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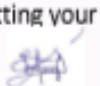

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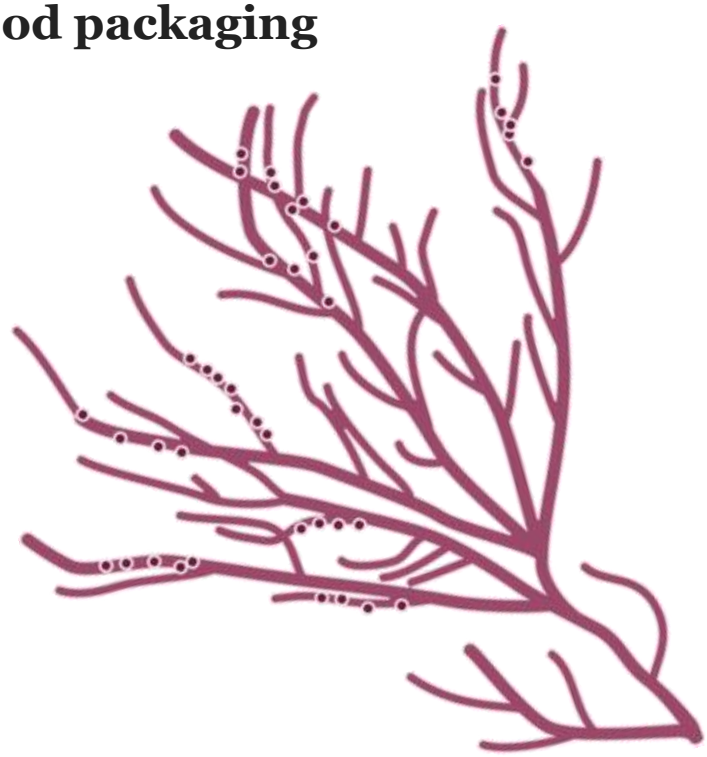
Optimization of the and antioxidant activity of *Gracilaria vermiculophylla* for use in an edible film for food packaging



Prepared for:

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Prepared by:

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Primary supervisor: Dr. Laurie C. Hofmann

Secondary supervisor: Bruno Freire Boa de Jesus

CONTENTS

Current work contains 6 Tables and 16 Figures. Sixty eight (68) literature sources were used as References. Work is written in 62 pages and includes 13531 words.

1. Abstract

Single-use food packaging contributes towards almost one-third of the global plastic waste. In an attempt to minimize environmental impacts, sustainable alternatives need to be developed. Seaweed is a renewable resource that can be utilized for packaging development for plastic replacement. *Gracilaria* is one of the most widely-cultivated algae, used in food and pharmaceutical industries as a valuable source of agar. Studies have succeeded in developing biopolymer plastics from *Gracilaria* containing high levels of antioxidants. Antioxidant properties are essential in biopackaging as they are beneficial to packaged food by limiting lipid oxidation, which is one the main causes of the food spoilage. This study focuses on the cultivation of *Gracilaria vermiculophylla* and optimizing its antioxidant properties for further use as an edible film for food packaging. Antioxidant compounds are synthesized in seaweeds as a response to oxidative stress that often occurs due to harsh environmental fluctuations. Therefore, antioxidant activity of the seaweed can be modified through exposure to different stress conditions. Three consecutive experiments were conducted in this study to better understanding on how different levels of light irradiance, differences in light : dark cycles, exposure to UVA radiation, hypersalinity, desiccation and the duration of exposure can affect the antioxidant profile of *Gracilaria vermiculophylla*. High light intensity increased the antioxidant levels of *G. vermiculophylla*, reaching a maximum of 33.45%. Moreover, the combination of the high light irradiances and UVA enhanced the antioxidant activity and had a positive survival effect on *G. vermiculophylla*. The study showed that increased antioxidant activity could be achieved after 3 days of exposure to different cultivating conditions. These findings may be beneficial to industrial scale seaweed cultivation, where favorable antioxidant levels could be reached shortly prior to harvesting.

2. Acknowledgements

Author would like to thank the Erasmus Mundus Joint Masters Degree Program for providing an opportunity to be a part of highly respected and life changing Joint Masters Degree in Aquaculture, Environment and Society (ACES). Than you to Prof. Elizabeth Cottier-Cook, Dr. Michalis Pavlidis, and Prof. Peter Beninger that are ACES Program leaders at Scottish Association for Marine Science (SAMS), University of Crete (UoC) and University of Nantes (UoN). Author gratefully thanks Laurie C. Hofmann and Bruno Freire Boa de Jesus for scientific supervision during the Master Project at Alfred Wegener Institute Helmholtz Center for Polar and Marine Research. Thank you to Prof. Bela H. Buck and Dr. Inka Bartsch for providing a laboratory equipment to perform experimental part of Master Project. This research is a part of the Mak-Pak project (Nachhaltige Verpackungslösung aus Makroalgen, Förderkennzeichen 28-1-A1.049-16) funded by the German Ministry of Agriculture and Nutritioin (BLE).

3. Introduction

3.1 Plastic pollution

Intensive use of the plastic material in a daily life has led to undeniable environmental pollution (Geyer, 2017; Siah et al., 2015). Since 1950, world plastic production has been constantly rising and in 2017, it was estimated at 374 million tones (Plastic Europe, 2018). The growth of plastic industry bypasses manufacturing of many other synthetic materials (Geyer et al., 2017). The commonly used plastic material is resistant to degradation in nature and consequently accumulates in landfills and marine environments (Barnes et al., 2009). Moreover, microplastics – small fragments of plastics, can cause harm to wildlife as well as enter in the food chain and further pose a threat to high trophic levels, including humans (Efferth & Paul, 2017; Prata, 2018; Sedayu et al., 2019). Therefore, over the last decade, there has been an increase in the research involving the replacement of plastics with sustainable and biodegradable materials (Sedayu et al., 2018). The food packaging industry is a major contributor to plastic waste (Jambeck *et al.*, 2015; Sedayu, Cran and Bigger, 2019) with 39.7% of the share to the total plastic waste (Plastic Europe, 2018), prompting the development of alternatives to combat the issue.

3.2 Biodegradable alternatives to plastic

Materials that were developed using renewable resources, such as: vegetable starch, vegetable protein, cellulose, chitosan, gluten, and guar gum among other renewable resources, are suitable alternatives to existing synthetic polymers (Gómez-Estaca *et al.*, 2014; Abdul Khalil *et al.*, 2017). However, several barriers prevent these biopolymers from the wide commercial distribution, among which are their source limited availability, cultivation or synthesis methods (Sedayu, Cran and Bigger, 2019). Moreover, commonly used plastics are relatively cheap compared to the alternative biopolymers

(Mekonnen et al., 2013). Furthermore, the mechanical and barrier properties of such biopolymers are relatively low, compared with the commonly used non-biodegradable materials (Abdul Khalil *et al.*, 2017).

Seaweed is one of the natural renewable sources, used for the development of packaging material. Marine algae are considered as one of the most promising materials due to their fast growth and relatively easy cultivation techniques, compared to the terrestrial natural sources (Sedayu, Cran and Bigger, 2019).

3.3 Seaweed-based films for food packaging: opportunities and drawbacks

Seaweed-derived polymers such as agar, carrageenan, and alginate have been used as a base for the biodegradable film development in food and pharmaceutical applications (Abdul Khalil et al., 2017; Farhan & Hani, 2017; Jumaidin et al., 2018; Sedayu et al., 2018, 2019). However, a complex technology and high costs involved in the polymer isolation combined with lower yield of polymer from biomass supplied are critical drawbacks to wider commercial use of such biofilms in the global market (Siah et al., 2015).

Seaweed-derived polymers have high oxygen vapor barrier properties along with impermeability to oils and fats. Nevertheless, biopolymers films often have lower mechanical and barrier capacity compared to synthetic counterparts (Jin and Min, 2010; Saurabh *et al.*, 2013; Abdul Khalil *et al.*, 2017). In the study of Siah et al., 2015 the mechanical properties of a biodegradable film, developed from the raw seaweed mass, was studied and the research highlighted a high mechanical strength of such seaweed-based biofilm, confirming a promising alternative to existing biopolymer films.

The red seaweed *Gracilaria* is one of the most widely-cultivated species (Abreu et al., 2011; Buschmann et al., 2008). It is used in food and pharmaceutical industries as a valuable source of agar (Sousa et al., 2010). The rapid growth and high yields of biomass makes this species viable for growth at commercial scale (Capo et al., 1999; Francavilla

et al., 2013). In addition, being a rich source of protein and non-starch polysaccharides, which determine the film-making properties in algae (Atef et al., 2014; Siah et al., 2015), *Gracilaria* appears to be a potential source for biofilm development (Baek and Song, 2018). Some studies have succeeded in developing biodegradable food packaging using agar extracts from *Gracilaria* (Sousa *et al.*, 2010; Baek and Song, 2018). However, agar films have relatively poor physical and mechanical properties thereby restricting its potential as food packaging source commercially (Rhim & Ng, 2007)

In the study of Siah et al., 2015 a novel technique was designed for film development from *Kapaphyccus* through utilization of whole seaweed material. Such films, when compared with the films from seaweed-derived polymers, do not require a complex process for polymer extraction and thus, are cheaper and easier to prepare. However, such technique has not been fully explored commercially and has not been used with *Gracilaria*-based films. Current work is a part of the Mak-Pak project that aims to create a biodegradable film for food packaging using whole seaweed, where *Gracilaria* is considered one of potential bases.

3.4 Improving the antioxidant properties of seaweed-based biofilms

Antioxidant profile is one of the valuable properties of biodegradable packaging as it can benefit packaged food through limiting lipid oxidation, which is one the main causes of the food spoilage. Existing strategies involve direct addition of antioxidants or packaging techniques that allow a limited oxygen access (Gómez-Estaca *et al.*, 2014). There is a high emphasis on research involving limited lipid oxidation of food packaged in the biodegradable film. Seaweed-based packaging offers an highly antioxidant rich packaging film (Abdul Khalil *et al.*, 2017). However, such properties might be modified by either reinforcing raw material with antioxidants or changing the antioxidant content of the algae during the cultivation stage. The technique of reinforcing the material was

developed for the biopolymer films (Jumaidin et al., 2018), but purely investigated for the whole seaweed-based films.

3.5 Factors that impact antioxidant activity of seaweed

The general mechanism of the stress response by an organism to environmental stress condition involves oxidative stress in the cellular pathway. Therefore, organisms developed a mechanisms to control oxidative stress by limiting the reactive oxygen species (ROS) production (Bischof and Rautenberger, 2012). Because of challenges produced by harsh and competitive environment, seaweeds developed highly efficient defense mechanisms by synthesizing antioxidant compound. For example, seaweeds surviving in the intertidal zones with fluctuating levels of irradiance and oxygen concentration combined with exposure to the air are able to cope with aforementioned stress by synthesis of a wide range of antioxidant compounds. These compounds include carotenoids, polyphenols, minerals, and vitamins among others. The rapid oxidative response of seaweeds to environmental stress provides an opportunity to control and modify the antioxidant capacity of cultured seaweed by exposing it to various stress conditions for a short period (Jiménez-Escrig et al., 2012).

3.6 Mak-Pak project

The present work is a part of the Mak-Pak project (Nachhaltige Verpackungslösung aus Makroalgen, Förderkennzeichen 28-1-A1.049-16) funded by the German Ministry of Agriculture and Nutritioin (BLE) that aims to create a biodegradable and/or edible packaging material for the food sector using macroalgae biomass. The goal is to develop a biodegradable package that will positively affect the packaged food and/or consumer health. Additionally, the package may be covered with a protective film to prevent the exchange of smell and taste between the packaging and the product.

The present work, is the first step in the film development and focuses on the seaweed material cultivation and optimizing its antioxidant properties for further use in the film making process.

4. Materials and methods

4.1 Macroalgal culture: material collection, conditions of cultivation

Gracilaria.vermiculophylla was collected from the intertidal zone during low tide on the island of Sylt (Wadden Sea, Germany, 54°54′ N, 8°20′ E) in March 2019 (water temperature 7°C, salinity 30 ppt). Harvested seaweed was transported with most towels in a cooler to the Alfred Wegener Institute Helmholtz Center for Polar and Marine Research in Bremerhaven for further cultivation. To obtain clean thalli for the experiments, single branches of *G. vermiculophylla* were removed from the field material, cleaned with cotton swabs dipped in 7.5% Iodine (Braunol 100 mL, B. Braun, Melsungen, Germany), rinsed in filtered seawater, and cultivated in clear 10-liter balloon flasks bubbled with compressed air in a green house. The temperature of cultivation was 17 °C which was shown to be the most favorable for *G. vermiculophylla* (Weinberger *et al.*, 2008). In order to avoid nutrient limitation artificial seawater was enriched by adding fertilizer (Blaukorn Garden Fertilizer 2.5 L, Münster, Germany) in a concentration of 40 µl of fertilizer in 1 L of seawater. The water in tanks was changed once per week to maintain appropriate levels of inorganic carbon. Germanium dioxide (70 mg/L) was added in the culture flasks to prevent growth of diatoms in the cultures. Algae were cultivated during March to May 2019 under natural light : dark cycle that varied from 10 to 13 hours.

4.2 Material preparation

G. vermiculophylla was acclimatized for 3 weeks before the start of the experiments under conditions mentioned above. Prior to every experiment, algal material

was cleaned with 7.5% Iodine solution (Braunol 100 mL, B. Braun, Melsungen, Germany) to remove epiphytes.

4.3 Algal growth, photosynthesis and thallus absorption

4.3.1 Growth performance

Growth performance of *G. vermiculophylla* in three experiments was measured by weighing wet algae material every three to four days. RGR were calculated using the logarithmic equation (1) proposed by Glenn and Doty, 1992. SGR were calculated using an equation (2) proposed by Luhan and Sollesta, 2010.

$$\frac{lnWt \ - \ lnW0}{t} \ g \ g-1 \ day-1 \qquad (1)$$

$$\frac{lnWt \ - \ lnW0}{t} \ x \ 100\% \ \ \% \ day-1 \qquad (2)$$

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). SGR were calculated using the logarithmic equation proposed

4.3.2 Pulse Amplitude Modulated fluorometry

Measure and analyses of the Chl fluorescence can provide knowledge on the photosynthetic organism performance, therefore, this technique was chosen to evaluate physiological state of *G. vermiculophylla* before and during experiments. The chlorophyll fluorescence of photosystem II (PS II) was measured by a portable pulse amplitude

modulation fluorometer (Junior-PAM, Walz, Effeltrich, Germany) as it is shown in the Figure 1. For the dark-adapted photosynthetic organisms, the ratio of variable (Fv) to maximal (Fm) fluorescence represents the potential electron transfer quantum yield of PS II. The effective quantum yield shows the probability of the absorbed photons to drive electrons through the open centers of PS II (Beer *et al.*, 2000). Therefore, it is an effective parameter to evaluate a photosynthetic organism viability to the different environmental conditions (Graiff et al., 2015) that were tested in three experiments.

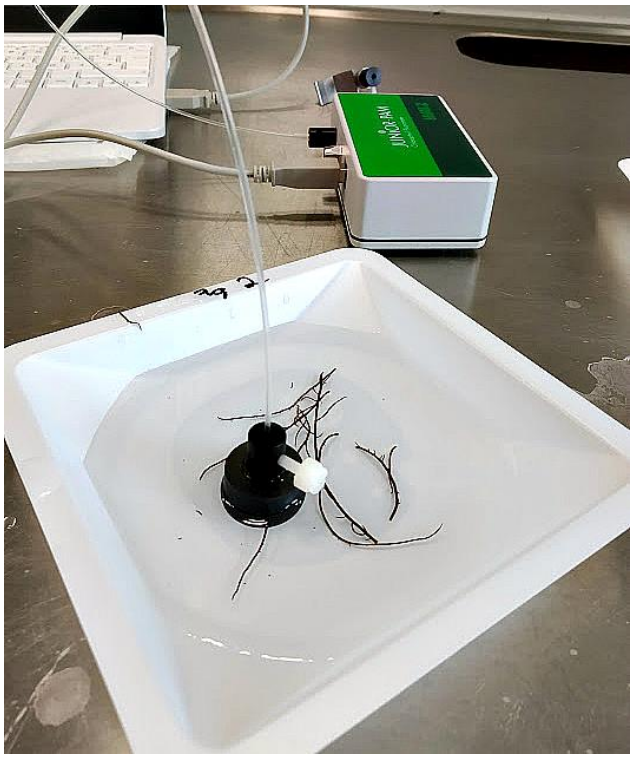


Figure 1. Set up for Fv/Fm measurments using Junior-PAM.

The maximum effective quantum yield of PS II (Fv/Fm ratio) was measured for samples from each experimental beaker. Samples were dark-adapted prior measurements as suggested by (Hanelt *et al.*, 1997; Figueroa *et al.*, 2010) for 15 minutes. After a dark adaptation, algae were exposed to a short (5s) far-red light pulse as reported by (Hanelt *et al*., 1997; Figueroa *et al*., 2010) and then irradiated with an increasing intensities of PAR (25; 45; 65; 90; 125; 190; 285; 420; 625 µmol photon sm^{-2} s^{-1}) with the

30 s interval between each PAR intensity. 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 (Hanelt *et al.*, 1997; Figueroa *et al.*, 2010) and thus, ensures steady fluorescence emission while measuring Fm (Hanelt *et al.*, 1997).

4.3.3 Algae thallus absorption

In order to determine macroalgae thallus absorption (A) following formula was used (Beer et al., 2000):

$$A = 1 - Et \,/\, E0,$$

where E_0 is the incident irradiance of PAR; E_t is the transmitted irradiance through the algae. To obtain E_0, E_t the light sensor (LI-189, LI-COR, Lincoln, USA) was covered with the black insulating tape leaving only 0.2 cm width space in the middle of the device. Incident irradiance (E_0) of PAR was measured at the top of the experimental beaker with the light sensor under the light conditions used in experiment. Transmitted irradiance E_t was determined by placing the same algae that was used for chlorophyll fluorescence measurements upon the light sensor in the narrow space that was not covered by the insolating tape. Both E_t and E_0 values were obtained by the sensor at the same point at the top of the beaker and at a strictly parallel position to the lamp.

4.4 Experiment setup

4.4.1 Experiment 1: The effect of salinity and desiccation on antioxidant properties

In this study, the first experiment aimed to examine the effect of desiccation and hyper-salinity conditions on the activation of the antioxidant system in *Gracilaria vermiculophylla*. The experiment was conducted in a walk-in climate room (15 °C). During a week, algae were cultivated in two different salinities with or without an application of

the desiccation treatment. Therefore, four different conditions including control were tested. Those conditions included desiccation of algal samples at both 30 and 40 ppt salinities (normal and hyper-salinity conditions respectively) and samples that did not include desiccation at both 30 and 40 ppt salinities.

In this study first experiment aimed to examine the effect of desiccation and hyper-salinity conditions on the activation of the antioxidant system in *G.vermiculophylla*. Experiment was installed in a walk-in constant cooling chamber (15 °C). During a week, algae were cultivated in two different salinities with or without a daily application of the desiccation treatment. Therefore, four different conditions including control were tested. Those conditions included desiccation of algal samples at both 30 and 40 ppt salinities (normal and hyper-salinity conditions respectively) and samples that did not include desiccation at both 30 and 40 ppt salinities. Control condition did not include desiccation of the algal samples at the salinity 30 ppt.

Each of the four treatments consisted of five 3 L beakers (n=5) (Figure 2). In each beaker 7 g of *G.vermicullophyla* was placed. The density for the algae cultivation was chosen using the stocking density recommended by Kim & Yarish, 2014 as follows: 2-4 g L^{-1}. The artificial seawater was used for the experiment and prepared by mixing salt (Seequasal GmbH) with tap water. Final salinity was measured with a waterproof pH/temperature/salinity meter (pH 3110, WTW GmbH, Weilheim, Germany). The 3 L beakers were filled with 2.6 L of 2 µm-filtered artificial seawater with the enrichment of 40 µL of commercial fertilizer (Blaukorn Garden Fertilizer 2.5 L, Münster, Germany) per 1 L of filtered seawater. All four treatments were cultured at the same irradiance of 150 µmol photon m^{-2} s^{-1} ±13.5% under LED lamps (Aquaruis 90, Aqua Medic Anlagenbau GmbH, Bissendorf, Germany) under a 16:8 h light : dark (L:D) cycle.

Figure 2. Five beakers with 7 g of Gracilaria vermiculophylla at day 0 of experiment 1 (salinity 30 ppt, without desiccation stress).

The desiccation was applied for two treatments (30 and 40 ppt) by elevating seaweed material above the beakers with a net for two hours daily. In the control treatment (30 ppt) and a treatment of hyper-salinity (40 ppt) without algal desiccation the material remained in the experimental beaker at the same culture conditions for 7 days. Five parameters were tested for four different experimental conditions. These parameters included: relative and specific growth rates (RGR and SGR respectively) (for day 3 and 7); Fv / Fm value measured by chlorophyll fluorescence; thallus absorptance; antioxidant activity. RGR, SGR, Fv/Fm value and thallus absorptance were measured on the wet material at days 3 and 7 (for RGR) and 0, 3, and 7 for Fv/Fm value and thallus absorptance. Three other parameters were calculated later using oven-dried material that was taken out from the beakers at a day 3 and 7 of experiment and from the stock culture

tank at the day 0. A schematic of the experimental setup that depicts sampling dates, experimental parameters and measurements, that were performed with the material is shown in Figure 3.

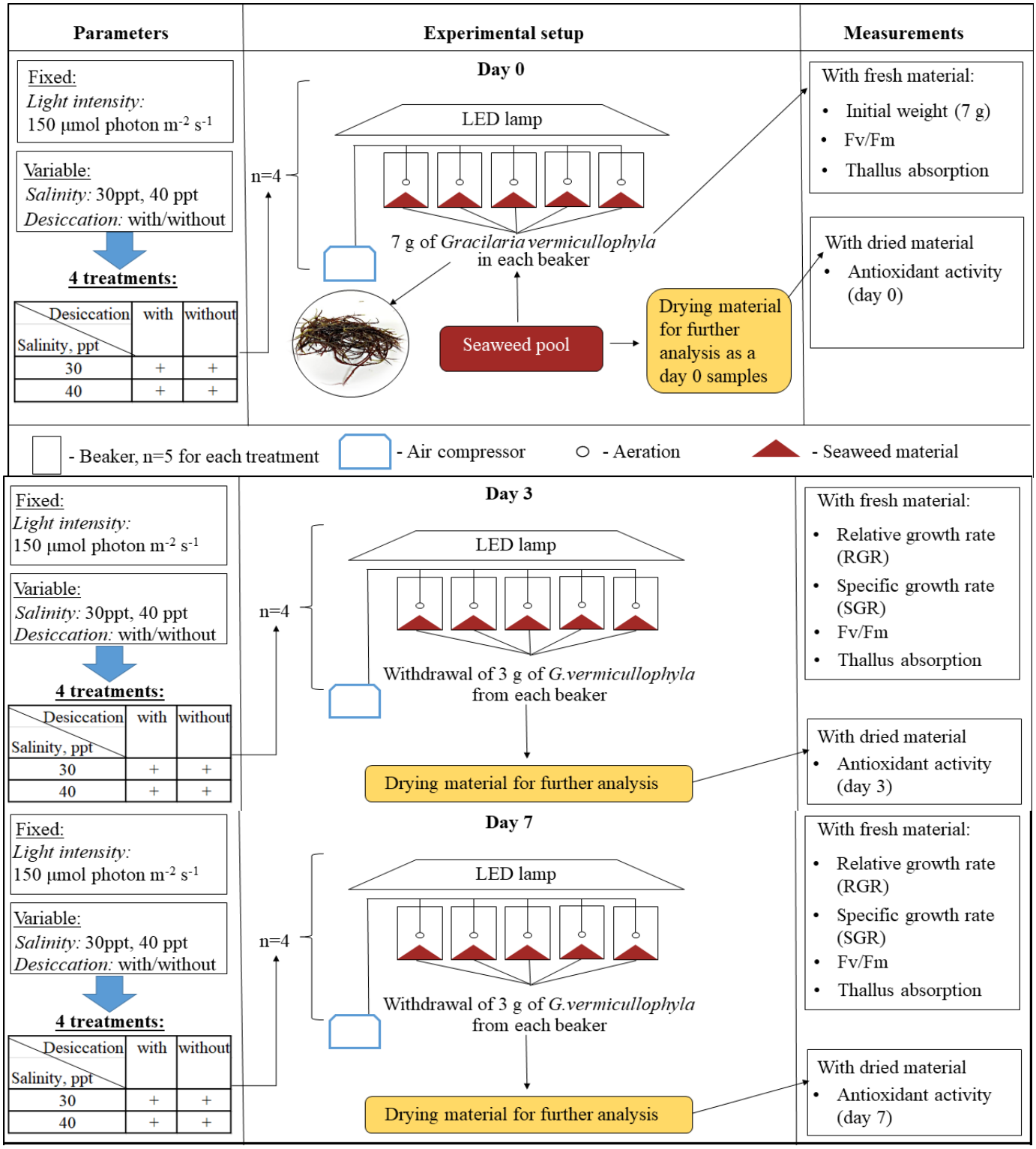


Figure 3. Schematic of the experiment 1 setup including used parameters and performed measurements.

Each of the four treatments consisted of five 3 L beakers (n=5). In each beaker 7 g of *G.vermiculophyla* was placed. The density for the algae cultivation was chosen using the stocking density recommended by Kim & Yarish, 2014 as follows: 2-4 g L^{-1}. The artificial seawater was used for the experiment and prepared by mixing salt (Seequasal GmbH) with tap water. Final salinity was measured with a waterproof pH/temperature/salinity meter (pH 3110, WTW GmbH, Weilheim, Germany). The 3 L beakers were filled with 2.6 L of 2 µm-filtered artificial seawater with the enrichment of 40 µL of commercial fertilizer (Blaukorn Garden Fertilizer 2.5 L, Münster, Germany) per 1 L of filtered seawater. All four treatments were cultured at the same irradiance of 150 µmol photon m^{-2} s^{-1} ±13.5% under LED lamps (Aquaruis 90, Aqua Medic Anlagenbau GmbH, Bissendorf, Germany) under a 16:8 h light : dark (L:D) cycle.

The desiccation was applied for two treatments (30 and 40 ppt) by elevating seaweed material above the beakers with a net for two hours daily. In the control treatment (30 ppt) and a treatment of hyper-salinity (40 ppt) without algal desiccation the material remained in the experimental beaker at the same culture conditions for 7 days.

Five parameters were tested for four different experimental conditions. These parameters included: relative and specific growth rates (RGR and SGR respectively) (for day 3 and 7); $Fv\ /\ Fm$ value measured by chlorophyll fluorescence; thallus absorptance; antioxidant activity. RGR, SGR, Fv/Fm value and thallus absorptance were measured on the wet material at days 3 and 7 (for RGR) and 0, 3, and 7 for Fv/Fm value and thallus absorptance. Three other parameters were calculated later using oven-dried material that was taken out from the beakers at a day 3 and 7 of experiment and from the stock culture tank at the day 0.

For drying in the oven, 3 g of material was collected each time. The material was dried in the oven at relatively low temperature of 30 ^{o}C for 48 hours. As one of the aims of the Mak-Pak project was to come up with a technology for the preparation of an edible

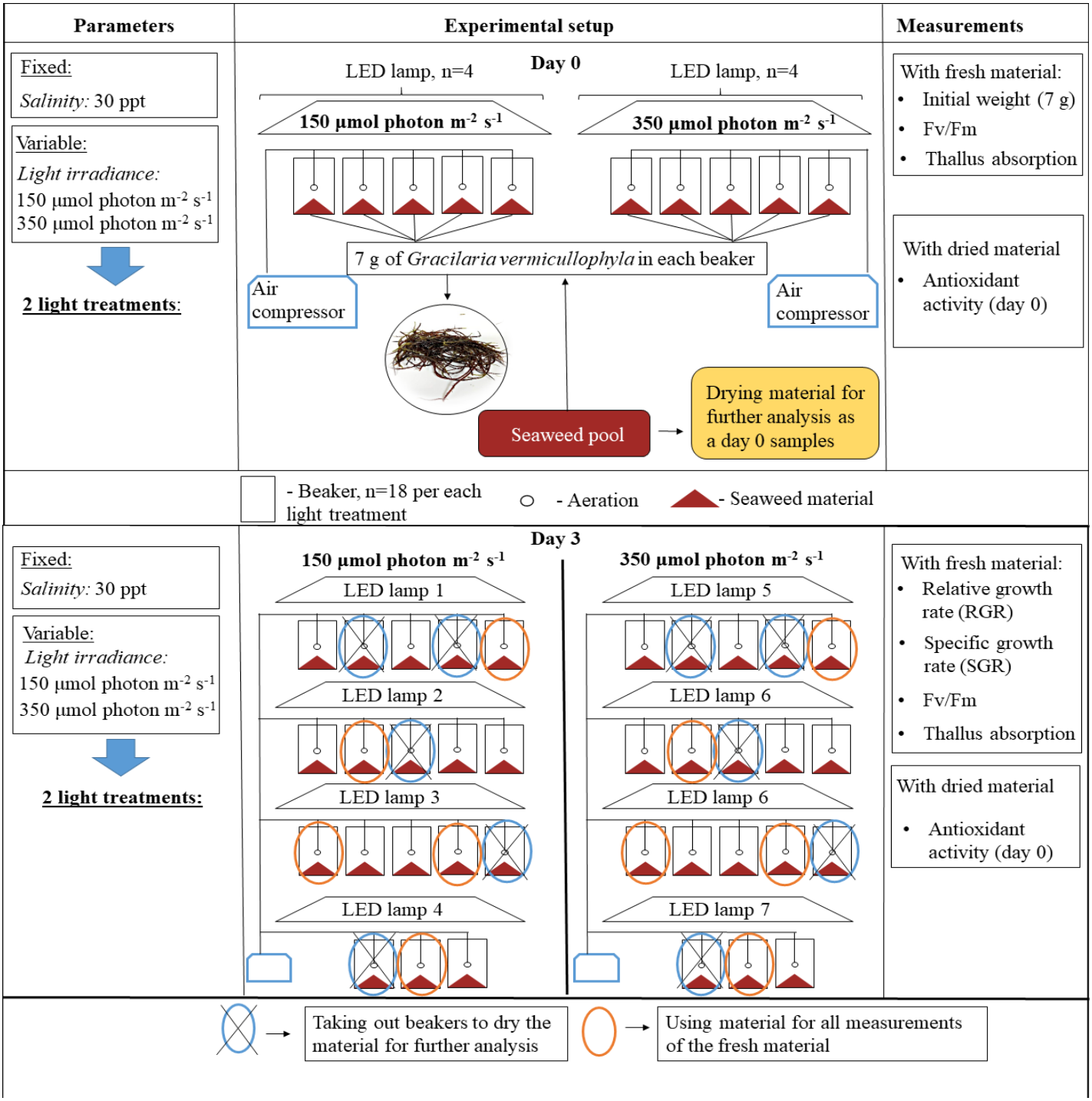
film for the industry, it was important to consider methods for preserving material for safe and long-term storage for later consumption. It is known that different methods of pre-processing, especially drying at high temperatures, significantly affects the physicochemical properties of algae. Thus, it was essential to test the antioxidant properties of algae after a particular type of the material pre-treatment that will be used in the future for edible film preparation in the industry. Studies have shown that freeze drying and drying at low temperatures are the techniques that are less destroying for the material (Stévant *et al.*, 2018). However, freeze-drying is a costly method and therefore not profitable for the industry scale. Therefore, drying at a low temperature such as 30 ^{o}C was chosen for *G.vermiculophylla* preservation.

4.4.2 Experiment 2: The effect of light (intensity, UVA radiation) on antioxidant activity

The second experiment aimed to examine the effect of light intensity as well as exposure to UVA radiation (360-400 nm) on the activation of the antioxidant system in *Gracilaria vermiculophylla*. Two different light irradiance levels were chosen: 150 µmol photon m^{-2} s^{-1} ± 13.5% (as in Experiment 1) and 350 µmol photon m^{-2} s^{-1} ± 14.2%. They were assigned as intermediate (IL) and high light (HL) treatments, respectively.

The second experiment was set at the same walk-in constant cooling chamber (15 °C), using the same LED lamps (Aquaruis 90, Aqua Medic Anlagenbau GmbH, Bissendorf, Germany) as in the first experiment, and under a 16:8 h L:D cycle. Each treatment was assigned 18 beakers (n=18), with 7 g of seaweed material in each of them. Thus, the same cultivation densities were used in the second experiment in a count of 2-4 g L^{-1} as proposed by Kim & Yarish, 2014.

A schematic of the experimental setup that illustrates sampling dates, experimental parameters and measurements that were performed with the material is shown in Figure 4 and Figure 5.



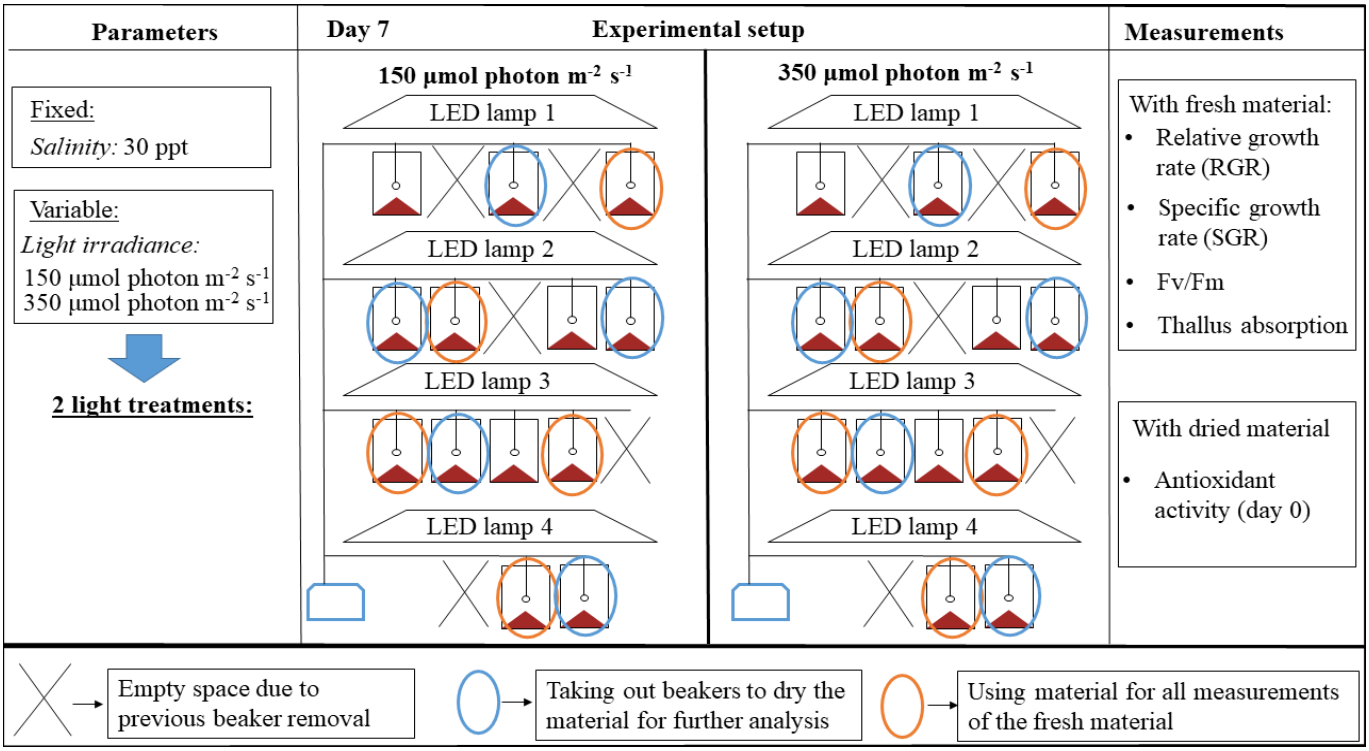


Figure 4. Schematic of the experiment 2 setup (from day 0 to day 7) including used parameters and performed measurements.

The UVA radiation was obtained by activation of an additional channel using the same lamps. The UV contributed a total of to 0.87 % ± 0.02 for IL treatment and 1.64 % ± 0.03 for HL treatment compared to the general light input. The salinity of the artificial water used for algae culture for all the treatments were 30 ppt with the nutrient enrichment of 40 µL of commercial fertilizer (Blaukorn Garden Fertilizer 2.5 L, Münster, Germany) per L of water.

The experiment lasted for 10 days. During the first seven days of the experiment algal material was exposed to the IL and HL treatments. The method of the material removal for the further biochemical analyses differed between the experiment 2 and experiment 1. In the experiment 2 after the material deduction from the beaker, this beaker was excluded for the further steps of the trial. Thus, the amount of the material in the beaker was not modified through the time of experiment.

On the day 7 of the experiment 8 beakers from each treatment were split into 2 groups as shown in the Figure3: one received additional UV treatment along with the same light irradiance used before (IL or HL accordingly); for the other group conditions remained the same and it performed as a control. Thus, by the end of the experiment, results were obtained from four different treatments: IL (control); IL plus UVA radiation; HL (control); HL plus UVA radiation. As for the first experiment, in the second experiment the same five parameters were measured: RGR and SGR, Fv / Fm, thallus absorbance, and antioxidant activity. Measurements were taken for each treatment at days 0; 3; 7; and 10.

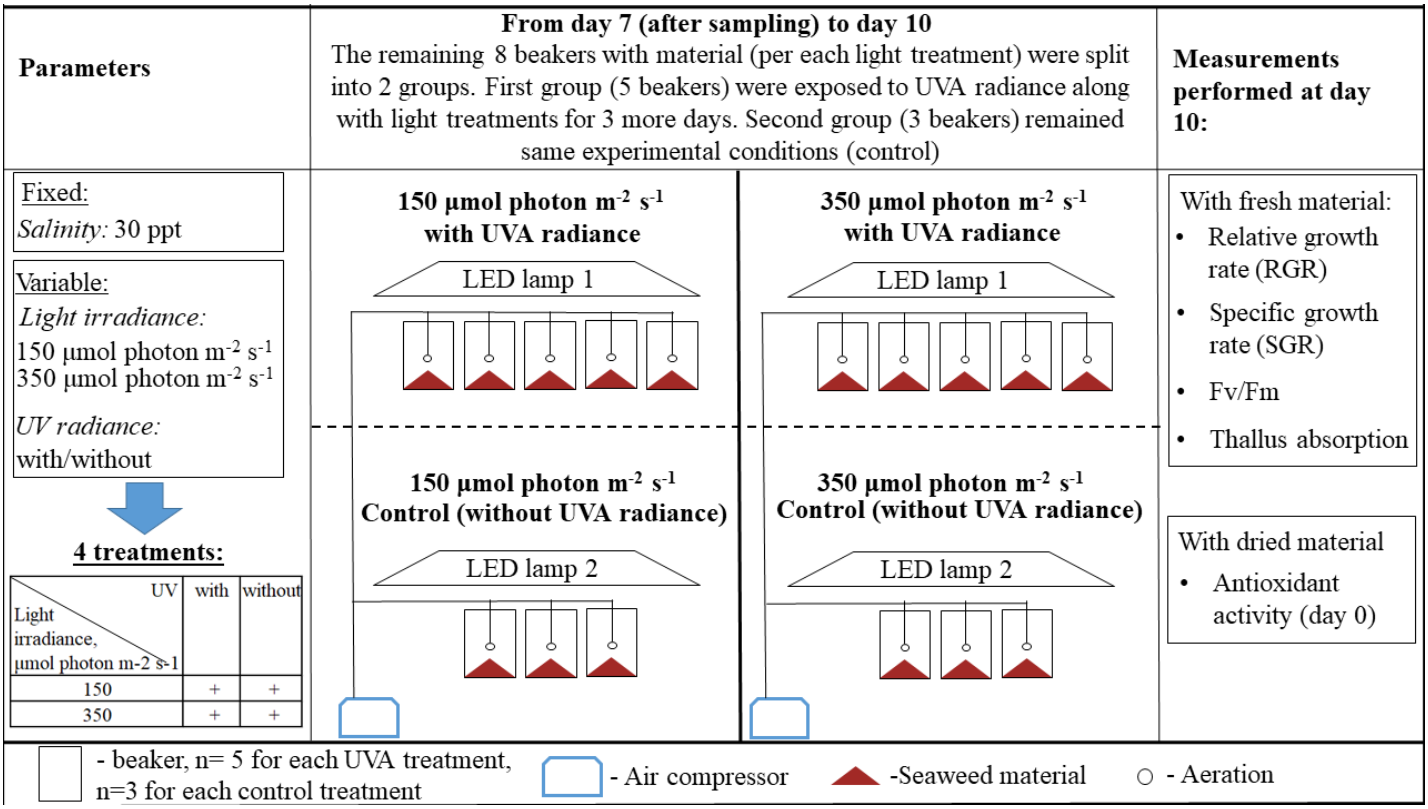


Figure 5. Schematic of the experiment 2 setup (from day 7 (after sampling to day 10) including used parameters and performed measurements

4.4.3 Experiment 3: The effect of day length, light intensity and UVA radiation on the antioxidant activity

The study of Weinberger *et al*., 2008 showed only slight difference in *G. vermiculophylla* growth when exposed to the same PAR doses in a day, while the light intensity and duration was different. However, there was lack of the information on the day length effects on the antioxidant properties of the red algae. Therefore, third experiment was conducted with the aim to examine possible effect of different combinations of the light intensity and the day length upon *G. vermiculophylla* antioxidant properties.

Experiment consisted of two parts: 4 and 3 days long. During the first part of the experiment, two treatments was exposed to the similar overall light input, while the light intensity and light : dark phases ratio vary considerably. First treatment consisted of 10 beakers (n=10) that were exposed to a low light condition (100 µmol photon m^{-2} s^{-1}±10 µmol photon m^{-2} s^{-1}) over the constant 24 h light cycle) consisted of 13 beakers (n=13). The second treatment (400 µmol photon sm^{-2} s^{-1}±30 µmol photon m^{-2} s^{-1}) consisted of 18 beakers (n=18) that had only 6 hours of light exposure. All beakers contained same amount of algae material (7 g) and water (2.8 L); the material deduction was performed the same way as described for the experiment 2. Thus, the overall PAR doses per day were equal for both treatments and were estimated at 2400 µmol photon sm^{-2} day^{-1}.

The second part of experiment aimed to investigate role of UV (for both treatments) and 24 hours exposure to the light (for 400 µmol photon sm^{-2} s^{-1} treatment) as well as combination of both (UV and 24 hours light exposure) on the antioxidant activity of *G. vermiculophylla*. Thus, the material from the low light treatment (100 µmol photon sm^{-2} s^{-1}) was split into two parts, where one was receiving additional UV A treatment along with the 100 µmol photon sm^{-2} s^{-1}±10% (n=5) for 24 hours, whereas the other part remained

similar conditions and performed as a control (n=3). The material from the second treatment (400 µmol photon sm^{-2} s^{-1}±7.5%) was split into three parts. The first part (n=5) switched to the 24 h light cycle with the same light intensity (400 µmol photon m^{-2} s^{-1}± 30 µmol photon m^{-2} s^{-1}). The second treatment (n=5) was exposed to both the same light condition (400 µmol photon m^{-2} s^{-1}± 30 µmol photon m^{-2} s^{-1}) along with the UV A for 24 hours. The third part (n=3) remained conditions that were tested in the first 4 days (400 µmol photon m^{-2} s^{-1}± 30 µmol photon m^{-2} s^{-1} for 6 hours). The UV contributed a total of to 0.74 % $\pm$ 0.02 for LL treatment and 1.92 % $\pm$ 0.03 for HL treatment compared to the general light input. The technology of the material removal for the further biochemical analyses was similar as performed in the experiment 2. The same physiological and biochemical parameters as in previous experiments were performed in the experiment 3. A schematic of the experimental setup that illustrates sampling dates, experimental parameters and measurements that were performed with the material is shown in Figure 6 and Figure 7.

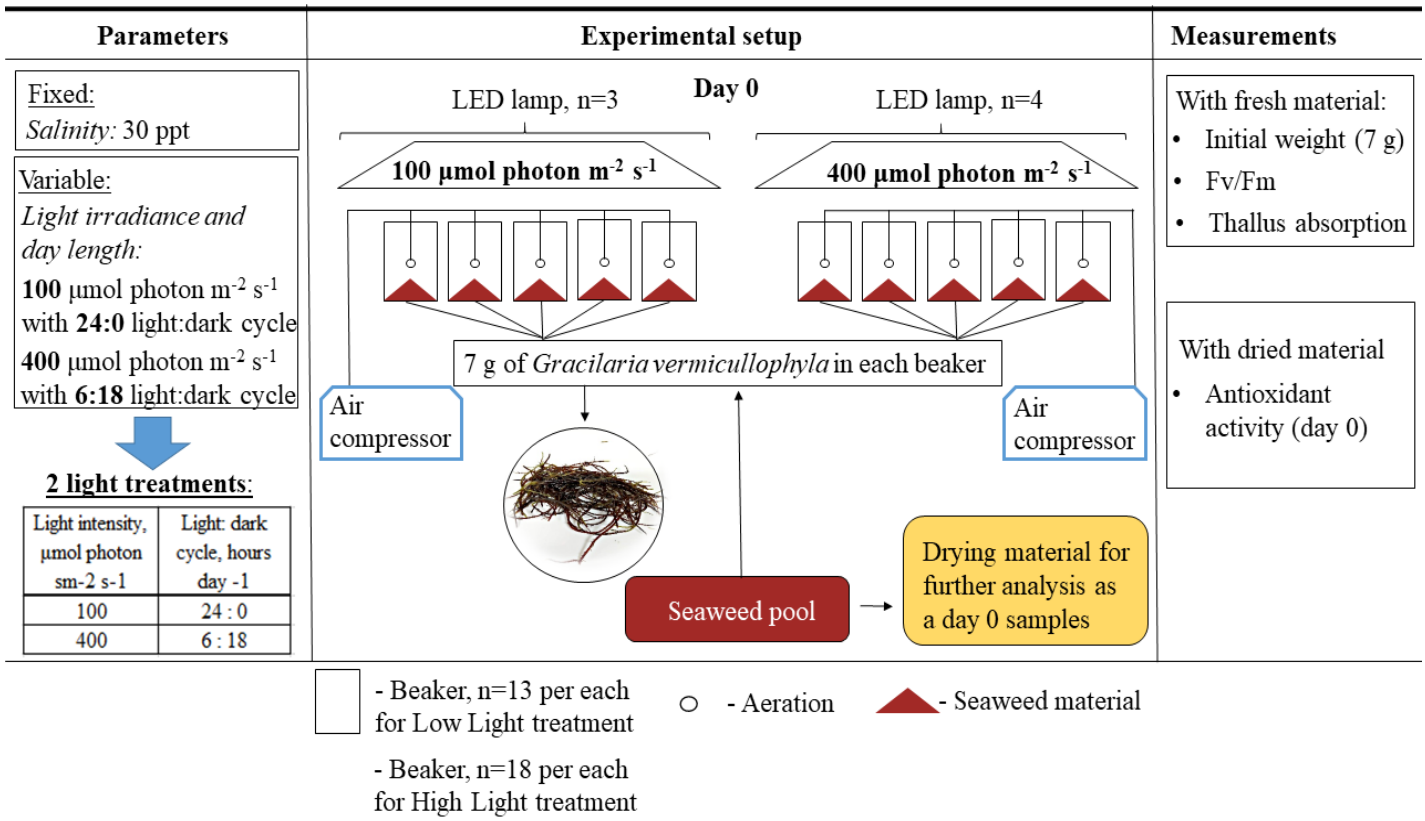


Figure 6. Schematic of the experiment 3 setup (from day 0 to day 4) including used parameters and performed measurements.

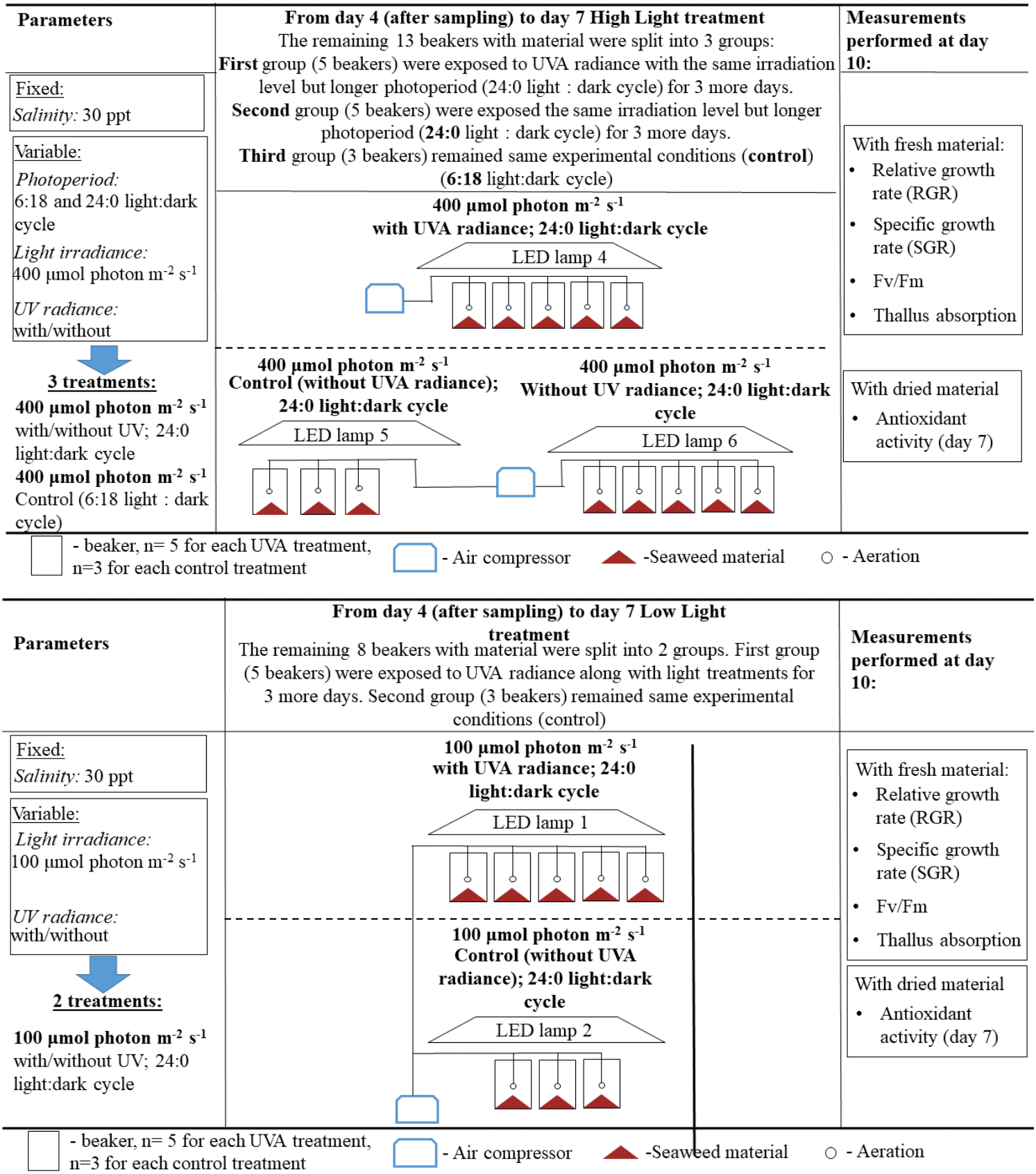


Figure 7. Schematic of the experiment 3 setup from day 4 after sampling, when additional UVA radiation was introduced to day 7.

4.5 Algae extract preparation

The amount of 0.1 g ± 0.01 g of dried *G. vermiculophylla* material was weight and then ground in the porcelain mortar with 2 mL of ethanol (70 %) (Vijayavel and Martinez, 2010) on the ice bedding (Figure 8a, 8b). The homogenous substance achieved after grinding was placed in the 15 mL falcon tubes (Figure 9a, 9b) and then incubated in a thermal bath (SS40-2, Grant Instruments, Cambridge, UK) at 45 ± 2 ºC, with a constant shaking at 130 rpm for 6 hours ºC (Alvarez-Gomez *et al*., 2016).

a) **b)**

Figure 8. Algae extract preparation: a) 1 g of dried Gracilaria vermiculophylla in the porcelain mortar on the ice bedding; b) adding ethanol (70%) to perform grinding in in the porcelain mortar on the ice bedding.

a) **b)**

Figure 9. Algae extract: a) obtained extract from 1 g of Gracilaria vermiculophylla; b) placing Gracilaria vermiculophylla extract into 15 mL Falcon tube.

Falcon tubes with the extracts and algae material were centrifuged (Beckman GS-15 R centrifuge) for 10 min at 4 ºC (Alvarez-Gomez *et al*., 2016) at 2500×*g* (Jiménez-Escrig *et al*., 2012). Then the supernatant were carefully transferred into empty Falcon tubes. Additional 2 mL of ethanol (70%) were add to the residue algae and incubation at a water bath for 1 hour followed by centrifugation were performed in order to obtain the second round of extraction. Both supernatants were mixed and used for the antioxidant analyses immediately.

4.6 Testing of antioxidant activity

ABTS radical cation decolorization assay was used to determine antioxidant activity in *G. vermiculophylla* experimental material. This assay was performed according to the method developed by Re *et al*., 1999 and was adapted by Torres et al., 2017 to the 96-well microplate. The $ABTS^{\bullet+}$ reagent was prepared by mixing 1 mL of 7 mM ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)]' solution with 0.5 mL of 2.45 mM potassium persulfate ($K_2S_2O_8$) (Re *et al.*, 1999). $ABTS^{\bullet+}$ solution was then allowed to incubate in the dark at a room temperature for at least 16 hours to reach maximal absorbance and a stable radical concentration (Re *et al*., 1999; Torres *et al*., 2019). The

$ABTS^{\bullet+}$ solution was diluted with 70 % ethanol until the absorbance of 0.70 at 734 nm was reached (Torres *et al*., 2019).

Sample extracts as well as Trolox solutions for the antioxidant standard and negative control were added to each well of the 96-well microplate at amount of 20 µL. After that 280 µL of ABTS•+ solution was added to each well (Torres *et al*., 2019). The microplate was incubated in the dark at a room temperature for 8 min (Alvarez-Gomez *et al*., 2016) and the absorbance was then recorded at 734 nm (Re et al., 1999; Torres et al., 2019) 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}, $R^2 > 0.95$).

4.7 Statistical Analysis

Significance of differences in antioxidant activity was determined by the analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) and Bonferroni tests. For the experiment 1 the duration of exposure to the treatment (days), salinity and application or not of the desiccation were considered as fixed factors. For the second and third experiments duration of exposure to the treatment (days), light intensity and additional application or not of the UV B were considered as fixed factors. Prior the ANOVA, SNK and Bonferroni tests data were tested for dispersion homogeneity using Levene's test and for normality of the distribution using Shapiro-Wilk test. Data were analyzed using SPSS Statistics 22 (IBM, Armonk, NY, USA).

5. Results

5.1 Experiment 1

5.1.1 Algal growth

In experiment 1 differences in growth rates between different treatments were observed already after three days exposure to the tested conditions. Repeated measures ANOVA test followed by *post hoc* SNK and Bonferroni tests showed that all factors (salinity, desiccation and duration of exposure) had a significant ($p<0.05$) influence on growth. Both desiccation and no desiccation groups at salinity 40 ppt as well as desiccation group at salinity 30 ppt showed negative RGR (-0.06847 $\pm$ 0.00327 g day^{-1}; -0.00421 $\pm$ 0.00514 g day^{-1}; -0.02888 $\pm$ 0.00327 g day^{-1} accordingly). The treatment with no desiccation at salinity 30 ppt showed a positive RGR (0.01354 $\pm$ 0.0062 g day^{-1}). Both groups of 30 and 40 ppt salinities that did not undergo desiccation had higher SGR values (1.354 $\pm$ 0.62 % day^{-1}; -0.421 + 0.51 % day^{-1} respectively) after 3 days from the experiment start, than those to which desiccation was applied (1.354 $\pm$ 0.62 % day^{-1}; -0.421 + 0.51 % day^{-1} for the 30 ppt and 40 ppt salinity groups respectively) (Figure 10).

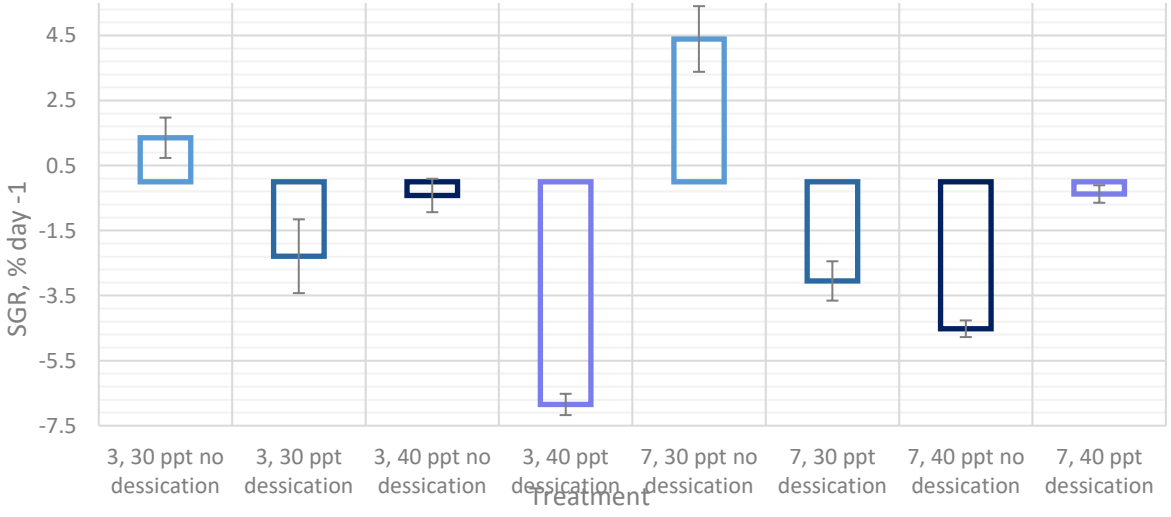


Figure 10. Specific growth rates of four tested treatments after 3 and 7 days of experiment.

The trend of G. vermiculophylla growth after 7 days of exposure followed the same pattern as at the 3d day of experiment: the only group with positive RGR was a group with no desiccation at salinity 30 ppt (0.04392 + 0.0101 g day-1). Three other groups: 30 ppt desiccation, 40 ppt desiccation and 40 ppt no desiccation had a negative RGR values (-0.03049 + 0.00605 g day-1; -0.00376 + 0.00269 g day-1; -0.0452 + 0.00256 g day-1 respectively).

5.1.2 Algal photosynthesis

Photosynthetic performance of the *G. vermiculophylla* samples was detected by measuring optimal quantum yield or Fv/Fm value. At the beginning of experiment Fv/Fm mean values were estimated at 0.607 $\pm$ 0.020; 0.610 $\pm$ 0.010; 0.615 $\pm$ 0.023; and 0.614 $\pm$ 0.020 for 30 ppt salinity without and with desiccation and 40 ppt salinity without and with desiccation respectively (Figure 11). After 3 days of exposure to the tested conditions for 3 groups: 30 ppt without desiccation, 30 ppt with desiccation, and 40 ppt without desiccation Fv/Fm value increased (0.641 $\pm$ 0.009; 0.652 $\pm$ 0.025; 0.640 $\pm$ 0.012). On the contrary, for the 40 ppt salinity with the desiccation Fv/Fm values significantly dropped (0.504 $\pm$ 0.024) ($p<0.05$). Thus, despite the growth rates decrease for the group of 30 ppt salinity with desiccation and 40 ppt without desiccation, their optimal quantum yield increased after 3 days exposure to the treatment.

After 7 days of exposure for both groups of 30 ppt salinity Fv/Fm value did not change significantly (0.642 $\pm$ 0.011; 0.655 $\pm$ 0.017 with and without desiccation respectively) comparing to parameters of day 3. The optimal quantum yield value changed significantly ($p < 0.05$) for the samples of the 40 ppt salinity without desiccation group at day 7 comparing to day 3 (0.579 $\pm$ 0.026). However, the overall change of Fv/Fm ratio in this group comparing with the initial state at day 0 was not significant.

The only group that showed a constant decline in photosynthetic performance was group with desiccation at 40 ppt salinity. At both 3 and 7th day of experiment Fv/Fm values significantly ($p < 0.05$) dropped (0.504 $\pm$ 0.024; 0.398 $\pm$ 0.013 for the 3^{d} and 7th days respectively).

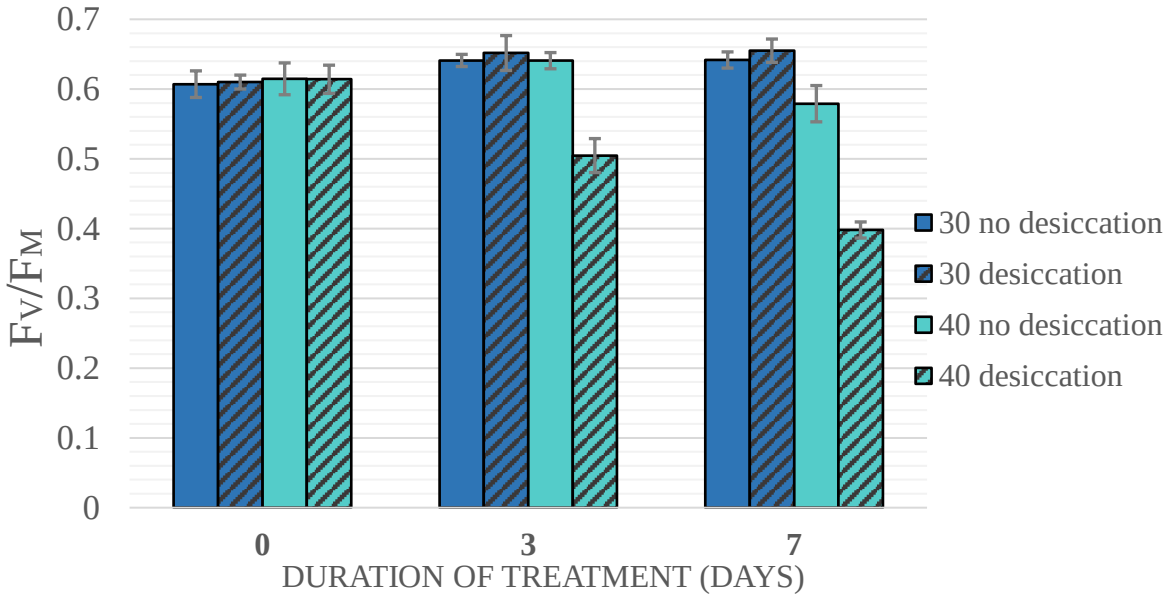


Figure 11. Fv/Fm value of four tested treatments at the beginning of experiment (day 0) and after 3 and 7 days of experiment.

5.1.3 Algal thallus absorption

Algal thallus absorption (A) was measured at the beginning of experiment and then after 3 of 4 days of exposure to the treatments. The day 0 measurements of A parameter did not significantly ($p<0.05$) differ for each experiment and the mean values was equal to 0.83 $\pm$ 0.04.

During the first experiment at day 3 only treatment of 40 ppt salinity along with application of desiccation showed a slight decrease in thallus absorption (0.71 $\pm$ 0.02) ($p<0.05$), whereas A factor for the other three groups did not change significantly ($p<0.05$) as demonstrated in the Table 1. The same trend can be observed for the thallus absorption at a day 7 of experiment: the group of 40 salinity with desiccation treatment

showed lower values, then those at the beginning of experiment (0.69 ± 0.02) ($p<0.05$), however those values did not differ significantly ($p<0.05$) from those of this group at a day 3. A value for other groups remained without any significant changes ($p<0.05$).

Table 1. Thallus absorption of four tested treatments at the beginning of experiment (day 0) and after 3 and 7 days of experiment. Data expressed as mean value ± standard deviation, n=5, p<0.05 (repeated measures ANOVA followed by post hoc SNK and Bonferroni tests)

Salinity, ppt	Dessication	Duration of exposure, days	Thallus absorption factor (*A*)
30	NO	0	0.84 ± 0.02
		3	0.88 ± 0.03
		7	0.87 ± 0.05
	YES	0	0.84 ± 0.02
		3	0.82 ± 0.02
		7	0.87 ± 0.01
40	NO	0	0.84 ± 0.02
		3	0.89 ± 0.07
		7	0.89 ± 0.07
	YES	0	0.84 ± 0.02
		3	0.70 ± 0/02
		7	0.69 ± 0.01

5.1.4 Antioxidant activity

Antioxidant activity of *G. vermiculophylla* was measured before the experiment (day 0) and at days 3 and 7 after exposure to the treatment. The initial antioxidant activity of the four tested groups were (10.75 ± 0.31; 11.04 ± 0.29; for 30 ppt salinity without and with desiccation respectively, and 11.52 ± 0.54; 11.32 ± 0.42 for 40 ppt salinity without and with desiccation respectively). After 3 days of exposure to the experimental conditions antioxidant activity dropped significantly ($p < 0.05$) after 3 days of exposure to

the experimental conditions as demonstrated in the Table 2. However, already after 7 day of the treatment all four groups significantly ($p < 0.05$) increased the antioxidant levels comparing to the day 3 measurements. After 7 days of exposure to the tested conditions both groups of 30 ppt salinity showed a significant ($p < 0.05$) increase in the antioxidant activity (10.81 ± 0.65; 11.50 ± 0.66 µmol TE/g for treatment without and with desiccation respectively) comparing to the activity of *G. vermiculophylla* at the beginning of experiment. Both treatments at 40 ppt salinity also showed a slight increase ($p < 0.1$) in the antioxidant activity (12.14 ± 1.27; 12.36 ± 0.89 µmol TE/g for treatment without and with desiccation respectively) comparing to the initial activity of algae samples.

Table 2. Antioxidant activity expressed in Trolox equivalents (µmol TE/g) of four tested treatments at the beginning of experiment 1 (day 0) and after 3 and 7 days of experiment. Data expressed as a mean value ± standard deviation, n=5, p<0.05

Salinity, ppt	Duration ofexposure	Dessication stress (yes/no)	Trolox equivalents (µmol TE/g)
30	0	NO	10.75 ± 0.31
	3		9.78 ± 0.58
	7		10.81 ± 0.65
	0	YES	11.04 ± 0.29
	3		9.02 ± 0.73
	7		11.50 ± 0.66
40	0	NO	11.52 ± 0.54
	3		8.10 ± 1.66
	7		12.14 ± 1.27
	0	YES	11.32 ± 0.42
	3		9.21 ± 0.28
	7		12.36 ± 0.89

5.2 Experiment 2

5.2.1 Algal growth

In experiment 2 there were no significant ($p<0.05$) differences in growth rates after 3 days of experiment between two light treatments (150 and 350 µmol photon sm^{-2} s^{-1} assigned intermediate (IL) and high light (HL) treatments accordingly). The mean RGR value of IL was 0.01342 $\pm$ 0.00361 g day^{-1} and of the HL treatment was 0.02406 $\pm$ 0.02101 g day^{-1}. There was no significant ($p<0.05$) difference in RGR values between IL and HL treatments after 7 day of experiment (0.01705 $\pm$ 0.00934 g day^{-1} and 0.03319 $\pm$ 0.01416 g day^{-1}).

Both HL treatments with and without additional exposure to UV showed significant ($p<0.05$) growth compared to the growth rates of the HL group at days 3 and 7 of experiment (0.04594 $\pm$ 0.02778 g day^{-1} and 0.07229 $\pm$ 0.01334 g day^{-1} with and without exposure to UV respectively). However, there was no significant ($p<0.05$) difference between the growth rates of both HL treatments at day 10 of experiment compared between each other. There was no significant ($p<0.05$) difference between RGR values of both IL groups (0.01098 $\pm$ 0.00825 g day^{-1} and 0.03437 $\pm$ 0.01144 g day^{-1} with and without exposure to UV respectively). Moreover, both groups of IL treatment did not differ significantly ($p<0.05$) from the measurements of this group at day 3 and 7 of experiment.

The SGR values of the IL treatment did not show a significant ($p<0.05$) fluctuations, whereas SGR values of the HL treatment was constantly increasing during whole time of experiment. Thus, the SGR value at day 10 compared to SGR at day 3 grew in 2.7 times for the HL with UV treatment and in 4.24 times for the HL without additional exposure to UV (Figure 12).

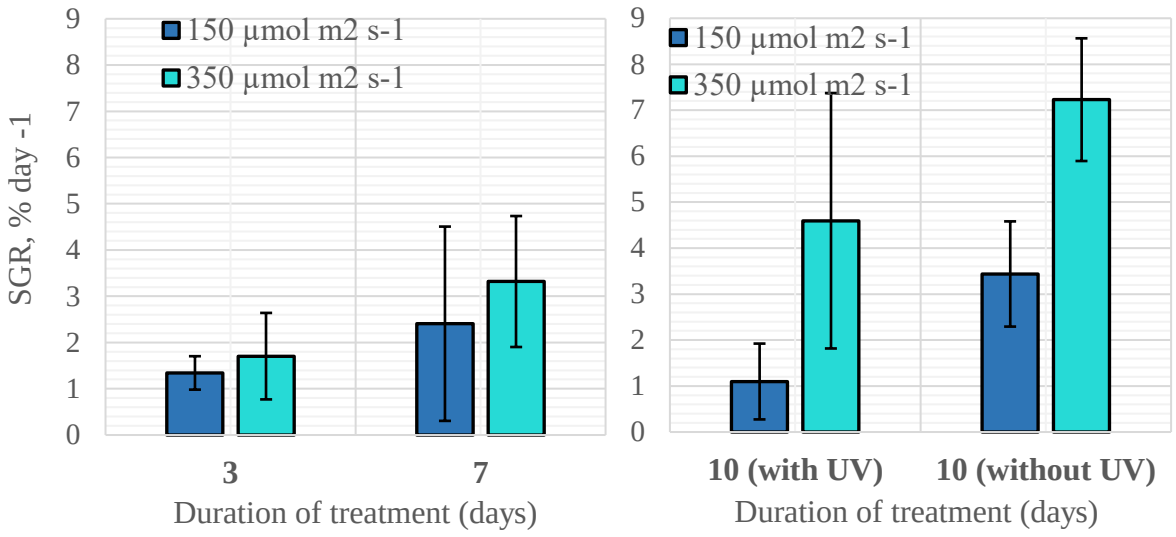


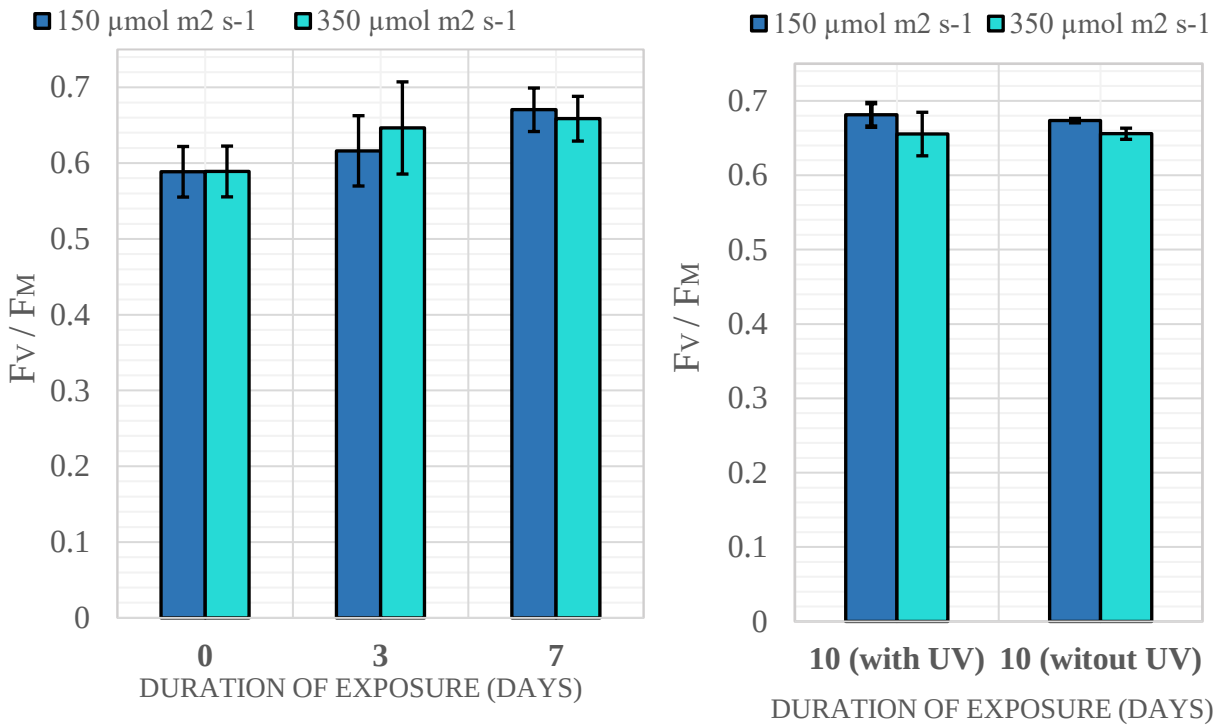
Figure 12. Specific growth rates of tested treatments after a) 3 and 7 days of experiment, b) 10 days of experiment.

5.2.2 Algal photosynthesis

During the first seven days of experiment material was exposed to two different light intensities: 150 µmol photon sm^{-2} s^{-1} and 350 µmol photon sm^{-2} s^{-1} (IL and HL treatments respectively). In the Figure 13a it is shown the average of the Fv/Fm value at day 0, 3, and 7 of the experiment. At the beginning of experiment the average value of the Fv/Fm ratio for the IL was 0.589 ± 0.033 and for the HL 0.589 ± 0.034. After three days of exposure to the experimental conditions the Fv/Fm mean value of both light treatments slightly increased (0.616 ± 0.046 and 0.646 ± 0.06 for the IL and HL respectively), however the change in the value was not significant ($p>0.05$). After 7 days exposure to the experimental conditions Fv/Fm mean value of the HL treatment did not change significantly ($p>0.05$) (0.658 ± 0.03 µmol photon sm^{-2} s^{-1}) comparing to both day 0 and day 3 values of this treatment. The Fv/Fm mean value of the IL treatment showed

the slight increase at a day 7 of experiment (0.670 ± 0.029) ($p<0.05$) to the Fv/Fm mean value of day 0.

Starting from the day 7 of the experiment, half of the material of both light intensities were exposed to UVA radiation (Figure 13b). Samples that were exposed to UV and light irradiance of 150 µmol photon m^{-2} s^{-1} did not show a significant ($p<0.05$) change in the mean of the Fv/Fm value (0.681 ± 0.016) than those that were not exposed to UV (0.673 ± 0.002). The similar trend was seen in the samples of the high light irradiance of 350 µmol photon sm^{-2} s^{-1}, where the Fv/Fm ratio for the UV treated samples (0.655 ± 0.03) ($p<0.05$) was very close to the mean Fv/Fm from the samples not treated with UV (0.656 ± 0.008).



a) **b)**

Figure 13. Fv/Fm value of two tested light treatments (150 µmol photon sm^{-2} s^{-1} and 350 µmol photon sm^{-2} s^{-1}) : a) at the beginning of experiment (day 0) and after 3 and 7 days of experiment, b) at a day 10 with and without exposure to UV

5.2.3 Algal thallus absorption

The average A factor at the beginning of the experiment 2 was 0.81 ± 0.02. The group that was under the high light conditions showed a significant ($p<0.05$) increase in thallus absorption values (0.89 ± 0.02) at day 3 (Table 3). However, thallus absorption values remained constant during further exposure to HL treatment.

The levels of thallus absorption at day 7 for the HL group that did not received additional UV treatment remained constant since day 3 of experiment.

The absorpbtion factor of the samples from LL treatment did not change significantly ($p<0.05$) compared to those at the beginning of experiment. At the day 7 the slight increase was observed for the IL group without UV treatment (0.85 ± 0.01). At the day 10 of experiment both IL treatments with and without additional exposure to UV, remained without any significant changes ($p<0.05$) (0.85 ± 0.03; 0.84 ± 0.03 with and without additional UV treatment respectively) in the thallus absorption compared to those at the day 7.

Table 3. Thallus absorption of tested treatments at the beginning of experiment 2 (day 0) and after 3, 7, and 10 days of experiment 2. Data expressed as mean value ± standard deviation, n=5 at p<0.05

Experiment	Light intensity, µmol photon m-2 s-1	Light: dark cycle, hours day -1	Exposure to UV (yes/no)	Duration of treatment, days	Thallus absorption factor (*A*)
Experiment 2	150	16:8	NO	0	0.81 ± 0.02
			NO	3	0.82 ± 0.01
			NO	7	0.85 ± 0.01
			NO	10	0.84 ± 0.03
			YES	10	0.85 ± 0.03
	350		NO	0	0.81 ± 0.02
			NO	3	0.89 ± 0.01
			NO	7	0.89 ± 0.02
			NO	10	0.84 ± 0.01
			YES	10	0.90 ± 0.01

5.2.4 Antioxidant activity

The experiment 2 was divided into two sub-parts (7 and 3 days each) that were evaluated separately. During the first part of experiment the effect of exposure to intermediate and high light intensities (150 µmol photon sm^{-2} s^{-1}± 20 µmol photon m^{-2} s^{-1} and 350 µmol photon m^{-2} s^{-1}± 40 µmol photon m^{-2} s^{-1} respectively) on the antioxidant properties of *G. vermiculophylla* were tested. Initial antioxidant activity of the IL treatment was: 7.85 ± 0.03 µmol TE/g and HL: 7.43 ± 0.13 µmol TE/g. After 3 days of exposure to the experimental conditions the antioxidant activity of the HL treatments remained without any significant changes (7.51 ± 0.18 µmol TE/g) ($p < 0.05$). However, similarly to the experiment 1, where the same conditions were used for one of the treatments (30 ppt salinity without desiccation), antioxidant activity of algae increased significantly (8.35 ± 0.49 µmol TE/g) ($p < 0.05$). After 7 days of exposure to the high light conditions the antioxidant activity of the samples decreased significantly (6.72 ± 0.03 µmol TE/g) ($p < 0.05$), whereas for the IL treatment antioxidant activity remained without any significant changes (7.97 ± 0.17 µmol TE/g) ($p < 0.05$). Generally, the antioxidant activity of the samples from the IL treatment had higher values then those of the HL treatment. Summary of antioxidant activity at days 0, 3, and 7 of experiment 3 is shown in the Figure 4.

In the second part of experiment both light treatment group were split into 2 subgroups, where one was exposed to additional UVA radiance along with irradiance used in the first half of experiment half of the samples. The other part of the samples from both treatments that did not undergo the additional UV treatment were cultivated at the similar conditions as at the first part of experiment and performed as a control. Antioxidant activity of both controls of IL and HL treatments significantly dropped during the second part of experiment (6.38 ± 0.2 µmol TE/g; 5.90 ± 0.15 µmol TE/g). Antioxidant activity of

samples from the HL increased drastically (8.18 ± 0.2 µmol TE/g) ($p < 0.05$), whereas the antioxidant activity of algae from the IL treatment slightly decreased (7.48 ± 0.39 µmol TE/g). Both UV treated samples of IL and HL groups showed significantly ($p < 0.05$) higher antioxidant properties than both control groups.

Table 4. Antioxidant activity expressed in Trolox equivalents (µmol TE/g) of experimental treatments at the beginning of experiment 2 (day 0) and after 3, 7, and 10 days of experiment. Data expressed as a mean value ± standard deviation, n=5, p<0.05

Light irradiance (µmol m2 s-1)	Duration of exposure (days)	UVA radiance (with or without)	Trolox equivalents (µmol TE/g)
150	0	NO	7.85 ± 0.03
	3	NO	8.35 ± 0.49
	7	NO	7.98 ± 0.69
	10	NO	6.38 ± 0.02
	10	YES	7.48 ± 0.39
350	0	NO	7.43 ± 0.13
	3	NO	7.51 ± 0.18
	7	NO	6.72 ± 0.03
	10	NO	5.90 ± 0.15
	10	YES	8.16 ± 0.20

5.3 Experiment 3

5.3.1 Algal growth

In the experiment 3 there were no significant ($p<0.05$) differences in growth rates after 4 days of experiment. Two-way ANOVA test followed by *post hoc* SNK and Bonferroni tests showed that all factors (low and high light irradiance and duration of experiment) did not have a significant ($p<0.05$) influence on growth. RGR of both low (LL) (100 µmol photon sm^{-2} s^{-1}) and high light (HL) (400 µmol photon sm^{-2} s^{-1}) treatment groups did not differ significantly ($p<0.05$) between each other (0.00931 $\pm$ 0.00540 g day^{-1} and 0.00938 $\pm$ 0.00670 g day^{-1} for LL and HL treatments accordingly). In the Figure 14 it is shown changes in specific growth rates at days 4 and 7 of experiment.

Measurements after 7 days of experiment were analyzed by three-way ANOVA test followed by *post hoc* SNK and Bonferroni tests, where light irradiance, duration of experiment and presence of UV treatment were considered as factors. Both LL groups with and without additional UV treatment did not show a significant ($p<0.05$) difference in the RGR between each other and compared to the RGR at day 4 of experiment (0.01472 $\pm$ 0.00490 g day^{-1} and 0.01788 $\pm$ 0.01209 g day^{-1} for LL groups without and with additional UV treatment respectively). The UV treatment as well as control treatment of the HL group did not show significant ($p<0.05$) difference in the RGR between each other and compared to the RGR at day 4 of experiment (-0.00771 $\pm$ 0.00490 g day^{-1} and 0.00219 $\pm$ 0.03778 g day^{-1} for HL control and with additional UV treatment respectively). The mean RGR value of the HL group, that was exposed to the constant light irradiance without additional UV treatment showed significant ($p<0.05$) decrease compared to day 4 of experiment (-0.04250 $\pm$ 0.04585 g day^{-1}).

SGR value of both HL and LL treatments at day 4, as well as both controls at day 7 and HL and LL treatments that were exposed to additional UV, did not fluctuate

significantly ($p<0.05$) and were in the range between -0.77 % day^{-1} to 1.47 % day^{-1} (Figure 10). The only group, that showed a significant ($p<0.05$) decrease in SGR was a group of HL without additional UV treatment, where the SGR were estimated at -4.25% ± 4.58 % day^{-1}.

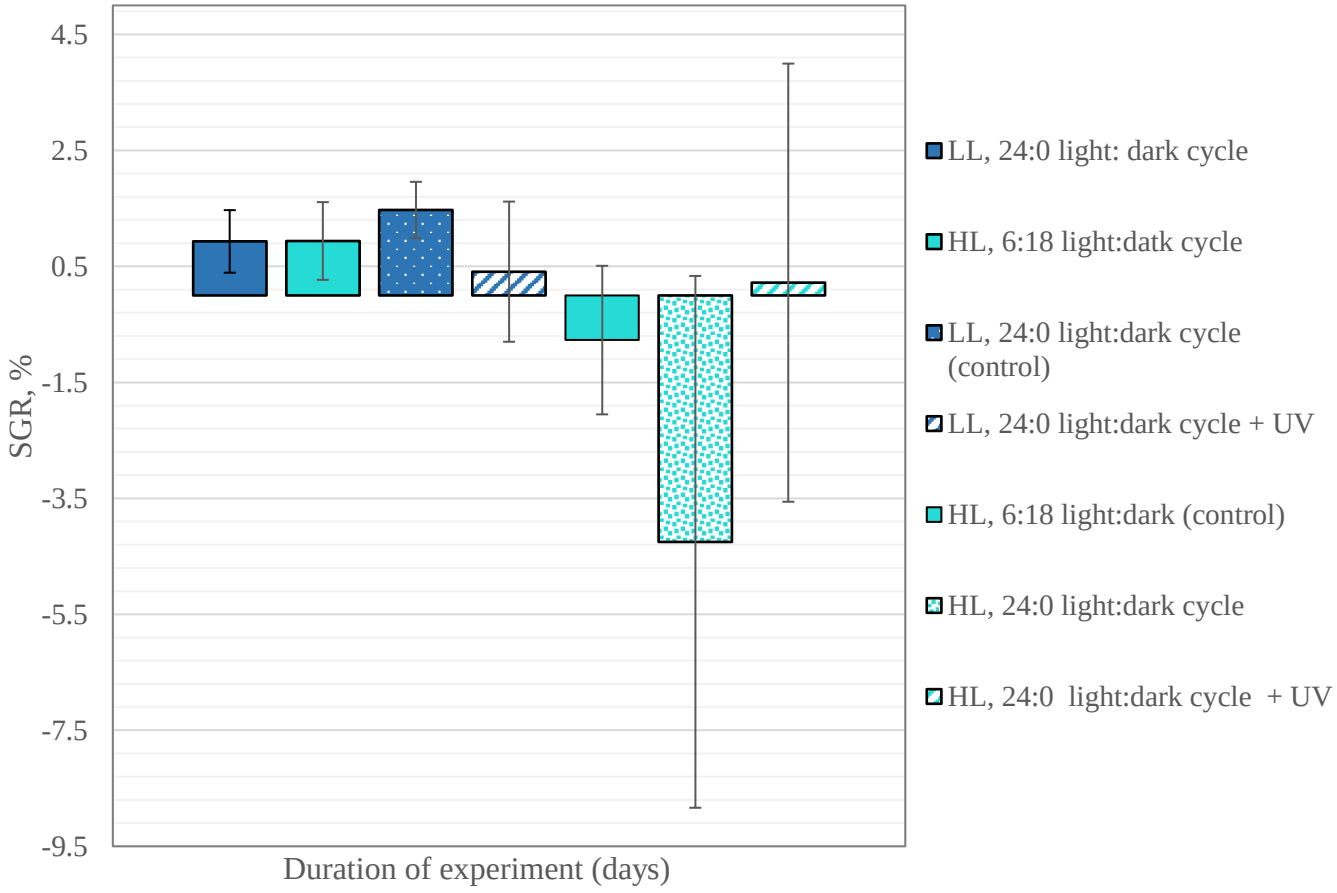


Figure 14. Specific growth rates of tested treatments (100 and 400 µmol photon sm-2 s-1) after 3 and 7 days of experiment.

5.3.2 Algal photosynthesis

The measurements of the Fv/Fm value were taken at days 0, 4, and 7 of the experiment. The initial average value of the Fv/Fm ratio was 0.636 ± 0.023 and 0.624 ± 0.021 (Figure 15). After 4 days of exposure to the experimental conditions the average of the Fv/Fm value of the 100 µmol photon sm^{-2} s^{-1} (LL) treatment did not change significantly ($p<0.05$) (0.653 ± 0.013) compared to the Fv/Fm ratio at day 0. The same pattern was recorded for the Fv/Fm ratio of the samples from the 300 µmol photon sm^{-2} s^{-1} (HL) treatment, where mean value at day 4 of experiment did not differ significantly ($p<0.05$) (0.601 ± 0.032) from the Fv/Fm value measured at the beginning of the experiment.

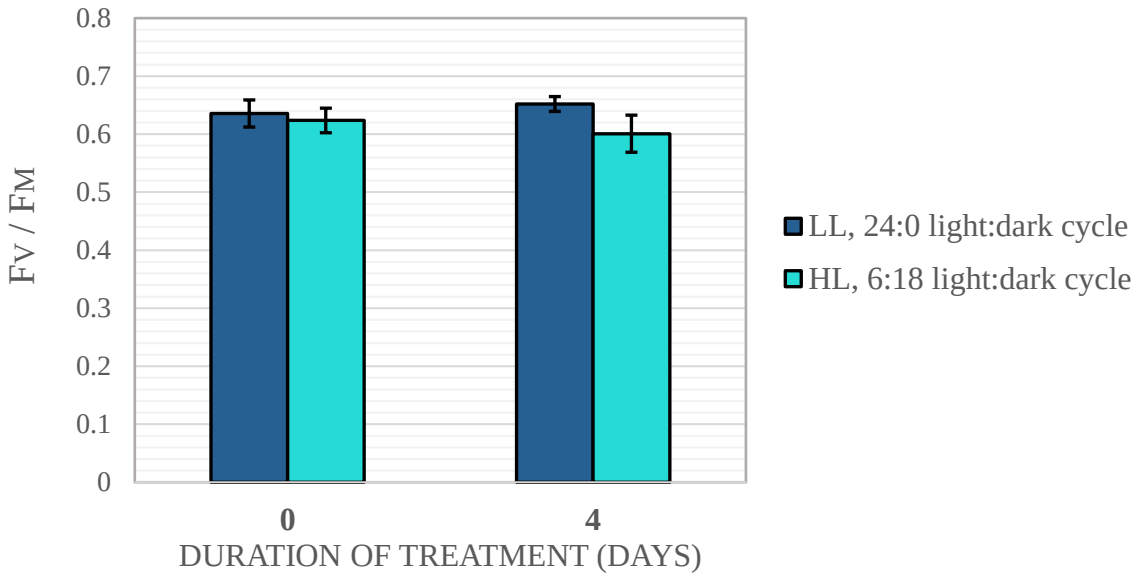


Figure 15. Fv/Fm value of two tested light treatments (100 µmol photon sm^{-2} s^{-1} and 400 µmol photon sm^{-2} s^{-1}) at a day 0 and 4 of the experiment.

As demonstrated in the Figure 16, after 7 days of exposure to the experimental conditions, the mean value of Fv/Fm ratio of the control samples from the LL treatment, that did not undergo the UV exposure significantly ($p<0.05$) increased compared to the day 4 measurements of this treatment group (0.675 ± 0.021). However, the mean Fv/Fm value for the LL samples that undergo the UV treatment did not change significantly compared to the day 3 measurements (0.652 ± 0.021). The Fv/Fm ratio of both control

and UV groups of the HL treatment did not change significantly ($p<0.05$) (0.589 ± 0.114 and 0.574 ± 0.031 for the control and UV group respectively) compared to Fv/Fm ratio of those groups at a day 4 of experiment. The group with the 24 h light cycle and no additional UV treatment showed a significant ($p<0.05$) decrease (0.0339 ± 0.034) in Fv/Fm value at a day 7 compared to day 4 and 0 measurements of this group. Overall, the Fv/Fm value increase compared to the beginning of experiment was observed only for the LL control group.

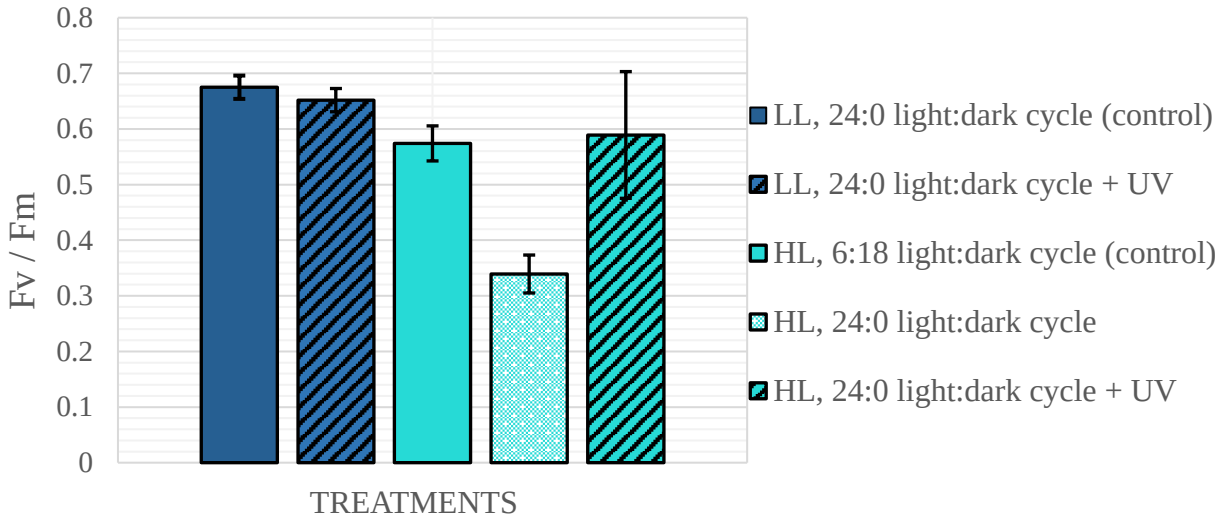


Figure 16. Fv/Fm value of two tested light treatments (100 µmol photon sm^{-2} s^{-1} and 400 µmol photon sm^{-2} s^{-1}) at a day 7 with and without exposure to UV.

5.3.3 Algal thallus absorption

The starting value of thallus absorption at the experiment 3 was 0.83 ± 0.03. All the samples at the day 4 and 7 of experiment, including UV treated samples, did not show any significant ($p<0.05$) change in the thallus absorption (Table 5). However, thallus absorption for HL samples was higher at day 3 (0.87 ± 0.13) ($p<0.05$) than those at the beginning of experiment. At day 7 HL treatment with the 24-hour exposure to the light and the HL control treatments showed similar thallus absorption values as those at day 4 of

experiment. The only group that showed a significant growth compared to the day 4 measurements was the HL group with the additional exposure to UV (0.92 + 0.02) ($p<0.05$).

Table 5. Thallus absorption of tested treatments at the beginning of experiment 2 (day 0) and after 4 and 7 days of experiment 3. Data expressed as mean value ± standard deviation, n=5 at p<0.05

Experiment	Light intensity, µmol photon m-2 s-1	Light: dark cycle, hours day -1	Exposure to UV	Duration of treatment, days	Thallus absorption factor (*A*)
Experiment 3	100	24 : 0	NO	0	0.83 ± 0.04
			NO	4	0.82 ± 0.02
			NO	7	0.82 ± 0.02
			YES	7	0.84 ± 0.02
	400	6:18	NO	0	0.83 ± 0.04
			NO	4	0.87 ± 0.01
			NO	7	0.89 ± 0.01
		24 : 0	NO	7	0.89 ± 0.01
			YES	7	0.92 ± 0.02

5.3.4 Antioxidant activity

The experiment 3 was divided into to sub-parts (4 and 3 days each) that were evaluated separately. Initial antioxidant activity of the LL (100 µmol photon sm^{-2} s^{-1}) treatment was: 6.184 ± 0.096 µmol TE/g and HL (400 µmol photon sm^{-2} s^{-1}): 6.753 ± 0.125 µmol TE/g (Table 6).

After 4 days of exposure to the experimental conditions the antioxidant activity of the LL treatments decreased significantly ($p<0.05$) (5.340 $\pm$ 0.058 µmol TE/g). Even though, after exposure to UV at day 7 the antioxidant activity increased significantly ($p<0.05$) (6.10 $\pm$ 0.14 µmol TE/g), it was lower than at the beginning of experiment. The LL control treatment at day 7 remained without significant ($p<0.05$) changes compared to the day 4 of experiment (5.44 $\pm$ 0.15 µmol TE/g).

The antioxidant activity of the HL treatment increased significantly ($p<0.05$) after 4 days of exposure to the experimental conditions (8.93 + 0.22 µmol TE/g). Three groups of the HL after 7 days of exposure did not show a significant ($p<0.05$) difference in antioxidant activity compared to the day 4 (8.68 + 0.06 µmol TE/g; 8.89 + 0.18 µmol TE/g and 9.01 + 0.34 µmol TE/g for the HL treatments with and without exposure to UV along with the control group respectively). Moreover, the antioxidant activity values did not differ significantly ($p<0.05$) between control and two treatment groups at day 7. To conclude, the increase in antioxidant activity compared to the day 0 was observed in all groups of the HL treatment at days 4 and 7. On the contrary, the antioxidant activity of the LL treatment at day 4 dropped, however LL group showed an increase in antioxidant activity, when treated with UV.

Table 6. Antioxidant activity expressed in Trolox equivalents (µmol TE/g) of different treatments at the beginning of experiment 3 (day 0) and after 3 and 7 days of experiment.

Light irradiance (µmol m2 s-1)	Duration of exposure (days)	UVA radiance (yes/no)	Trolox equivalents (µmol TE/g)
100	0	NO	6.18 ± 0.10
	4	NO	5.34 ± 0.06
	7	YES	6.10 ± 0.14
	7	NO	5.44 ± 0.15
400	0	NO	6.75 ± 0.13
	4	NO	8.93 ± 0.22
	7	YES	8.68 ± 0.06
	7	NO	8.89 ± 0.18
	7	NO	9.01 ± 0.34

6. Discussions

In this study the three experiments performed have shown that a wide range of conditions, including irradiance fluctuation, salinity variation and exposure to UV A radiation induces change in the growth rate of *G. vermiculophylla* along with photosynthetic capacity and level of antioxidant compounds. The initial values of antioxidant activity of *G. vermiculophylla* samples as well as values obtained after

different treatments corresponded to the values of the antioxidant activity of *Gracilaria spp* reported in the studies of Vijayavel and Martinez, 2010 as well as Alvarez-Gomez et al., 2017.

6.1 Experiment 1

The physiological response of *G. vermiculophylla* to subjected to the given experimental conditions was significant, during the first experiment, a combination of 30 ppt salinity without application of desiccation stress was the only condition, where the seaweed showed positive growth rates. Exposure to the hyper-salinity condition as well as a daily application of desiccation stress induced a decrease in growth of *G. vermiculophylla.* However, negative growth rate measurements did not necessarily induce decrease in photosynthetic activity. The Fv/Fm ratio, increased in three out of four treatments after three days of exposure to experimental conditions. The Fv/Fm ratio is used to indicate the stress levels that algae suffer (He *et al*., 2015), where a decrease in the Fv/Fm ratio is observed for the stressed plant (White, Anandraj & Bux, 2011). Therefore, a slight increase in the Fv/Fm ratio of the 3 treatments indicate their positive effect on efficiency of PSII. The light irradiance used in this experiment was slightly higher than the one used for algae culture in the greenhouse combined with different light : dark cycle ratio, which could promote photosynthetic activity. Moreover, algae had access to fresh nutrients at the beginning of experiment, that also could have effect on the increase in Fv/Fm ratio (White, Anandraj & Bux, 2011).On the contrary, high salinity along with desiccation represent a stress condition for *G. vermiculophylla* culture. This can be explained by photoxidation defense mechanism of *G. vermiculophylla* damage, when exposed to both increased light intensity, longer photoperiods (light : dark cycle ratio increase compared to the culture conditions in the greenhouse) as well as fluctuating water levels inducing dessication (Magnusson, 1997).

The mean values of Fv/Fm for those three groups (30 ppt with and without desiccation, and 40 ppt without desiccation) corresponded to a slightly stressed condition. However, those values were very close to the values, reported for the non-stressed organisms (Björkman & Demmig 1987). This confirms that *G. vermiculophylla* has a high survival threshold and explains its widespread occurrence around the globe (Nyberg & Wallentinus, 2009).

Hyper salinity conditions were shown to negatively impact *G. vermiculophylla* light absorption through thallus. The present study shows a trend of decreased light absorption through algal thallus in high salinity conditions. This could be potentially attributed to a decrease in chlorophyll abundance in a response to salinity-induced stress as it was shown in the study of Kumar *et al*., 2010.

The growth of *G. vermiculophylla* significantly decresed on exposure to hyper salinity conditions showing a further decline in growth rate with increased duration of stress conditions(Kumar et al., 2010). However, such extreme salinity conditions elevated antioxidant activity in *Gracilaria vermiculophylla* after 7 days of exposure. The same trend was observed by Kumar et al., 2010, where after 6 days of exposure Gracilaria *spp* had increased antioxidant activity. This can be explained by *Gracilaria* stress-response to high salinity conditions by activating its antioxidant machinery to limit ROS production (Kumar *et al.*, 2010; Bischof and Rautenberger, 2012).

6.2 Experiment 2

The growth of the IL treated *G.vermiculophylla* did not differ significantly ($p>0.05$) between the duration of light exposure as well as additional UV radiation. The growth rates of *Gracilaria vermiculophylla* were higher under increased light intensity. Growth rates showed constant increase for HL treated samples during 10 days of experiment.

Thus, increased light intensities showed a favorable effect on algae growth (Narasimhan et al., 2013) . Increase in light intensity showed higher absorption of light through thallus in *G. vermiculophylla* . This can be due to plasticity of pigments as an adaptive mechanism longer photoperiods (Aguilera et al., 2008) (comparing to the light : dark cycle in the greenhouse where algae were cultivated prior to the experiment) along with the increased light intensity. Exposure to UV radiance did not show a significant impact ($p>0.05$) on algal growth or thallus absorption. This could be due to the fact that UV radiance accounted for only about 0.01 fraction of the total light intensity

Both IL and HL treatments showed an increasing trend in the PSII efficiency as Fv/Fm ratio increased already after 3 days of exposure to the experimental conditions. However, after three days of exposure Fv/Fm ratio did not differ significantly between two light treatments. This may be a result of relatively small difference between HL and IL irradiance levels.

In both treatments UVA treated samples had higher antioxidant activity than the ones without UV radiation treatment. An explanation for this observation is the synthesis of mycosporine-like amino acids (MAAs) that produce in algae in a response to UV radiation as a defense mechanism to oxidative stress (Karsten & Wiencke, 1999; Rastogi & Incharoensakdi, 2013; 2014). MAAs substances act as sunscreens that protect algae from harmful UV radiance (Dunlap and Shick, 1998). In addition, MAAs also have a secondary photoprotective function as antioxidants (De La Coba *et al.*, 2009; Torres *et al*., 2018; Wada *et al*., 2015). Therefore, the UV radiance could act as an inducer of MAAs synthesis and thus, increase the antioxidant activity of seaweed (De La Coba *et al.*, 2009). The maximum antioxidant activity in IL treated samples occurred on 3d day of experiment, whereas in HL it happened after additional UV radiance at the 10^{th} day. It may be due to higher UV radiance fraction in the HL treatment and thus, higher effect of UVR on MAAs synthesis.

6.3 Experiment 3

After 4 days of exposure to LL and HL treatments, with overall equal PAR (Photosynthetically Active Radiation in the range 400-700 nm) doses per day, growth rates of *Gracilaria vermiculophylla* did not differ significantly among each other. Moreover, PSII had similar effectiveness in both HL and LL treated samples. The study of Weinberger et al., 2008 showed that growth of *G. vermiculophylla* can be impacted not only by irradiance levels, but also by daily PAR dose received by algae. This could explain no sigificant differences between growth rate and photosynthetic activity of HL and LL treated samples after 4 days of exposure. The Fv/Fm ratio of HL treated samples was lower than the one of the low light treated samples. This may be due to *G.vermiculophylla* sensitivity to photoinhibition when grown under high light conditions, through formation of ROS. The light absorption through thalli was higher in HL treated samples, which can be attributed to superior algal adaptivity to longer photoperiods and higher irradiance, which may involve plasticity of pigments (Aguilera *et al*., 2008).

However, when exposed to HL during 24 hours Fv/Fm ratio of algae corresponded to the critical health state reported for *Gracilaria* (Figueroa *et al*., 2006). On the contraty, samples that undergo additional UV treatment during 24 hour light : dark cycle of the HL treatment had similar Fv/Fm values as HL control ones (6:18 light : dark cycle). This could suggest that combination of low levels of UVA and high irradiance could positively affect algal defense mechanism against oxidative stress.

The antioxidant activity of *G.vermiculophylla* did not increase in the LL treatment with 24 : 0 light : dark cycle. Moreover, antioxidant capacity of algae decreased during the experiment, compared to the initial antioxidant capacity of *G.vermiculophylla.* It may be due to extremely low irradiance level that have a negative effect on MAAs synthesis in algae (Barceló-Villalobos et al., 2017). However, after exposure to UV radiance

antioxidant activity of algae increased, reaching initial values of antioxidant activity of *G. vermiculophylla*. This confirms findings of experiment 2, where UVR had a positive effect on antioxidant activiti of *G. vermiculophylla*.

Rapid increase in the antioxidant activity of *G. vermiculophylla* was observed already after 4 days of experiment. However, antioxidant activity of *G. vermiculophylla* after changing photoperiod from 6 to 24 hours did not show a significant affect on antioxidant activity of seaweed. This confirms effectiveness of antioxidant defence system of algae as a rapid response to the short-term UV induced stress (Aguilera *et al*., 2002; Bischof *et al*., 2007).

Some studies have shown UV inhibiting effect on plants physiological processes, including the photosynthesis (Xu & Gao, 2015; Cai *et al.*, 2016). However, UV radiance does not always have a negative effect on algae physiological performance (Bautista-Saraiva *et al*., 2018; Figueroa *et al*., 2009; Roleda *et al*., 2012). Some algae are genetically adapted to certain levels of UV radiance (Flores-Molina *et al*., 2016; Van Donk *et al*., 2001). Tolerance to UV radiance can be a result of a genetical adaptation (Flores-Molina *et al*., 2016; Van Donk *et al*., 2001), algae life stage (Navarro et al., 2010), as well as MAAs synthesis efficiency (Roleda, Nyberg and Wulff, 2012). Consequently, when certain levels of UV radiance are not reached, some physiological pathways in algae organism could not be triggered (Holzinger & Lütz, 2006). This may explain the fact that algae under high irradiance (24:0 light:dark cycle) were in a critical condition, while those, which received a small dose of UV along with HL treatment (24:0 light:dark cycle), were able to overcome harmful impact of high irradiance coupled with UV. This could suggest that *G.vermiculophylla* used in the experiment could have a certain level of tolerance to UVA radiance and could require it in order to trigger some essential physiological processes.

A study of Roleda *et al*., 2012) showed a positive effect of moderate levels of UVA radiance on growth and photosynthesis of *G.vermiculophylla*. This could explain a relatively high Fv/Fm ration of UV treated samples (24:0 light:dark cycle) similar to Fv/Fm mean value of HL control group (6:18 light:dark cycle).

To conclude, findings from this experiment could suggest that *G.vermiculophylla* used in the experiment could have a certain level of tolerance to UVA radiance and could require it in order to trigger some essential physiological processes

6.4 Conclusion

In this study *Gracilaria vermiculophylla* was cultured under various environmental conditions in order to optimize its antioxidant activity. The rapid defense machinery of *G.vermiculophylla* against oxidative stress allows obtaining favorable antioxidant capacity of algae by exposing it to harsh conditions short-term period. The study showed that antioxidant activity of *G. vermiculophylla* can very significantly among different treatments that involve fluctuation in light intensity, UV radiation, variable photoperiod, and desiccation stress. The antioxidant activity was shown to increase when *G .vermiculophylla* was grown in hyper salinity or high light conditions. In particular, the irradiance of 400 µmol photon m^{-2} s^{-1} was shown to have the highest positive effect on the antioxidant capacity of *G.vermicullophylla*. The trend of UVA radiance enhancing effect on antioxidant levels was observed in the study. The proposed methods could be further applied in industrial scale seaweed cultivation for food or pharmaceutical purposes, where high antioxidant levels are highly essential.

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