TITLE

Physiological potential of the chlorophyte *Caulerpa prolifera* for proliferation across the Mediterranean−Atlantic basins in a warmer ocean

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Physiology of *Caulerpa prolifera* and warming

ABSTRACT

Ocean warming is altering the metabolic balances of organisms, favouring the expansion of thermo-tolerant individuals. The fast-growing macroalga *Caulerpa prolifera* is rapidly expanding in the Ria Formosa lagoon (Portugal), a connection area between Mediterranean and Atlantic basins. We investigated the metabolic capacity of *C. prolifera* to cope with ocean warming, to elucidate its expansion potential. The photosynthetic and respiratory plasticity of 4 populations of *C. prolifera* spread along the Mediterranean−Atlantic basins was assessed under a temperature range of 20 to 30°C. In addition, molecular markers were used to investigate the genetic identity of the strain found in Ria Formosa, which confirmed its Mediterranean origin. All examined populations showed large physiological thermo-tolerance and metabolic plasticity to warming. The photosynthetic efficiency of *C. prolifera* improved by 50% with temperature, and the maximum photosynthetic production doubled along the temperature range tested. Respiration did not vary with temperature, whereas the metabolic quotient increased by more than 70%when temperature increased from 20 to 25−30°C. Minor differences in the photosynthetic descriptors were detected among populations, reflecting light- and dark-adapted physiology of Mediterranean and Atlantic populations, respectively. Our results show that all tested populations of *C. prolifera* have the physiological potential to cope with temperature increases up to 30°C, which indicates that ocean warming may contribute to the expansion of *C. prolifera* in the Mediterranean−Atlantic basins.

KEY WORDS

Caulerpa prolifera; Global warming; Metabolism; Thermal tolerance; Physiological plasticity; Photosynthesis; Respiration.

INTRODUCTION

Marine systems are being exposed to pressures linked to global climate change. Among these, global warming is a major threat affecting marine life and ecosystem functioning (Pörtner et al. 2014). The warming trend in the ocean as well as the frequency and intensity of extreme events (e.g. heat waves) have increased in the last decades (Oliver et al. 2018), and projections estimate they will continue to do so over the next century (Oliver et al. 2019).

Warming is globally affecting the functioning of all marine systems, but coastal areas are particularly sensitive habitats where the effects of temperature and heat waves are more extreme than in the open ocean (Helmuth et al. 2006). Associated with longlasting warming conditions, changes in the structure of coastal populations and in the geographic distributions of species have also been predicted (Smale & Wernberg 2013).

Rising water temperature may pose a challenge for species living at their physiological tolerance limit since they may be less competitive and become displaced by more tolerant species. In contrast, warmadapted species and organisms living closer to their colder limits are expected to improve growth and increase their abundance under future warming conditions (Smale & Wernberg 2013). Warming may thus create new suitable habitat niches for these species and boost their colonization potential. The arrival of new species with higher temperature tolerance in high and temperate latitudes is expected in association with warming (Lima et al. 2007, Bates et al. 2013). Evaluation of the thermal tolerance of a species is therefore a critical step for a more realistic assessment of the impact of global warming on coastal marine organisms.

Ocean warming is predicted to affect the physiology and metabolic balance of organisms (Koch et al. 2013) by altering the energetic costs of metabolic processes and forcing energetic trade-offs (Yvon- Durocher et al. 2010). This may have subsequent impacts on the functioning of the whole ecosystem to which they belong (Pörtner et al. 2014). Moderate temperature increases can benefit the productivity of primary producers, stimulating photosynthesis, growth and biomass accumulation (Harley et al. 2012). However, since respiration is expected to increase more than photosynthesis with temperature (Brown et

al. 2004), once a given thermal threshold is exceeded, the metabolic balance becomes less favourable. A decrease in net metabolic balance may compromise the fitness of an organism, leading to deleterious effects such as carbon imbalance, reduced growth and even mortality (Harley et al. 2012). Thermal tolerance and metabolic thresholds largely depend on the optimum thermal range, acclimation capacity and genetic adaptation potential (Stillman 2003), which remain unknown for most species.

The local environment also shapes thermal thresholds and sensitivity of organisms to temperature. Exposure to extreme and more frequent thermal events may enhance resilience to stress, pushing adaptation and expansion of tolerance thresholds (Schoepf et al. 2015). Within European waters, similar warming trends and multidecadal oscillations have been described for the Mediterranean Sea and the Atlantic Ocean (Marullo et al. 2011). However, a gradient of temperature naturally exists along the Mediterranean-Atlantic basins, with higher sea surface temperature and frequency of heat waves in the Mediterranean basin (Oliver et al. 2018). It may thus be hypothesized that organisms thriving in Mediterranean waters will cope better with higher temperatures than their Atlantic counterparts and that a physiological pattern in response to temperature could be found along the Mediterranean-Atlantic axis.

The chlorophyte *Caulerpa prolifera* (Forsskål) J.V. Lamouroux is a clonal, fast-growing macroalga that is globally distributed and is considered an autochthonous species on the Mediterranean and Atlantic coasts (Varela-Álvarez et al. 2015, Cacabelos et al. 2019). A recent biogeographical study identified 3 main biogeographical areas for this species: (1) the West Atlantic, (2) the East Atlantic and (3) a larger area representing the Mediterranean, the transition zone towards the Atlantic and some sites in the Indo- Pacific (Varela-Álvarez et al. 2015). Large phenotypic plasticity has been attributed to the genus Caulerpa, with high intra- and inter-specific physiological variability among species (Robledo & Freile- Pelegrin 2005). Niche modelling analysis demonstrated that different genetic lineages of the species C. taxifolia (M. Vahl) C. Agardh showed dissimilar responses to environmental drivers, space colonization and invasive ranges that are not detectable by species-based analyses (Chefaoui & Varela-Álvarez 2018). Physiological plasticity and genetic identity of populations

are thus key factors to be considered when studying the acclimation and adaptation capacities of Caulerpa spp. to global warming and its colonization potential.

The spatial niche of *C. prolifera* overlaps with other benthic macrophytes growing in soft-bottom shallow subtidal coastal areas. The high proliferation and expansion potential of this species may pose serious challenges for coastal foundation species with high ecological value, such as seagrasses, severely impacting coastal biodiversity and eco-social functioning (Occhipinti-Ambrogi & Savini 2003, Bennett et al. 2016). On the southern coasts of Portugal, at the Atlantic edge of the Mediterranean−Atlantic transition zone, occasional sightings of *C. prolifera* have been documented since the 19th century. However, no further records of *C. prolifera* in southern Portugal were documented in surveys regularly carried out in Ria Formosa in the last decades (Cunha et al. 2013). However, it is plausible that the original population persisted, remaining cryptic and contained in small areas until favourable conditions triggered its expansion. In 2011, a single small patch (~10 m2) was first observed in the Ria Formosa lagoon, adjacent to Fuseta Island (Cunha et al. 2013), and in the following years a number of small patches were found in a nearby area (i.e. Armona inlet) (Alexandre & Santos 2020). Since 2016, large subtidal meadows of *C. prolifera* have proliferated in Ria Formosa around Culatra Bay (Alexandre & Santos 2020), expanding further west towards the Atlantic Ocean. At present, the genetic origin, physiological performance and spread potential of the strain recently rediscovered in Ria Formosa remain unclear.

In the context of climate change, the capacity of *C. prolifera* to cope with future warmer scenarios is poorly understood. Despite its ecological relevance in shallow coastal systems, few studies have addressed the physiology of *C. prolifera* (Terrados & Ros 1992, Häder et al. 1997, Malta et al. 2005, Vergara et al. 2012), and the intra-specific physiological plasticity of *C. prolifera* remains largely unknown. To date, no study has compared the physiology of different populations growing throughout the Mediterranean−Atlantic distribution range of this species.

We investigated the capacity of *C. prolifera* to cope with global warming by evaluating the physiological tolerance of different populations of *C. prolifera* spread along the

Mediterranean−Atlantic basins to elucidate the proliferation potential of this species under future ocean warming. This is the first study evaluating the physiological response of *C. prolifera* from Portuguese coasts.

We hypothesized that the *C. prolifera* strain recently rediscovered in Culatra, Ria Formosa (Portugal), has a Mediterranean origin and that Mediterranean lineages are physiologically more thermo- tolerant than those from the Atlantic, since populations in the Mediterranean are exposed to higher temperatures than those in the Atlantic (Pérez-Ruzafa et al. 2005, Tuya et al. 2014). To test these hypotheses, we conducted a physiological laboratory experiment to study the metabolic response to temperature of 4 different populations of *C. prolifera* growing along the Mediterranean-Atlantic basins representing different genetic lineages within the 3 main biogeographical areas previously described (Varela-Álvarez et al. 2015). We measured the photosynthetic and respiratory responses of individuals exposed to a temperature gradient, from 20 to 30°C, covering present-day and future warming scenarios (IPCC 2014). We also applied molecular tools sequencing the chloroplast DNA (cpDNA) tufA gene of the population recently developed in Ria Formosa (Culatra) to confirm its genetic identity and origin.

MATERIALS AND METHODS

Algal collection and acclimation

Four different populations of *Caulerpa prolifera* spreading along the Mediterranean-Atlantic basin, including the population recently rediscovered in Ria Formosa (Culatra), were selected to study their thermal physiological plasticity.

Specimens of *C. prolifera* (i.e. stolons bearing several fronds each) from Murcia, Spain (Mar Menor, 37° 48’ 48.7” N, 0° 47’ 05.6” W), Cádiz, Spain (Santibañez, 36° 28’ 09.2” N, 6° 15’ 00.9” W), Ria Formosa, Portugal (Culatra, 37° 00’ 00.3” N, 7° 49’ 53.4” W) and Las Palmas de Gran Canarias, Spain (Arinaga, 27° 50’ 59.8” N, 15° 23’ 26.4” W) were collected by snorkelling and scuba diving during June and July 2017 (Fig. 1). Mean temperatures at the collection sites ranged between 18 and 21°C (see Table S1 in the Supplement).

Once collected, specimens were gently stored in seawater in darkness and shipped to the Ramalhete experimental field station (CCMAR, Portugal) within 48 h. Specimens of *C. prolifera* from each population were potted in 100 l outdoor mesocosms (n = 4) with a layer of clean sand at the bottom and a neutral light shadow mesh (ca. 50% surface irradiance attenuation) on top. Mesocosms were connected to an open- water system with natural light, temperature and photoperiod. Specimens were kept in the outdoor mesocosms for 6 wk. The daily mean ± SE temperature during the experimental period was 25.88 ± 1.75°C. Maximum surface irradiance at noon reached 2000 µmol photon m^{-2} s^{-1}. During this period, physiological laboratory experiments and molecular identification were conducted.

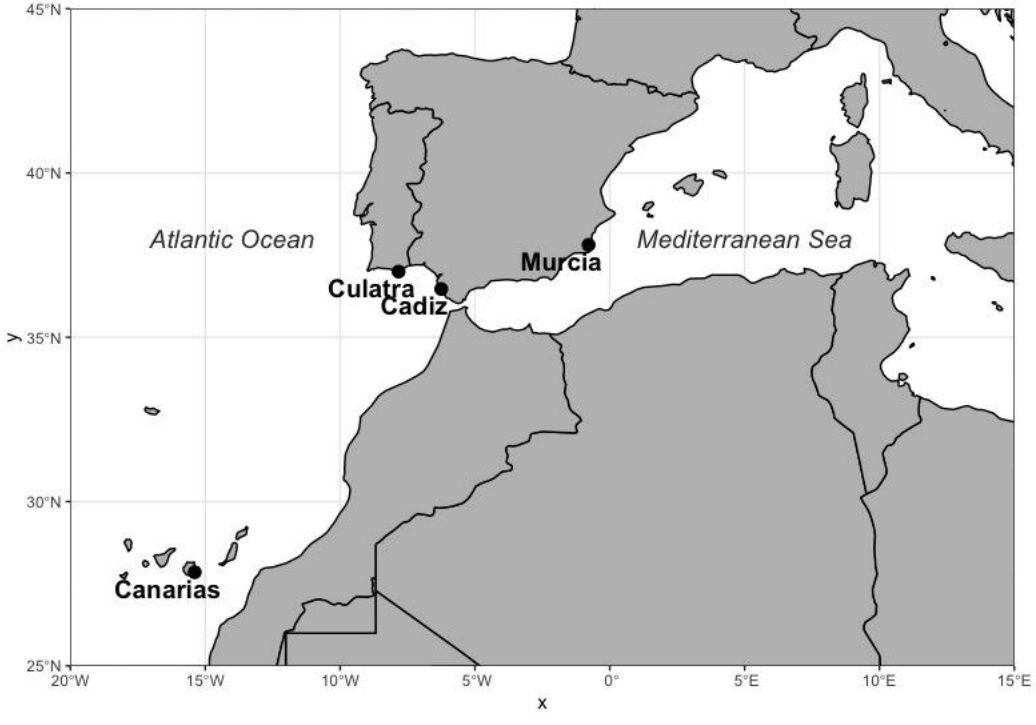

Figure 1. Map of the eastern Atlantic and western Mediterranean region. Black dots indicate the locations of the *Caulerpa prolifera* populations studied.

Physiology: fluorescence and photosynthesis vs. irradiance curves

The physiological performance of *C. prolifera* was evaluated via fluorescence and photosynthesis vs. irradiance curves (P−E curves) in the laboratory under temperature- and

light-controlled conditions. Three different temperature treatments (20, 25 and 30°C) were chosen, where 20°C represents the mean temperature at the collection sites (Table S1). The 25°C treatment represents the mean summer temperatures among the populations studied (Tuya et al. 2014, Garcias-Bonet et al. 2019), while 30°C represents the projected mean summer temperature by the end of the century under a scenario of moderate greenhouse gas emissions equivalent to RCP6.0 (Garcias-Bonet et al. 2019).

The afternoon before the laboratory experiments were conducted, 2 specimens from each population were selected from the outdoor acclimation mesocosms, covered with dark shades, and kept in their own acclimation mesocosm in order to achieve full photosynthetic relaxation prior to physiological measurements (Enríquez & Borowitzka 2010). On the day of the measurements, the dark-acclimated specimens from each population were collected from their mesocosm, kept in seawater and darkness and taken to the laboratory where measurements of fluorescence and P−E curves took place.

The photochemical photosynthetic performance of C. prolifera, i.e. the capacity to convert light into energy, was studied via fluorescence in the same specimens later used for the P−E curves. The fluorescence of chlorophyll from photosystem II was measured with a pulse amplitude modulated fluorometer (IMAGING-PAM, Walz). The maximum photochemical efficiency (maximum quantum yield, Fv/Fm) (Krause & Weis 1991) was measured in the specimens kept in darkness overnight (dark-acclimated) prior to starting the P−E curves. The effective photo- chemical efficiency (effective quantum yield, Fv'/Fm') was measured right after specimens were exposed to the maximum irradiance in the P−E curve, i.e. at the end of the P−E curve. The maintenance of the photo- chemical conversion was estimated by the % yield drop, calculated as the percentage of reduction in the maximum quantum yield (Fv/Fm) at the end of the P−E curve (Fv'/Fm').

Once the dark-adapted specimens were collected from the mesocosms and maximum quantum yield was measured, they were kept in darkness and placed in vials filled with seawater at the experimental temperature set for day, and P−E curves started right after. Photosynthetic and respiratory oxygen flux was evaluated with P−E curves conducted on each population. The P−E curves were performed in rounds, where each round tested 1

temperature (i.e. 20, 25 or 30°C) and included 1 specimen from each population. Five P−E curves (n = 5 rounds) were performed at each temperature tested. Each day, one P−E curve was run (i.e. 15 d of measurements). The laboratory was kept at constant temperature coincident with the temperature tested in the P−E curve.

Specimens tested in the P−E curves were incubated in small incubation chambers (Erlenmeyer, 200 ml) closed with air-tight plastic stoppers and were subjected to a series of 10 increasing irradiances under temperature-controlled conditions (i.e. 20, 25 and 30°C). Irradiances ranged from initial darkness up to 1000 µmol photon m^{-2} s^{-1}. The maximum irradiance was set in order to simulate the higher irradiances reaching Caulerpa meadows (Morris et al. 2013, Mishra et al. 2018). Erlenmeyer chambers were placed on a shaking plate to stimulate water movement and avoid oxygen saturation at the diffusion layer around the specimens during the incubations. Specimens were incubated in filtered seawater (GF/F filter) collected from the open-flow system supplying the acclimation mesocosms tanks. Salinity (35.5) was checked using a hand-held refractometer (Atago). A control incubation, containing only filtered seawater, was run randomly within several P−E curve rounds to check the gas-tightness of the incubation chambers and the potential contribution of the microbial community in the water mass. Gas losses and microbial contributions were negligible (data not shown).

Oxygen evolution within the incubation chamber during the P−E curves was quantified using optode sensors (spot sensors for Microx 4 meter, PreSense) which allowed determining the O2 concentration without opening the chambers. At each irradiance, oxygen (concentration and % saturation) and temperature were recorded in each incubation chamber for at least 5 min in order to obtain a stable slope. Whenever necessary during the P−E curves, seawater inside the chambers was partially renewed with N_2-bubbled seawater to avoid O2 oversaturation.

Net photosynthesis and respiration rates were derived from oxygen measurements. Dark respiration (Rd) was calculated from the oxygen consumption rate in darkness. The photosynthetic efficiency (alpha) was estimated from the initial slope of the P−E curves by linear least squares regression analysis. Net maximum photosynthetic rates (Pmax) were

obtained from the average maximum values above saturating irradiance. The light saturation point (Ek) was computed as the ratio between maximum photosynthetic rate and photosynthetic efficiency (Pmax/ alpha), and the light compensation point (Ec) as the intercept of the P−E curve with the x-axis (Rd/alpha). Once P−E curves and fluorescence measurements were performed, the volume in the incubation chamber was measured, incubated specimens were collected, scanned for area analysis and dried at 65°C over 2 d for dry weight assessment. Area and weight were used for photosynthesis and respiration normalization.

DNA extraction, PCR amplification, sequencing and molecular identification

A pooled blade sample of C. prolifera collected from the Culatra population was taken for genetic identification and origin. DNA was extracted using the Nucleospin 96 Plant II kit (Macherey-Nagel). A partial region of the cpDNA tufA gene was amplified by PCR following Famà et al. (2002) and Varela-Álvarez et al. (2015) using primers from Famà et al. (2002). PCR conditions were hot start (95°C, 5 min); 40 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 1 min 30 s; and final extension of 5 min at 72°C. PCR reactions were performed in a 20 µl volume containing buffer (10×), dNTPs (2 mM), MgCl2 (50 mM), primers (10 mM), 0.3 U Taq polymerase and approximately 5−10 ng of template DNA. Amplified products were visualized on a 1% agarose gel. Purified PCR products were sent for sequencing to the CCMAR sequencing unit. The resulting sequences were compared first with cpDNA tufA sequences of C. prolifera available in GenBank, and second, with the 3 distinct haplotypes (Mediterranean, East Atlantic and West Atlantic) associated with the 306 nt common region in tufA sequences defined by Varela-Álvarez et al. (2015).

Statistics

A preliminary exploratory data analysis was performed on data to detect and remove outliers when necessary. A 2-way ANOVA was applied to physiological descriptors obtained from P−E curves and fluorescence considering temperature and population as fixed factors. Parametric conditions were tested by Shapiro and Levene's tests, for normality and

homoscedasticity, respectively. The significance level was set at $p = 0.05$. When parametric conditions were not satisfied, statistical significance was set at $p = 0.01$ to minimize type I error (Underwood 1997). Statistical analysis was performed with R Version 1.1.453 (R Core Team 2018). Values within the text are given as mean ± SD unless otherwise indicated.

RESULTS

Physiology of *Caulerpa prolifera*

The maximum photochemical performance, maximum quantum yield (Fv/Fm), of C. prolifera ranged between 0.71 and 0.78 and did not vary among populations (Table 1), indicating a good physiological status of all *C. prolifera* specimens selected for P−E curves (Malta et al. 2005). The effective photochemical yield (Fv'/Fm') measured after P−E curves significantly increased with temperature ($p < 0.001$) but the response to warming differed among populations (temperature × population, $p < 0.05$) (Fig. 2, Table 1). Populations from the Mediterranean edge (Murcia and Cádiz) showed higher fluorescence effective yield at 30°C, while in the Atlantic populations (Canarias and Culatra), the effective yield peaked at 25°C (see Table S2 in the Supplement for the complete database). The % yield drop before (Fv/Fm) and after (Fv'/Fm') the P−E curve gives information on the maintenance of the photochemical conversion in *C. prolifera* after the P−E curve. The decrease in the photochemical conversion (% yield drop) after the P−E curve was around 80%, but the decrease was significantly reduced with temperature ($p < 0.05$), indicating an optimization of the photochemical conversion as temperature increased (Fig. 2, Table 1). Significant differences in both the effective yield and the % yield drop were detected between 20 and 25−30°C (Table 1).

The metabolic performance, i.e. the photosynthetic capacity to convert light into oxygen and its consumption by respiration, of *C. prolifera* was estimated from the P−E curves. P−E curves were expressed on both an area and a weight basis of specimens (Fig. S1 in the Supplement). Photosynthetic and respiratory parameters were only calculated on an area basis since a significant linear relationship was found between area and dry weight (DW)

among specimens used in this study (weight [g DW] = -0.0002 + 0.0023 area [cm^2]; R_2 = 0.78; F_{73} = 257.9, $p < 0.0001$).

The photosynthetic efficiency in light conversion (alpha) of *C. prolifera* increased by > 50% between 20 and 30°C (from 0.023 ± 0.014 to 0.036 ± 0.010 µmol O_2 µmol $photon^{-1}$, $p < 0.001$), with significant differences detected between 20 and 30°C (Fig. 3, Table 1). Differences among populations were also detected ($p < 0.001$), with Atlantic populations (i.e. Canarias) consistently showing higher photosynthetic efficiencies than the other populations (Fig. 3, Tables 1 & S2).

Table 1. ANOVA results for the effect of temperature (Temp) and population (Pop; Can: Canarias, Mur: Murcia, Cad: Cadiz, Cul: Culatra) on the variables derived from fluorescence and photosynthesis vs. irradiance (P−E) curves. Maximum yield (Fv/Fm,), effective yield (Fv'/Fm'), % maximum quantum yield drop, photosynthetic efficiency (alpha), compensation irradiance (Ec), saturation irradiance (Ek), maximum photosynthesis (Pmax), respiration (R) and metabolic quotient (P/R). Significance is indicated as
***p < 0.001; **p < 0.01; *p < 0.05

Variable	Parametric	Factors	df	Sum sq	Mean sq	F	Pr(>F)	Tukey HSD
Fv/Fm	Yes	Population	3	0.000782	0.0002607	1.25	0.302	
		Residuals	47	0.009803	0.0002086			
Fv'/Fm'	Yes	Temperature	2	0.02150	0.010748	9.238	0.00052***	20°C < 25 and 30°C
		Population	3	0.00569	0.001898	1.632	0.19770	
		Temp × Pop	6	0.02193	0.003654	3.141	0.01310*	

		Residuals	39	0.04538	0.001163			
% yield	Yes	Temperature	2	243.4	121.68	4.673	0.0159*	20°C > 25 and 30°C
drop		Population	3	191.1	63.71	2.447	0.0801	
		Temp × Pop	6	356.3	59.38	2.280	0.0581	
		Residuals	35	911.3	26.04			
Alpha	No	Temperature	2	0.001558	0.0007789	8.956	0.000519***	20°C < 30°C
		Population	3	0.002385	0.000795	9.142	< 0.0001***	Can > Mur, Cad & Cul
		Temp × Pop	6	0.000250	0.0000416	0.478	0.820903	
		Residuals	46	0.004000	0.0000870			
Ec	No	Temperature	2	16.56	8.281	2.710	0.0782	
		Population	3	13.72	4.573	1.496	0.2294	
		Temp × Pop	6	45.80	7.634	2.498	0.0371*	
		Residuals	42	128.34	3.056			
Ek	Yes	Temperature	2	4186	2093.0	9.633	0.000329***	20°C < 25 and 30°C
		Populatio	3	5259	1752.9	8.06	0.000208	Can < Mur & Cul

		n				7	***	
		Temp × Pop	6	2411	401.8	1.849	0.110904	
		Residuals	45	9778	217.3			
Pmax	Yes	Temperature	2	17.593	8.797	50.513	< 0.0001***	20°C < 25°C < 30°C
		Population	3	2.567	0.856	4.913	0.00481**	Mur > Cad & Cul
		Temp × Pop	6	0.656	0.109	0.628	0.70688	
		Residuals	46	8.011	0.174			
R	No	Temperature	2	0.00550	0.002752	1.065	0.353789	
		Population	3	0.06125	0.020415	7.896	0.000263***	Cul < Mur & Can; Cad < Can
		Temp × Pop	6	0.05122	0.008537	3.302	0.009219**	
		Residuals	43	0.11117	0.002585			
P/R	No (normal-ity failed)	Temperature	2	466.8	233.39	16.788	< 0.0001***	20°C < 25 and 30°C
		Population	3	92.4	30.80	2.215	0.101	
		Temp × Pop	6	142.4	23.73	1.707	0.144	
		Residuals	41	570.0	13.90			

Compensation irradiance (Ec) was consistently low (5 ± 2 µmol photon m^{-2} s^{-1}) and was neither affected by temperature nor differed among populations (Tables 1 & S2). The saturation irradiance (Ek) also remained low (<100 µmol photon m^{-2} s^{-1}) but significantly increased with temperature ($p < 0.001$), rising from 54 ± 21 to 75 ± 20 µmol photon m^{-2} s^{-1} at 20 and 30°C, respectively. Paralleling the photosynthetic efficiency, Ek also differed among populations ($p < 0.001$), with Atlantic populations saturating photosynthesis at lower irradiances (Tables 1 & S2).

The maximum photosynthetic capacity (Pmax) of C. prolifera doubled with temperature, from 1.13 ± 0.44 to 2.48 ± 0.42 µmol O2 m^{-2} s^{-1}, within the 20 to 30°C range ($p < 0.001$) (Fig. 3 Table 1). Differences in Pmax were also detected among locations ($p < 0.01$), with Mediterranean populations (i.e. Murcia) showing the highest photosynthetic rates, particularly when compared to populations from the Mediterranean− Atlantic transition area (i.e. Cadiz and Culatra) (Fig. 3, Tables 1 & S2).

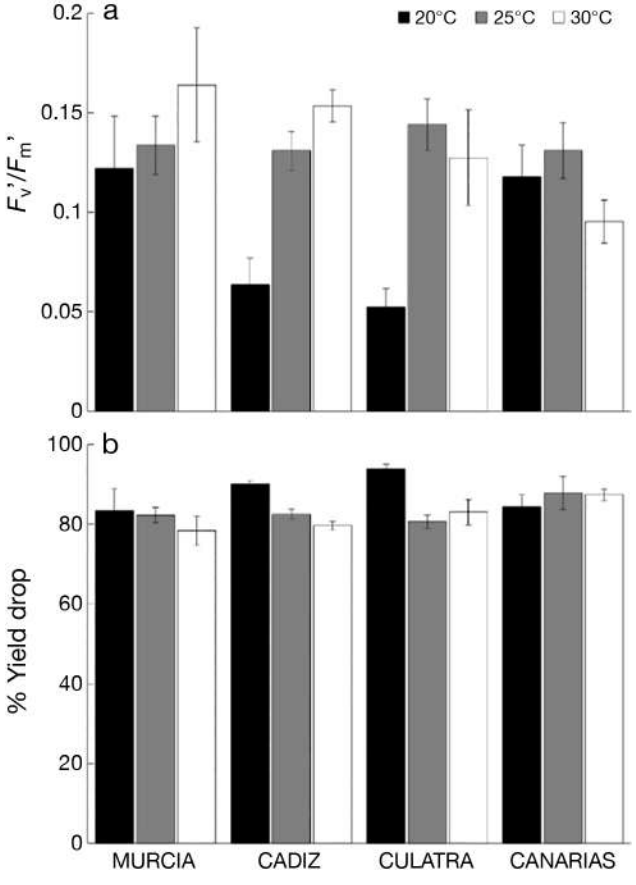

Fig. 2. Fluorescence variables. (a) Effective photochemical yield (Fv'/Fm'). (b) Percentage of maximum quantum yield drop. Bars represent mean ± SE

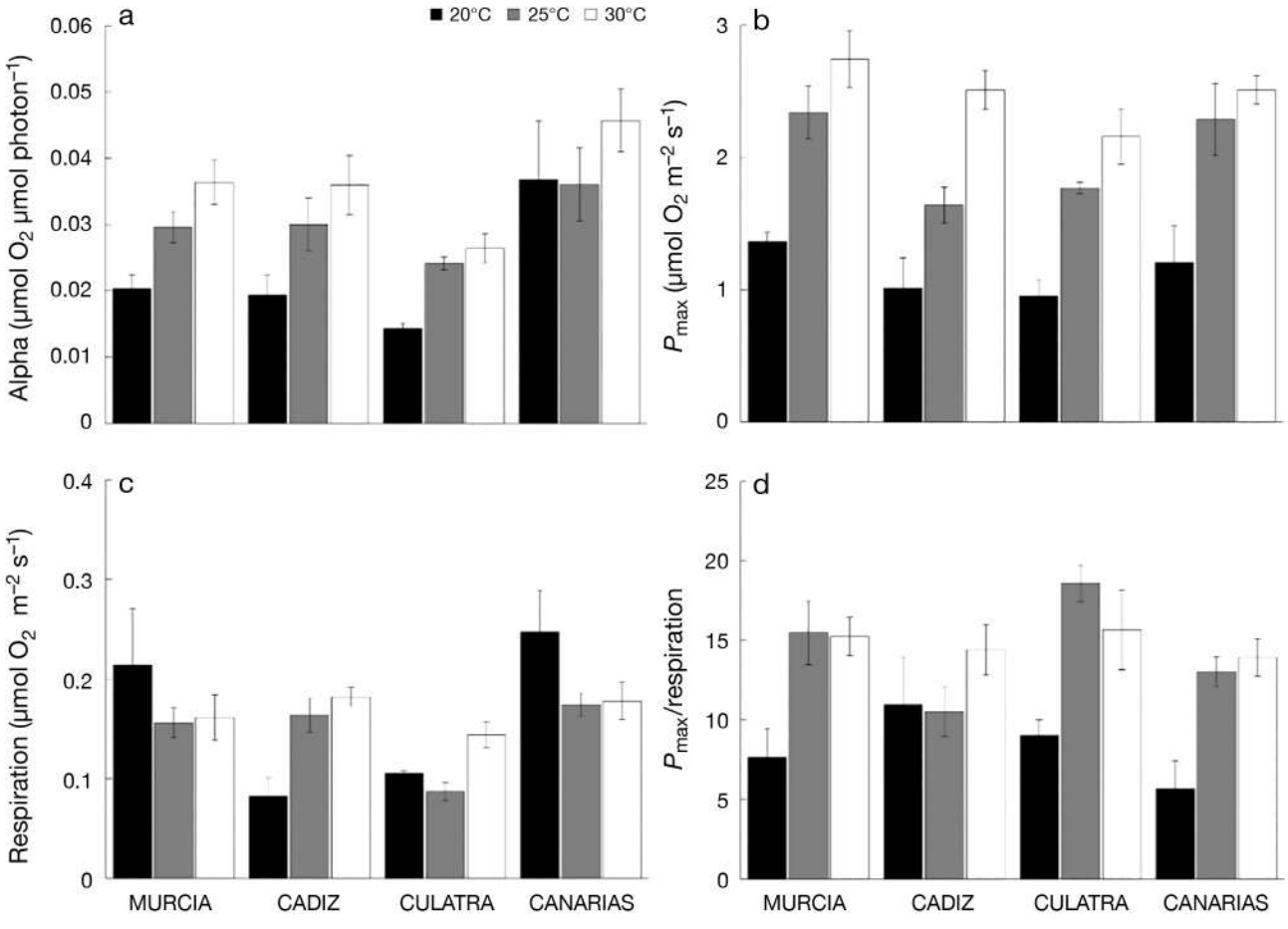

Fig. 3. Photosynthesis vs. irradiance (P–E) curve parameters. (a) Alpha (µmol O_2 µmol photon^{-1}); (b) Pmax (µmol O_2 m^{-2} s^{-1}); (c) respiration (R; µmol O_2 m^{-2} s^{-1}); (d) metabolic quotient (P/R). Bars represent mean ± SE

Contrastingly, respiration of *C. prolifera* did not reveal any pattern with temperature ($p = 0.354$) but differed among the studied populations ($p < 0.001$) (Fig. 3, Table 1). The variability in respiration within populations was higher at 20°C than at 25 or 30°C (Fig. 3, Table S2). Globally, Atlantic (i.e. Canarias) and Mediterranean (i.e. Murcia) populations showed higher respiration rates than populations in the Atlantic–Mediterranean transition

zone.

The metabolic status of *C. prolifera* was evaluated as the ratio between photosynthesis and respiration. The metabolic quotient (Pmax/respiration, P/R) was clearly autotrophic, with photosynthetic rates an order of magnitude higher than respiration (Fig. 3). Temperature significantly increased the metabolic quotient ($p < 0.001$, Table 1), which increased by more than 70%, rising from 8.18 ± 4.21 to 14.19 ± 4.23 between 20 and 25°C, respectively, and remaining high at 30°C (Table 1).

Genetic identity of a new strain of *C. prolifera*

In order to investigate the source of colonization and verify the genetic identity of the strain recently rediscovered in Culatra (Ria Formosa, Portugal), a cpDNA region (tufA gene) was sequenced. The tufA sequences produced in the present study (GenBank accession numbers: MT702819, MT702820, MT 702821) were identical to one another and were also identical to the sequences of *C. prolifera* described for a close location (Fuseta, Portugal) collected in 2011 (Cunha et al. 2013). The sequences from Culatra, Portugal, were also identical to ones within the Mediterranean clade (Varela-Álvarez et al. 2015), confirming the species identity and the Mediterranean origin of the Culatra strain.

DISCUSSION

Our results show a large metabolic thermo- tolerance plasticity in the chlorophyte *Caulerpa prolifera*, revealing the potential of this species to cope with future seawater temperature rise associated with global warming. The photosynthetic performance and the metabolic quotient of *C. prolifera* increased with temperature in all of the populations studied across the Mediterranean−Atlantic border, suggesting a favourable scenario, in terms of productivity and expansion potential, for this species under future warming scenarios.

Physiology of *C. prolifera*

From a photosynthetic perspective, *C. prolifera* has been considered to be a shade-adapted

alga (Häder et al. 1997, García-Sánchez et al. 2012). The high photosynthetic efficiency and low compensation and saturation irradiances measured in our study support this assumption and fall within the range of values previously reported for this species (Terrados & Ros 1992, Enríquez et al. 1995, Vergara et al. 2012). However, maximum photosynthetic performance remained high at irradiances above saturation, with values similar to light-adapted algae species (Longstaff et al. 2002). Despite the high irradiances applied in the P−E curve (circa 1000 µmol photon m^{-2} s^{-1}), no signs of photoinhibition were detected in any of the 4 populations studied.

As expected, photochemical yield dropped at the end of the P−E curves, but the decrease in the effective yield was mainly due to a decrease in maximal fluorescence (F_m) rather than an increase in minimal fluorescence (F_0) (Fig. S2 in the Supplement). These results suggest photoprotection, via an increase in non-photochemical quenching (i.e. heat loss) (Bolhar-Nordenkampf et al. 1989), rather than photoinhibition, as the main energy dissipation mechanism (Demmig-Adams & Adams 1992), challenging the dark-adapted physiology attributed to this species. The high photosynthetic efficiency and sustained photosynthetic rates together with the consistent low respiration rates recorded lead to a high autotrophic balance (P/R), supporting the high productivity attributed to this species (Vergara et al. 2012).

Response of *C. prolifera* to warming

Within the context of ocean warming, rising temperature is expected to affect the activation energy of enzymes involved in global metabolism altering the physiology and metabolic balance of organisms (Yvon- Durocher et al. 2010). Photosynthesis is strongly affected by temperature and is expected to increase as long as temperature rises below the thermal optimum (Way et al. 2015). Optimum Pmax around 30°C has been previously reported in Mediterranean populations of *C. prolifera* during spring−summer periods, while the optimum temperature for photosynthetic efficiency (alpha) has been observed around 20°C (Terrados & Ros 1992). Our results showed how both photosynthetic efficiency (alpha) and photosynthetic potential (Pmax) increased with temperature within the range of

temperatures tested in all *C. prolifera* populations studied with optimum temperatures around 30°C. In line with the photosynthetic improvement, the photochemical conversion (Fv'/Fm') also improved with temperature. The reduction in the yield drop at higher temperatures further indicates an optimization of the electronic flow through Photosystem II with temperature, probably due to maximization of enzymatic reactions linked to the electron transport chain (Farquhar et al. 1980).

Metabolic theory predicts that warming will increase both photosynthesis and respiration in photosynthetic organisms but will decrease the net metabolic quotient (P/R) since respiration is expected to increase more than photosynthesis with temperature (Brown et al. 2004). Terrados & Ros (1992) reported increments in both respiration and photosynthesis with warming, and a relatively stable metabolic quotient, within the 15 to 30°C range. Our results also showed that *C. prolifera* consistently increased photosynthesis with temperature within the range tested, but respiration remained low and unaffected by temperature. Both photosynthesis and respiration traits showed similar plasticity (37 and 41% coefficient variation for Pmax and Rd, respectively), suggesting that the low respiration recorded at the higher temperatures may be a physiological response for energy optimization (partial or negative acclimation) (Larigauderie & Körner 1995, Zha et al. 2003). It is also plausible that the higher temperature tested in our study (30°C) is not high enough to trigger a significant increase in respiration (Hüve et al. 2012). Although a photosynthetic and metabolic thermal threshold above 30°C has been reported for *C. prolifera* communities (Terrados & Ros 1992), our results suggest physiological potential for metabolic optimization beyond this threshold. Further research is needed to confirm the limits and stability of physiological thresholds for *C. prolifera*.

Besides inherent species plasticity, local environment may also contour the population acclimation and adaptation potential shaping thermal thresholds and sensitivity to temperature (Schoepf et al. 2015). Thus, similar increases in seawater temperature may differently affect populations of the same species locally adapted to different thermal environments (Vinagre et al. 2018). The higher mean sea surface temperatures of the Mediterranean, between 25 and 27°C in summer (Garcias-Bonet et al. 2019), compared to

the Atlantic, around 24°C in the Canary Islands (Tuya et al. 2014), lead to our hypothesis that organisms thriving in Mediterranean waters (i.e. the Murcia population) could cope better with higher temperatures than Atlantic counterparts (i.e. the Canary population) and that a physiological pattern in response to temperature could be found along the Mediterranean−Atlantic axis. Slightly different physiological acclimation strategies were detected among populations along the Mediterranean−Atlantic basins with both sun and shade-adapted physiological strategies attributed to Caulerpa spp. (Robledo & Freile-Pelegrin 2005). Mediterranean individuals from Murcia sustained sun-adapted higher photosynthetic rates, while the Atlantic counterparts (i.e. Canarias) showed a shade-adapted high photosynthetic efficiency and lowest saturation irradiance. The population recently rediscovered in Portugal within the Mediterranean-Atlantic transition zone (i.e. Culatra) showed the lowest respiration. The lower metabolic demand of the newly established population can be a physiological strategy for energy optimization that contributed to the colonization of new space. Despite these differences, all populations optimized and increased the metabolic quotient with temperature revealing large physiological plasticity and thermo-tolerance of *C. prolifera* regardless of its biogeographic origin. Our results suggest that all populations of C prolifera studied here have the physiological potential to cope with warming, up to 30°C, regardless of their origin.

Genetic origin and expansion potential of *C. prolifera* in Ria Formosa

The Mediterranean-Atlantic transition zone is a controversial biogeographical boundary for *C. prolifera* expansion between the Mediterranean and the East Atlantic bioregion (Varela-Álvarez et al. 2015). Despite being a limit for biota expansion (Alberto et al. 2008), gene flow of biota from the Mediterranean towards the Atlantic Ocean has been demonstrated in this area (Masucci et al. 2012). The Mediterranean origin of a new *C. prolifera* strain found in Ria Formosa (Culatra) supports the hypothesis that *C. prolifera* reached Portugal from an expansion of a Mediterranean source, as was the case for a strain previously found in Ria Formosa (Fuseta) (Cunha et al. 2013). The population discovered in 2011 in Fuseta has since disappeared, probably as a result of anthropogenic activities (dredging) that destroyed the

meadow (R. Santos pers. obs.). Because of the capacity of this genus to establish from small fragments (Ceccherelli & Cinelli 1999), it is plausible that the population remained alive by re-attaching and spreading westwards towards Culatra. Since occasional sightings of *C. prolifera* have been reported in the last few years on the coast of Huelva (south-western Spain) (de la Rosa-Álamos & Altamirano 2011), it is also plausible that *C. prolifera* strains coming from the Mediterranean expanded towards the Atlantic coast in different waves of colonization. Further research is needed to verify the introduction vectors of *C. prolifera* in Portugal and towards the Atlantic.

Ecological implications

Global warming may benefit species with high physiological plasticity growing in areas with temperatures lower than their thermal optimum. It may create new habitat niches and boost their colonization potential to the detriment of less competitive foundation species (Lima et al. 2007, Harley et al. 2012, Bates et al. 2013). *C. prolifera* is considered an opportunistic species that can tolerate high nutrient and suspended sediment loads, spreading widely in subtidal areas (Cunha et al. 2013). The shadeadapted physiology and metabolic optimization of *C. prolifera* reported in this study could explain its expansion and colonization through the bare deep areas of Ria Formosa channels observed in recent years (Alexandre & Santos 2020). Besides this physiological advantage, the high productivity and energy optimization reported in this study contribute to the fast proliferation and colonization potential of this species and may confer a competitive advantage over other coastal macrophytes, such as seagrasses (Ceccherelli et al. 2000, Stafford & Bell 2006). Considering the high physiological plasticity described in this study and the current expansion of *C. prolifera* in Ria Formosa, it is likely that ocean warming will further favour the expansion of this species. The Ria Formosa strain could act as a thermo-tolerant colonization vector for *C. prolifera* along the Portuguese coast potentially expanding towards the Atlantic basin. This expansion may challenge the functioning and stability of coastal habitats dominated by other foundation species (Marbà & Duarte 2010, Thomsen et al. 2019) and the associated ecosystem services (Bennett et al. 2016). Global warming poses

a favourable scenario for the spread of *C. prolifera* in future years. Further studies are required to track the expansion of this species and to evaluate its ecological implications.

ACKNOWLEDGEMENTS

We thank A. García-Lazzati, supported by an ERASMUS+ traineeship, for his contribution in collecting specimens from Canarias and his assistance in mesocosm maintenance and laboratory experiments. We also thank J. Reis for assistance at the Ramalhete field station and I. Barrote for generously providing the Imaging-PAM. We also thank the 3 reviewers for their comments that helped to improve the manuscript. This study received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 752250, and from the Portuguese Foundation for Science and Technology (FCT) through the national projects RIAVALUE (PTDC/MAR-EST/3223/2014), GRASSMET (PTDC/MAR-EST/4257/2014) and UIDB/ 04326/2020. I.O. was supported by 2 postdoctoral grants (CCMAR/BPD/004/ 2017 and H202-MSCA-IF-EF-ST-752 250), E.V.A. was supported by a postdoctoral grant (SFRH/BPD/ 109452/2015) and a research contract (DL 57/ 2016/ CP1361/CT0037), and E.A.S. was supported by SFRH/ BSAB/ 150485/2019.

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SUPPLEMENTARY INFORMATION

Table S1. Mean, minimum and maximum in situ temperatures reported at locations close to the collection sites.

Population	Mean temperature (ºC)	Min - Max temperature (ºC)	Reference
Murcia	20.5	14.2 - 28.5	Fraile-Nuez et al. (2018)
Cádiz	21.3	11.5 - 27.4	Egea et al. (2019)
Culatra	18.5	10.0 - 27.0	Cravo et al. (2020)
Canarias	21	18.0 - 24.0	Tuya et al. (2014)

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Table S2. Database of variables derived from fluorescence and P-E curves. Maximum quantum yield (Fv/Fm,), effective quantum yield (Fv'/Fm'), % yield drop, Pmax (µmol $O_2 \cdot m^{-2} \cdot s^{-1}$), alpha (µmol O_2 mmol $photon^{-1}$), Ec (µmol $photon \cdot m^{-2} \cdot s^{-1}$), Ek (µmol $photon \cdot m^{-2} \cdot s^{-1}$), Maximum photosynthesis (Pmax, µmol $O_2 \cdot m^{-2} \cdot s^{-1}$), respiration (µmol $O_2 \cdot m^{-2} \cdot s^{-1}$), metabolic quotient (Pmax/respiration, P/R). Data represent mean ± standard deviation (number of replicates).

		Murcia	Cádiz	Culatra	Canarias
Parameter	Temp	Mean ± SD (n)	Mean ± SD (n)	Mean ± SD (n)	Mean ± SD (n)
Fv/Fm	-	0.758 ± 0.013 (13)	0.755 ± 0.014 (13)	0.749 ± 0.013 (13)	0.750 ± 0.017 (12)
Fv'/Fm'	20º C	0.122 ± 0.046 (3)	0.064 ± 0.023 (3)	0.052 ± 0.018 (4)	0.118 ± 0.032 (4)
	25º C	0.134 ± 0.033 (5)	0.131 ± 0.019 (4)	0.144 ± 0.029 (5)	0.131 ± 0.028 (4)
	30º C	0.164 ± 0.057 (4)	0.153 ± 0.018 (5)	0.127 ± 0.053 (5)	0.095 ± 0.024 (5)
% yield drop	20º C	83.36 ± 8.06 (2)	90.17 ± 1.08 (2)	93.87 ± 2.06 (3)	84.44 ± 5.21 (3)
	25º C	82.29 ± 4.24 (5)	82.54 ± 2.49 (4)	80.70 ± 3.69 (5)	87.86 ± 8.39 (4)
	30º C	78.35 ± 7.34 (4)	79.65 ± 2.39 (5)	83.04 ± 7.13 (5)	87.40 ± 3.10 (5)
Alpha	20º C	0.020 ± 0.004 (4)	0.019 ± 0.006 (5)	0.014 ± 0.002 (4)	0.037 ± 0.020 (5)
	25º C	0.030 ± 0.005 (5)	0.030 ± 0.009 (5)	0.024 ± 0.002 (5)	0.036 ± 0.012 (5)
	30º C	0.036 ± 0.007 (5)	0.036 ± 0.010 (5)	0.026 ± 0.005 (5)	0.046 ± 0.010 (5)
Ec	20º C	5.925 ± 4.308 (4)	2.291 ± 1.637 (4)	4.808 ± 1.874 (4)	5.006 ± 1.488 (4)
	25º C	5.658 ± 0.629 (5)	4.658 ± 0.553 (5)	2.876 ± 1.237 (5)	4.511 ± 1.330 (5)
	30º C	5.334 ± 0.987 (5)	7.062 ± 1.207 (4)	5.148 ± 1.868 (5)	5.191 ± 1.754 (4)

Ek	20º C	69.270 ± 15.901 (4)	50.767 ± 18.383 (5)	67.361 ± 19.538 (4)	33.776 ± 11.997 (5)
	25º C	79.415 ± 8.589 (5)	56.047 ± 7.073 (5)	73.724 ± 6.993 (5)	64.556 ± 6.771 (5)
	30º C	76.771 ± 15.534 (5)	84.237 ± 28.485 (4)	82.737 ± 17.351 (5)	56.631 ± 10.824 (5)
Pmax	20º C	1.363 ± 0.140 (4)	1.011 ± 0.517 (5)	0.953 ± 0.234 (4)	1.206 ± 0.615 (5)
	25º C	2.339 ± 0.448 (5)	1.637 ± 0.306 (5)	1.768 ± 0.100 (5)	2.286 ± 0.610 (5)
	30º C	2.741 ± 0.485 (5)	2.509 ± 0.332 (5)	2.158 ± 0.472 (5)	2.509 ± 0.237 (5)
Respiration	20º C	0.214 ± 0.114 (4)	0.082 ± 0.037 (4)	0.105 ± 0.005 (4)	0.247 ± 0.093 (5)
	25º C	0.156 ± 0.033 (5)	0.164 ± 0.038 (5)	0.087 ± 0.020 (5)	0.174 ± 0.026 (5)
	30º C	0.161 ± 0.051 (5)	0.182 ± 0.018 (4)	0.144 ± 0.028 (5)	0.178 ± 0.037 (4)
P/R	20º C	7.666 ± 3.612 (4)	10.968 ± 5.940 (4)	9.026 ± 1.977 (4)	5.686 ± 3.903 (5)
	25º C	15.480 ± 4.457 (5)	10.515 ± 3.491 (5)	18.065 ± 2.281 (4)	13.038 ± 2.139 (5)
	30º C	15.251 ± 2.391 (4)	14.422 ± 3.188 (4)	15.675 ± 5.605 (5)	13.926 ± 2.373 (4)

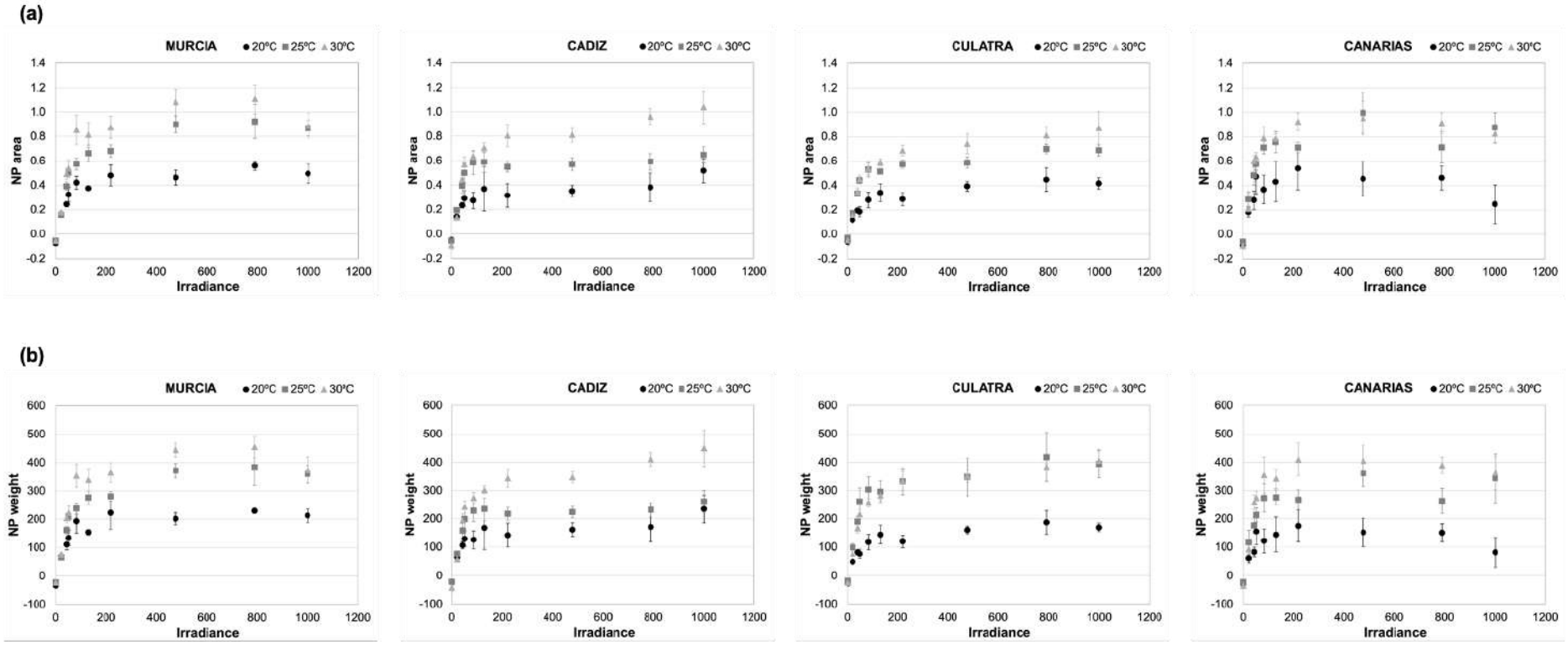

Figure S1. Photosynthesis vs Irradiance (P-E) curves for the four populations of *Caulerpa prolifera* studied. Above row (a) shows the net photosynthesis expressed on area basis (µmol O_2 cm^{-2} h^{-1}). Below row (b) shows the net photosynthesis expressed on dry weight basis (µmol O_2 gDW^{-1} h^{-1}). Irradiance units are µmol photon m^{-2} s^{-1}. Black circles, dark grey squares, and light grey triangles refer to temperature treatments of 20, 25 and 30º C, respectively. Error bars indicate standard error.

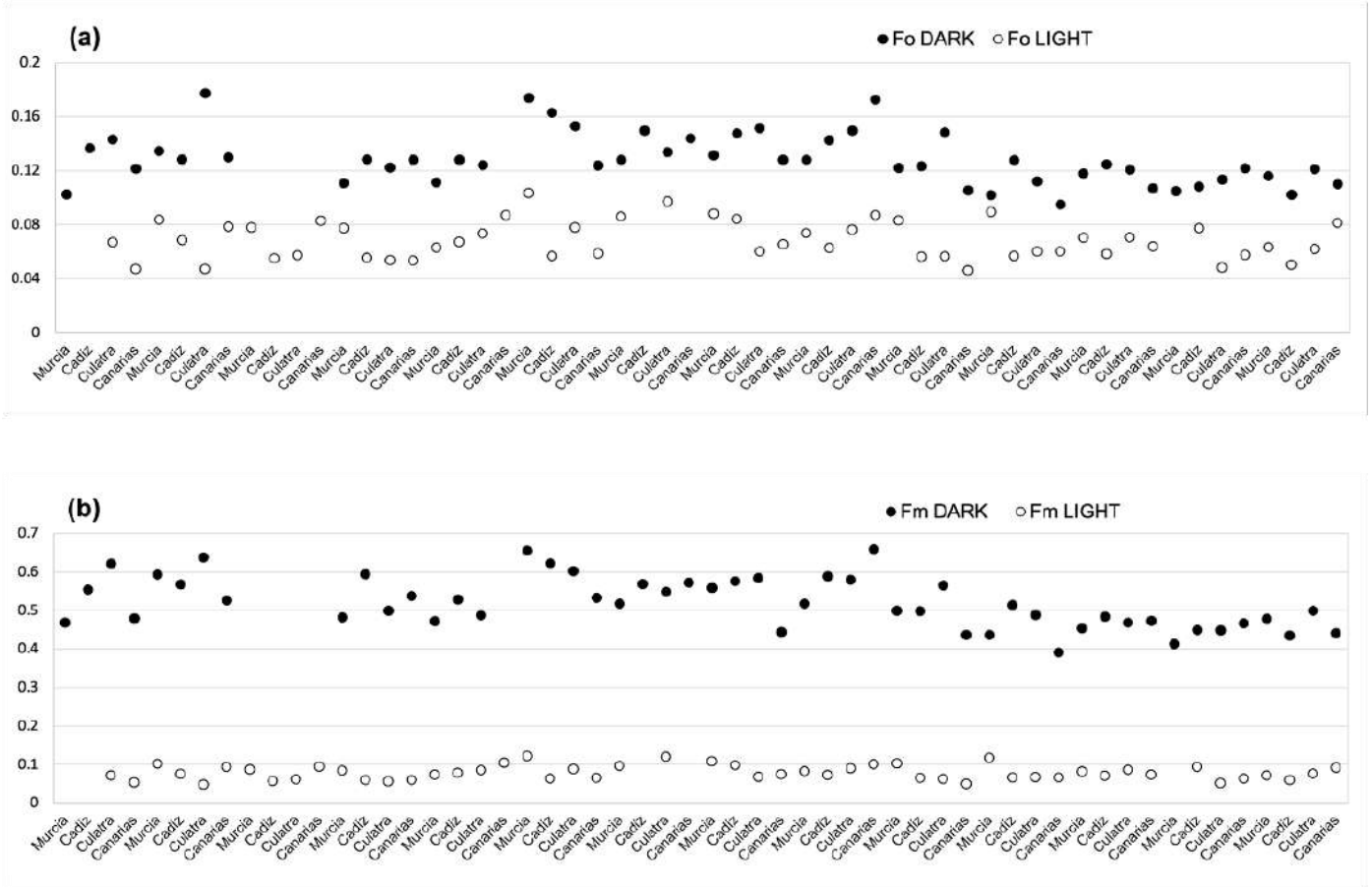

Figure S2. Fluorescence signal in *C. prolifera* specimens from the four populations studied. Minimal fluorescence, F_0, (a); and maximal fluorescence, F_m, (b) measured in darkness before the P-E curves (black circles) and in light after the P-E curves (white circles).