0.4335	0.667
Residual	115.92			4.432		
α_{ETR}						
De	0.0204	3.348	0.266	0.0002	2.250	0.2106
Ho	0.0068	9.800	0.0108	0.0001	8.000	0.0144
De × Ho	0.0061	8.792	**0.0086**	0.0001	8.000	**0.0166**
Residual	0.0007			0		
E_k						
De	29.660	257.537	0.038	516.947	0.0801	0.943
Ho	0.1364	0.0617	0.8065	1445.607	0.4225	0.5235
DexHo	0.1152	0.0521	0.9505	6453.963	1.886	0.204
Residual	2.210			3421.342		

to 11 h (Table 2), despite the ANOVA did not indicate a significant effect. At 11 h, the α_{ETR} did not vary between the shallow and middle levels (pairwise tests, $p > 0.05$); the α_{ETR} was, however, higher in the deep level (Table 2, pairwise tests, $p < 0.05$). In the afternoon, the α_{ETR} showed larger values at the shallow and deep levels relative to the middle level (Table 1). For *D. simplex*, α_{ETR} was higher in the shallow level at 11 h (Table 2). At 13 h, α_{ETR} did not vary between depths (pairwise tests, $p > 0.05$). The saturation light intensity (E_k) of both *C. humilis* and *D. simplex* did not vary between depths from 11 h to 13 h ('De × Ho', $p > 0.05$, Table 1). For *C. humilis*, we found

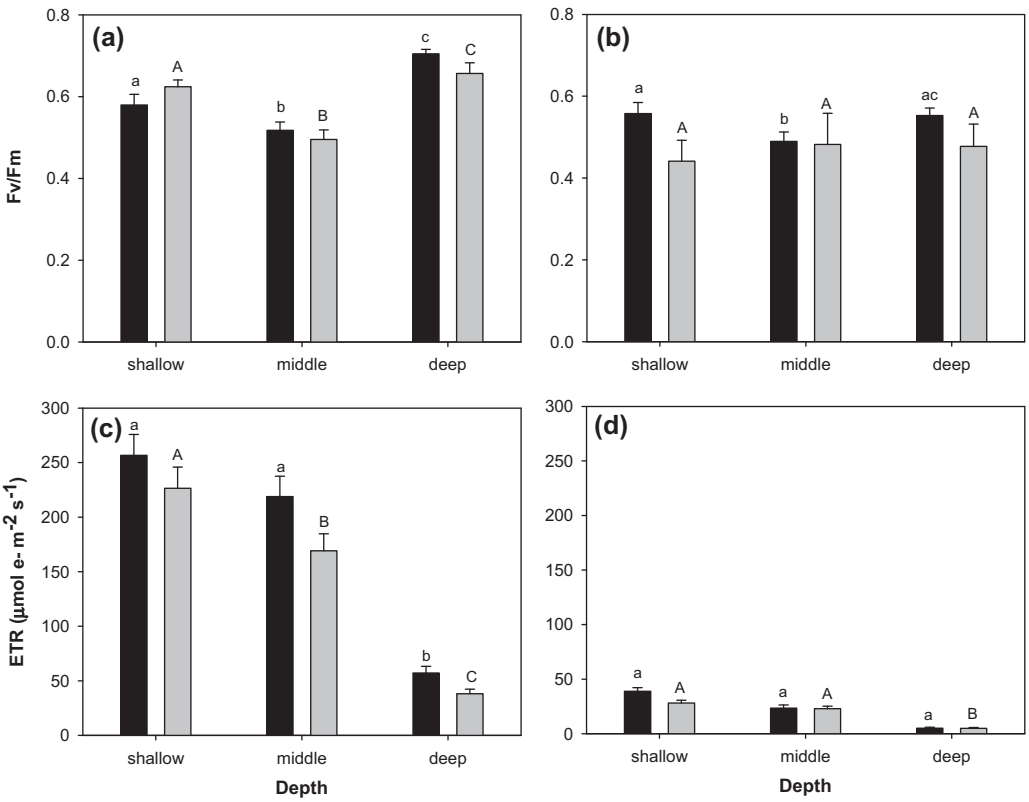

Fig. 1. Maximal quantum yield of (A) *C. humilis* and (B) *D. simplex* at 11 h and 13 h (black and grey bars, respectively) ($n = 8$) and photosynthetic capacity (ETR) of (C) *C. humilis* and (D) *D. simplex* at the different depths ($n = 3$). Data show means ± SE. Significant differences between depths are denoted by lowercase (11 h) and capital (13 h) letters above bars.

Table 2
Photosynthetic parameters obtained from Rapid Light Curves (RLCs) for *C. humilis* and *D. simplex* at different depths. Data show means ± SE; $n = 3$. Different superscripts denote significant differences.

Algae	11 h			13 h		
	ETR_{max}	α_{ETR}	E_k	ETR_{max}	α_{ETR}	E_k
C. humilis						
Shallow	48.11 ± 4.25	0.29 ± 0.01^{a}	205.87 ± 47.74	75.65 ± 6.12	0.40 ± 0.00^{A}	188.68 ± 16.54
Middle	33.78 ± 1.95	0.29 ± 0.03^{a}	117.08 ± 15.39	32.49 ± 2.53	0.28 ± 0.01^{B}	118.44 ± 7.91
Deep	38.17 ± 6.51	0.39 ± 0.01^{b}	95.55 ± 14.34	38.41 ± 4.26	0.41 ± 0.01^{AB}	91.53 ± 8.41
D. simplex						
Shallow	9.73 ± 1.06	0.05 ± 0.00^{a}	201.31 ± 24.99	9.33 ± 2.25	0.03 ± 0.00^{A}	281.08 ± 56.72
Middle	8.13 ± 0.36	0.03 ± 0.00b	270.87 ± 12.15	6.61 ± 0.80	0.03 ± 0.00^{A}	220.00 ± 26.86
Deep	7.40 ± 0.16	0.03 ± 0.00^{b}	246.53 ± 5.48	8.14 ± 1.37	0.03 ± 0.00^{A}	271.40 ± 45.84

that the E_k values decreased significantly with depth ('De', $p < 0.05$, Table 1).

3.3. Photosynthetic pigments

Despite the contents of photosynthetic pigments of both seaweeds increased with depth throughout the day (Fig. 2), differences were not statistically significant as 'main effects' ('De'; 'Ho' $p > 0.05$, Table 3). However, the pigment contents of *D. simplex* varied between depths inconsistently through times ('De × Ho', $p < 0.05$, Table 3). In the morning (11 h), the Chl *a*, Chl *d* and carotenoid contents increased with depth (pairwise tests, $p < 0.05$). In the afternoon, the pigment contents of *D. simplex* did not vary between depths (Fig. 2d–f, pairwise tests, $p > 0.05$).

3.4. Photoprotective compounds

The total phenolic content of *C. humilis* did vary between depths inconsistently through the day ('De × Ho', $p < 0.01$, Table 3). In the morning (11 h), the phenolic content was higher in the shallow level

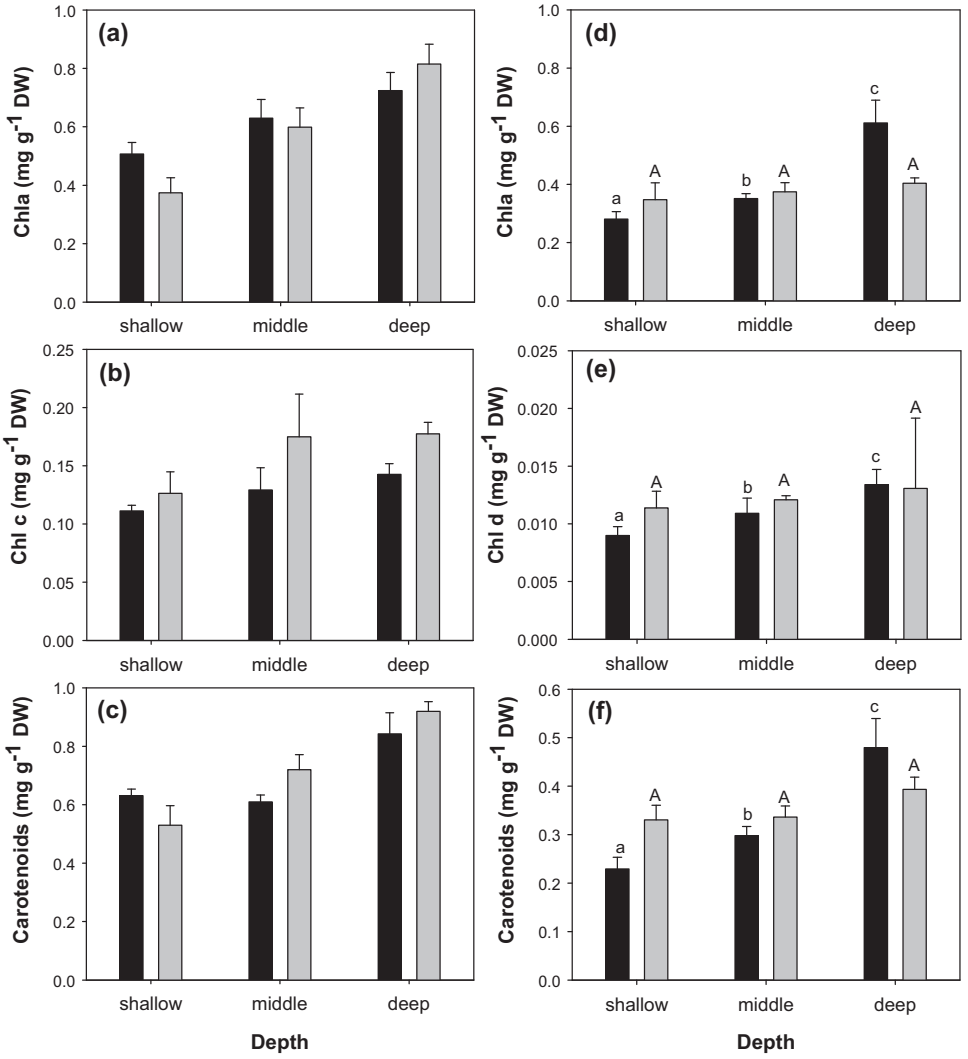

Fig. 2. Photosynthetic pigment contents: Chl *a* (A), Chl *c* (B) and carotenoids (C) for *C. humilis* and Chl *a* (D), Chl *d* (E) and carotenoids (F) for *D. simplex* at the different depths at 11 h and 13 h (black and grey bars, respectively). Data show means ± SE; $n = 3$. Significant differences between depths are denoted by lowercase (11 h) and capital (13 h) letters above bars.

Table 3
Results of two-way ANOVAs testing the effect of 'Depth' and 'Hours' on the photosynthetic pigments, the total phenolic content and antioxidant activity (EC_{50}) of *C. humilis* and MAAs of *D. simplex*. Significant values are highlighted in bold.

	C. humilis			*D. simplex*		
	MS	*F*	*p*	MS	*F*	*p*
Chl *a*						
De	1.946	0.8974	0.5058	0.0919	1.652	0.4474
Ho	0.1562	0.0646	0.7918	0.0009	0.1571	0.7072
De × Ho	2.168	0.8965	0.4188	0.0556	9.450	**0.0034**
Residual	2.418			0.0059		
Chl *c*						
De	0.5062	0.2947	0.8244	–	–	–
Ho	2.858	30.478	0.0002	–	–	–
De × Ho	1.718	18.316	**0.0014**	–	–	–
Residual	0.0938			–	–	–
Chl *d*						
De	–	–	–	0	0.1117	0.8244
Ho	–	–	–	0.0001	3.992	0.0354
De × Ho	–	–	–	0.0001	3.126	**0.0358**
Residual	–	–	–	0		
Carotenoids						
De	3.321	12	0.2106	0.038	2.106	0.3912
Ho	0.2858	1.574	0.2236	0.0076	2.300	0.1576
De × Ho	0.2858	1.574	0.2478	0.0181	5.462	**0.0184**
Residual	0.1816			0.0033		
Phenolic content						
De	0.9752	10.431	0.1152	–	–	–
Ho	0.1882	9.965	0.0084	–	–	–
De × Ho	0.0935	4.951	**0.0258**	–	–	–
Residual	0.0189			–	–	–
EC_{50}						
De	3.311	0.8433	0.6034	–	–	–
Ho	1.150	1.257	0.2726	–	–	–
De × Ho	3.926	4.291	**0.038**	–	–	–
Residual	0.915			–	–	–
% Total MAAs						
De	–	–	–	0.975	10.431	0.1152
Ho	–	–	–	0.188	9.965	0.0084
De × Ho	–	–	–	0.093	4.951	**0.0258**
Residual	–	–	–	0.018		
% Shinorine						
De	–	–	–	3.311	0.8433	0.6034
Ho	–	–	–	1.150	1.257	0.2726
De × Ho	–	–	–	3.926	4.291	**0.038**
Residual	–	–	–	0.915		
% Porphyre-334						
De	–	–	–	1.946	0.8974	0.5058
Ho	–	–	–	0.156	0.0646	0.7918
De × Ho	–	–	–	2.168	0.8965	0.4188
Residual	–	–	–	2.418		
% Palytine						
De	–	–	–	0.506	0.2947	0.8244
Ho	–	–	–	2.858	30.478	0.0002
De × Ho	–	–	–	1.718	18.316	**0.0014**
Residual	–	–	–	0.093		
% Asterine						
De	–	–	–	3.321	12	0.2106
Ho	–	–	–	0.285	1.574	0.2236
De × Ho	–	–	–	0.285	1.574	0.2478
Residual	–	–	–	0.181		
% MAAs-glycine						
De	–	–	–	5.354	4.053	0.0526
Ho	–	–	–	2.541	4.399	0.0636
De × Ho	–	–	–	1.321	2.286	0.1418
Residual	–	–	–	0.577		

than in the middle and deep levels (Fig. 3a, pairwise tests, $p < 0.01$). However, at 13 h, the phenolic content in the shallow and middle level was similar, and higher than the deep level (Fig. 3a, pairwise tests, $p < 0.01$).

The mycosporine-like amino acid (MAAs) concentration varied between depths inconsistently through the day for *D. simplex* ('De × Ho', $p < 0.05$, Table 3). At 11 h, a larger MAAs content was detected at the shallow and middle levels relative to the deep level. At 13 h, the MAAs concentration was higher in the shallow level, decreasing with depth. The MAAs concentration was higher in the afternoon (13 h) than in the morning (11 h) (Fig. 4, 'Ho' $p < 0.05$, Table 3). The dominant MAAs in *D. simplex* during the morning and in the shallow level was Shinorine, followed by Palythine and Mycosporine-glycine. In the afternoon, the Mycosporine-glycine and Shinorine concentration was similar. Furthermore, Asterina-330 appeared in the shallow specimens, while its concentration

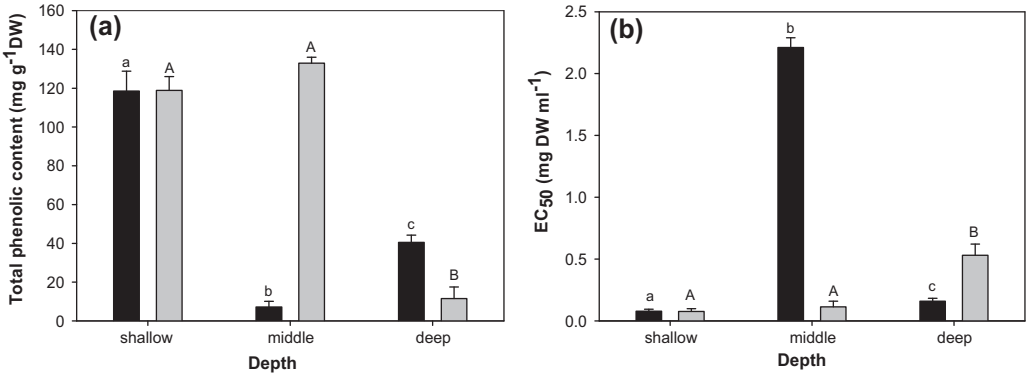

Fig. 3. Total phenolic content (A) and antioxidant activity, measured by the EC_{50} index (B), for *C. humilis* at the different depths at 11 h and 13 h (black and grey bars, respectively). Data show means ± SE; $n = 3$. Significant differences between depths are denoted by lowercase (11 h) and capital (13 h) letters above bars.

Table 4
Mycosporine-like amino acids for *D. simplex* at different depths in the intertidal rock pool at 11 h and 13 h. Data show means ± SE; $n = 3$. Different superscripts denote significant differences.

	% Shinorine	% Porphyra-334	% Palythine	% Asterina-330	%MAA-glycine
Shallow					
11:00	40.48 ± 5.80^{a}	17.96 ± 2.55^{a}	27.55 ± 3.31^{a}	3.45 ± 0.37^{a}	11.55 ± 2.67^{a}
13:00	33.1 ± 3.51^{A}	7.53 ± 1.52^{A}	25.72 ± 1.63^{A}	2.51 ± 0.98^{A}	31.17 ± 2.11^{A}
Middle					
11:00	14.66 ± 0.8^{b}	14.40 ± 1.18^{a}	36.25 ± 1.46^{a}	–	34.78 ± 2.20^{a}
13:00	37.81 ± 3.97^{A}	8.52 ± 0.89^{A}	16.27 ± 2.52^{B}		37.40 ± 4.61^{A}
Deep					
11:00	23.43 ± 1.05^{c}	13.36 ± 2.09^{a}	32.18 ± 2.85^{a}	–	31.06 ± 3.20^{a}
13:00	$22.95 \pm 3.^{C}$	13.59 ± 2.27^{A}	29.28 ± 3.0^{AC}		34.18 ± 3.16^{A}

was negligible for specimens from the middle and deep levels (Table 4). The dominant MAAs in *D. simplex* during the morning and in the middle level was Palythine and Mycosporine-glycine. In contrast, in the afternoon, the Shinorine concentration increased with similar values than Mycosporine-glycine. Finally, at the deep level, during the morning, there was a dominance by the MAAs Palythine and Mycosporine-glycine. In the afternoon, the MAAs concentration did not vary respect the values observed in the morning (Table 4).

3.5. Antioxidant activity

For both algae, the antioxidant activity did vary between depths throughout the day ('De × Ho', $p < 0.01$, Table 3). In the morning, algae from the shallow and deep levels had higher antioxidant capacity (lower EC_{50} values) relative to the middle level (higher EC_{50} values). In the afternoon, the antioxidant activity in the shallow level remained high; in the middle level, the antioxidant activity increased respect the values observed in the morning, while in the deep level the antioxidant activity decreased (Fig. 3b). We obtained a positive correlation between the phenolic concentration and the antioxidant activity ($1/EC_{50}$) for *C. humilis* ($R = 0.89$, $p < 0.03$).

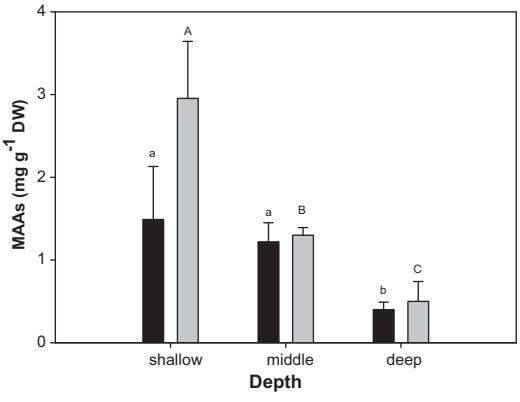

Fig. 4. Mycosporine-like amino acids concentration, expressed in mg g^{-1} DW, for *D. simplex* at 11 h and 13 h (black and grey bars, respectively) at the different depths. Data show means ± SE; $n = 3$. Significant differences between depths are denoted by lowercase (11 h) and capital (13 h) letters above bars.

4. Discussion

Organisms living in intertidal pools experience fluctuations in their physical environment (Harley and Helmuth, 2003). The high irradiance and transparency of shallow waters in the Canaries (Häder et al., 2001) suggests that macroalgae growing there should have efficient photoprotective mechanisms (high dynamic photoinhibition and photoprotection strategies) to tolerate light-induced stress, relative to species from biogeographical regions with less daily integrated irradiance (Abdala-Díaz et al., 2006; Figueroa et al., 2009). Macroalgae growing at shallow depths are often tolerant to UV and recover well after periods of high UV radiation. Morphologically, species with tougher and thicker thalli may also be less sensitive to UV, as a result of more protective tissues (Roleda et al., 2006). In the present study, although *D. simplex* has thicker thallus that *C. humilis*, the latter showed a better photoprotection strategy, coinciding with Roleda et al. (2005), where thicker thalli showed higher DNA damage.

The ability to show dynamic photoinhibition during exposure to high light conditions, as well as the general acclimation of photosynthesis, is directly related to macroalgal zonation (Hanelt, 1998). In this study, the photoinhibition potential differed between species. The brown algae *C. humilis* showed a high photoinhibition in the deep level, which decreased toward the shallow level, where photoinhibition was not observed. Similarly, Hanelt and

Roleda (2009) showed a greater photoinhibition for brown algae collected from deep relative to shallow areas. In contrast, *D. simplex* showed a high photoinhibition potential in the shallow level, which decreased with depth, where minimum photoinhibition was observed. These results show that zonation patterns are physiologically determined, and vary according to species specific life-traits. In this case, *C. humilis* showed a better acclimation to high irradiances than *D. simplex*.

The variation of ETR showed a clear vertical pattern; shallow-water algae were more productive, i.e. increased ETR from 11 h to 13 h, which was otherwise not observed for algae from the middle and deep levels, with the exception of *D. simplex* from the deep level. The high values of ETR_{max} and E_k, in conjunction with low values of α_{ETR}, of both macroalgae from shallow-water are indicative of a typical sun-adapted behavior (Betancor et al., 2014). These results reveal better stress acclimation of both seaweeds in the shallowest level. In this sense, Bischof et al. (1998) reported a decreasing ETR_{max} for the brown algae *Laminaria saccharina* with depth, and Sagert et al. (1997) showed an increased α_{ETR} values and decreased P_{max} values with depth for the red algae *Chondrus crispus*.

The irradiance has a pronounced effect on algal pigment composition (Carnicas et al., 1999). In our study, the pigment concentration showed an increase with depth for both macroalgae, consistent with reports for *Chondrus crispus* and *Laminaria saccharina* (Sagert et al., 1997; Borum et al., 2002). The increase of accessory pigments in *D. simplex*, such as the carotenoids, at high irradiances can be explained by their photoprotective role (Carnicas et al., 1999). On the other hand, the minimum variation in the content of accessory pigments of *C. humilis* showed the better adaptation of this macroalga relative to the former, being more resistant to high light conditions.

Similar to dynamic photoinhibition, production of phenolic compounds may act as a photoprotection mechanism against high solar irradiance by absorbing incident photons, or indirectly as a result of their antioxidant activity (Abdala-Díaz et al., 2006; Connan et al., 2006). In our study, the phenolic compounds concentration did vary between depths. The phenol content in *C. humilis* was very high at shallow water regardless of timing. In contrast, in the middle level, the phenol content drastically increased in the afternoon (Abdala-Díaz et al., 2006) and decreased in the deep level at high irradiances, as reported by Abdala-Díaz et al. (2006) for *C. tamariscifolia*. In *C. humilis*, the high phenol content and the positive correlation with its antioxidant activity suggests that these compounds have a photoprotective role (Zubia et al., 2009).

The harmful effects of UVR can be ameliorated through photoprotective mechanisms, e.g. the accumulation of UV-screen substances and the activation of antioxidant systems (Korbee et al., 2006). In our study, the MAAs concentration in *D. simplex* varied with depth; thalli at the shallow level showed higher MAAs content. The production of MAAs is often connected with the amount of solar radiation (Karsten et al., 1999; Korbee et al., 2006). In our study, the MAAs content was related to irradiance levels, i.e. the content was lower in the morning than in the afternoon. This result is consistent with those reported by Karsten et al. (1999) for *Devaleraea ramentacea*, where the MAAs content decreased with depth and increased with irradiance levels. In this study, around 30% of the total MAAs of *D. simplex* was Mycosporine-glycine, while above 25% were Palythine, Shinorine and Porphyra-334. Importantly, thalli from shallow water had relevant amounts of Asterina-330, the MAAs with higher protector capacity (De la Coba et al., 2009).

5. Conclusion

Macroalgal zonation has been often related to the ability to resist high light stress (Hanelt, 1998). In our study, we observed that even the same species at different depths change their photosynthetic and pigment apparatus to adapt to different light conditions. In conclusion, our results reinforce the notion that macroalgal zonation is primarily physiological determined, where shallow-water thalli have more active acclimation mechanisms at different environmental stress.

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Appendix A. Appendix A

Fig. A1.

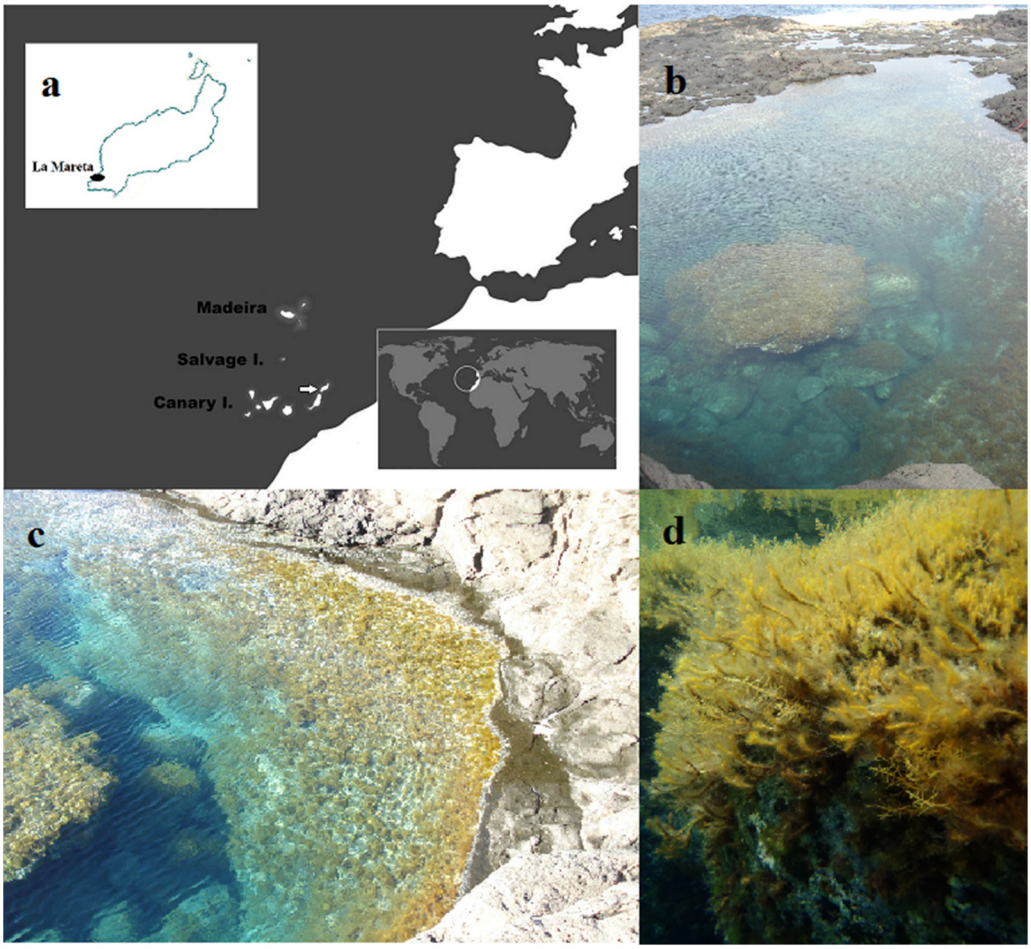

Fig. A1. (A) Geographical situation of the study location, La Mareta, Lanzarote Island. (B) Intertidal rock pool where assays took place. (C) Vertical distribution of *Cystoseira humilis* and *Digenea simplex* in the intertidal rocky pool. (D) Thalli *C. humilis* and *D. simplex*. 3.

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