Physiological responses to variations in grazing and light conditions in native and invasive fucoids

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ABSTRACT

Poor physiological acclimatization to climate change has led to shifts in the distributional ranges of various species and to biodiversity loss. However, evidence also suggests the relevance of non-climatic physical factors, such as light, and biotic factors, which may act in interactive or additive way. We used a mechanistic approach to evaluate the ecophysiological responses of four seaweed species (three dominant intertidal fucoids, *Fucus serratus*, *Ascophyllum nodosum*, *Bifurcaria bifurcata,* and the invasive *Sargassum muticum*) to different conditions of grazing, light irradiance and ultraviolet (UV) radiation. We performed a large-scale mesocosm experiment with a total of 800 individual thalli of macroalgae. The factorial experimental design included major algal traits, photoacclimation, nutrient stoichiometry and chemical defence as response variables. Few significant effects of the factors acting alone or in combination were observed, suggesting a good capacity for acclimatization in all four species. The significant effects were generally additive and there were no potentially deleterious synergistic effects between factors. *Fucus serratus*, a species currently undergoing a drastic contraction of its southern distribution limit in Europe, was the most strongly affected species, showing overall lower photosynthetic efficiency than the other species. The growth rate of *F. serratus* decreased when UV radiation was filtered out, but only in the presence of grazers. Moreover, more individuals of this species tended to reach maturity in the absence of grazers, and the nitrogen content of tissues decreased under full-spectrum light. Only the phlorotannin content of tissues of *B. bifurcata* and of exudates of *A. nodosum*, both slow-growing species, were positively affected by respectively removal of UVB radiation and the presence of grazers. The findings for *S. muticum*, a well-established invasive seaweed across European coasts, suggested similar physiological response of this fast-growing species to different levels of grazing activity and light quality/intensity. As expected, this species grew faster than the other species. *Bifurcaria bifurcata* and *A. nodosum* only showed minor effects of light quality and grazing on phlorotannins content, which suggests good resistance of these two long-lived species to the experimental conditions. Mechanistic approaches that are designed to analyse interactive effects of physical and biotic factors provide an understanding of physiological responses of species and help to improve the confidence of predictive distribution models.

1. Introduction

Intertidal species, which inhabit the interface between land and sea, endure highly variable oceanic and atmospheric environmental conditions (e.g. Helmuth et al., 2002) such as changing levels of light, salinity and temperature (reviewed in Lobban and Harrison, 1994; Hurd et al., 2014). Canopy-forming macroalgae are the most important structural engineers of temperate intertidal ecosystems and play important biotic roles such as providing habitat, shelter and food for the accompanying flora and fauna (Wikström and Kautsky, 2007).

Environmental stress associated with climatic and non-climatic factors and biotic stressors can affect macroalgae at biochemical, ecophysiological, morphological and population levels (Weidner et al., 2004; Martínez et al., 2012a; Celis-Plá et al., 2014; Fernández et al., 2015). Although light promotes photosynthetic activity, seaweeds may be physiologically damaged by excessive solar irradiance, including increased levels of UV radiation, particularly during low tide (Figueroa and Viñegla, 2001; Gao and Xu, 2010; Figueroa et al., 2014). The increased levels of solar radiation associated with weaker radiative effects of clouds and aerosols in temperate latitudes, already observed in

temperate latitudes (Wild, 2012; Sanchez-Lorenzo et al., 2013), may compromise the performance and/or survival of seaweeds (Quintano et al., 2013), especially in intertidal habitats. Regarding biotic constraints, the actions of grazers may compromise growth, photosynthesis and reproduction of macroalgae (Svensson et al., 2009; Rothäusler et al., 2009; Kraufvelin, 2017) and thus strongly affect the distribution and biomass of the latter. In some areas, grazers can remove up to 100% of macroalgae present (e.g. Weidner et al., 2004). Not only direct grazing, but also release of chemical cues by grazers may have strong effect on population dynamics and physiological performance of macroalgae (Rohde and Wahl, 2008), although responses may vary depending on the identity of grazers (Moore et al., 2007).

Macroalgae show different levels of sensitivity to stress depending on species, morpho-functional group, ecological strategy and life cycle (Figueroa et al., 2014; Hurd et al., 2014). Overall, the physiological vulnerability and acclimatization of seaweeds to UV radiation will depend on the balance between photoprotective mechanisms, accumulation of antioxidants and activation of antioxidant enzymes, as well as the accumulation of UV-screening photoprotective compounds, such as phlorotannins in brown macroalgae (Abdala-Díaz et al., 2006; Figueroa et al., 2014). These compounds have been associated with various cell functions and in particular, as deterrents to grazers (e.g. Pavia and Toth, 2000). The protective role of phlorotannins may be important both intracellularly and extracellularly, mainly *via* exudation (Koivikko et al., 2005). Nevertheless, the role of phlorotannins in inducible or constitutive defence against grazers is highly controversial (e.g. Kubanek et al., 2004; Toth et al., 2005; Long et al., 2013), and it is possible that factors such as phlorotannin structure, grazer gut environment or geographical variation may mediate the effects (Kubanek et al., 2004).

Although intertidal macroalgae are characterized by a plastic ecophysiology, responses to stress are species-specific and vary depending on the environmental context (Einav et al., 1995; Figueroa et al., 2014). This is of particular importance when comparing native and invasive species because the latter are expected to show greater physiological plasticity and a broader physiological tolerance to abiotic and biotic factors (reviewed in Schaffelke et al., 2006; Pansch et al., 2008; Engelen et al., 2011).

A mechanistic understanding of the factors that determine the performance of different species is essential for studying their resilience to environmental changes (Fernández et al., 2015; Martínez et al., 2015). Poor physiological acclimatization in response to climate change has resulted in the decline of populations in numerous species worldwide, in association with shifts and retreats in the distribution ranges of marine and terrestrial species and the spread of invasive species of warm affinity (Hickling et al., 2006; Perry et al., 2005; Mieszkowska et al., 2006; Lima et al., 2007; Tylianakis et al., 2008). However, nonclimatic physical and biotic factors also play a fundamental role in shaping the distribution of species (Martínez et al., 2012a; Hansen and Stone, 2015; Godsoe et al., 2017).

Interactive effects among environmental and biotic factors on physiological performance of species can cause unexpected changes in species distribution (Darling and Côté, 2008; Godsoe et al., 2017). Although it has been often assumed the prevalence of synergistic interactive effects, additive and antagonistic effects are more common than expected, which suggests highly idiosyncratic responses (Crain et al., 2008; Darling and Côté, 2008). Potentially synergistic effects between physical and biotic factors in the intertidal zone have been reported (Harley et al., 2006; Kubicek et al., 2011). However, scant evidence of non-additive effects between physical factors involved in the ongoing decline of canopy-forming species at their geographic distributional limits has been obtained in several previous experimental studies (Martínez et al., 2012a; Ferreira et al., 2014; Celis-Plá et al., 2014; Fernández et al., 2015). Previous research on the interactive effects of solar radiation and grazing activity on brown macroalgae did not yield conclusive results (Lotze et al., 2002; Macaya et al., 2005; Kubicek

et al., 2011; Rothäusler et al., 2011). While both factors may severely affect the productivity of early recruits (Lotze et al., 2002), adults can show efficient photoacclimation and be less affected by grazers (Macaya et al., 2005; Rothäusler et al., 2011).

In the present study, we aimed to investigate the interactive effects of solar radiation and grazing activity on the ecophysiological responses of intertidal fucoids, including a well-known invasive species that dominate European coasts. We focused on four habitat-forming fucoid species: the cold-temperate *Ascophyllum nodosum* (Linnaeus) and *Fucus serratus* Linnaeus, the southern lusitanic *Bifurcaria bifurcata* R. Ross, and the invasive Asiatic species *Sargassum muticum* (Yendo) Fensholt *Ascophyllum nodosum* has a scattered but persistent geographic distribution in southern Europe, although some populations have recently retreated, at least in the Bay of Biscay and on the coast of Asturias, N Spain (Viana et al., 2014). *Fucus serratus* has been undergoing a drastic contraction of its distribution range on the northern Spanish coast during the last decade (Viejo et al., 2011; Duarte et al., 2013), whereas the distribution of *B. bifurcata* and *S. muticum* are currently expanding towards southern Portugal and Morocco (Lima et al., 2007; Martínez et al., 2012b; Sabour et al., 2013). The latter species is now successfully established on European coasts (Farnham et al., 1973). Overall, these distributional trends suggest that these species show different levels of sensitivity to climatic and non-climatic physical factors (Svensson et al., 2009; Martínez et al., 2012a; b; Fernández et al., 2015; Martínez et al., 2015).

We performed a factorial mesocosm experiment in order to evaluate the ecophysiological response of the above-mentioned macroalgae to the combined effects of light irradiance, UV radiation and grazing by intertidal invertebrates. Specifically, we assessed major vital traits (growth rate and reproductive output), photoacclimation (by PAM fluorometry), nutrient stoichiometry (total C and N), and chemical defence (phlorotannin contents of tissues and exudates). These variables were considered functional indicators of the physiological responses to stress conditions and consequently of the capacity for acclimatization (see Figueroa et al., 2014). We expected that the ecophysiological performance of the four species, in relation to their current distributional trends, would be affected differently and that the successful invader *S. muticum* would show an enhanced acclimatization capacity. On the basis of our previous findings about physiological responses of fucoids to diverse environmental factors (Martínez et al., 2012a; Ferreira et al., 2014; Fernández et al., 2015), we also expected additive effects to be of greater importance than interactive effects.

2. Material and methods

2.1. Collection sites and organisms

One week before the start of the experiment (31 August 2012), vegetative fronds of *B. bifurcata* and *S. muticum* were collected from intertidal shores at Cabo Estai (42°11′ N, 8° 48′ W), whereas *A. nodosum* was collected at Rande (42°17′ N, 8°39″ W), both in the Ria de Vigo, NW Spain. Vegetative fronds of *F. serratus* were collected at Amorosa (41° 38′ N, 8°49′ W), N Portugal. The fronds were immediately transported to the laboratory in a cool icebox and submerged in seawater to a depth of about 8 cm in a 300 L aerated tank outdoors until required. Before being weighed, all experimental fronds were carefully cleaned to remove grazers and epiphytes, and excess water was removed with absorbent paper.

Two very abundant mesograzers, the snails *Gibbula umbilicalis* (da Costa) and *Littorina obtusata* (Linnaeus), which are naturally associated with the selected macroalgae, were chosen for this study. The snails were collected by hand from the intertidal zone of Cabo Estai and Rande (both in the Ría de Vigo. In the laboratory, the gastropods were maintained outdoors in a 30 L aerated tank and fed ad libitum with the four macroalgae until the start of the experiment. Snails of similar size were used to minimize size-specific differences in feeding behaviour

(Pavia et al., 1999).

2.2. Experimental set-up and design

The experiment was conducted between 9 September and 23 November 2012 (46 days). The macroalgae were incubated in small plastic tanks (20 L), i.e. incubators (N = 20), placed inside larger PVC tanks (250 L). The large tanks were used as insulating water baths to prevent abrupt temperature shifts in seawater inside the incubators (see Fig. S1 in Appendix A). Four interconnected PVC tanks were filled with fresh water, which was maintained at a constant temperature of 15.8 °C ± 0.17 (mean ± S.E., n = 56) by using seawater chillers (Aqua Medic Titan®). The system was located outdoors at the CIIMAR (Porto, Portugal).

In each 20 L incubator, 10 individual fronds of each species (i.e. a total of 40 specimens and an initial total number of fronds across experimental treatments of 800), chosen at random, were tagged, attached to a square plastic frame with lines, and submerged in seawater to a depth of about 8 cm (Fig. S1). An aluminium frame was placed on top of each PVC tank, and two UVA-340 fluorescent tubes (3M Company, USA) were hung from the frame. These lights were turned on for 6 h d^{-1} (09:00 to 15:00 h). The natural day length during the induction experiment was about 10 h. The distance from the frames to the algae was ~0.5 m, and the lamps increased the UVA irradiance by approximately 0.5% above ambient. UVA can be an important driver in regulating variations of physiological performance of macroalgae (Quintano et al., 2013). To prevent nutrient limitation, the seawater in the incubators was enriched every two days by adding inorganic N (NaNO$_3$) and P (NaH$_3$PO$_4$) to final concentrations of respectively > 50 μM N and 5 μM P. This nutrient enrichment protocol has been shown to enhance macroalgal growth in previous cultivation trials (Martínez et al., 2012a). The salinity of the water was monitored daily with a refractometer. When the salinity reached > 35 ppt (standard seawater salinity), fresh water was added, and when it reached < 35 ppt, seawater was added. An air circulating system with air pumps was used to provide agitation and oxygenation to each incubator. The macroalgae were allowed to acclimatize to these conditions for 6 days before the start of the experiment.

Different cut-off filters were chosen for the 5 light treatments (see Figueroa et al., 2010, 2014): (1) treatment PAB allowed transmission of UV radiation (280–700 nm, full sunlight without filter), (2) treatment PC was a procedural control for filters, allowing full transmittance (280–700 nm, Ultraphan 295 filter), (3) treatment 33% PAB reduced the photosynthetically active radiation (PAR) by 33% (double neutral mesh), (4) treatment PA blocked UVB < 320 nm (320–700 nm, LEE 130 filter), and (5) treatment P blocked all UVR < 400 nm (400–700 nm, LEE 226 filter). Grazers were either present (grazing treatments, 10 incubators) or absent (no grazing treatment, 10 incubators). Grazing consisted of two treatments: (1) a test for waterborne cues from nearby grazed co-specific macroalgae and grazing gastropods, and (2) a test for additional grazers attack. For tests of waterborne cues, 13 grazers of each species together with ~10 g of each macroalga were placed in a net breeder (14 × 13 × 17 cm) lining the bottom of each incubator, thus preventing contact with the experimental fronds. Densities of grazers in the experimental treatments were chosen to represent realistic densities as found in the field (5.17 ± 0.52 individuals per 0.04 m^2). Pieces of macroalgae and grazers in the net breeders were replaced every 7 days. Previous studies have shown that defence induction by mesograzers occurs within 7–14 days (Weidner et al., 2004; Rohde and Wahl, 2008). For the additional grazer attack treatment, half of the fronds subjected to the waterborne cues treatment were transferred to 4 small tanks. In these tanks, grazers (13 of each species) were allowed to attack the fronds for 4 h every third day throughout the entire experimental period, i.e. a total of 10 times. The fronds were returned to the original incubator after each grazing period to ensure that they were exposed to the same

concentration of waterborne cues as those individuals not directly exposed to grazers. Before returning to the original incubators, thalli were checked for feeding scars to ensure that the treatment was effective.

2.3. Environmental measurements

Radiation measurements. PAR (400–700 nm), UVA (315–400 nm) and UVB (280–315 nm) were monitored daily every 5 min with an on-site meteorological station (HOBO® U30 station). The spectral characteristics (280–800 nm) of the incubators (cages constructed with white plastic and covered with neutral and UV cut-off filters) were also determined and calibrated every day at solar noon, with the aid of a photo-radiometer (DeltaOHM®, HD 2012.2) connected to cosine PAR, UVA and UVB quantum sensors (n = 46).

2.4. Biological measurements

2.4.1. Biochemical composition of macroalgae

The total carbon and nitrogen contents of the macroalgae were determined in five samples of each species prior to the experiment and in two samples randomly chosen from the different treatments at the end of the experiment. Samples were dried at 60 °C for 48 h and ground to a fine powder, and the total carbon and nitrogen contents were determined using a CHN elemental auto-analyser (Perkin Elmer Analytical Instruments, Shelton, CT, USA).

The phlorotannin contents of the macroalgal tissue and exudates of two randomly chosen individuals from the different treatments were determined at the end of the experiment. Pieces of tissue of each species were transferred to 2 mL Eppendorf tubes and frozen at −80 °C until analyses. Phlorotannins were extracted from macroalgal tissue with 70% aqueous acetone (Koivikko et al., 2005) and samples were centrifuged (10 min at 7680 g). The total phlorotannin contents of the extracts were determined according to a modification of the Folin–Ciocalteu method (Waterman and Mole, 1994), with phloroglucinol (ICN Biomedicals Inc.) as a standard agent. Samples were diluted taking into account the measurable range of the spectrophotometer. A 0.5 mL aliquot of the diluted sample was mixed in a test tube with 2.0 mL of 1 N Folin-Ciocalteu reagent (Merck). The mixture was allowed to stand for 3 min, after which 2.0 mL of 20% Na$_2$CO$_3$ was added. Samples were incubated in darkness at 40 °C for 15 min, and the absorbance of the supernatant was then measured at 765 nm in a Jasco UV-500 spectrophotometer. Phlorotannin exudates were obtained by enclosing small pieces of tissue in 10 mL clear plastic tubes filled with seawater, for 6 h to concentrate the exudate. All samples were frozen at - 80 °C until further analyses. In this case, a spectrophotometric method based on UV absorption was used because salts were precipitated by the Folin-Ciocalteu reagent. Phlorotannins and phlorogucinol show a characteristic peak at 267–270 nm with the left-hand height proportional to phloroglucinol concentration. The phloroglucinol concentration was therefore calculated as a linear function of differences in absorbance at 267 nm and 250 nm (absorbance [peak 267 nm, trough 250] = 0.0018 [phloroglucinol mg L^{-1}]- 0.0005, R^2 = 0.9993, p < 0.001).

2.4.2. Growth of macroalgae and reproductive output

All individual fronds were weighed once a week to the nearest 0.1 g (N = 800). The growth response was estimated as the change in wet weight by using equation 1:

$$\text{Growth} = (P_o - P_i)/t$$

where P_o is the initial wet weight, measured at beginning of the experiment, P_i is the wet weight at time *I*, and *t* is the time since P_o, in days. As the weekly growth rate was similar to the growth rate calculated between the start and the end of the experiment, only the latter was finally used in the analysis (weekly values not shown).

Reproductive output was only determined in individuals of *F.*

serratus, *B. bifurcata* and *A. nodosum* as maturity was reached in a relatively large number of individuals of these species. Individuals were considered capable of reproduction when reproductive structures, i.e. receptacles, were present. The individuals were classified as reproductive male, reproductive female, reproductive with unidentified sex or non-reproductive. In mature individuals, the portion of the thallus bearing the reproductive structures was excised and the wet biomass of reproductive and vegetative parts was determined separately after the tissues were dried with absorbent paper.

2.4.3. Chlorophyll fluorescence measurements

In situ chlorophyll fluorescence associated with photosystem II (PAM 2000 and MINIPAM, Heinz Walz GmbH, EVeltrich, Germany) was measured to characterise the photosynthetic status of the macroalgae. Maximum quantum yield (F_v/F_m) was used as an indicator of physiological stress and potential damage of the algae (Maxwell and Johnson, 2000). Algae were dark adapted for a minimum of 15 min to allow complete relaxation or oxidation of reaction centres and determination of basal fluorescence (F_o). A saturation pulse was then applied to determine the maximal fluorescence (F_m), with $F_v = F_m - F_o$. Maximum quantum yield was measured in 15 individuals of each species during the acclimatization period, i.e., to describe the initial baseline response, and after 26 and 39 days under experimental conditions (n = 5 and n = 3, respectively), i.e., to investigate the final response to the treatments. Additionally at the end of the experiment, rapid photosynthesis *versus* irradiance curves (PI curves) were recorded in macroalgae subjected to the different combinations of treatment (n = 3). Samples were irradiated with increasing irradiances of actinic red light (650 nm; PPFD = 15–1500 µmol m^{-2} s^{-1}). A saturating pulse was applied every 30 s to measure effective quantum yield of PSII (ΔF/Fm′) before actinic irradiation was further increased. The relative electron transport (rETR) was calculated by multiplying quantum yield by photon irradiance (Walz, 2014, and references therein). Relative ETRs *versus* irradiance function curves were fitted according to the model proposed by Platt and Gallegos (1980), to obtain the maximal relative ETR (ETR$_{max}$), the photoinhibition (β), the initial slope of rETR *versus* irradiance function (α) and the irradiance for half saturation of rETR (E_K) calculated from the intercept between ETR$_{max}$ and α.

2.5. Statistical analysis

General linear models were used to explore the influence of grazing and light quality on the different physiological response variables. All analyses were performed separately for each species due to intrinsic differences between species, as revealed by preliminary analyses carried out before the experiment.

Both grazing treatments were placed in the same incubators to be exposed to the same concentration of waterborne cues (i.e. the two treatments only differed in the exposure to bites by grazers), which may have led to spatial autocorrelation in the data. We therefore performed one-way analyses of variance to evaluate the effect of both grazing treatments on the diverse response variables. There were no significant differences between the two grazing treatments in any of the comparison (p > 0.25 in most cases, data not shown), and these two treatments were therefore pooled in the analyses. To test whether growth, F_v/F_m, ETR$_{max}$, α, β or E_k of the species varied in relation to grazing and light quality at the end of the experiment, we applied a model with Grazing (2 levels: pooled grazing treatments and no grazers) and Light quality (5 levels) as orthogonal fixed factors, and Incubator as a random factor nested within the interaction (Grazing x Light quality). To test whether the total nitrogen and carbon contents of tissues and phlorotannin contents of exudates and tissues varied with grazing and light quality at the end of the experiment, we applied a model with Grazing and Light quality as orthogonal fixed factors. We used a Type II Sum of Squares ANOVA (Langsrud, 2003) to deal with unbalanced data. When significant main factors or interactions were indicated, a posteriori

Student Newman Keuls tests were applied. Normality of variances of all response variables was checked using normal quantile-plots. In addition, the homogeneity of variance was tested using Cochran tests (Underwood, 1997) and by visual inspection of the residuals.

To test whether the reproductive stage (binary response 0 or 1) of individuals was affected by grazing and light quality, we applied a logistic model with Grazing and Light quality as orthogonal fixed factors. A binomial distribution of the error terms was assumed and logit-link function was used. Analyses were restricted to those species that reached maturity (*F. serratus*, *B. bifurcata* and *A. nodosum*). All analyses were conducted using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Irradiance conditions in mesocosms

The weather was generally cloudy during the experiment. The mean (± S.E.) photosynthetically active radiation (PAR) during daylight hours (from 8:30 a.m. to 7:00 p.m.) was 479 (± 93) µmol m^{-2} s^{-1}, and UVA and UVB irradiance, 0.96 (± 0.18) and 84 (± 21) W m^{-2}, respectively. The UV/PAR ratios ranged from 0.06 to 0.21. During the experiment, the maximum mean irradiance (± S.E.) reached at around 12:00 h (local time) was respectively ∼900 (± 81) µmol m^{-2} s^{-1} of PAR, and 1.75 (± 0.26) and 184 (± 18) W m^{-2} for UVA and UVB. The irradiance close to the fronds was lower than the incident solar irradiance due to light absorption by the experimental incubators and filters used, which successfully manipulated the light spectrum (Fig. 1).

3.2. Ecophysiological response of species

3.2.1. Sargassum muticum

This species showed the fastest growth rate of the four species considered. The growth was constant across treatments and was not significantly affected by either light quality/intensity or grazing treatment (Fig. 2a, Table 1). Individuals did not reproduce during any of the treatments as the reproductive period occurs during spring (Olabarria et al., 2009). The parameters characterizing the *in vivo* photosynthetic performance did not vary significantly across treatments (non-significant ANOVA results, not shown). Despite the high growth rate, this species did not show the highest photosynthetic potential, with intermediate values of ETR$_{max}$ and α, and the saturation plateau occurred at a lower irradiance, i.e. a lower value of E_k than for the other studied species (Fig. 3, Table 2). The high F_v/F_m value suggested optimal performance of the photosynthetic machinery (as in all target species; not mentioned hereafter).

As observed for the growth and the photobiological response, none of the biochemical compounds (i.e. total N and C, and phlorotannin contents of tissue and exudate) varied in response to the treatments (Table 3, non-significant ANOVA results not shown). The tissue concentration of phenolic compounds was lower than in the other species, but not the exudate concentration that was similar to that of *B. bifurcata* (Table 2). Overall, the results suggest the physiological tolerance of this fast-growing invasive species to variations in grazing activity and light quality/intensity.

3.2.2. Fucus serratus

Fucus serratus was the species most strongly affected by both grazing activity and light quality, and interactive effects were observed in some cases. It grew slower than *S. muticum*, but faster than the other longer-lived perennial species (Fig. 2b). The net growth rate was higher when individuals were exposed to natural light conditions, i.e. treatments PC and PAB, in respectively the presence and absence of grazers (SNK tests, p < 0.05; Fig. 2b, Table 1). However, when UV radiation was filtered, the growth rate decreased, especially in the presence of grazers (SNK tests, p < 0.05). Although individuals tended to become fertile in the absence of grazers, the effect of grazers was not significant (Fig. 4a).
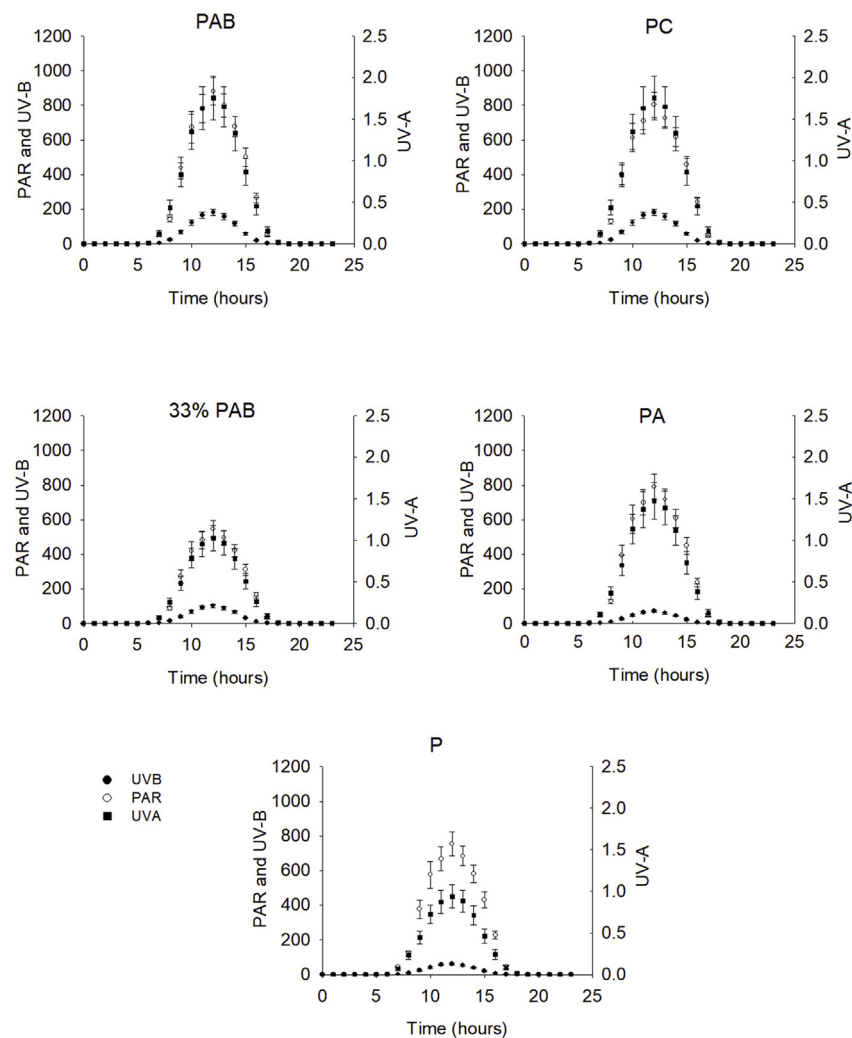
Fig. 1. Mean (± S.E.) transmitted irradiance of photosynthetically active radiation (PAR, 400–700 nm), UVA radiation (315–400 nm) and UVB radiation (280–315 nm) in different treatments: PAB (full sunlight, no filter), PC (procedural control treatment allowing full transmittance, Ultraphan 295 filter), 33% PAB (treatment reducing PAR in 33%, double neutral mesh), PA (treatment blocking UVB, LEE 130 filter) and P (treatment blocking all UVR, LEE 226 filter); n = 46.

The N content was maximal when PAR was reduced by 33% with the neutral mesh filter (SNK tests, p < 0.05; Fig. 5a, Table 3). Accordingly, the mean E_k value for this treatment, irrespective of the grazing activity, was significantly lower, suggesting that algae achieved maximal ETR at the lowest PAR irradiance treatment. The mean E_k was even lower for individuals exposed to the full light spectrum in the absence of grazers (SNK tests, p < 0.05, Table 4). The F_v/F_m also varied significantly with light conditions, and the lowest value was reached when individuals were exposed to the full-light spectrum, but under the filter (PC) (SNK tests, p < 0.05, Table 4). Overall, data on photosynthetic parameters suggest that the photosynthetic potential of this species for absorbing and quenching incoming light, i.e. low α and ETR$_{max}$, is lower than that of the other species under all experimental conditions. Nevertheless, this species showed greater resilience to high light conditions, as the rETR was relatively low, but constant under all experimental types of irradiance, suggesting low inhibition i.e. the lowest β and the highest E_k (Fig. 3, Table 2).

The C content was again constant across treatments (in this and all the target species, hereafter not mentioned). Although the phlorotannins content was not significantly influenced by experimental treatments in this species, the mean phlorotannins concentrations in both tissues and exudates were higher compared to the other species. (Table 2).

3.2.3. Bifurcaria bifurcata

This long-lived species showed a slow growth rate, which was not affected by the experimental treatments (Fig. 2c, Table 1). A large number of algae reached reproductive maturity during the experiment (between 40 and 100%), which suggests optimal conditions for growth and reproduction in all treatments. Although not significant, the proportion of individuals that reached reproductive maturity tended to be greater in the absence of grazers, except when PAR was reduced by 33% (Fig. 4b). Photosynthetic performance was not significantly affected by any of the experimental treatments. The light curves indicated high photosynthetic efficiency under low lighting i.e. high α values for high maximal ETR rate, and an intermediate inhibition rate (β, Fig. 3, Table 2).

Of all biochemical compounds considered, only phlorotannins in tissues varied significantly with light quality, and the content was higher when UVB was filtered out, i.e. P and PA treatments (SNK tests, p < 0.05; Fig. 5b, Table 3). Again, the results indicated that this species acclimatized well to experimental conditions.

3.2.4. Ascophyllum nodosum

This perennial, very long-lived (up to decades) species was the slowest growing of the four species, with no significant effect of experimental treatments (Fig. 2d, Table 1). Regarding its photosynthetic potential the species showed the highest α and ETR$_{max}$, which suggests
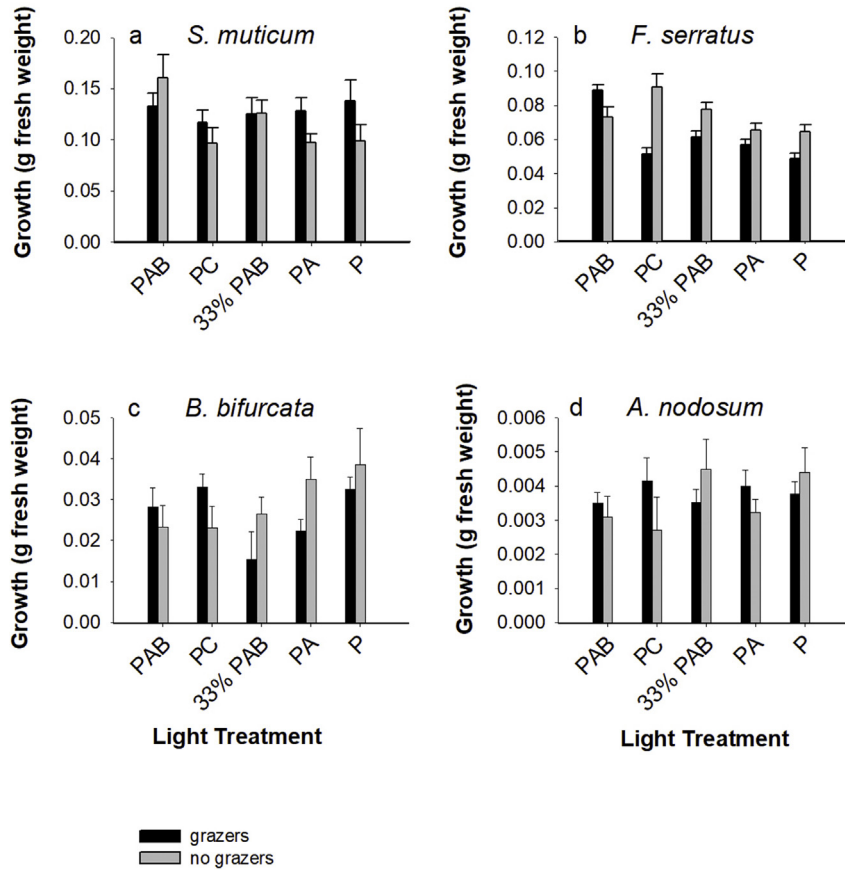
Fig. 2. Mean (+S.E.) growth rates (the difference between the initial and final fresh weights in g) of each species at the different experimental treatments after 7 weeks; n = 10. Data from the two incubators and the two grazing treatments were pooled.

good performance, as also shown by *B. bifurcata* relative to the other two macroalgae (Fig. 3, Table 2). The inhibition coefficient (β) was therefore also highest for this species. A few of the plants reached reproductive maturity, and although a larger number of individuals tended to do so in the absence of grazers, this effect was not statistically significant (Fig. 4c).

The phlorotannins content of the exudates was low, but was significantly higher in the presence of grazers (SNK tests, p < 0.05; Fig. 5c; Table 3).

4. Discussion

Overall, the target macroalgae showed a good physiological performance under the experimental conditions, although with variable success, which suggests different degrees of sensitivity and ecophysiological strategies. Our study encompassed levels of grazing and

irradiance that the target species experience naturally during immersion in the field although maximal solar radiation during emersion can be higher than that used in our experiment and, therefore, impacts on physiological performance may be greater than those observed. All species, especially *S. muticum*, grew during the experiment, became mature when the study period overlapped with their reproductive period, and exhibited high F_v/F_m values (above 0.7), suggesting good physiological potential for acclimatization. However, we observed some significant inhibitory responses, especially on the declining canopy-forming fucoid *F. serratus*.

We mainly observed additive effects (4 out of 6) on ecophysiological responses of macroalgae for the values tested. Light quality affected significantly the maximal quantum yield (F_v/F_m) and the tissue nitrogen content of *F. serratus*, and the tissue phlorotannins content of *B. bifurcata*, whereas grazing activity had a significant effect on the exudate phlorotannins content of *A. nodosum*. These findings reinforce

Table 1
General linear models of the effect of grazing and light quality on the growth of the four macroalgae. Grazing and Light quality were orthogonal fixed factors and Incubator was a random factor nested within the interaction Grazer x Light quality. Variances were homogeneous.

	df	*S. muticum*		*F. serratus*		*B. bifurcata*		*A. nodosum*	
		F	p	F	p	F	p	F	p
Intercept	1	195.99	**< 0.001**	175.27	**< 0.001**	130.63	**< 0.001**	652.14	**< 0.001**
Grazing (G)	1	0.49	0.498	2.07	0.222	0.475	0.504	0.314	0.584
Light quality (L)	4	0.35	0.833	1.14	0.452	1.141	0.396	0.471	0.756
G x L	4	0.49	0.744	3.75	**0.038**	0.866	0.504	1.925	0.166
Incubator (G x L)	10	0.48	**< 0.001**	3.88	**< 0.001**	2.316	**0.016**	0.758	0.668
Residual	130								

Significant p-values are indicated in bold.
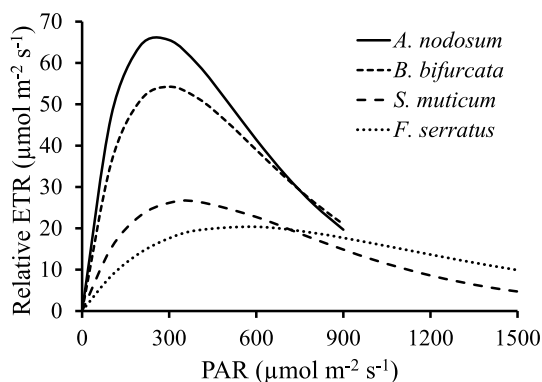
Fig. 3. P-I curves for each species. Regressions were fitted according to Platt and Gallegos (1980). Data from different treatments were pooled, n = 90, except for *F. serratus* (n = 87) and *A. nodosum* (n = 88).
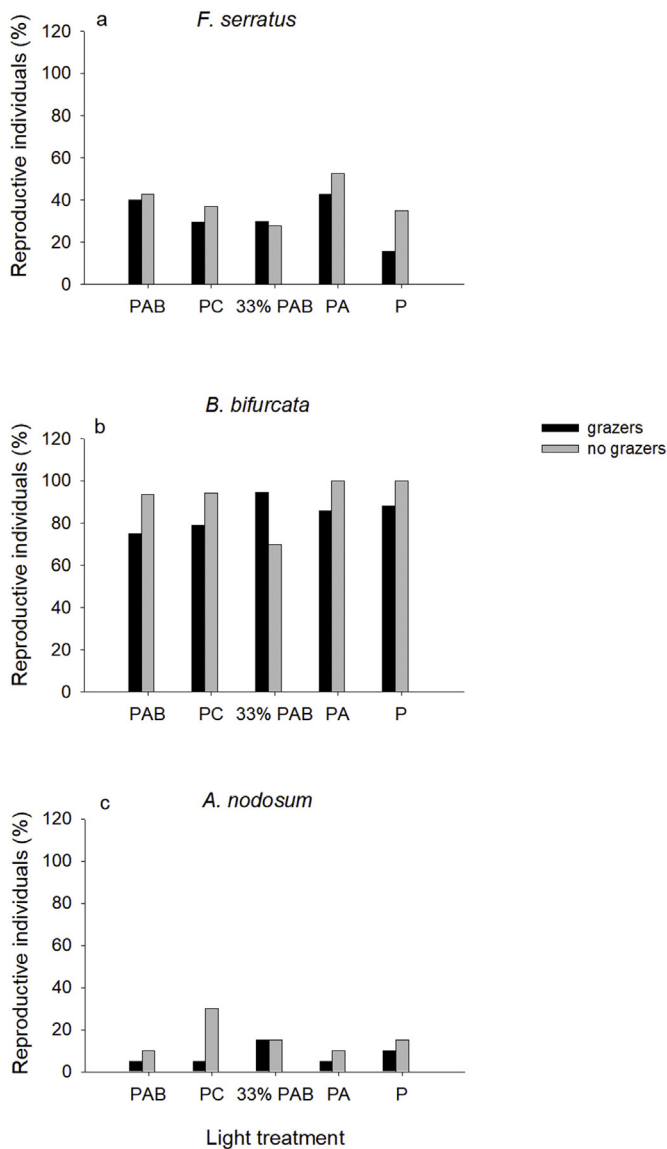
Table 3
General linear models of the effect of grazing and light quality on the nitrogen content and phlorotannin contents of tissues and exudates of three macroalgae. Grazing and Light quality were orthogonal fixed factors. Variances were homogeneous.

	df	*F. serratus*		*B. bifurcata*		*A. nodosum*	
		Nitrogen content		Phlorotannin content of tissue		Phlorotannin content of exudate	
		F	p	F	p	F	p
Intercept	1	1278.94	< 0.001	465.92	< 0.001	19.76	< 0.001
Grazing (G)	1	0.60	0.447	1.59	0.221	6.58	**0.018**
Light quality (L)	4	3.37	**0.028**	6.49	**< 0.002**	0.71	0.592
G x L	4	1.97	0.136	0.76	0.562	0.69	0.060
Residual	20						

Significant p-values are indicated in bold.

those of studies carried out by other authors (e.g. Dethier et al., 2005; Macaya et al., 2005) and of our previous research indicating the predominance of additive effects of physical stressors in intertidal macroalgae at the limits of their geographical distribution in the Atlantic Ocean and Mediterranean Sea (Martínez et al., 2012a; Ferreira et al., 2014; Celis-Plá et al., 2014; Fernández et al., 2015). This is particularly important when attempting to predict shifts of species distribution in response to climate change, as the inclusion of biotic stressors into the predictive models may enhance their performance (Araujo and Luoto, 2007), although statistical methods often assume additivity, especially in species distribution models (SDMs) (Darling and Côté, 2008). For reliable prediction of the effect of climate change on species distribution shifts, the greatest challenge will be to determine how often additive effects occur when both biotic and abiotic factors are considered.

The invasive species *S. muticum* displayed the fastest growth rate, irrespective of the experimental treatment. The lack of response to grazing activity may be due to a compensatory growth strategy, i.e. rapid growth to counteract grazing pressure (see Pansch et al., 2008). Indeed, this species shows growth rates of up to 46 and 80 cm per month in the presence of a high density of grazers (Young et al., 2012). This life trait is frequently associated with successful invaders (Pyšek and Richardson, 2007). *Bifurcaria bifurcata* and *A. nodosum* displayed the slowest growth rates, consistent with their perennial strategy and long lifespan, especially that of *A. nodosum* (Dudgeon and Petraitis, 2005). As found for *S. muticum*, the experimental treatments had not significant effect on growth and reproductive output of these two species. However, the photosynthetic efficiency was higher at low irradiance than in *S. muticum*. Although not widely reported, grazing activity and light quality can have minor effects on growth of certain seaweed species (e.g. Michler et al., 2002; Rothäusler et al., 2011). This indicates the potential of these species to adapt to a wide range of grazing pressure and light conditions in the rocky intertidal system (as in Rothäusler et al., 2011).

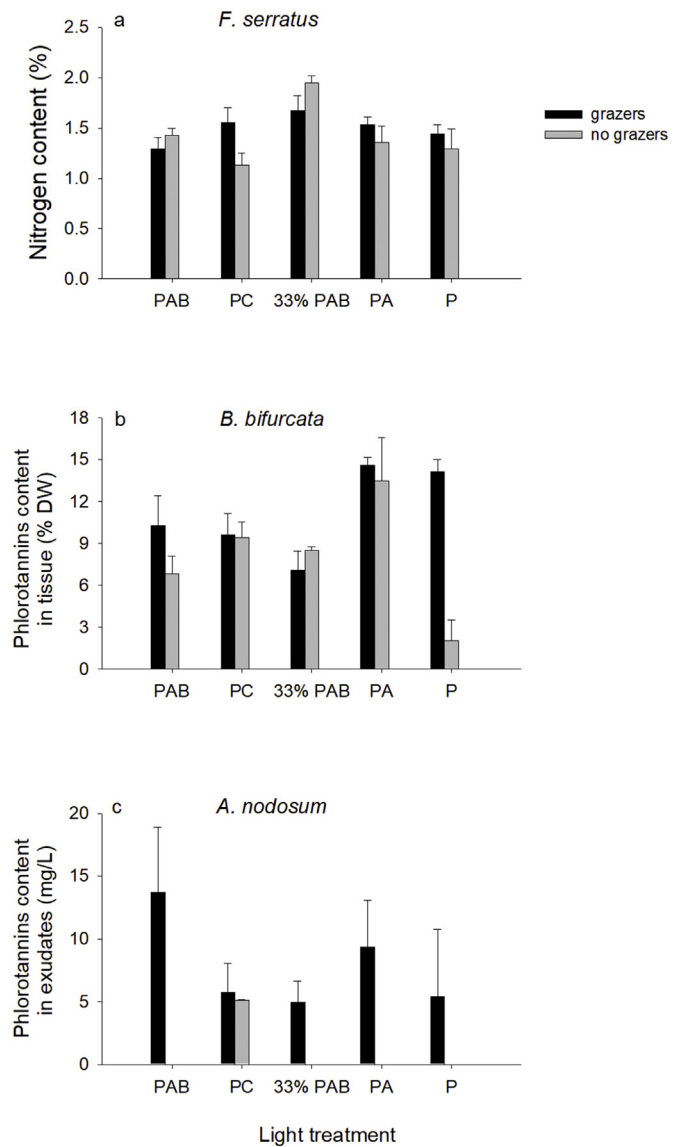By contrast, *F. serratus* showed intermediate growth rates consistent

with its perennial, but shorter-life span (of 3–4 years) (Arrontes, 1993). The growth rate of *F. serratus* was affected by the interaction between light quality and intensity and grazing activity. Although intertidal species that inhabit the low shore are generally more sensitive to UV radiation (Gómez et al., 2004; Figueroa et al., 2014), this was not the case here as the growth rate decreased when the UV radiation was filtered out. Although UVB may positively affect macroalgal growth (Hanelt et al., 2006), our findings are not consistent with those of previous research indicating negative effects of UV radiation on adults and zygotes of *F. serratus* (Michler et al., 2002; Altamirano et al., 2003). Moreover, a previous study showed that growth of individuals from different populations, living close to those used in this study, was inhibited by high solar radiation (Martínez et al., 2012a). The difference may be due to the fact that the individuals in the earlier study were exposed to high levels of radiation stress during low tide, whereas in our study individuals were always immersed. During immersion, macroalgae experience cooler temperatures and lower UV and light levels (i.e. more benign conditions), while during emersion, macroalgae are exposed to increased irradiance and UV stress and also to elevated air temperatures and increased desiccation, which may affect their performance (Guenther and Martone, 2014). In general, the presence of grazers had a negative effect on growth, except under natural light conditions. The negative effects of grazers (e.g. gastropods, sea urchins) on net growth rates of macroalgae have been reported for diverse fucoids and red algae (Dethier et al., 2005; Toth et al., 2007; Hawkins et al., 2009; O'Leary and McClanahan, 2010), although the effects can be modulated by light *via* consumption rates (Ramalhosa et al., 2016). The growth rate and also the reproductive output both tended to be negatively affected by the presence of grazers. Reduced fitness due to lowered growth rates and reproductive output during grazing is common in brown seaweeds, including *Fucus* species (e.g. Pavia et al., 2002; Dethier et al., 2005). If seaweeds allocate resources to wound

Table 2
Mean (± S.E) biochemical (n = 30) and photosynthetic responses (n = 87–99) of each species at the end of the experiment. Data are pooled across treatments. E_k varied significantly depending on light quality and grazing treatments in *F. serratus* and F_v/F_m varied depending on light quality in *F. serratus*; nitrogen and phlorotannin contents of tissue varied depending on light quality in *F. serratus* and *B. bifurcata*, respectively; phlorotannins in exudates also varied across grazing treatments in *A. nodosum*. Phl $_{tissue}$: phlorotannins content of tissue; Phl $_{exudate}$: phlorotannins content in exudates; ETR $_{max}$ (µmol electrons m^{-2} s^{-1}): maximal electron transport rate; F_v/F_m: quantum yield; α: the initial slope of ETR *versus* irradiance function; β: the photoinhibition; E_k (µmol electrons m^{-2} s^{-1}): the irradiance for half saturation of ETR.

Species	N (%)	C (%)	Phl $_{tissue}$ (%)	Phl $_{exudate}$ (mg/L)	(F_v/F_m)	ETR$_{max}$	α	β	E_k
S. muticum	1.57 ± 0.05	26.72 ± 0.29	4.99 ± 0.28	22.30 ± 3.65	0.72 ± 0.00	26.6 ± 1.13	0.22 ± 0.00	20.5 ± 0.92	138.21 ± 4.54
F. serratus	1.42 ± 0.05	32.52 ± 0.33	13.67 ± 0.55	50.11 ± 6.19	0.76 ± 0.00	20.5 ± 0.82	0.15 ± 0.01	15.6 ± 0.91	197.74 ± 8.04
B. bifurcata	1.46 ± 0.07	31.39 ± 0.49	10.71 ± 0.57	19.50 ± 1.61	0.78 ± 0.00	54.2 ± 1.99	0.32 ± 0.01	28.3 ± 1.15	183.41 ± 11.67
A. nodosum	1.54 ± 0.05	33.46 ± 0.25	10.41 ± 0.56	5.55 ± 1.14	0.74 ± 0.00	66.2 ± 1.65	0.38 ± 0.01	29.1 ± 0.61	176.40 ± 5.28

Fig. 4. Mean (+S.E.) percentage of mature individuals of *F. serratus*, *B. bifurcata* and *A. nodosum* in the different experimental treatments after 7 weeks; n = 10. Data are shown as percentages and correspond to pooled values for both incubators and the two grazing treatments.

Fig. 5. Mean (+S.E.) of: a) nitrogen content (%) in tissue of *F. serratus*; b) phlorotannin content (% dry weight) in tissue of *B. bifurcata*; c) phlorotannin content (mg/L) in exudates of *A. nodosum* at the different experimental treatments after 7 weeks; n = 4. Data from both incubators and the two grazing treatments were pooled.

healing and to producing chemical or morphological defence compounds, then these resources will not be available for growth or reproduction.

The macroalgae also displayed different responses in relation to nutrient stoichiometry, phlorotannin content and photosynthetic activity. *Sargassum muticum* contained the lowest amount of phlorotannins in tissues, but the amounts did not vary significantly. Assuming that phlorotannins have a protective role, this finding is consistent with the hypothesis that fast-growing plants invest fewer resources in constitutive/inducible defences than slower-growing plants (Karban, 2011). Although *S. muticum* was the fastest growing of the species considered, it did not display the highest photosynthetic potential and activity. However, in many macroalgae, changes in photosynthetic activity are not always consistent with changes in growth, and light quality seems to have a greater effect on growth than on photosynthesis, indicating that physiological processes leading to inhibition of growth may act independently of changes in photosynthetic activity (Michler al., 2002). For example, diverse studies on seaweeds have revealed that effects of UVB on growth rate are often evident at lower doses than effects on photosynthetic activity (Franklin and Forster, 1997).

Bifurcaria bifurcata and *A. nodosum* presented similar ecophysiological strategies, and the experimental conditions only affected the phlorotannin content of respectively tissues and exudates. Contrary to expectations, the phlorotannin content of *B. bifurcata* increased when UVB was filtered out. Phlorotannin production in this species is complex and depends on diverse factors such as the reproductive period, light intensity, etc. (Le Lann et al., 2012). Our results are, however, consistent with those of a study that indicated fewer phlorotannins in stressed thalli of a fucoid species (Dethier et al., 2005). Phlorotannin production in *A. nodosum* responded to grazing activity, and production was highest in the presence of grazers, as previously reported for this species (Pavia and Åberg, 1996; Pavia et al., 1997). Although growth of the two species was not directly affected by grazing and/or light, effects may be manifested indirectly through an increased production of costly secondary metabolites that function as defences against UV-radiation or grazers (Pavia and Toth, 2000; Figueroa et al., 2014). The two species

Table 4

General linear models of the effect of grazing and light quality on the photosynthetic performance (E_k and F_v/F_m) of the four macroalgae. Grazing and Light quality were orthogonal fixed factors. Variances were homogeneous; *p < 0.05, ***p < 0.001. F_v/F_m: quantum yield; E_k (µmol electrons m^{-2} s^{-1}): the irradiance for half saturation of ETR.

	df	S. muticum		F. serratus		B. bifurcata		A. nodosum	
		E_k	F_v/F_m	E_k	F_v/F_m	E_k	F_v/F_m	E_k	F_v/F_m
		F	F	F	F	F	F	F	F
Intercept	1	**339.01*****	**13 318.8*****	**456.34*****	**49 278.7*****	**264.9*****	**35 492.7*****	**821.52*****	**18 367.1*****
Grazing (G)	1	0.113	0.65	2.13	3.20	0.601	0.65	0.306	0.24
Light quality (L)	4	0.946	1.46	2.70	**5.84***	0.752	0.49	0.268	0.57
G x L	4	0.591	0.28	**3.54***	0.80	0.889	0.14	0.488	0.72
Incubator (G x L)	10	**4.412*****	**4.39*****	**2.16***	1.77	0.947	**2.40***	1.297	**3.92*****
Residual	67	(E_k)							
	79	(F_v/F_m)							

Significant p-values are indicated in bold.

showed a better performance than *S. muticum* under higher irradiance, which suggests that the invader has a lower photosynthetic capacity, contrary to expectations. These results are consistent with previous findings that indicated poorer fitness traits in this species compared with native species (Engelen and Santos, 2009; Vaz-Pinto et al., 2014). For example, the native *Cystoseira humilis* Schousboe ex Kützing has a better nutritional strategy than *S. muticum* to cope with limiting nutrient conditions (Vaz-Pinto et al., 2014).

Fucus serratus, which showed an intermediate ecophysiological strategy between that of the invasive *S. muticum* and the perennial native fucoids, contained the highest concentrations of phlorotannins in tissues and exudates, which did not vary significantly with grazing activity and/or light quality and intensity. By contrast, the nitrogen content increased when light intensity was reduced. This result is consistent with the potential accumulation of N-rich photosynthetic pigments under shaded conditions (Figueroa et al., 2003). Light reduced the photosynthetic performance of *F. serratus* because F_v/F_m decreased significantly under the full light spectrum. These results are in line with previous studies that indicated a negative effect of solar radiation on the physiological performance of diverse brown macroalgae, including *F. serratus* (Abdala-Díaz et al., 2006; Martínez et al., 2012a; Fernández et al., 2015). This species also showed a lower photosynthetic potential (i.e. low α and ETR_{max}) than the other species under study, although the experimental conditions did not significantly affect the photosynthetic activity. The high cost associated with phlorotannin production (see Jormalainen and Ramsay, 2009), together with the lower photosynthetic capacity, may at least partly explain the vulnerability of this species to changes in light quality and intensity.

In conclusion, the results suggest more sensitivity to experimental conditions of the declining *F. serratus,* rapid growth and good acclimatization potential of the well-established invasive species *S. muticum*, and great resistance of the native perennials *B. bifurcata* and *A. nodosum*. The study findings indicate that grazing and light are important variables affecting the physiological performance of *F. serratus*, and to a lesser extent that of *B. bifurcata* and *A. nodosum*. An increased and/or prolonged grazing activity by invertebrates in southern European shores due to large-scale climate change (Jenkins et al., 2001) and changes in solar radiance that are already being noted in the Iberian Peninsula (Sánchez-Lorenzo et al., 203) may have important effects on the distribution of these three species. These two variables should be considered in an additive way for successful projections of future distributional shifts of these habitat-forming species.

Conflicts of interest

None.

Authors' contributions

CO, FA and BM conceived the study and designed the experiment, CO and FA collected the samples, CO, FA, AF and JST performed the experiment, CO, BM interpreted data, performed the statistical analysis and wrote the manuscript. All authors reviewed the manuscript. All authors read and approved the final draft of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2018.05.016.

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