Research Article

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The effect of irradiance versus light dose on the antioxidant activity of two strains of *Ulva lacinulata*

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Abstract: The genus *Ulva*, described as a good source of antioxidants known for its antibacterial properties and associated with the capacity to adapt to different environments and high growth rates, has justified the increasing interest in its large-scale production. While extensive research has been done on optimizing the extraction of *Ulva*'s bioactive compounds, few studies were conducted on increasing or optimizing antioxidant activity (AA) of *Ulva* spp. during cultivation. Our study aimed to investigate an optimization method of *Ulva lacinulata* by testing the impact of light dose and irradiance on its AA. Two geographically different strains (NE-Atlantic and Mediterranean) were observed for 5 days under two irradiances (70 or 185 µmol photons m^{-2} s^{-1}) with the same light dose (4 mol photons m^{-2} d^{-1}). Samples were collected at different times (0, 3, 24, 48 and 120 h) to evaluate their antioxidant activity (with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical decolorization assay) and photosynthetic performance (with Pulse Amplitude Modulated fluorometer). A strain-dependent response was observed in the NE-Atlantic strain which had significantly higher AA after 5 days (89 %) under the photosynthetic saturating irradiance, while the Mediterranean strain was not impacted, suggesting that light dose may significantly affect AA in certain *Ulva* spp.

Keywords: ABTS assay; antioxidants; irradiance; daily light integral; *Ulva*

1 Introduction

Land-based recirculating aquaculture systems (RAS) are usually associated with fish aquaculture and, to our knowledge, only a few attempts have been made to cultivate seaweeds using these systems. To this day, the only RAS associated with seaweeds are systems working mainly with fish where seaweeds are then integrated with the original recirculating fish tanks (Bambaranda et al. 2019; Cahill et al. 2010; Deviller et al. 2004; Mata et al. 2016; van Khoi and Fotedar 2011). The exclusive use of RAS for seaweed cultivation is therefore new and despite the considerable advantages associated with this system (Cardoso et al. 2023; Ed-Idoko 2021; Malone 2013), RAS is still associated with its high costs of maintenance (Lüning and Pang 2003; Mata et al. 2016; Sebök et al. 2019; Steinhagen et al. 2021). Therefore, guaranteeing consistent and profitable seaweed production is essential. To increase profits, several steps can be taken: 1) reduce costs (e.g., electricity usage, water treatment, salt concentration, nutrient quality); 2) increase the quality of the biomass to increase the seaweed's value (e.g., high antioxidant concentration); 3) increase the number of harvesting periods (e.g., producing species with high relative growth rates, RGR). Each step can be reached with more efficiency if prior to cultivation a process of species and strain selection is completed (Cardoso et al. 2023; Ed-Idoko 2021; Fort et al. 2020; Malone 2013). By species/strain selection, it is possible to determine the optimal conditions in which each species/strain will present the highest RGR and content of interesting compounds (e.g., antioxidants, protein content, lipid content). At the same time, it allows the farmer to select the species and strains that show their best qualities in the least costly settings (e.g., lower irradiance, lower salinity).

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The green seaweeds of the genus *Ulva* have the potential to be utilized in several industries because of their polysaccharides that have been shown to have high bioactivity (Amin 2020; Mo'o et al. 2020; Shao et al. 2013), including high antioxidant and antimicrobial activity (Amin 2020; Shao et al. 2013). Examples of those industries are the food (Amin 2020; Gomaa et al. 2022; Morelli et al. 2019), feed (Martínez-Antequera et al. 2021), packaging (e.g. by prolonging the shelf life of packaged perishables; Bosse and Hofmann 2020; Gomaa et al. 2022; Tretiak et al. 2021), and pharmacy and cosmetics industries (Leyva-Porras et al. 2021; Lomartire et al. 2022; Mo'o et al. 2020; Perera et al. 2021). Therefore, interest in *Ulva* production has been increasing (Dominguez and Loret 2019; Fleurencel et al. 1995; J. Li et al. 2018; Lordan et al. 2011; McCauley et al. 2018), and of particular importance for improving future production of *Ulva* spp. is strain selection and strain optimization for large scale production of *Ulva* to reduce costs and achieve a high-quality product (Cardoso et al. 2023; Fort et al. 2020). Nevertheless, few studies have been conducted on increasing or optimizing antioxidant activity (AA) of *Ulva* spp. during cultivation (Steinhagen et al. 2022), in particular in RAS (Cardoso et al. 2023). Because it is known that saturating irradiances can cause the formation of reactive oxygen species (ROS), and thus the increase of antioxidant activity in plants and algae (Bischof and Rautenberger 2012; Collén and Pedersén 1996), we attempted to cause an positive antioxidant response to two strains of *Ulva* by exposing them to saturating irradiance for photosynthesis. In order to be sure that any change in antioxidant activity was due to the irradiance, and not the light dose, we used the same daily light integral for both irradiance treatments. We hypothesised that irradiance would be the main contributing factor for the increase of antioxidants if variations between treatments were found. Additionally, we hypothesised that the lack of visible differences between treatments would mean that light dose had a stronger impact on the antioxidant activity than irradiance. It was expected that the seaweed grown under saturating irradiance would have a higher antioxidant activity than the control group.

In addition to assessing the antioxidant activity of the macroalgae, the photosynthetic performance was also analysed throughout the experiment. It was expected that the macroalgae exposed to the higher irradiance would acclimate to the saturating irradiance, which would be expressed through a decrease in the initial slope of the light curve (alpha) and therefore an increase in the light saturation point (I_k) (Foy and Gibson 1982).

The main goal of this work was to investigate quality optimization, and establish an easy to reproduce method by increasing the antioxidant activity of *Ulva lacinulata* in large scale land-based cultivation systems (such as RAS). Two strains of *U. lacinulata* from different origins (Mediterranean and NE-Atlantic) were investigated to determine if there is a strain-dependent response to irradiance and/or light dose. Furthermore, we aimed to select from the two strains the one with the strongest response to the saturating irradiance treatment, as the most promising one for cultivation in a land-based recirculating aquaculture system.

2 Materials and methods

2.1 Biomass collection

The NE-Atlantic *Ulva lacinulata* (Kützing) Wittrock was collected in Lagoa de Óbidos, Portugal (39°23′41.5″N 9°12′48.9″W) in January 2021 and cleaned by rinsing the seaweed through running seawater several times to eliminate epiphytes and small organisms on the surface of the blades. The Mediterranean strain of the same species originated from Thessaloniki Bay, Greece (40°33′57.4″N 22°57′28.0″E) and arrived as a clean unialgal culture (isolated in 2017, AWI culture 1290, from Sotiris Orfanidis, Hellenic Agricultural Organisation – Demeter, Greece).

The two strains had been previously identified as *Ulva lacinulata* by using the plastic-encoded marker *tufa* (Cardoso et al. 2023).

2.2 Pre-cultivation at the Alfred Wegener Institute (AWI)

The material used for this work was grown in 5-l glass cultivation bottles which were filled with artificial seawater (Seequasal-Salz, Seequasal Salz Production and Trade GmbH, Germany) at a salinity of 30 ± 2 (Refractometer, Atago, Japan). The bottles were placed in a climate-controlled chamber at 15 °C and were illuminated on a 16:8 light/dark cycle at an irradiance of 70 µmol photons m^{-2} s^{-1} (measured in air; LI-250A, Li-cor, Germany), with illumination from LEDs (white light; Lamps: Aquarius 90 LED, Aqua Medic, Germany; Lamps regulated by Spot Control, Aqua Medic, Germany). Nutrient supply was provided by 56 µl l^{-1} Blaukorn garden fertilizer (14 % total nitrogen, 6 % nitrate, 8 % ammonium, 5.5 % water soluble phosphate; COMPO SANA®, Germany) twice a week and the seaweed received fresh artificial seawater once a week. All bottles were aerated with compressed air via tubes connected with 0.2 µm air filters (Chromafil A-20/25, Macherey-Nagel GmbH & Co. KG, Germany).

2.3 Experimental set-up

Ulva lacinulata from the 5-l bottles was collected and 1.5 g of *Ulva* was cut into five 0.3-g pieces and separated into 1-l beakers under the two light treatments: saturating irradiance treatment or the control (70 µmol photons m^{-2} s^{-1}; $n = 3$). The beakers were filled with artificial seawater at 30 ± 2. Besides the irradiance, the abiotic conditions stayed the same as described previously.

Three beakers of each strain were placed under a lamp emitting light at a saturating irradiance of 185 µmol photons m^{-2} s^{-1} ($n = 3$), measured in air. This part of the cooling chamber was completely covered with dark cloth to prevent cross contamination of light from other lamps. To achieve this irradiance with the lamp used, it was

necessary to combine light of different colours including white, blue and red light. To guarantee that the light colour would not impact the results of our experiment, the same quality of light was used for the control group ($n = 3$) set-up at an irradiance of 70 µmol photons $m^{-2} s^{-1}$. The light quality was kept between the two treatments by maintaining the ratio of different light colours used. This was possible by adjusting the irradiance (in percentage) of each colour in the computer controlling the lights (Spot Control, Aqua Medic, Germany). Additionally, prior to the start of the experiment, the macroalgae were acclimated to the coloured light for one week.

To keep the light dose, also known as daily light integral (DLI), of both treatments similar, the macroalgae were illuminated with the saturated light for 6 h a day, while the control group was illuminated for 16 h, resulting in a DLI of 4 mol photons $m^{-2} d^{-1}$ (see Supplementary Material, Calculation of Daily Light Integral). The experiment lasted for five days, and 0.3 ± 0.05 g of macroalgae were taken out of each beaker at five different sampling points to measure the antioxidant activity after 0, 3, 24, 48 and on the last day of the experiment at 120 h. Those times were chosen based on previous experiments, which had found an increase in antioxidant activity in *Agarophyton vermiculophyllum* after three days of exposure to a saturated irradiance (Tretiak et al. 2021). Since *Ulva* acclimates quickly to environmental conditions such as illumination, more measuring points were chosen in the beginning of the experiment (Cruces et al. 2019). Upon sampling for analysis, the macroalgae were rinsed with distilled water and dried in an oven at 30 °C for 48 h. The low drying temperature was used in order to assess the antioxidant activity in macroalgal biomass following the protocol that is currently used for producing macroalgae-based packaging material (Bosse and Hofmann 2020).

2.4 Determination of antioxidant activity

To determine the antioxidant activity (AA) of the dried seaweed, the ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation decolourisation assay) was carried out according to Re et al. (1999) and the extraction protocol of Tretiak et al. (2021) was followed. Prior to the experiment, different Trolox (water-soluble vitamin E derivate; Barclay et al. 1984; Forrest et al. 1994) concentrations were measured ranging from 0 to 100 µg ml^{-1} (diluted in ethanol) to measure the absorption of the different Trolox concentrations and generate a standard Trolox curve. The standard Trolox curve was later used as a reference to evaluate the antioxidant activity in the samples. The equation ($y = ax + b$) obtained by the Trolox curve (that showed a linear trend) was used to obtain the values for a (0.357) and b (–1.0028), that were later used for the calculation of the antioxidant activity of the samples in Trolox equivalents (TE) in µg ml^{-1} (Equation (1)), where A_{734nm} is the measured absorption from each sample at 734 nm.

$$x = \frac{y - b}{a} \qquad (1)$$

$$\mathrm{TE} = \frac{A734\,\mathrm{nm} - b}{a}$$

Ethanol was used as a negative control and Trolox solution (100 µg ml^{-1}) was used as a positive control.

The antioxidant extracts were obtained by grinding 0.06 ± 0.005 g of the dried *Ulva* with 0.3 g of sand (SiO_2 Merck KGaA, Darmstadt, Germany) in a mortar on ice until achieving a fine paste. During the grinding process 1.2 ml of 70 % ethanol (EtOH) were added to the mixture. Extracts were collected into 15-ml centrifugation tubes and were incubated for 6 h in a shaking water bath (45 °C and 130 rpm; GFL 1086, GFL, Germany), and were centrifuged (Eppendorf 5810 R, Eppendorf, Germany) at 4 °C and 2500 *g* for 10 min. The supernatant was collected and the pellet resuspended once in 1.2 ml of ethanol. All samples were incubated for 1 h in the same water bath and the centrifugation process repeated once more. The two supernatants obtained from each replicate were mixed together and the pellets were discarded.

The ABTS solution was diluted in ethanol to an absorption of 0.7 at 734 nm (Re et al. 1999) before being added into the 96-multiwell plate where each well was filled with 20 µl of extract. Absorption of the samples at 734 nm was measured with a microplate reader (Infinite 200 Microplate Reader, Tecan Trading AG, Männedorf, Switzerland). The absorption of the plates was eliminated by subtracting the values of absorption from blank wells. The results from the ABTS assay were given in A_{734nm} that were then used to calculate the Trolox Equivalents in µg dry weight per ml.

To facilitate the comparison of our data with the results presented in the literature, the antioxidant activity presented in Trolox Equivalents in µg ml^{-1} was later transformed into µmol g^{-1} DW (dry weight) based on the totality of AA found in each 0.06 g of dried *Ulva* samples.

Because this work aimed to evaluate each strain by its capacity to increase its antioxidant activity when exposed to the irradiance treatments, the results were then transformed into percentage data by using the AA of each strain at 0 h as 100 %. This follows the assumption that both strains were acclimated to the culture conditions prior to the experiment but not to the saturating irradiance treatment and, therefore, AA at 0 h represents the normal levels of AA under non-stressful conditions.

2.5 Photosynthetic performance

In addition to measuring the antioxidant activity, the photosynthetic performance of the macroalgae exposed to the two light treatments was measured using a Pulse Amplitude Modulated (PAM) fluorometer (Imaging PAM, Heinz Walz GmbH, Germany) every 2 days.

As an indicator of the photosynthetic activity, the relative electron transport rate (rETR) was measured using rapid light curves with light pulses emitted every 30 s. The initial slope of the light curves (alpha-value) was calculated from the relative electron transport rate. In addition, the maximum rETR (rETRmax) and the point of light saturation (I_k) were determined. In this work, the established ETR should be seen as a relative value (rETR) since the absorptivity value used is also used for land plants, and can differ remarkably between different parts of the same sample (Heinz Walz GmbH 2019; Higo et al. 2017).

Additionally, the photosynthetic efficiency (Fv/Fm) was measured. Fv/Fm accounts for the maximal quantum yield of photosystem II after dark adaptation for 5–10 min and was calculated via the equation reported by Maxwell and Johnson (2000).

2.6 Statistical analysis

The statistical significance of the independent variables, light treatment and time, on the antioxidant activity and the rapid light curves parameters rETRmax, I_k and alpha were analysed through 3- and 2-way PERMANOVAs (9999 permutations), investigating the effect of experiment duration and treatment on the AA (TE in µmol g^{-1} DW) and photosynthetic performance of the seaweed. For smaller data sets

(e.g., evaluation of significant differences between treatments at a certain time point), Kruskal-Wallis tests were performed followed by a post-hoc test (Dunn test). The data was processed in the software R studio ("PERMANOVA", "Vegan" packages; R Core Team 2022). A statistically significant difference between treatments was assumed whenever the *p*-value was below 0.05.

Regression analyses between time and antioxidant activity were analysed based on the AA data in TE in µmol g^{-1} DW as well as on the percentage (%) of initial AA. The percentage data was calculated assuming that the average antioxidant activity from both control and HL treatments at 0 h was 100 %. Additionally, the relation between AA (TE in µmol g^{-1} DW) and the alpha values obtained by the PAM measurements, were evaluated through a regression analysis. Linear regressions were tested as well as different degrees of polynomial equations (2nd, 3rd, 4th and 5th degree), using the function "lm()", in R, to determine the best fit. The "geom_smooth" function with the method "lm" (package "Ggplot2") in the R studio software (R Core Team 2022) was used to create graphical representations of the results.

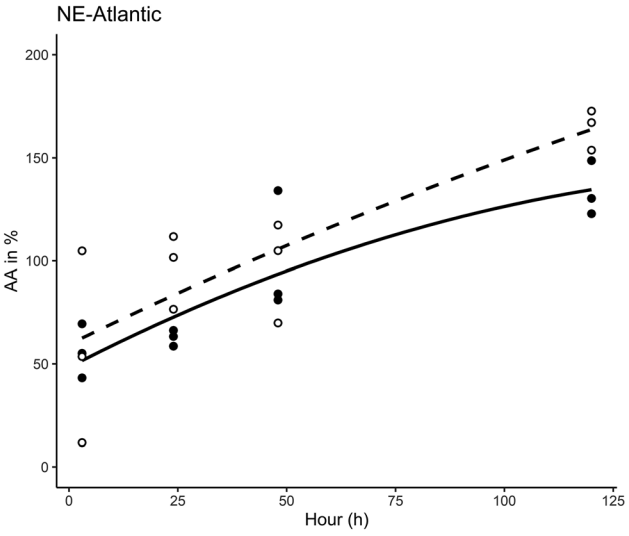

Figure 1: Antioxidant activity (% of initial) of the NE-Atlantic strain of *Ulva lacinulata* as a function of time. White circles: saturating light irradiance (SL) treatment (185 µmol photons m^{-2} s^{-1}); black circles: control (70 µmol photons m^{-2} s^{-1}). The broken line represents the regression analysis performed for the SL treatment; the solid line represents the trend obtained by the regression analysis performed with the control group.

3 Results

The NE-Atlantic strain was the strain with the highest increase in AA (TE in µmol g^{-1} DW) at the end of the experiment, with AA increasing by 1.4 TE (µmol g^{-1} DW), while the Mediterranean strain increased its AA by 1.1 TE (µmol g^{-1} DW; Figures S2 and S3). At 120 h, the NE-Atlantic strain showed significantly higher AA (TE in µmol g^{-1} DW) in the SL treatment compared to the control (p = 0.049), while no significant difference was observed between treatments in the Mediterranean strain, for the same time point. An interaction between time and strain was found that impacted the antioxidant activity (p = 0.01). From the start, the Mediterranean strain presented higher concentrations of AA (TE in µmol g^{-1} DW) than the NE-Atlantic strain. Both treatment and strain had a significant impact at 0 h ($F_{(1,\ 11)\text{treatment}}$ = 5.5 $p_{\text{treatment}}$ = 0.047; $F_{(1,\ 11)\text{strain}}$ = 30.67, p_{strain} = 0.0004). While variations in AA were observed in both strains, the Mediterranean strain kept its levels of AA higher than the other strain tested. Because of the interaction found between time and strain and the significant difference found between strains at 0 h, the results of this work will further be presented separated by strain and the percentage data will be used to evaluate the impact of the saturating treatment in each strain.

In the NE-Atlantic strain, the AA (TE in µmol g^{-1} DW) increased significantly in both treatments over time ($F_{4,29}$ = 6.6036, p = 0.0001, Figure S2, Table S2). A linear increase in AA (% of initial) over time was observed in both treatments (Figure 1; Table S1). The results suggest that the treatments had no impact on the AA. However, in Figure 1, it is possible to see that the AA increased faster over time in the SL treatment than in the control group. In the last day of the experiment, the SL reported a significantly higher AA compared to the control group (p = 0.049). Furthermore, in the control group no significant differences between sampling time points were found. However, in the SL treatment a significant increase in AA was observed between 3 h and 120 h (p = 0.0338).

Significant differences in AA (TE in µmol g^{-1} DW) in the Mediterranean strain (Figure S3; Table S2) were observed over time ($F_{4,29}$ = 14.5932, p = 0.0001), but no significant differences were found between treatments ($F_{1,29}$ = 0.4251, p = 0.5615; Figure S3; Table S2). Non-linear trends for both light treatments were found in the experiment with the Mediterranean strain (Figure 2; Table S1), where AA (%) increased until 48 h and then levelled off. No significant differences were found between treatments at the end of the experiment (120 h). However, in the control group, the AA increased significantly between 3 h and 48 h (p = 0.0338).

Regarding the photosynthetic performance of the *Ulva* strains (Table 1), significant differences between strains were observed in the I_k values at the start of the experiment (0 h; $F_{1,10}$ = 8.66093, p = 0.0186) and in the alpha values at the end of the experiment (120 h; $F_{1,10}$ = 9.7851, p = 0.0126). The NE-Atlantic strain presented significantly higher I_k values at the beginning of the experiment than the Mediterranean strain. However, at the end of the experiment (120 h) the Mediterranean strain presented significantly higher alpha values than the NE-Atlantic strain. In both cases, it was in the control groups that the results differed the most.

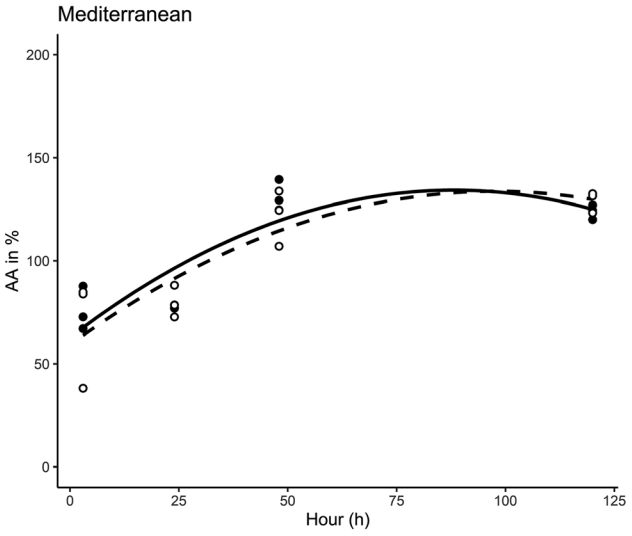

Figure 2: Antioxidant activity (% of initial) of the Mediterranean strain of *Ulva lacinulata* as a function of time. For other details see Figure 1.

The alpha values of the NE-Atlantic strain decreased significantly throughout the experiment in the control ($F_{2,6}$ = 12.392, p = 0.027; Table 1). No significant difference between the SL treatment and the control group was detected over time ($F_{1,12}$ = 0.0713, p = 0.7962).

Contrary to the NE-Atlantic strain, the interaction between Time and Treatment had a significant effect on the alpha values of the Mediterranean strain ($F_{2,12}$ = 9.4331, p = 0.0034). The alpha values decreased from the beginning to the end of the experiment in the SL treatment but increased in the control group (Table 1). Additionally, a negative correlation, based on linear regression analysis, was found between the AA (TE in µmol g^{-1} DW) and the alpha values in the Mediterranean strain under the SL treatment (Figure S4, Table S3).

The maximum rETR was significantly influenced by the interaction between time and treatment in the samples of the NE-Atlantic strain ($F_{2,12}$ = 17.6772, p = 0.0006; Table 1). The maximum ETR from the SL treatment increased throughout the experiment, while the maximum ETR from the control group decreased from 0 h to 48 h and then increased until the end of the experiment (Table 1). In the samples of the Mediterranean strain, both the time of exposure and the different treatments lead to an increase of maximum ETR (Time: $F_{2,12}$ = 12.8167, p = 0.0018; Treatment: $F_{1,12}$ = 5.0234, p = 0.0429). The two treatments registered an increase in the maximum ETR during the experiment. The increase was significant in the SL treatment between 0 h and 120 h (p = 0.0338).

A significant interaction between time and treatment was observed for the light saturation point (I_k) in the NE-Atlantic strain ($F_{2,12}$ = 12.123, p = 0.0019). The I_k of the samples of the SL treatment increased throughout the experiment, whereas, in the control group, they decreased for the first 48 h (Table 1).

Both time ($F_{2,12}$ = 36.2630, p = 0.0001) and treatment ($F_{1,12}$ = 18.1279, p = 0.0022) lead to significant changes in the I_k of the Mediterranean strain. The I_k of the SL treatment significantly increased throughout the experiment (from 0 to 120 h; p = 0.0219) but the same was not observed in the control group (p = 0.0681). The I_k of the samples exposed to saturated irradiance were significantly higher than the I_k of the control group at 120 h (p = 0.04953; Table 1).

The Fv/Fm values (Table 2) of the NE-Atlantic strain were significantly impacted by time (p = 0.0008; Table 2). Regardless of treatment, the Fv/Fm values of the samples of the NE-Atlantic strain increased throughout the experiment (p = 0.0045; Table 2). However, only the increase in the control group was significant (p = 0.0338) between 0 h and 120 h.

Table 1: The average initial slope (alpha), maximum rETR (rETRmax) and light saturation points (I_k in µmol quanta m^{-2} s^{-1}) with associated range values (n = 3) of the two groups after 24, 48 and 120 h of the experiment.

	SL			C		
	24 h	**48 h**	**120 h**	**24 h**	**48 h**	**120 h**
NE-Atlantic						
Alpha	0.17 ± 0.02	0.15 ± 0.03	0.15 ± 0.03	0.18 ± 0.01	0.15 ± 0.01	0.14 ± 0.02
rETRmax	26.39 ± 3.97	32.19 ± 4.97	32.84 ± 1.70	28.41 ± 2.23	22.96 ± 1.67	27.32 ± 3.60
I_k	145.99 ± 20.97	203.62 ± 12.83	210.20 ± 54.54	154.08 ± 17.37	144.12 ± 9.87	180.28 ± 7.43
Mediterranean						
Alpha	0.20 ± 0.00	0.17 ± 0.01	0.16 ± 0.02	0.17 ± 0.00	0.19 ± 0.01	0.19 ± 0.04
rETRmax	28.28 ± 2.20	31.39 ± 3.13	35.76 ± 4.40	22.56 ± 1.90	31.72 ± 5.47	32.39 ± 11.10
I_k	140.15 ± 12.33	182.86 ± 10.13	213.74 ± 2.79	126.87 ± 13.08	164.73 ± 35.14	168.38 ± 32.53

SL, saturated light treatment; C, control.

Table 2: Average Fv/Fm values and associated range from the samples of the light experiment measured after 24, 48 and 120 h.

		Time		
Strain	**Treatment**	**24 h**	**48 h**	**120 h**
NE-Atlantic	SL	0.693 ± 0.027	0.727 ± 0.021	0.722 ± 0.020
	C	0.687 ± 0.018	0.707 ± 0.026	0.734 ± 0.023
Mediterranean	SL	0.695 ± 0.029	0.734 ± 0.024	0.722 ± 0.020
	C	0.704 ± 0.017	0.738 ± 0.014	0.735 ± 0.002

SL, saturated light treatment; C, control.

Similar to the NE-Atlantic strain, the duration of the experiment had a significant impact on the Fv/Fm values of the Mediterranean strain ($F_{2,12}$ = 23.1665, p = 0.0002). The Fv/Fm values increased significantly between 0 h and 120 h (p = 0.0087).

4 Discussion

We compared the impact of irradiance versus light dose on the antioxidant activity of two strains of *Ulva lacinulata*. Such investigations are important for optimizing land-based cultivation systems and improving biomass quality and value. Most experimental designs with *Ulva* spp. have tested different irradiance treatments, but keep the same photoperiod, thus causing the light dose to vary between treatments as well (Fortes and Lüining 1980; Olsson et al. 2020; Toth et al. 2020; Xiao et al. 2016). Thus, a clear separation between the effects of irradiance and light dose cannot be made. Therefore, we designed our experiment to keep the light dose constant in both treatments (within non-saturating daylengths for *Ulva* species; Fortes and Lüining 1980).

The I_k values in both strains in the SL treatment increased through the experiment, while alpha decreased and the maximum ETR was reached at a higher I_k (Foy and Gibson 1982), suggesting that the strains acclimated to the higher irradiance. The most significant increase in the electron transport rate (rETR) was observed in the Mediterranean strain (both treatments) at 48 h, suggesting that acclimation to the experimental conditions at the beginning of the experiment was necessary. This was corroborated by the AA levels that dropped in 3 h in both treatments and strains, most likely due to the transfer of the macroalgal biomass from large bottles (high density and self-shading) to the smaller beakers with lower density. However, Fv/Fm values showed no decline and slightly increased throughout the experiment in all groups, indicating good health and effective photosynthesis, with values ranging from ~0.69 to 0.74 (Cruces et al. 2019; Higo et al. 2017; Masojídek et al. 2010). Healthy green algae usually show Fv/Fm values close to 0.7 (Magnusson 1997; Ünal et al. 2010). Considering only the results from the last two days, the NE-Atlantic strain showed 30 % higher AA in the SL treatment than in the control group, suggesting that irradiance can be used to increase AA in this strain. In contrast, the Mediterranean strain showed no significant response, suggesting that light dose was more important than irradiance for this strain.

The photosynthetic analysis combined with the AA results suggest that the NE-Atlantic strain is better acclimated to rapidly changing intertidal conditions in its natural habitat, therefore responding quickly to higher irradiance by increasing AA under the SL treatment (Zhao et al. 2016; Zhuo et al. 2019).

The negative correlation between the alpha values and the AA (in TE μmol g^{-1} DW) in the Mediterranean strain under the SL treatment (Figure S4, Table S3) suggests that this strain was, from the start, adapted to lower irradiances. The increase in alpha represents an acclimation to low irradiance and the decrease in antioxidants can be explained by the reduced risk of ROS production at low irradiance conditions. Under the SL treatment, this strain showed the highest increase in I_k, proving its plasticity and capacity to adapt to the higher irradiance. We expected a similar change in photosynthetic efficiency in lower irradiance, but no differences in AA were detected between treatments. This similarity between treatments can be justified by *Ulva* being an intertidal species, dependent on its plasticity to adapt to tidal variations and wave disturbance (Zhao et al. 2016), as the saturating irradiance used was possibly not above the necessary threshold to cause an upregulation of AA.

Different methods to measure AA of the same extract (e.g., DPPH, FRAP or the ABTS assay) result in different outcomes, as different types of antioxidants will have a different affinity to each method (Chakraborty and Paulraj 2010; Magnusson et al. 2015). The extract type (e.g., alcoholic, or aqueous) is also important, as it determines the kind of antioxidants extracted from sample (Chakraborty and Paulraj 2010; Heo et al. 2005; Mezghani et al. 2013; Srikong et al. 2017). Both extract types should be examined for a more

comprehensive overview of the total antioxidant content. A study of the AA of the genus *Umbraulva* using the ABTS assay, did not lead to significant results (Belter 2021). But, in the red seaweed *Agarophyton vermiculophyllum*, Tretiak et al. (2021) found a significantly higher AA after four days under a saturating irradiance.

We used an ethanolic extract to determine the AA of two *Ulva* strains. Ethanolic extracts of different *Ulva* species are characterized by the presence of chlorophylls (*a* and *b*), carotenoids, flavonoids and phenolic compounds (El-Baky et al. 2009; Pappou et al. 2022; Wulanjati et al. 2020). Pappou et al. (2022) compared different extracts and determined that pure ethanolic extracts presented the best extraction capacity for carotenoids. Phenolic compounds were also found in the ethanolic extracts of *Ulva* and associated with antibacterial and antioxidant activity, and *Ulva*'s photoprotective mechanisms (Cabello-Pasini et al. 2011; Wulanjati et al. 2020). Therefore, we can suppose that carotenoids and phenolic compounds were present in the extracts used in our work. A comprehensive study of *Ulva* carotenoids by Eismann et al. (2020), showed that total carotenoid yields are species and strain-dependent, which may account for the differences observed in our work. Still, the lack of specificity of the ABTS assay, does not allow corroboration of this possibility or detect variations between the antioxidant compounds. As well, only relative ETR (which does not account for the specific absorptivity of each sample) was measured, and possible changes in the pigment content of the seaweed influencing the absorptivity could not be addressed.

The similarities in AA between treatments in the Mediterranean strain might have been caused by the maintenance of the pigment ratio between the different pigments, while the concentration of each pigment varied in response to the treatments. This hypothesis is based on the work of Ramus et al. (1976) that suggests that intertidal seaweeds such as *Ulva lactuca* adapt to the sun and shade like higher plant species by varying the total pigment concentration but not the ratio of accessory pigments. In the same work it is suggested that intertidal species present a "classic intensity adaptation" justified by the need to adapt to the low and high tide conditions, in which the seaweeds can be exposed to high irradiances for some hours. However, a direct comparison between our work (AA) and that of Ramus et al. (1976) cannot be made.

As the drop in AA that occurred in 3 h in all different treatments suggests there might be changes in AA throughout the day. This daily change of AA was also found in brown seaweeds (Abdala-Díaz et al. 2006; Connan et al. 2007). Taking samples periodically at shorter intervals would be useful to obtain more accurate data on daily changes in AA and for better comparison of changes from one day to another.

The similarities between the treatments in the Mediterranean strain suggest that light dose had a stronger impact on AA than irradiance. In a study on *Codium tomentosum*, the results showed that longer days (16 h light:8 h dark; higher light dose) produced higher growth rates and higher concentration of pigments (Marques et al. 2021), particularly chlorophyll *a* (Shiu and Lee 2005; Yildiz et al. 2012). Work with *Agarophyton vermiculophyllum* (as *Gracilaria vermiculophylla*) showed that, when offered with the same total dose of PAR, the seaweed can present similar growth rates when grown either in short or longer days (Weinberger et al. 2008). Studies on *Ulva* have shown that longer days can be associated with higher relative growth rates, reproductive area sizes, and concentrations of chlorophyll (Y. Li et al. 2018; Schwoerbel 2019; Yue et al. 2019). But the growth rates of *Ulva lactuca* only increased until exposed to a daylength of 16 h, after which the growth stabilized (i.e. became saturated; Fortes and Lüning 1980). Thus, increasing the irradiance to 185 µmol photons m^{-2} s^{-1} but reducing the daylength by 10 h will have a similar impact as a normal culture condition of 16 h:8 h day:night cycle at 70 µmol photons m^{-2} s^{-1}.

The NE-Atlantic strain is better acclimated to and more effective in high light conditions, suggesting that antioxidant activity could be increased further if the irradiance was higher. This strain could therefore be relevant to produce high-quality biomass with higher levels of antioxidants. Harvesting the material after 5 days under SL conditions would result in seaweed biomass with higher AA content, which could be a useful functional property in certain industries, for example for use in packaging material (Bosse and Hofmann 2020).

The Mediterranean strain showed a strong resistance (no variation in AA between treatments) and acclimation to low irradiance. Robust growth rates and resistance to strong changes in irradiances makes this strain a good fit for land-based cultivation (Cardoso et al. 2023).

In a previous work with these strains, the AA was similar between the two at the beginning of the experiment (Cardoso et al. 2023). The fact that a significant difference was found in this work suggests that an external factor, likely light colour, impacted the two strains differently, as this was the only factor (besides irradiance) that differed between this and the previous work. We used the same light quality (colour) between treatments to guarantee that the only influencing factor was the irradiance, but exposure of *Ulva* sp. to blue light was found to increase AA within 44 days (Schwoerbel 2019). Further studies with light colours would be beneficial to understand their impacts and applicability to the growing seaweed industry.

In this work and that of Cardoso et al. (2023), the results were obtained through small-scale experiments under

laboratory conditions. However, artificial seawater was used to replicate the water in the large-cultivation facility and commercial fertilizer was used instead of Provasoli's (1968). Future work should be done to confirm the assumptions presented by testing the different strains in a large-scale facility once the conditions are optimized for each strain.

5 Conclusions

Strain selection and optimization are important steps for improving the profitability of seaweed aquaculture, especially in costly land-based recirculating systems. We tested whether light dose or irradiance could be used to enhance the AA in two *Ulva* strains and hence produce a higher value product. Combining a saturating irradiance with a short day (6 h) did not prove to be a successful method to increase AA in the Mediterranean strain, which was resistant to changes in irradiance. The NE-Atlantic *U. lacinulata* was found to be more responsive to irradiance variations, after 5 days under the higher irradiation treatment. Our findings suggest that short-days with high irradiance would have the same impact on the NE-Atlantic strain as long days with low irradiance had on the Mediterranean strain. Therefore, high quality biomass from the NE-Atlantic strain could be obtained by increasing the irradiance for 5 days at the end of the cultivation cycle, while a similar quality of biomass can be obtained in the Mediterranean strain by cultivating at low irradiance with a long photoperiod, which is also ideal for optimal vegetative growth. Further work is required to determine if higher irradiances for a brief period would impact the Mediterranean strain. For a more cost-effective production, different light dose and light quality treatments should be tested, as further adjustments can amount to a reduction in production costs or to an increase in biomass quality.

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Author contributions: LCH, IC and AM conceptualized and designed the studies, AM and IC drafted the manuscript, LCH and IC supervised the studies, AM carried out the studies, collected and analysed the data. LCH and IC provided technical and scientific supervision, LCH provided lab facilities and administrative support, LCH obtained funding for this project, and LCH critically revised the manuscript. The authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Data availability: The raw data used in this work was submitted to PANGAEA (Cardoso et al. 2024a,b). The molecular sequences of the species presented in this work are deposited in GenBank with the accession numbers: OP778143 (NE-Atlantic *Ulva lacinulata*), OP778144 (Mediterranean *Ulva lacinulata*). The raw data can be obtained on request from the corresponding author.

Nagoya Protocol: We have written confirmation by the *Instituto da Conservação da Natureza e das Florestas (ICNF)* in its function as ABS National Focal Point as well as Competent National Authority that although Portugal is a party to the Nagoya Protocol no national legislation nor any regulatory requirements drawing from the Nagoya Protocol for access to genetic resources in mainland Portugal exist presently. As the samples for this project were collected in mainland Portugal, there are no applicable prior informed consent requirements. The Greek *Ulva* material was isolated in 1986 (AWI culture number 1262) and in 1967 (AWI culture number 1290). Hence, the samples were taken before the Nagoya Protocol came into force in 2014. Although Regulation EU-No. 511/2014 does not apply accordingly, we complied with

our due diligence by asking the Greek National Focal Point about national ABS permit requirements and were granted access with a research permit for flora (RECALL/Δ ΡΔ/12548/797) issued by Ministry of The Environment & Energy-GDD & DP – Forest Protection Directorate.

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