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Optimizing antioxidant activity in *Agarophyton vermiculophyllum* for functional packaging

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ABSTRACT

The value of macroalgae in a healthy human diet is becoming increasingly recognized and supported throughout Europe. Macroalgae provide a rich source of vitamins, minerals, proteins, fatty acids, and antioxidants that also support the functionality of macroalgae in other industries, including cosmeceuticals, pharmaceuticals, and more recently, packaging. Sustainable aquaculture of macroalgae will be necessary to supply the increasing demand for macroalgae as a functional material, considering that natural harvests are limited and cannot keep up with demand. Different methods can be used to cultivate macroalgae, including flow-through systems or recirculating aquaculture systems (RAS) with natural or artificial seawater. The latter provides strict control over the growth conditions and water quality in order to provide a high quality and traceable product. Additionally, environmental conditions such as salinity, temperature, and light can be modified to optimize the concentration of functional ingredients in macroalgae. While most research efforts have focused on seasonal and geographic trends in concentrations of functional ingredients in wild macroalgae, there is less information available on optimizing these functional ingredients in aquaculture. Therefore, we performed controlled experiments to optimize the activity of antioxidants in *Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N.Norris et Fredericq comb. nov. (formerly *Gracilaria vermiculophylla*) grown in RAS with artificial seawater and commercial fertilizer. We show that the free radical scavenging activity could be increased by 13% via high salinity, and up to 34% by increasing the light intensity, but not daily light dose, for a period of 7 days. We also monitored growth rates and the maximum quantum yield of photosystem II (F_v/F_m) and show that the conditions for optimizing antioxidant activity are not optimal for growth or photosynthesis. We therefore suggest an optimization period of 4–7 days exposure to high light on a 6:18 hour light:dark cycle prior to harvesting in order to increase antioxidant activity.

1. Introduction

Intensive use of plastic material in daily life has led to undeniable environmental pollution [1–3]. Since 1950, world plastic production has been constantly rising, and in 2017, it was estimated at 374 million tonnes [2]. The growth of the plastic industry bypasses manufacturing of many other synthetic materials [1]. However, plastic is resistant to degradation in nature and consequently accumulates in landfills and marine environments [3]. Moreover, microplastics – small fragments of plastic – can cause harm to wildlife and accumulate up the food chain and further pose a threat to high trophic levels, including humans [4–6]. Therefore, over the last decade, there has been an increase in research involving the replacement of plastics with sustainable and biodegradable materials [7]. Macroalgae provide a natural, renewable resource that could potentially be used in a lot of different sectors, including the packaging industry (e.g. Evoware, OOHO). Marine algae are considered a promising raw material due to their fast growth, bioactive contents and natural, biodegradable polymers [6]. Additionally, macroalgae

contain a wide variety of functional ingredients with antioxidant activity that have the potential to prolong the shelf-life of materials stored in macroalgae-based products [8]. For example, an application in the food industry could reduce the main causes of food waste due to spoilage by lipid oxidation and microbial contamination. Existing strategies for preventing spoilage involve direct addition of antioxidants or packaging techniques that allow limited oxygen access [9]. Alternatively, macroalgae-based products, including functional packaging (packaging that provides benefits to the consumer) offer an antioxidant rich, natural, sustainable, biodegradable material that could reduce plastic waste, increase shelf-life and provide a unique customer experience. Macroalgae-derived polymers such as agar, carrageenan, and alginate have already been used as a base for biodegradable film development in food and pharmaceutical applications [6,7,8,10,11]. However, the complex technology and high costs involved in the polymer isolation, combined with low yields and large amounts of wasted biomass, are critical drawbacks to wider commercial use of such films in the global market [12]. Therefore, industrial solutions using the whole macroalgae biomass could be more useful [12], and additional investigations in this direction are currently underway (data not published). Either way, there is a knowledge gap regarding optimizing the functionality of seaweeds for industrial applications, particularly in the functional packaging industry. Consequently, we focused on optimizing the antioxidant activity in the whole macroalgae biomass, rather than in extracts.

Macroalgae have developed mechanisms to suppress or scavenge the oxygen radicals that are inevitably produced during photosynthesis and electron transport [13]. Macroalgae encounter particularly high levels of stress in the harsh and competitive intertidal environment, and have therefore developed highly efficient defense mechanisms via the synthesis of antioxidant compounds. For example, macroalgae inhabiting the intertidal zones with fluctuating levels of irradiance and oxygen concentration combined with exposure to the air are able to cope with stress by synthesizing of a wide range of metabolic compounds with radical scavenging activity. These compounds include carotenoids, polyphenols, polysaccharides, squalenes and vitamins, among others [14–16]. The rapid oxidative response of macroalgae to abiotic stress [17,18] provides an opportunity to control and modify the antioxidant capacity of cultured macroalgae by exposing it to various stress conditions for a short period. Many studies have reported the seasonal fluctuations of antioxidant activity in natural macroalgae or in macroalgal extracts [8,13,19–24]. These studies provide sufficient evidence that salinity, tidal emersion, and light stress can induce oxidative stress responses in macroalgae. Therefore, we manipulated salinity, desiccation, light intensity and light quality in order to increase the antioxidant activity of *Agarophyton vermiculophyllum* in culture in an effort to increase the functionality of this species for further industrial applications. We chose *A. vermiculophyllum* because it is an agarophyte that is already used in the food industry as a thickening agent and in films and coatings [25,26]. Although it is a non-native species in the Wadden Sea, it has established stable populations throughout northern Europe and can be the most abundant macroalgal species in some areas. Additionally, we focus here only on closed, recirculating land-based aquaculture systems, where propagation of additional unwanted biomass into the ecological systems is not a threat. Its broad salinity and temperature tolerance, fast growth rate, and vegetative propagation [27–29] make it an ideal candidate for aquaculture and optimization studies. The novelty of this study lies in the optimization of antioxidant activity in a cultured macroalgal species by manipulating the environmental conditions, which to our knowledge, has only been done indirectly by reducing the stocking density of the green alga *Derbesia tenuissima* in culture [30]. We hypothesized that short-term exposure to hypersalinity, desiccation, high light intensity, and ultraviolet radiation (UVR) would result in elevated levels of antioxidant activity in *A. vermiculophyllum*.

2. Materials and methods

2.1. Macroalgal culture

Agarophyton vermiculophyllum was collected from the intertidal zone during low tide on the Wadden Sea island of Sylt (List, Germany, close to the Sylter Hafen, 55°00′49.9″N 8°25′53.3″E) in March 2019 (water temperature 7 °C, salinity 30 ppt). Harvested seaweed was transported in a cooler to the Alfred Wegener Institute Helmholtz Center for Polar and Marine Research (AWI) in Bremerhaven for further cultivation. The epiphytes were cleaned manually from the seaweed thalli using cotton swabs and a diluted iodine solution (7.5%, Braunol, B. Braun, Melsungen, Germany) in filtered artificial seawater and then placed in tanks (600-L, 1.4 m wide, 1.20-m deep) for further cultivation. Each tank contained a stand pipe with a perforated bottom where compressed air was pumped from below to keep algal biomass suspended in the water column. *A. vermiculophyllum* was cultured in a RAS with artificial seawater at 17 °C, salinity 30 ppt, under natural irradiance in a greenhouse. The artificial seawater was enriched by adding fertilizer (Blaukorn Garden Fertilizer 2.5 L, Münster, Germany) bi-weekly at a concentration of 40 µL·L^{-1} of seawater, which resulted in a concentration of approximately 300 µM N. For each experiment, 7 g (wet weight) of algae were cultivated in a climate room at 15 °C in 3 L clear plastic beakers (VITLAB GmbH, Grossostheim, Germany) with filtered artificial seawater enriched with Blaukorn. With warming from the lamps, the temperature inside each beaker in our experiments reached about 17 °C, which is close to the optimal growth temperature (20 °C) for *A. vermiculophyllum* originating from the Baltic Sea of Denmark [27,29]. Furthermore, this was the same temperature used for maintaining cultures in the greenhouse. It was decided not to use a higher temperature in order to prevent high epiphyte growth. The density for the algae cultivation was chosen using the stocking density recommended by Kim &Yarish [31]. Artificial light was provided by LED lamps (Aquarius 90, Aqua Medic Anlagenbau GmbH, Bissendorf, Germany). The intensity and day length were changed depending on the experiment (see Sections 2.2–2.4). The water in the beakers was changed once per week to maintain appropriate levels of inorganic carbon. Germanium dioxide (7 mg/L) was added in the culture tanks to prevent growth of diatoms on the algal thalli. Three separate experiments were conducted to investigate the effect of 1) salinity and desiccation, 2) light intensity and UVR, and 3) light dose and UVR on the free radical scavenging activity of *A. vermiculophyllum*.

2.2. Manipulation of salinity and desiccation

Artificial seawater was prepared by mixing salt (Seequasal-Salz, Seequasal Salz Production and Trade GmbH, Münster, Germany) with tap water. The final salinity was measured using a multiparameter meter (Orion VERSA STAR Pro, Thermo Scientific). Twenty 3 L beakers containing 7 g of algae were distributed to four treatments (n = 5): (1) salinity 30 psu with no desiccation, (2) salinity 30 psu with desiccation, (3) salinity 40 psu with no desiccation, and (4) salinity 40 psu with desiccation. The desiccation was applied by elevating the macroalgal material above the beakers with a net for 2 h daily. All treatments were exposed to the same irradiance of 150 µmol photon m^{-2} s^{-1} ± 13.5% under a 16:8 h light:dark (L:D) cycle for 7 days. These culture conditions were similar to those used in a previous study with *A. vermiculophyllum* from Denmark [27], which showed that this light intensity is saturating for growth at 15 °C. Relative growth rates (RGR) and free radical scavenging activity were measured after 3 and 7 days of exposure (see Sections 2.5–2.6). Three grams of macroalgae was removed from each beaker for analysis of free radical scavenging activity. Chlorophyll fluorescence parameters (F_v/F_m) were measured at the beginning of the experiment at T_0 (Day 0), T_3 (Day 3) and T_7 (Day 7; see Section 2.7 and Fig. S1 for experimental design).

2.3. Manipulation of light intensity and UVR

Two light intensities (150 and 350 µmol photons $m^{-2} s^{-1}$ on a 16:8 h L:D cycle) were tested for their effects on radical scavenging activity. Both of these light intensities were saturating to photosynthesis (data not shown) based on initial rapid light curves measured using pulse amplitude modulated chlorophyll fluorescence (see 2.6). The different light intensities were obtained by changing the brightness settings on the LED lamps. These conditions were maintained in 36 three-liter beakers (n = 18) for 7 days. Then 8 beakers from each treatment were split into 2 groups (Fig. S2): one received additional UV treatment along with the same light irradiance used before; for the other group conditions remained the same as a control. Thus, by the end of the experiment, results were obtained from four different treatments: low light; low light + UVA; high light; and high light + UVA. While this design resulted in an unbalanced treatment on day 10, we used a balanced design for statistical analysis (n = 3). The UVR was obtained by activation of channel 6 (UV; when UV light was added, all 6 channels on the LEDs were activated) on the LED lamps using the 6-channel Aquarius Control accessory (Aqua Medic Anlagenbau GmbH, Bissendorf, Germany), which had a spectrum ranging from 359 to 398 nm (measured using a RAMSES UV/VIS radiometer, TriOS Mess- und Datentechnik GmbH, Rastede, Germany) and therefore fell within the UVA range (see Fig. S3). The UVR intensity was 0.26 W m^{-2} and 0.16 W m^{-2} in the high and low light treatments, respectively. This contributed a total of 0.87% ± 0.02 of the intensity to the low light treatment and 1.64% ± 0.03 of the intensity to the high light treatment. Measurements of RGR, F_v/F_m, and free radical scavenging activity were conducted at T_0, T_3, T_7, and T_{10}. As biomass was removed for sampling at each time point, the corresponding beaker was excluded from further analysis during the experiment. Thus, the amount of biomass in the beaker was not artificially modified throughout the experiment and did not affect the light exposure of the macroalgae, with the exception of natural growth.

2.4. Manipulation of light dose and UVR

Thirteen beakers were exposed to 100 µmol photons $m^2 s^{-1}$ on a 24-hour cycle, while 18 beakers were exposed to 400 µmol photons $m^{-2} s^{-1}$ for 6 h (6:18 L:D). Despite different intensities, the long day in the low light treatment and short day in the high light treatment resulted in a similar daily photosynthetically active radiation (PAR) dose of 8.64 mol photons $m^{-2} day^{-1}$. In this experiment, we used slightly different light intensities (100 and 400) in the low and high light treatments so that we could compare our results to previous experiments that have looked at PAR dose effects on *Gracilaria* spp. [29]. After four days, UVR was added to 5 beakers from each light intensity treatment. The remaining 5 beakers (after removal of some beakers for sampling) from the high light treatment were switched to a 24-hour cycle, resulting in a PAR dose of 34.56 mol photons $m^{-2} day^{-1}$. The UVR contributed a total of 0.74% + 0.02 and 1.92% + 0.03 for the low and high light intensities, respectively (see Fig. S4 for experimental design).

2.5. Growth rates

Growth performance of *A. vermiculophyllum* was measured by weighing wet algae material every three to four days. Relative growth rates (RGR) were calculated using Eq. (1):

$$RGR = \frac{ln(W_t) - ln(W_0)}{t} \times 100\% \qquad (1)$$

where W_0 is the initial algae wet weight (g), W_t is the final wet weight (g), and t is a duration of culture (days).

2.6. Chlorophyll fluorescence

Measure and analyses of chlorophyll *a* fluorescence can provide knowledge on the photosynthetic organism performance. Therefore, this technique was chosen to evaluate the physiological state of *A. vermiculophyllum* before and during experiments. The chlorophyll *a* fluorescence of photosystem II (PS II) was measured by a portable pulse amplitude modulation fluorometer (Junior-PAM, Walz, Effeltrich, Germany). The effective quantum yield of photosystem II (F_v/F_m ratio) was measured for samples from each experimental beaker. Samples were dark-adapted prior to measurements as suggested by Hanelt et al. and Figueroa et al. [32,33] for 15 min. After dark adaptation, algae were exposed to a short (5 s) far-red light pulse [32,33] and then irradiated with increasing intensities of PAR (25; 45; 65; 90; 125; 190; 285; 420; 625 µmol photons $m^{-2} s^{-1}$) with 30 s intervals between each PAR intensity. The short far-red light pulse before the rapid light curve measurements is required for red algae, as this pulse ensures a full oxidation of the electron transport chain, and thus, ensures steady fluorescence emission while measuring F_m [32,33].

2.7. Free radical scavenging activity

Agarophyton vermiculophyllum material used for analysis was rinsed in fresh water to remove salt and dried at 30 °C in an oven for 48 h. This drying protocol was chosen to mimic the handling that would most likely be used to process macroalgae in industrial applications. An extraction was prepared by weighing 0.1 ± 0.001 g of dried material and grinding it in a porcelain mortar with 2 mL of ethanol (70%) [34]. From preliminary tests comparing different concentrations of ethanol used in the literature, we found that 70% ethanol produced the best extraction efficiency. The ground material was placed in 15 mL falcon tubes and then incubated in a thermal bath (SS40-2, Grant Instruments, Cambridge, UK) at 45 ± 2 °C, with a constant shaking at 130 rpm for 6 h [35]. The extracts were then centrifuged (Beckman GS-15 R centrifuge) for 10 min at 4 °C at 2500 × *g* [35,36]. Then the supernatants were carefully transferred into empty Falcon tubes. The extraction of the residue algae was repeated with an additional 2 mL of ethanol (70%) followed by the water bath for 1 h and centrifugation in order to increase extraction efficiency. Both supernatants were mixed and used for the antioxidant analyses of each sample immediately.

The ABTS radical cation (ABTS•+) decolourization assay was used [37] and adapted to the 96-well microplate [38] to determine radical scavenging activity in *A. vermiculophyllum*. The ABTS•+ reagent was prepared by mixing 1 mL of 7 mM ABTS [2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulphonic acid)] solution with 0.5 mL of 2.45 mM potassium persulfate ($K_2S_2O_8$) [37]. The ABTS•+ solution was then allowed to incubate in the dark at a room temperature for at least 16 h to reach maximal absorbance and a stable radical concentration [37,38]. The ABTS•+ solution was diluted with 70% ethanol until the absorbance of 0.70 at 734 nm was reached [38].

Sample extracts as well as Trolox solutions for the antioxidant standard and negative control were added (20 µL) to each well of the 96-well microplates. Three aliquots from each extract were added to separate wells. Then 280 µL of ABTS•+ solution was added to each well [38]. The microplate was incubated in the dark at a room temperature for 8 min [35] and the absorbance was then recorded at 734 nm [37,38] by the microplate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland). A standard curve was conducted with different dilutions of Trolox with ethanol (0 to 600 µg mL^{-1}, $R^2 > 0.95$). Antioxidant activity was expressed as µmol Trolox equivalents (TE)·g^{-1} dry weight (DW).

2.8. Statistics

Due to the small sample size and complexity of our experiments, standard ANOVA tests were not appropriate for statistical analysis. Therefore, the data were analyzed using the aligned rank transform

(ART) for nonparametric factorial analyses. Using this method, we could perform factorial nonparametric analyses and include repeated measures where appropriate. The ARTool package was used to conduct these analyses in R version 3.6.1. Repeated Measures was used for RGR and F_v/F_m parameters, as they were measured on the same individuals over time. Time was treated as a repeated measures factor, while salinity, desiccation, light intensity, UVR, and PAR dose were treated as fixed factors. Because free radical scavenging activity required destructive sampling and was not measured on the same individuals over time, repeated measures was not applied for this response variable. When only two groups were tested (e.g. light intensity on a single day) a *t*-test (Welch test) was conducted. A Principle Component Analysis was applied to some data to clarify patterns in the physiological responses of the algae to environmental variables. The data were centered and scaled using the prcomp function in the package ggfortify in R version 3.6.1 and the results were visualized using the function autoplot.

3. Results

3.1. Effects of salinity and desiccation

The free radical scavenging activity of *A. vermiculophyllum* ranged from 8.6 to 14.0 µmol TE g^{-1} DW. There was a significant interaction between exposure time, desiccation and salinity (ART: F = 5.8, p = 0.0054) on the percent of initial Trolox equivalents (Fig. 1A). The free radical scavenging activity first decreased in all treatments within the first 3 days, and then increased in all treatments after 7 days of exposure, but they were highest in the 40 ppt salinity treatments after 7 days, regardless of whether or not the algae were exposed to desiccation. The high salinity treatment resulted in a 13% increase compared to the initial conditions.

There was a significant interactive effect of salinity, desiccation and time on the RGR (ART: F = 52.3, p = 8.9e^{-5}). The macroalgae only grew in the 30 ppt salinity treatment without desiccation, and growth was higher after 7 days than after 3 days (Fig. 1B). In all other treatments, the macroalgae exhibited no growth or a negative growth rate.

There was a significant interactive effect of salinity and desiccation (F = 48.7, p = 0.00012) and salinity and time (ART: F = 44.2, p = 0.00016) on the F_v/F_m. Desiccation severely decreased the F_v/F_m in the high salinity treatment, and this became more severe over time (Fig. 1C). Additionally, desiccation caused an immediate decrease in the F_v/F_m in the high salinity treatment, while the negative effect of high salinity was delayed in the treatment without desiccation. In contrast,

desiccation had no effect on F_v/F_m in the 30 ppt salinity treatment.

3.2. Effects of light intensity and UVR

The free radical scavenging activity of *A. vermiculophyllum* ranged from 5.8 to 8.7 µmol TE g^{-1} DW. Within the first 7 days of exposure to the two light treatments, the percent of initial Trolox equivalents was significantly higher in the algae exposed to 150 µmol photons m^{-2} s^{-1} than those exposed to 350 µmol photons m^{-2} s^{-1} (ART: F = 22.4, p = 0.00049, Fig. 2A) and the values generally decreased over time (ART: F = 4.8, p = 0.029). Therefore, high light intensity in the absence of UVR did not result in increased free radical scavenging activity. After UVR was added, there was a significant interaction between light intensity and UVR (ART: F = 17.0, p = 0.0033). UVR exposure combined with high light intensity resulted in an 8% increase in free radical scavenging activity compared to the low light intensity with UVR exposure (but only 4% higher than the initial activity). In the absence of UVR exposure, the algae exposed to high light intensity had the lowest free radical scavenging activity (Fig. 2A).

Neither light intensity nor time of exposure had a significant effect on RGR after seven days of exposure. After the addition of UVR for three days, there was a significant effect of both light intensity (ART: F = 23.3, p = 0.00041) and UVR (ART: F = 8.7, p = 0.012). The RGR was highest in the algae exposed to high light and UVR, while it was lowest in the algae exposed to low light with no UVR (Fig. 2B). In both light treatments, the exposure to UVR increased the RGR.

There was a significant treatment effect of light intensity on F_v/F_m after 10 days of exposure (ART: F = 5.4, p = 0.039), such that F_v/F_m was higher at 150 µmol photons m^{-2} s^{-1} than at 350 µmol photons m^{-2} s^{-1} (Fig. 2C), but no significant effect of UVR (ART: F = 0.15, p = 0.70).

3.3. Effects of PAR dose and UVR

The free radical scavenging activity of *A. vermiculophyllum* ranged from 5.3 to 9.3 µmol TE g^{-1} DW. The macroalgae exposed to the high light intensity had significantly higher free radical scavenging activity than the algae exposed to the low light intensity (ART: F = 113.3, p = 1.6e^{-8}) on both day 4 and day 7, and this was 32% higher than the initial activity (Fig. 3A). There was a significant interactive effect between light intensity and UVR on antioxidant activity (ART: F = 5.4, p = 0.049, Fig. 3A). In the absence of UVR, the antioxidant activity decreased in the low light treatment, but when UVR was applied, the antioxidant activity returned to initial levels. In comparison, UVR had no effect on
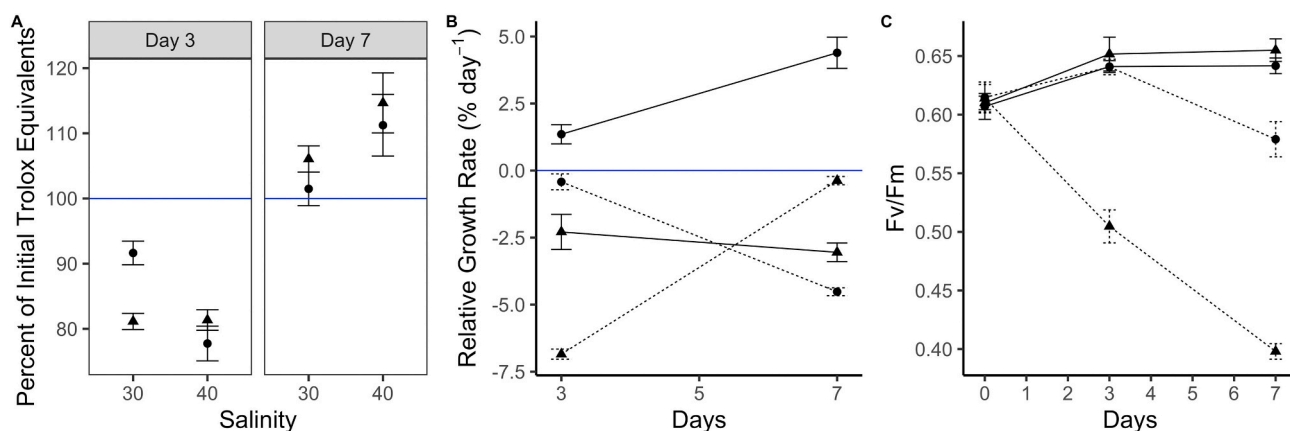

Fig. 1. The response of A. vermiculophyllum to salinity and desiccation. A) The free radical scavenging activity (expressed as mean ± SE percent of initial, n = 5) of A. vermiculophyllum after 3 and 7 days of exposure to 30 and 40 ppt salinity with (triangles) and without (circles) desiccation B) the mean (±SE, n = 5) relative growth rate and C) mean (±SE, n = 5) effective quantum yield of photosystem II (F_v/F_m) of A. vermiculophyllum after 3 and 7 days of exposure to 30 (solid lines) and 40 (dotted lines) ppt salinity with (triangles) and without (circles) desiccation. The solid blue line in A represents 100%, such that values above that line indicate an increase in antioxidant activity compared to the initial values. The solid blue line in B represents 0 growth, such that values above that line indicate positive growth, and below indicate negative growth.
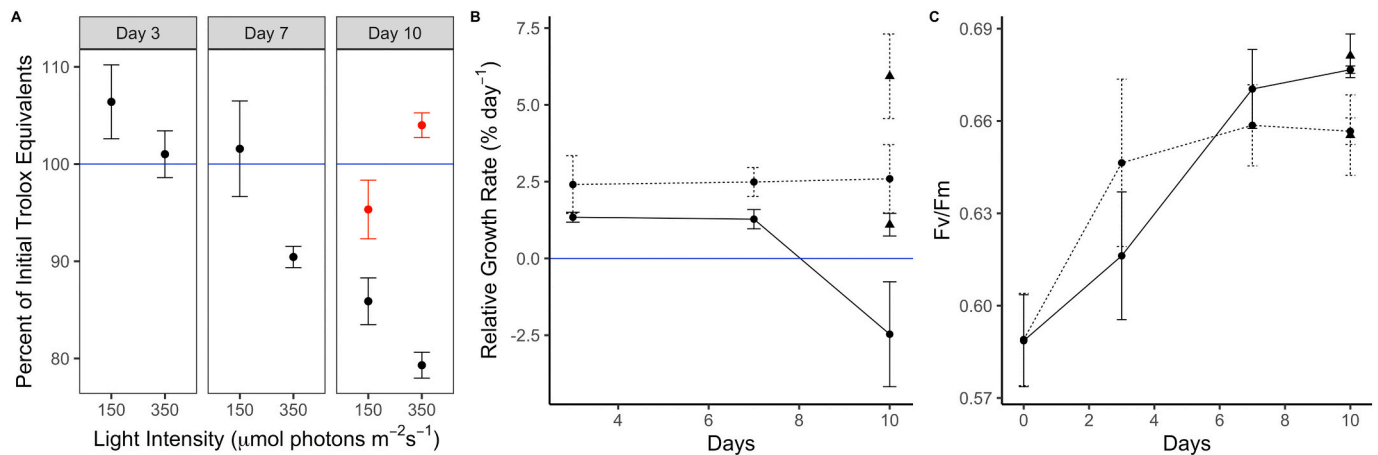
Fig. 2. The response of A. vermiculophyllum to different irradiances. A) free radical scavenging activity (expressed as mean percent of initial, n = 3) of A. vermiculophyllum after 3, 7 and 10 days of exposure to low (150) and high (350) light intensity (μmol photons m^{-2} s^{-1}) and after the addition of a 3-day UVR treatment (red symbols) on the 7th day B) mean (±SE, n = 5) relative growth rate and C) mean (±SE, n = 5) effective quantum yield of photosystem II (F$_v$/F$_m$) of A. vermiculophyllum after 3, 7 and 10 days of exposure to low (150; solid lines) and high (350; dotted lines) light intensity (μmol photons m^{-2} s^{-1}) and after the addition of a 3-day UVR treatment (triangles) on the 7th day. The solid blue line in A represents 100%, such that values above that line indicate an increase in antioxidant activity compared to the initial values. The solid blue line in B represents 0 growth, such that values above that line indicate positive growth, and values below it indicate negative growth.
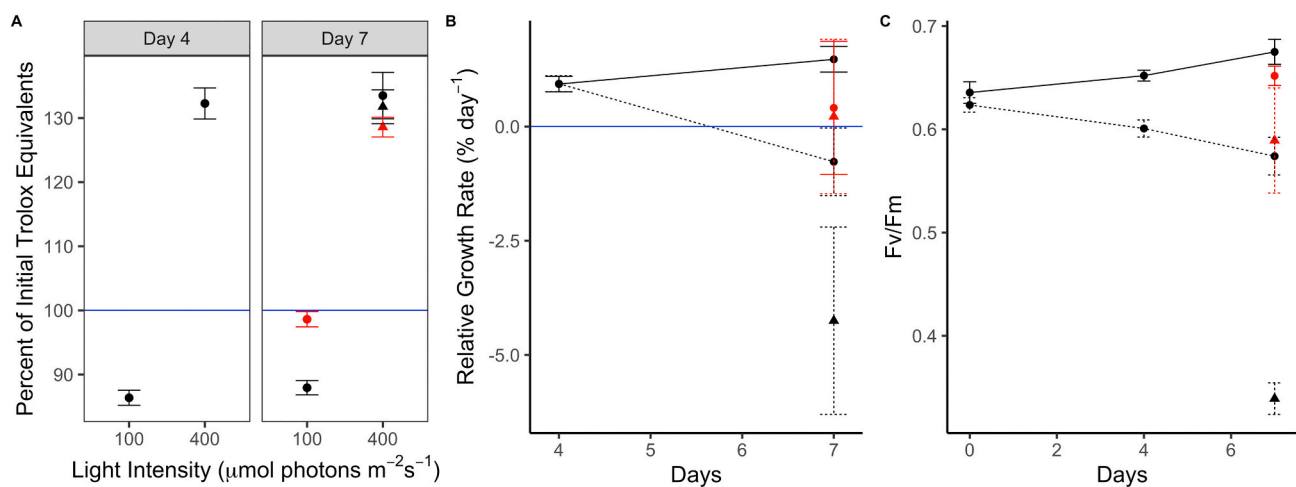

Fig. 3. The response of A. vermiculophyllum to PAR dose versus light intensity. A) Mean (±SE, n = 3) percent of initial Trolox equivalents after 4 days of exposure (left panel) to low (100) and high (400) light intensities, but the same PAR dose (8.6 mol photons m^{-2} day^{-1}). A high PAR dose treatment (400 μmol photons m^{-2} s^{-1} for 24 h; 34.6 mol photons m^{-2} day^{-1}; triangles) was added after 4 days and the effect of UVR (red symbols) for 3 days (right panel) on the percent of initial Trolox equivalents is shown. B) Mean (±SE, n = 5) relative growth rates and C) mean (±SE, n = 5) effective quantum yield of photosystem II (F$_v$/F$_m$) after exposure to low (100; solid lines) and high (400; dotted line) light intensities, but the same PAR dose (8.6 mol photons m^{-2} day^{-1}). A high PAR dose treatment (400 μmol photons m^{-2} s^{-1} for 24 h; 34.6 mol photons m^{-2} day^{-1}; triangles) and UVR (red symbols) were added after 4 days. The solid blue line in A represents 100%, such that values above that line indicate an increase in antioxidant activity compared to the initial values. The solid blue line in B represents 0 growth, such that values above that line indicate positive growth, and values below it indicate negative growth.

antioxidant activity in the high light treatment.

There were no significant treatment effects of light intensity or UVR on RGR (Fig. 3B). There was a significant interactive effect of light intensity and time (ART: F = 10.4, p = 0.032) on RGR, such that growth rates decreased over time in the high light treatment, and increased slightly in the low light treatment. There was a significant main effect of light intensity on F$_v$/F$_m$ (ART: F = 21.6, p = 0.0097, Fig. 3C), such that the F$_v$/F$_m$ was higher in algae exposed to 100 μmol photons m^{-2} s^{-1}, despite the fact that they received the same PAR dose (Fig. 3C). In contrast to the antioxidant activity, the significant interaction between UVR and light intensity (ART: F = 12.2, p = 0.0036) showed no effect of UVR on F$_v$/F$_m$ in the low light treatment. In the high light treatment during 24-hour exposure, the F$_v$/F$_m$ was significantly reduced in the absence of UVR, while the addition of UVR maintained F$_v$/F$_m$ values

similar to those in the algae exposed to the lower PAR dose without UVR (Fig. 3C).

A Principle Component Analysis of antioxidant activity, growth rates, and F$_v$/F$_m$ explained 91% of the variance in the first two components (Fig. 4). The first axis separated the algae exposed to low versus high light dose based on different growth rates and F$_v$/F$_m$. The second axis separated the algae exposed to different light intensities based on different antioxidant activity, and to a small extent F$_v$/F$_m$.

The effect of UVR on antioxidant activity differed depending on light intensity and dose. At low to moderate light intensities (100–350 μmol photons m^{-2} s^{-1}) and dose (8–20 mol photons m^{-2} day^{-1}), UVR had a significant impact on antioxidant activity, such that algae exposed to UVR had higher antioxidant activities than those not exposed to UVR (Fig. 5). On the other hand, algae exposed to high light intensity (400
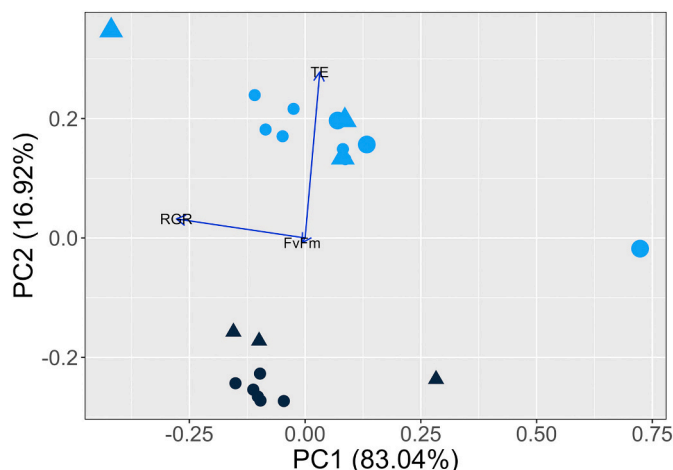
Fig. 4. Principle Component Analysis of the response variables measured (Trolox equivalents, relative growth rate, and Fv/Fm). The environmental factors light intensity (100: dark blue, 400: light blue), light dose (8.64: small symbols, 34.6: large symbols) and UVR (no UVR: circles, UVR: triangles) are used to help visualize the variance in the data. The percent of the variance explained by each principle component are shown on each axis label in parenthesis.
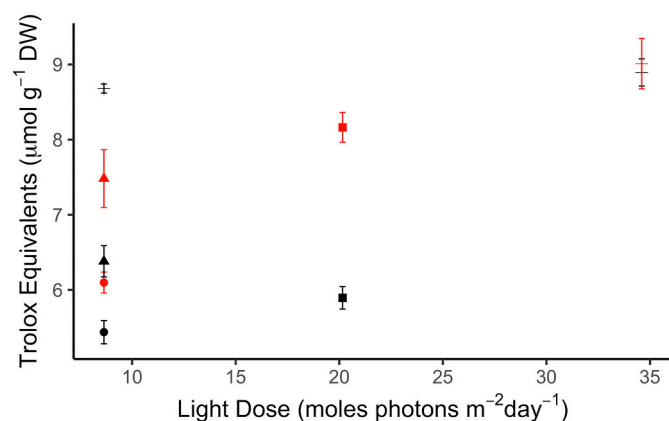

Fig. 5. The effect of light dose on antioxidant activity. Mean antioxidant activity (±SD, n = 3) of A. vermiculophyllum expressed as Trolox equivalents as a function of light dose. The effect of UVR exposure (red symbols) and light intensity (circles = 100, triangles = 150, squares = 350, cross = 400) are shown.

μmol photons m^{-2} s^{-1}) at both low and high light doses had the highest antioxidant activity, and UVR did not have a significant effect.

4. Discussion

The free radical scavenging activity of *A. vermiculophyllum* in our study ranged from 5.3 to 14.0 μmol TE g^{-1} DW, which corresponds to those reported in previous studies for *Gracilaria* spp. and other macroalgae species [34,35]. In fact, these values are very similar to the range of antioxidant activities reported for seven macroalgal species from Spain, the highest of which (*Hydropuntia cornea*) was approximately 14.5 μmol TE g^{-1} DW [35].

Our results demonstrate that the free radical scavenging activity of *A. vermiculophyllum* can be increased after short-term exposure to environmental stressors such as salinity and high light intensity, but that light intensity was more important for increasing the free radical scavenging activity than PAR dose or time of exposure. Exposure to high salinity for one week (but not 3 days) resulted in a 13% increase, high

light intensity on a long day cycle for seven days combined with a three-day UVR treatment resulted in a 4% increase, and high light intensity on a short day (6 h) cycle for 4–7 days resulted in a 32% increase in free radical scavenging activity. Therefore, short-term exposure to high light intensity on a short-day cycle was the most efficient way to increase free radical scavenging activity in *A. vermiculophyllum*. Exposure to high light intensity for short days induced higher antioxidant activity than low light intensity for long days, even when the PAR dose was the same, suggesting that high light intensity, rather than the total amount of photons absorbed over time, triggers a response in antioxidant activity. This increase in antioxidant activity corresponded to a decrease in photosynthetic efficiency, but growth rates were unaffected by light intensity and remained the same when the algae received the same PAR dose. A similar trend in growth rates of *A. vermiculophyllum* in the Baltic Sea was reported by [29]. While few studies have investigated the different effects of PAR dose versus PAR intensity, this trend complements the fact that higher rates of productivity have been found in macroalgae exposed to lower photon flux densities over a longer period [39], perhaps because the macroalgae invest more energy into photoprotection at high light intensities. Nevertheless, it is unclear why we observed higher antioxidant activity at 150 compared to 350 μmol photons m^{-2} s^{-1} in one experiment. The initial F_v/F_m was slightly lower in these algae compared to those that were exposed to 100 and 400 μmol photons m^{-2} s^{-1}, suggesting that the photosynthetic efficiency and the general physiological condition of the algae differed slightly at the start of the two experiments, perhaps due to seasonal differences. It has been shown that antioxidant activity in macroalgae follows seasonal patterns [22,23], but we did not observe differences in the initial antioxidant activity based on ABTS•+ of the algae before each experiment.

In addition to light intensity, light quality also affects antioxidant activity in macroalgae. Ulvan extracted from *Ulva pertusa* had significantly higher antioxidant activity when the algae were grown under blue light compared to white light [40]. Additionally, *Ulva* sp. grown under blue light had higher ABTS•+ radical scavenging activity when grown under blue light compared to white light, regardless of day length [41]. Therefore, it seems both intensity, duration, and quality are important parameters that influence antioxidant activity in macroalgae, and further research should investigate whether high intensity blue light could further enhance the antioxidant activity of *A. vermiculophyllum*.

In a study where stocking density of *Derbesia tenuissima* was manipulated to test the effects of light availability on antioxidant activity, the authors found that stocking density, and thus, light intensity, had a significant impact on the total phenol content, DPPH radical scavenging ability, and ferric reducing antioxidant capacity, but not ABTS•+ radical scavenging activity [30]. They also showed that photosynthetic efficiency decreased with increasing antioxidant production, which we also observed in *A. vermiculophyllum*. The authors suggest monitoring the F_v/F_m to manage culture conditions for optimizing antioxidant activity in land-based macroalgal culture. The highest increase in antioxidant activity we observed (32%) corresponded to an 11.5% reduction in F_v/F_m values (88.5% of the highest F_v/F_m values). Similarly, Magnusson et al. [30] found that an increase in total phenol content of 20% corresponded to a 15% reduction in F_v/F_m (85% of maximum F_v/F_m). Monitoring F_v/F_m has also been proposed as a method to detect stress early in *Gracilaria cornea* cultivation [42]. Therefore, monitoring F_v/F_m can be used as a tool to maintain crop health during biomass production, as well as a management tool during the optimization of antioxidant activity.

UVR had a strong effect on antioxidant activity at low light intensities, but not at high light intensity (>350 μmol photons m^{-2} s^{-1}). In contrast to antioxidant activity, growth rates and photosynthetic efficiency were not significantly affected by UVR. The UVR intensity used in our study was quite low compared to studies that have investigated UVR effects on macroalgae [32,43,44], but the time of exposure was longer (up to 16 h day^{-1}) in our study, and clearly had an effect on antioxidant activity. It would be necessary to increase the UVR in future studies to determine if the antioxidant activity could be increased even further.

In conclusion, we showed that a four-day exposure period to high light intensity for short days (6 h) showed the highest increase in free radical scavenging activity in *A. vermiculophyllum*. Hypersalinity also increased free radical scavenging activity, but to a lesser extent. We therefore suggest an optimization period of 4–7 days exposure to high light on a 6:18 h light:dark cycle prior to harvesting in order to increase antioxidant activity. More research is required to investigate if higher light intensities or UVR could further increase antioxidant activity in *A. vermiculophyllum*, but here we provide a good starting point for increasing the functionality of *A. vermiculophyllum* in diverse industrial applications, including the potential extension of shelf-life for food products.

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CRediT authorship contribution statement

LCH & ST designed the study and drafted the manuscript, ST carried out the study and collected the data, LCH & ST analyzed the data, JS provided technical support, BB & JH provided lab facilities and administrative support, RB, FR, IE, DH, JH and BB obtained funding for this project and revised and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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