Optimisation of land-based cultivation conditions and enhancement of antioxidant activity in *Umbraulva* sp. (Chlorophyta, Ulvaceae)

submitted for acquisition of the academic degree

Master of Science in Marine Biological Resources

by

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Executive Summary

This thesis is part of the 'Mak-Pak Scale-Up' project (Industrietaugliche Verfahrensoptimierung zur Herstellung einer nachhaltigen Verpackungslösung aus Makroalgen für den Lebensmittel-Handel, Förderkennzeichen 28-1-DL-02-B2-0), funded by the German Ministry of Food and Agriculture (BMEL). Aim of the project is to produce a sustainable, compostable and edible food packaging alternative to plastic on an industrial scale. The product is going to be made from green algae belonging to the Ulvaceae family which will be cultivated in land-based re-circulating aquaculture systems. In order to provide a high-quality product, suitable species and cultivation conditions need to be identified. This thesis intends to answer a series of questions regarding the long-term cultivation conditions and the enhancement of antioxidant activity in an *Umbraulva* species. The effects of different seawater types and stocking densities on the growth and photosynthetic activity of *Umbraulva* sp. were assessed. Both filtered natural seawater and re-used artificial seawater from a show-casing aquarium were identified as suitable cultivation media. No conclusive results were obtained regarding the optimal stocking density. In the second part of the thesis an enhancement of antioxidant activity through application of different light qualities and quantities was attempted. Antioxidant activity was measured using an $ABTS^{\bullet+}$ de-colourisation assay. No increased antioxidant activity was caused by the application of blue light compared to the standard cultivation white light. No increased antioxidant activity was measured in the light quantity experiment either, although high light intensity treatment and high daily photo-dose treatments lead to an induction of light stress in *Umbraulva* sp. Further research into physiological characteristics of the *Umbraulva* sp. is necessary before an industrial application as raw material for packaging material becomes realistic.

Abstract

This thesis is part of the 'Mak-Pak Scale-Up' project, funded by the German Ministry of Food and Agriculture (BMEL, Förderkennzeichen 28-1-DL-02-B2-0). Aim of the project is to produce a sustainable, compostable and edible food packaging alternative to plastic on an industrial scale. The product is going to be made from green algae belonging to the Ulvaceae family that will be cultivated in land-based re-circulating aquaculture systems. Suitable species and cultivation conditions need to be identified in order to provide a high-quality product. This thesis intends to answer questions regarding the long-term cultivation conditions and the enhancement of antioxidant activity in *Umbraulva* sp. Both filtered natural seawater and re-used artificial seawater from a show-casing aquarium were identified as suitable cultivation media. The enhancement of antioxidant activity through the application of blue light was unsuccessful. No increased antioxidant activity was measured in the light quantity experiment either, although high light intensity treatment and high daily photo-dose treatments lead to an induction of light stress in *Umbraulva* sp. Follow-up research is necessary in order to identify exact nutrient compositions, stocking densities and light settings in order to cultivate *Umbraulva* sp. with the desired results in biomass production and antioxidant activity.

Contents

Nomenclature

Abbreviation

AA	Antioxidant activity
ABTS$^{\bullet+}$	[2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation
ASW	Artificial seawater
AWI	Alfred-Wegener-Institute, Helmholtz-Center for Polar and Marine Research
BMEL	Bundesministerium für Ernährung und Landwirtschaft
	(German Ministry for Food and Agriculture)
EtOH	70% Ethanol
LED	Light emitting diode
NSW	Natural seawater
PAM	Pulse-amplitude modulated (fluorometer)
PETC	Photosynthetic electron transfer chain
PSII	Photosystem II
RAS	Re-circulating aquaculture system
rASW	Re-used artificial seawater
ROS	Reactive oxygen species
RT	Room temperature (ca. 20°C)
SW	Seawater

Variables

dGR	Daily growth rate $[cm^2 \cdot d^{-1}]$
DLI	Daily light integral $[molphotons \cdot m^{-2} \cdot d^{-1}]$
DW	Dry weight $[g]$
F_0	Minimum chlorophyll *a* fluorescence level $[nm]$
F_m	Maximum chlorophyll *a* fluorescence $[nm]$
F_v/F_m	Effective quantum yield of Photosystem II
$PPFD$	Photosynthetically active photon flux density $[\mu molphotons \cdot m^{-2} \cdot s^{-1}]$
RGR	Relative growth rate $[\%]$
SA	Surface area $[cm^2]$
SD_{SA}	Stocking density in reference to thallus surface area $[SA_{algae} \cdot SA_{vessel}^{-1}]$
SD_{bm}	Stocking density in reference to algae biomass $[g_{DW} \cdot SA_{vessel}^{-1}]$
TE	Trolox Equivalent $[\mu g \cdot mL_{Extract}^{-1}]$
tSA	Total surface area $[cm^2]$
WW	Wet weight $[g]$

1 Introduction

1.1 The problem with plastic

Solid polymer materials, or plastics, possess a number of characteristics that make them attractive to many industries. Their form, rigidity, elasticity and heat resistance can be adjusted according to their intended use. They are furthermore durable, chemically inert and can be supplemented with additives, which makes them attractive as packaging material for products ranging from food and medical supplies to chemicals (Busse and Rechenberg, 2020). In Germany alone 11.82 million tons of plastic were produced in 2017, the largest part (26.6 %) of which were produced for packaging materials.
Unfortunately, from an environmental point of view, some of their characteristics present major drawbacks. An often short-term or single-use application of products paired with a high durability entails that approximately 79% of plastic products will end up in landfills or find their way into the environment (Geyer et al., 2017). Macro-plastics (>25 mm) can accumulate, as for instance in the Great Pacific Garbage Patch (Lebreton et al., 2018), or break down into microplastics (1 μm - 1 mm). The later are known to be ingested by animals, like filter feeders, and are accumulated throughout the food chain (Fernández and Albentosa, 2019).
The causes and effects of plastics in the environment have been and are still being researched worldwide. Many questions are still being discussed, but one statement seems to be conclusive: We need to find solutions to remove plastics from our environment and dispose them more efficiently while ceasing to produce more and more new material.

1.2 In search of a solution: 'Mak-Pak Scale-Up'

One project that aims to provide a sustainable, compostable and ideally edible food packaging alternative to plastic is 'Mak-Pak Scale-Up' (Industrietaugliche Verfahrensoptimierung zur Herstellung einer nachhaltigen Verpackungslösung aus Makroalgen für den Lebensmittel-Handel, Förderkennzeichen 28-1-DL-02-B2-0), funded by the German Ministry of Food and Agriculture (BMEL). Their final product is furthermore supposed to improve the shelf-life of packaged

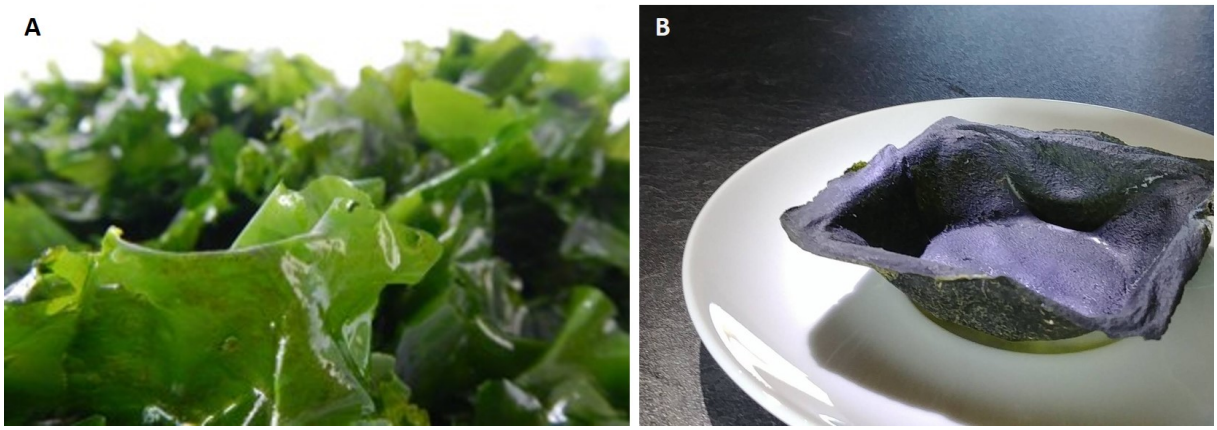

Figure 1.1 **A)** *Ulva lactuca* raw material grown under laboratory conditions and **B)** resulting final food packaging. (Photo: Laurie C. Hofmann; BLE, 2020)

food and provide a health benefit to the consumer through an increased antioxidant content (Abd El-Baky et al., 2009).

'Mak-Pak Scale-Up' produces a pulp from the entire algae as raw material (Figure 1.1 BLE, 2020), instead of utilising single components for bio-polymers (i.e. starch, cellulose) (Reichert et al., 2020). The procedure is therefore less wasteful, overall easier to apply and more accessible to potential producers.

The project looks to produce a material from algae that can be found locally and grown in land-based re-circulating aquaculture systems (RAS) (AWI, 2021). A prototype has already been produced from green macro-algae of the Ulvaceae family during the previous project 'Mak-Pak' (Nachhaltige Verpackungslösung aus Makroalgen, Förderkennzeichen 28-1-A1.049-16), funded by the German Federal Office for Agriculture and Food. As the name suggests, 'Mak-Pak Scale-Up' aims to identify further suitable Ulvaceae species as well as cultivation and production methods, that can be scaled up from the laboratory setting to an industrial scale.

The various project partners are focused on different aspects of the process (Figure 1.2). Cultivation conditions and reliable reproduction methods are researched by the team at Alfred-Wegener Institute, Helmholtz Center for Polar and Marine Research (AWI, Bremerhaven, Germany). Their results are then shared with and applied by the agricultural partner 'ROVAL GbR' (Rockstedt, Germany). Processing of the algae into pulp and production of the final product are researched by a team at 'Hochschule Bremerhaven' (Bremerhaven, Germany) and communicated to the industrial partners 'FGW Fasergusswerk Polenz GmbH & Co. KG' (Neustadt, Germany) and 'Hengstenberg GmbH & Co. KG'(Esslingen, Germany). Marketing and sale of the finalised product are conducted by the fast-food chain 'NORDSEE GmbH' (Bremerhaven,

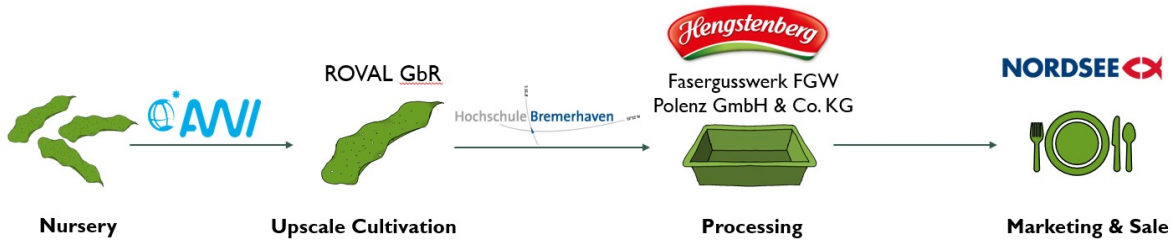

Figure 1.2 Division of tasks and responsibilities between 'Mak-Pak Scale-Up' partners. Alfred-Wegener Institute (AWI) is in charge of research on reproduction and cultivation conditions which are then applied by agricultural partner ROVAL GbR in re-circulating aquaculture systems. Methods for processing of the algal biomass to pulp and final packaging product are researched by Hochschule Bremen and applied by Hengstenberg GmbH & co. KG and FWG Fasergusswerk Polenz GmbH & Co. KG. The packaging product is finally marketed and sold by Nordsee GmbH. (Illustration: Laura S. Belter; BLE, 2020)

Germany).

1.3 The raw material: *Umbraulva* sp. (Chlorophyta, Ulvaceae)

The green-algal genus *Umbraulva* has only recently been defined as a monophyletic taxon, closely related to the genera *Percursaria*, *Ulvaria*, and *Ulva* (Maggs et al., 2007; Wynne and Furnari, 2014; Kawai et al., 2021). Prior to molecular analyses *Umbraulva* were considered to belong to the *Ulva* genus, due to the striking similarities in morphology and life cycles.
Species from both genera undergo an isomorphic, biphasic life cycle (Mantri et al., 2020) in which the alternating sporophytic and gametophytic phases are morphologically identical. Algae from both genera consist of thin thalli that are connected to a substrate by a rhizoid holdfast. The adult stages of *Ulva* spp. can consist of either tubular or blade-like, as well as mono- or distromatic thalli. *Umbraulva* spp., however, only form soft, distromatic and folious thalli with elliptical holes (Maggs et al., 2007). Ulvaceae are known to form 'green tides' when these thalli become detached from their substrate and wash up on shores while continuing to grow (Coelho et al., 2016).
Apart from genetic analysis, it is possible to differentiate between *Ulva* and *Umbraulva* based on two main characteristics. While *Ulva* spp. often float below the sea surface and inhabit intertidal environments with high fluctuations in temperature, light and water currents, *Umbraulva* spp. preside mostly in deeper subtidal habitats (8-40 m) with less turbulence (Maggs

Figure 1.3 Partial thallus of *Umbraulva* sp. with characteristic olive-green colour and small elliptical holes. (Photo: Laura S. Belter

et al., 2007; Wynne and Furnari, 2014; Steinhagen et al., 2019). *Umbraulva* spp. possess the carotinoid pigment siphonaxanthin, which helps them utilise green light for photosynthesis, as an adaptation to the lack of longer-wave light in those deeper habitats, (Kageyama et al., 1977; Maggs et al., 2007). Siphonaxanthin is more prevalent with Codiales and *Umbraulva* is the only genus within the Ulvaceae family with this pigment. It causes the thallus to have a dark olive-green colour that is easily distinguishable from the characteristic bright green of *Ulva* spp.
Umbraulva was most likely introduced to the European marine environment from the Indo-Pacific region and can now be found throughout the North-East Atlantic and Mediterranean Sea (Maggs et al., 2007; Kawai et al., 2021). Only one species, *Umbraulva dangeardii*, has recently been identified in German waters around the North Sea island Heligoland (Steinhagen et al., 2019).
To date *Umbraulva* has not been studied well, apart from its morphology, distribution and pigment composition. Other Ulvaceae, with *Ulva* spp. at its forefront, are already being cultivated and applied for bioremediation, bio-refinery, functional food supplements (i.e., trace-elements, vitamins, antioxidants), aquaculture feed and packaging materials (Abd El-Baky et al., 2009; Coelho et al., 2016; Mantri et al., 2020; BLE, 2020). It is only logical to investigate the physiol-

ogy, ecological behavior and possible uses of *Umbraulva*, considering that it is likely to become more abundant in European waters.

1.4 Aims & Hypotheses

The *Umbraulva* sp. material used in this thesis was provided by the 'Nordsee-Aquarium' in Bremerhaven and originally acquired from Heligoland. This thesis intends to clarify whether it can be used as raw material for food packaging while simultaneously collecting information about the general physiological characteristics of the species.
The optimal cultivation conditions need to be identified in order to grow a large amount of biomass on an industrial scale without sustaining any loss in quality. Within the 'Mak-Pak Scale-Up' project this study focuses on the step of cultivating the green macro-algae (Figure 1.2). It can be divided into two main topics: cultivation conditions and enhancement of antioxidant activity in *Umbraulva* sp.

1.4.1 Cultivation conditions

Long-term cultivation conditions are researched for application in the RAS at ROVAL GbR. In a first experiment growth and photosynthetic activity of *Umbraulva* sp. were monitored in three types of water: natural seawater (NSW) from Heligoland, freshly produced artificial seawater (ASW) and re-used artificial seawater (rASW) from a showcasing aquarium at 'Nordsee-Aquarium'.

> Hypothesis 1: The natural seawater is most likely to promote optimal growth in *Umbraulva* sp., followed by the re-used artificial seawater, as these conditions resemble the natural environment more closely.

A study by Oca et al. (2019) on *Ulva ohnoi* revealed that the relationship between stocking density in a cultivation vessel and growth is not strictly linear. The thin green algae often float at the sea surface (Hiraoka et al., 2004) and require a certain amount of self-shading to protect themselves against excessive light damage. Once that threshold is reached, the algae grow exponentially until the limits of space and nutrient availability are reached.
In an artificial setting, like a RAS, this balance between self-shading and space limitation can be maintained by monitoring and adjusting the stocking density within a culturing vessel. The second experiment experiment tests whether this effect of stocking density on growth and overall well-being can also be measured in *Umbraulva* sp.

Hypothesis 2: It is expected that *Umbraulva* sp. grows better in a smaller cultivation vessel that permits self-shading.

1.4.2 Enhancement of antioxidant activity

Antioxidants are a counter measure of algae and plants against 'reactive oxygen species' (ROS) (Shanura Fernando et al., 2016). These oxygen compounds contain radicals, making them highly reactive and harmful to biomolecules, including nucleic acids and components of the photosynthetic electron transfer chain (PETC). During abiotic stress, such as high light intensities, the regular PETC is interrupted and can lead to the production of ROS. Severe damage to the cells is prevented through ROS scavenging measures, including non-chemical quenching (release of excess light energy as heat or light), chloroplast packing (increase of chloroplast and therefore available PETCs) and antioxidant activity (Maxwell and Johnson, 2000; Shanura Fernando et al., 2016; Khorobrykh et al., 2020).
In Ulvaceae ROS are reduced by antioxidants such as phenolic compounds (Abd El-Baky et al., 2009), photosynthetic pigments like carotinoids (Amorim et al., 2020) and ulvan, a polysaccharide only found in this green algae family (Le et al., 2018).
The second part of this thesis aims to identify light settings that cause an increase in antioxidant activity. Successful settings are then supposed to be applied in the RAS right before harvest, when enough biomass has already been produced.
Previous studies have shown that cultivation of *Ulva* spp. under blue light results in an increased antioxidant activity (Le et al., 2018; Schwoerbel, 2019). The first experiment will test, whether a similar effect can be measured in *Umbraulva* sp. by comparing the general cultivation conditions with white light against blue light cultivation.

Hypothesis 3: It is expected that an application of blue light will lead to an increased activity of antioxidants, similar to that measured in *Ulva* spp.

The second and last experiment tests three light intensity settings against each other. Here, the cultivation conditions are compared with a treatment that subjects the algae to a higher photosynthetic photon flux density ($PPFD$) but unchanged amount of provided photons throughout the day (daily light integer DLI). A second treatment uses the same higher $PPFD$ combined with a higher DLI than in the cultivation conditions and first treatment.

Hypothesis 4: Both, increased PPFD and increased PPFD plus increased DLI are expected to cause light stress in *Umbraulva* sp. which will most likely lead to a measurable increase in antioxidant activity.

The thesis follows the approach of previous master students Sofiia Tretiak (Tretiak, 2019) and Jakob Schwoerbel (Schwoerbel, 2019). The results will be shared with and applied by ROVAL GbR, aiming to further advance the development of a sustainable, algae-based packaging material.

2 Materials and Methods

2.1 *Umbraulva* sp material collection, identification and cultivation conditions

The macro-algae material used in this study was collected from show-casing aquaria at the 'Nordsee Aquarium' in Bremerhaven, Germany. The algae, along with other biota, were introduced to the aquarium system with the initially used natural seawater (NSW) from Heligoland, Germany (54°11'N/ 7°53'E). The 'Nordsee Aquarium' has since switched to using artificial seawater (ASW; Sea Salt CLASSIC, Tropic Marin AG, Hünenberg, Switzerland) in their tanks. Both types of water have a salinity of 32 ± 2 *psu*.

Samples were cleaned in distilled fresh water, dried in silica gel and sent to Sophie Steinhagen at University Gothenburg, Sweden for genetic identification. The genus *Umbraulva* was verified without any further specification.

Algae were transported to the lab with seawater (SW) from the aquaria in styrofoam boxes, rinsed three times in filtered NSW from Heligoland ($5\,\mu m$ polypropylene filter candle (PP 5 254 63 27), Graver Technologies Europe GmbH, Esslingen-Sirnau, Germany), carefully wiped clean with cotton balls and then transferred into $5\,L$ glass cultivation bottles with a surface area of $226.98\,cm^2$ (Duran, DWK Life Science GmbH, Wertheim/Main, Germany) with filtered NSW.

The initially applied culturing and standard experimental conditions were modelled after experiments with *Ulva* spp. by Tretiak et al. (2021) and Schwoerbel (2019). These settings were then adjusted to the needs of *Umbraulva* sp. after the first experiment (seawater types) and a nutrient uptake analysis (see Appendix).

The macro-algae cultures were kept in a climate-controlled room at $15 \pm 1\,^{\circ}$C with initially $80 \pm 10\,\mu mol photons \cdot m^{-2} \cdot s^{-1}$ (Schwoerbel, 2019; Tretiak et al., 2021) white light (Aquarius 90, Aqua Medic Anlagenbau GmbH, Bissendorf, Germany).

This was increased to $100 \pm 10\,\mu mol photons \cdot m^{-2} \cdot s^{-1}$ after the seawater type experiment. A diurnal cycle of 16h:8h light:dark was applied, resulting in a daily light integer (*DLI*) of $4.61\,mol photons \cdot m^{-2} \cdot d^{-1}$ and $5.76\ mol photons \cdot m^{-2} \cdot d^{-1}$, respectively.

Water was exchanged every 7 days. 40 $\mu L/L_{sw}$ Blaukorn garden fertiliser (COMPO GmbH, Münster, Germany) were initially added every 4 days as a nutrient supplementation, this was increased to 100 $\mu L/L_{sw}$ alongside the light adjustment in order to avoid nutrient limitation. 2.5 mL/L_{SW} of saturated Geranium(IV) Dioxide solution (Merck KGaA, Darmstadt, Germany) were added as a measure against diatom growth (Markham and Hagmeier, 1982) after transfer of the *Umbraulva* material from 'Nordsee Aquarium' to the lab. Two weeks of acclimatisation were granted under these conditions before proceeding with the experiments.

2.2 Experimental Setup

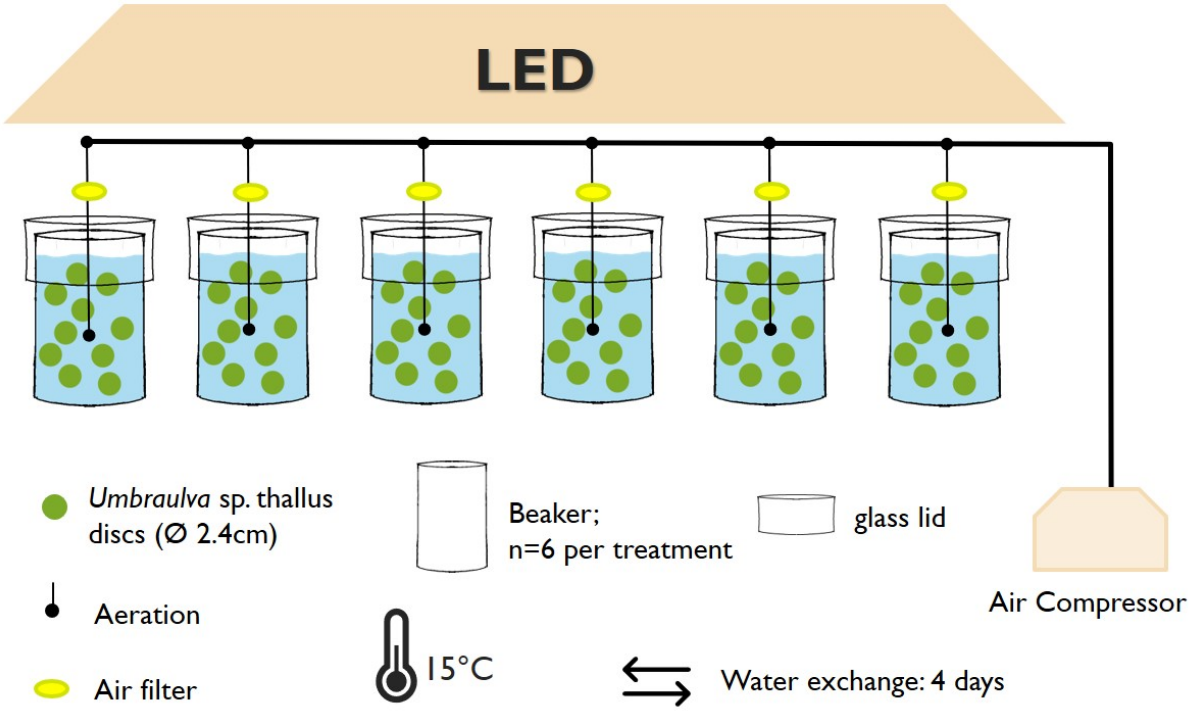

Figure 2.1 Standard experimental setup used for all cultivation and antioxidant enhancement experiments. 6 Replicates were placed under light emitting diodes at 15 °C. Water was aerated through glass tubes and exchanged every 4 days during experiments and every 7 days during cultivation. Vessel volume, Photosynthetic photon flux density, daily light integral, seawater type and number of thallus discs per replicate were adjusted according to the specific experimental setups and measurements.

The standard experimental setup was modelled after Tretiak et al. (2021) and Schwoerbel (2019) and used for all four experiments (Figure 2.1). The exact conditions, treatments and applied measurements are summarised in (Table 2.1).

At the start of each experiment discs of 2.4 *cm* diameter were cut from *Umbraulva* sp thalli with

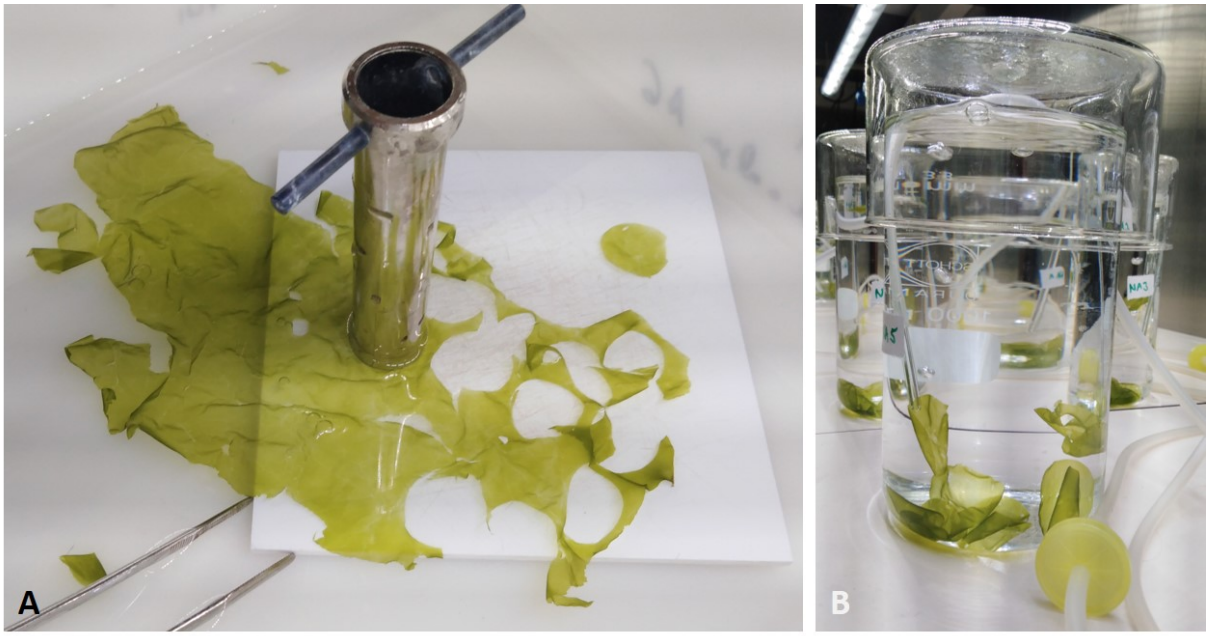

Figure 2.2 **A)** Discs with a 2.4 *cm* diameter were cut from *Umbraulva* sp. thalli *Umbraulva* sp. before each experiment and **B)** either 10 or 15 discs were added to each replicate for the cultivation and enhancement experiments, respectively.

a cork borer (Figure 2.2). 10 discs were allocated to each one of of six replicates per treatment in the cultivation condition experiments. 15 discs were needed for the two antioxidant enhancement experiments, as a minimum of 0.01g dry weight (*DW*) was needed for quantification of the antioxidant activity (Re et al., 1999; Torres et al., 2017).

The discs were kept in $1\,L$ glass beakers with a surface area of $70.88\,cm^2$ (Duran, DWK Life Science GmbH, Wertheim/Main, Germany) during the experiments and seawater was exchanged every 4 days. $40\,\mu L/L_{sw}$ Blaukorn were added after each water exchange for the seawater type experiment. This was increased to $100\,\mu L/L_{sw}$ fertiliser for all remaining experiments in order to match the cultivation conditions and avoid nutrient depletion.

White light was provided by light emitting diodes LED (Aquarius 90, Aqua Medic Anlagenbau GmbH, Bissendorf, Germany). A 16h:8h light:dark diurnal cycle at $80 \pm 10\,\mu mol photons \cdot m^{-2} \cdot s^{-1}$ was applied for the seawater type experiment and $100 \pm 10\,\mu mol photons \cdot m^{-2} \cdot s^{-1}$ for all subsequent experiments.

Seawater type, cultivation vessel size, light quality and light quantity were adjusted according to the aim of each experiment (Table 2.1).

A malfunction of the climate-controlled cultivation room occurred on day 6 of the light quality experiment, raising the temperature from 15 °C to 23 °C over several hours. The values

measured during the second sampling point after 7 days were therefore left out of the further analysis.

Table 2.1 Settings of the cultivation conditions (Standard) and the four experiments. The manipulated factor is printed bold for each experiment and lists the applied treatments separated by 'vs'. Units are printed within square brackets. PPFD stands for photosynthetic photon flux density, DLI for daily light integer, NSW for natural seawater, ASW for artificial seawater and rASW for re-used artificial seawater. The additional PPFD, DLI and nutrient values in round brackets indicate the values used until the conclusion of the seawater type experiment. The additional value for the duration in the light quality experiment indicate a measurement that had to be excluded from analysis due to technical problems.

	Cultivation			**Enhancement**	
	Standard	Seawater type	Vessel size	Light quality	Light quantity
Light:dark cycle [h]	16:8	16:8	16:8	16:8	**16:8 vs 6:18**
PPFD [µmol photons $\cdot m^{-2} \cdot s^{-1}$]	(80)100	80	100	100	**90 vs 240**
DIL [mol photons $\cdot m^{-2} \cdot d^{-1}$]	(4.61) 5.76	4.61	5.76	5.76	**5.18 vs 13.82**
Light quality	White	White	White	**White vs blue**	White
Temperature [°C]	15	15	15	15	15
Seawater type	NSW	**NSW vs ASW vs rASW**	NSW	NSW	NSW
Vessel volume [L]	5	1	**1 vs 5**	1	1
Nutrients [$\mu L \cdot L^{-1}$]	(40) 100	100	100	100	100
Duration [d]	/	14	14	4 (7)	4

2.3 Data Collection

Measurments were done on days 0 ('Initial'), 4, 7, 11, 14 or a selection of these, depending on the experiment. Measured factors include thallus disc size in cm^2, sample weight in g, photosynthetic activity in F_v/F_m. Antioxidant activity was measured as Trolox equivalents (TE) in $\mu g/mL_{Extract}$. The rates at which *Umbraulva* sp. takes up nutrients was also assessed (see Appendix). The data collection protocols and calculations are described within this section.
The sampling protocol varied between experiments, depending on the respective research question and the manageability of the sampling schedule. It is also important to note, that not all measured factors were analysed for each experiment, as they would not have contributed meaningfully to the aim of this thesis.
Table 2.2 summarises which variables were measured and analysed in each of the experiments. It is important to note, that during the chronologically first experiment on seawater types, measurements were repeatedly conducted on the exact same replicates. This approach was changed

for all remaining experiments, where extra beakers were added to the experimental setup for each sampling day

It furthermore needs to be pointed out that not all measured or calculated variables were analysed in this thesis. A focus was instead put on those variables, that were most suitable to answer the research questions. Raw data and calculations of all variables can be viewed in the Appendix section A.3.

Table 2.2 Overview of the sampling protocol for each of the four experiments. X and - indicate that a measurement of that factor was or wasn't, respectively. It is furthermore specified which factors have been measured but have not yet been analysed.

	Seawater type		**Vessel size**		**Light quality**		**Light quantity**	
	Measured	Analysed	Measured	Analysed	Measured	Analysed	Measured	Analysed
Size	X	X	X	X	X	-	X	-
Weight	-	-	X	X	-	-	X	-
Photosynthetic activity	X	X	-	-	X	X	X	X
Antioxidant activity	-	-	-	-	X	X	X	X
Measuring interval	Initial + day 4, 7, 11, 14		Initial + day 7, 11, 14		Initial + day 4 (+ 7)		Initial + day 4	

Size

The size of *Umbraulva* sp discs was measured as **surface area** (*SA*). The discs were laid out next to a 3x3 cm reference square on an LED tablet and photographed from above (Canon Deutschland GmbH, Krefeld, Germany). The pictures were analysed in the Image J software (v 1.52 Rasband, 2021). The pictures were first converted into 8-bit grey-scale images and then turned into binary black and white images. *SA* of the black disc areas was then outlined and calculated by the software (Figure 2.3 Schwoerbel, 2019).

It was initially intended to compare discs sizes between treatments. The discs, however, tended to break apart into smaller pieces, making individual comparisons impossible. The combined *SA* of all discs in one replicate was used instead for further analysis. This variable was then used to calculate other variables of size and growth. *SA* was doubled in order to calculate the **total surface area** tSA available for photosynthesis (Equation 2.1).

$$tSA = 2 \cdot SA \tag{2.1}$$

Two growth rate variables were calculated always in relation to the previous sampling point t. The **daily growth rate** dGR expresses how many cm^2 *Umbraulva* sp grew per day (Equation

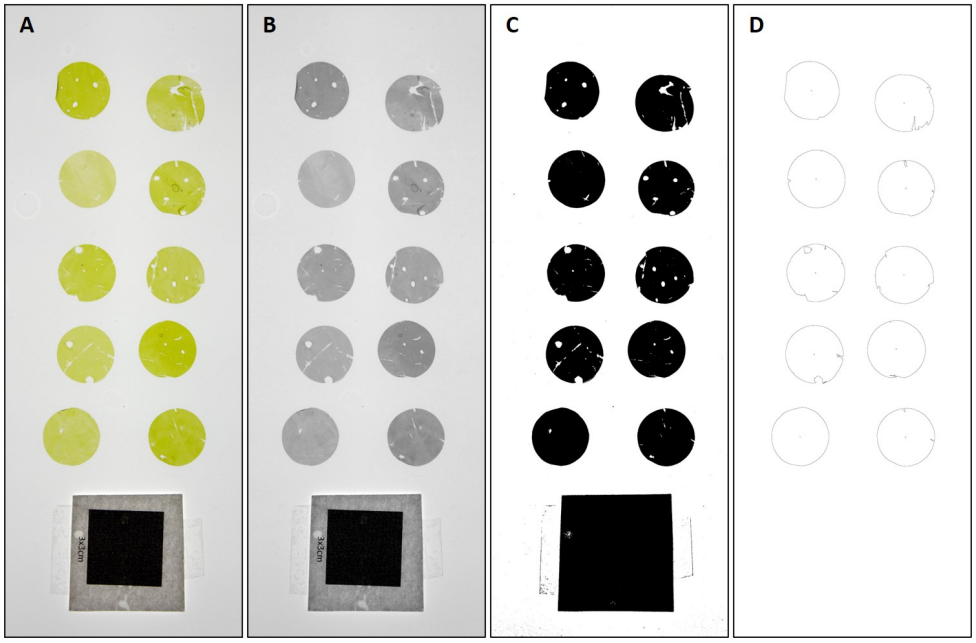

Figure 2.3 Progress of image analysis in ImageJ software. **A)** The original photograph of ten thallus discs at the start of the seawater experiment with a 3x3cm reference square next to them. **B)** The same picture after conversion to grey-scale. **C)** The grey-scale threshold was increased until the thallus discs appeared entirely black on a white background. **D)** Illustration of the outlines of the discs as computed by the software.

2.2 Oca et al., 2019).

$$dGR = \frac{SA_2 - SA_1}{t_2 - t_1} \tag{2.2}$$

Relative growth rate *RGR* expresses by which percentage *SA* increased per day, in reference to the previous sampling point *t* (Equation 2.3 Oca et al., 2019).

$$RGR = \frac{ln(SA_2) - ln(SA_1)}{t_2 - t_1} x100\% \tag{2.3}$$

Another variable that potentially influences the cultivation of macro-algae is **stocking density** *SD*. This variable represents the ratio between available surface area of the cultivation vessel and macro-algae biomass (Oca et al., 2019). Originally this factor was calculated using the weight in *g* of biomass (SD_{bm}, Equation 2.4). For the experiments in which weight was not measured, the equation was modified to calculate the ratio between surface area SA_{vessel} of the cultivation vessel the and surface area of the thallus disc SA_{algae} (SD_{SA}, Equation 2.5).

$$SD_{bm} = \frac{m_{algae}}{SA_{vessel}} \tag{2.4}$$

$$SD_{SA} = \frac{SA_{algae}}{SA_{vessel}} \tag{2.5}$$

Weight

Wet weight WW and **dry weight** DW were measured during the vessel size and light quantity experiments. Thallus discs were rinsed in distilled water and afterwards patted dry with a tissue. The samples were transferred into a plastic weigh boat and WW was determined in g with a precision of $0.1mg$ (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany). Dw was measured after oven-drying the samples at 30°C for 48h and used for the calculation of SD_{bm} (Equation 2.4)

Photosynthetic activity

Photosynthetic activity can be measured through chlorophyll *a* fluorescence using a pulse-amplitude modulated (PAM) fluorometer (Heinz Walz GmbH, 2020). Chl *a* fluorescence occurs, when the chloroplasts are light saturated and no longer able to transform absorbed light energy into photo-chemical energy (photosynthesis). The excess energy is re-emitted as either heat or light, as it might otherwise cause formation of cell-damaging ROS (Shanura Fernando et al., 2016; Heinz Walz GmbH, 2020).
Photo-chemical quenching (photosynthesis), non-photo-chemical quenching (heat dissipation) and chlorophyll *a* fluorescence balance each other out. If photo-chemical and non-photo-chemical quenching are kept at a minimum during the fluorescence measurement, the obtained value can be equated to the maximum photosynthetic efficiency of that organisms (Büchel and Wilhelm, 1993; Maxwell and Johnson, 2000).
This mechanism is used for the measurement of photosynthetic activity with a JUNIOR-PAM fluorometer and the associated WinControl 3.30 software (Heinz Walz GmbH, Effeltrich, Germany), as applied in this thesis.
In the first step, *Umbraulva* thallus discs are kept in the dark for 5-10 minutes. This leads to a reduction of photo-chemical and non-photo-chemical pathways to a negligible minimum. The light light guide of the JUNIOR-PAM fluorometer is then applied to the algae surface with the help of magnetic leaf clips (Heinz Walz GmbH, 2020). The minimum chlorophyll *a* fluorescence F_0 is measured. The intensity of the measuring light is adjusted until F_0 reaches a value between 200 and 400. An actinic light flash is then administered, causing photosynthetic reactions to take place. The Chl *a* fluorescence reaches it's maximum F_m once the photosynthetic electron transport chain (PETC) is saturated causing all additionally absorbed light energy to be re-emitted.

These values are then used to calculate F_v/F_m (Equation 2.6. It expresses the maximum photosynthetic efficiency of photosystem II (PSII) in the PETC (Büchel and Wilhelm, 1993; Maxwell and Johnson, 2000) and is widely used as an indicator of health for plants and algae (Higo et al., 2017).

$$F_v/F_m = \frac{F_0 - F_m}{F_m} \tag{2.6}$$

Antioxidant activity

The ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation de-colourisation assay was carried out for the quantification of antioxidant activity *AA* (Re et al., 1999; Torres et al., 2017).

Thallus discs were rinsed twice in distilled fresh water and then oven-dried for 48 h at 30 $^{\circ}C$. This imitates the drying process during the industrial production of the packaging material and ensures, that the measured antioxidant levels can actually be found in the final product.

ABTS reagent was made and incubated at 20°C in the dark for at least 16 h before measuring the samples. ABTS (Hoffmann - LaRoche AG, Basel, Switzerland) and potassium persulfate $K_2S_2O_8$ (Honeywell International Inc., Charlotte, NC, USA) were mixed in distilled water with final concentrations of 7 mM and 2.45 mM, respectively. The reagent was then diluted with 70 % ethanol until it reached an absorption of 0.70 at 734 nm.

0.1 g of dried algae and 0.3 g of sea sand SiO_2 (Merck KGaA, Darmstadt, Germany) were mixed and transferred into a mortar on ice. Under addition of mL 70 % ethanol (EtOH) the mixture was ground into a fine paste (Figure 2.4). Another 1 mL EtOH was used to rinse the extract into a 15 mL centrifugation tube (Eppendorf, Hamburg, Germany). The samples were then incubated in a shaking water-bath (130rpm, 45°C) for 6 h.

The extracts were then centrifuges for 10 minutes at 4°C and 2500g. The supernatant was transferred into a new 15 mL centrifugation tube and another 2 mL EtOH were added to the pellet. All samples underwent a second incubation at 130rpm and 45°C for 1 h. Centrifugation for 10 minutes at 4°C and 2500g was repeated. The supernatant of the initial tube was added to the extract in the second tube and immediately measured. 20 μL of each sample were added to a 96-well plate (Nunc A/S, Roskilde, Denmark). EtOH was used as a negative control and the vitamin E equivalent Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as positive control with a concentration of 100 $\mu g/mL$. Solvent blanks were measured with each plate and used to eliminate the absorption of plate and solvent.

280 μL of ABTS reagent were added to each well, except for the solvent blanks. Plates were incubated in the dark for 8 minutes at room temperature (RT) before measuring the absorption of

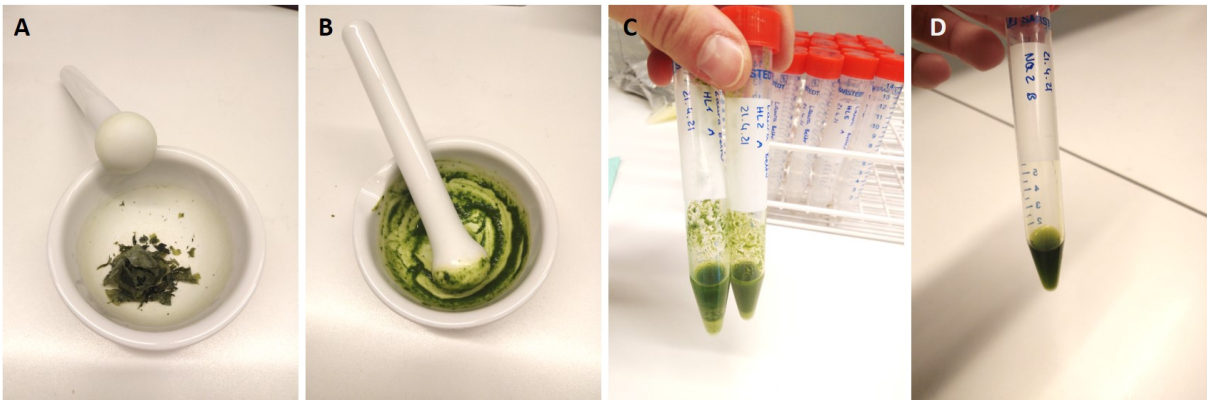

Figure 2.4 Sample preparation for the ABTS radical cation de-colourisation assay. **A)** dry algae and sand in mortar **B)** Algae are ground into paste with 70 % ethanol using pestle and mortar **C)** The mixture is transferred into a 15 *mL* centrifugation tube and incubated. **D)** The samples are centrifuged and supernatant is extracted and measured. (Photos: Laura S. Belter| Re et al., 1999; Torres et al., 2017)

each sample with the Infinite 200 micro-plate reader (Tecan Trading AG, Männedorf, Switzerland).

A Trolox standard curve (Equation 3.1 was created by measuring different dilutions of Trolox in EtOH (0-600 $\mu g/mL$, $R^2 > 0.95$). The measured sample absorption A_{734nm} was used to calculate AA as Trolox equivalent TE in $\mu g/mL$ (Equation 2.8).

$$y = a \cdot x + b \tag{2.7}$$

$$TE = \frac{A_{734nm} - b}{a} \tag{2.8}$$

2.4 Statistical Analysis

The previously described calculations were conducted in Microsoft Excel (Microsoft Corporation, Redmont, USA) and are linked in the Appendix. Statistical analysis was performed with R software (Version 4.0.5 (R Core Team, 2021)).

A linear regression was analysed for the Trolox standard curve and its formula used for further calculations.

Anova assumptions of each measured variable were assessed for the experiments. Distribution of the data was checked via QQ-plot and Shapiro-Wilk test. Extreme outliers were identified and removed from the analysed data set. Homoscedasticity, or equal variance between groups, was analysed using Levene's test. Descriptive statistics were calculated and are summarised for each variable in tables A.2-A.15 in the Appendix.

Repeated measures two-way Anova compared the effects of experiment duration and seawater treatment on the different variables in the first experiment. Regular two-way Anova was conducted for the vessel size experiment. One-way Anova was chosen for the antioxidant enhancement experiments, comparing the initial values with the treatments after 4 days.
A significant difference between groups was assumed, whenever the p-value was below 0.05. In cases where a significant influence of treatment or duration on the measured variable was detected, a post-hoc TukeyHSD test was conducted for pairwise comparison.
The results were displayed using boxplots with letters above the boxes indicating whether significant differences were detected.

3 Results

3.1 Cultivation conditions

3.1.1 Seawater Types

Total surface area

The total, photosynthetically active surface area *tSA* of *Umbraulva* sp. was significantly influenced by seawater type and experiment duration ($p=1.35 \cdot 10^{-9} < 0.05$). A significant increase in *tSA* can be observed until day 11 in both, natural and artificial seawater, reaching a maximum average area of $453.17 \pm 64.69\, cm^2$ and $304.15 \pm 59.71\, cm^2$ on day 14, respectively (Table A.2). *Umbraulva* sp. cultivated in NSW outgrew those in rASW seawater by day 7 and those in ASW by day 11 (Figure 3.1). TSA of thallus discs in rASW, however, did not stagnate like the other treatments, and reached a similar size as those in NSW on day 14.
Visual observations during the experiment revealed, that *Umbraulva* discs in NSW became very thin and fragile towards the end of the experiment. *Umbraulva* sp. grown in ASW and rASW on the other hand remained thicker and more robust.

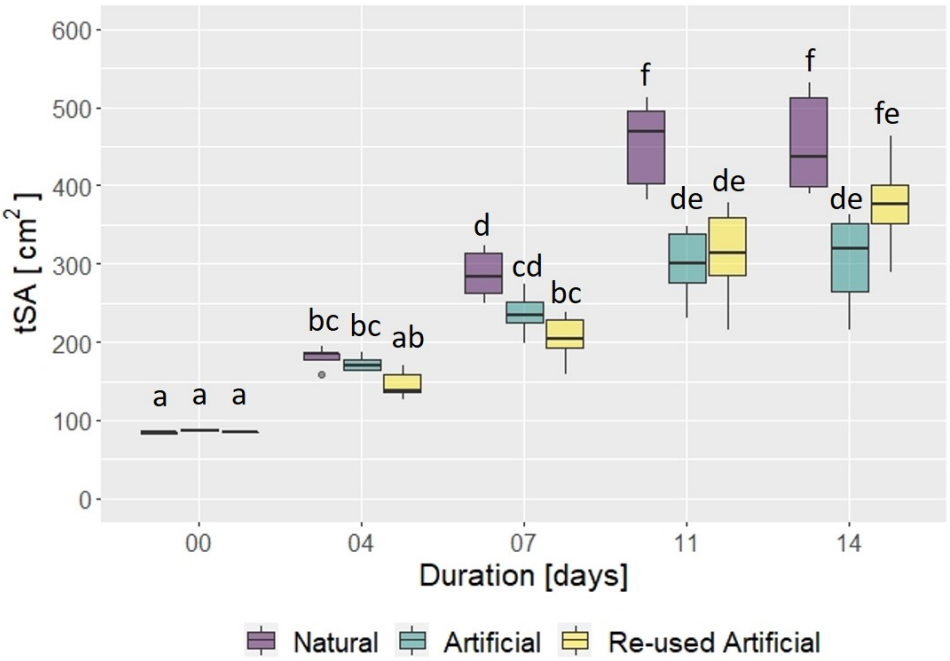

Figure 3.1 The effect of experiment duration and seawater treatment (natural, artificial or re-used artificial) on the total photosynthetically available surface area *tSA* is illustrated here. Letters above the boxes indicate the results of the Analysis of variance. Groups that are labeled with differing letter sequences are significantly different from each other.

Growth rate

Two variables for growth were calculated. Both, daily growth rate *dGR* (Table A.4) and relative growth rate *RGR* (Table A.3), were influenced by the seawater treatment and experiment duration. A decline in growth rate was measured in all treatments over time.
Algae grown in NSW started out with the highest growth rate of $24.04 \pm 2.82\, cm^2 \cdot d^{-1}$ after 4 days, which equals an increase of $18.98 \pm 1.5\% \cdot d^{-1}$. Growth rates of *Umbraulva* sp. approached 0 in NSW and ASW, while the decline was less steep for discs in rASW (Figure 3.2).

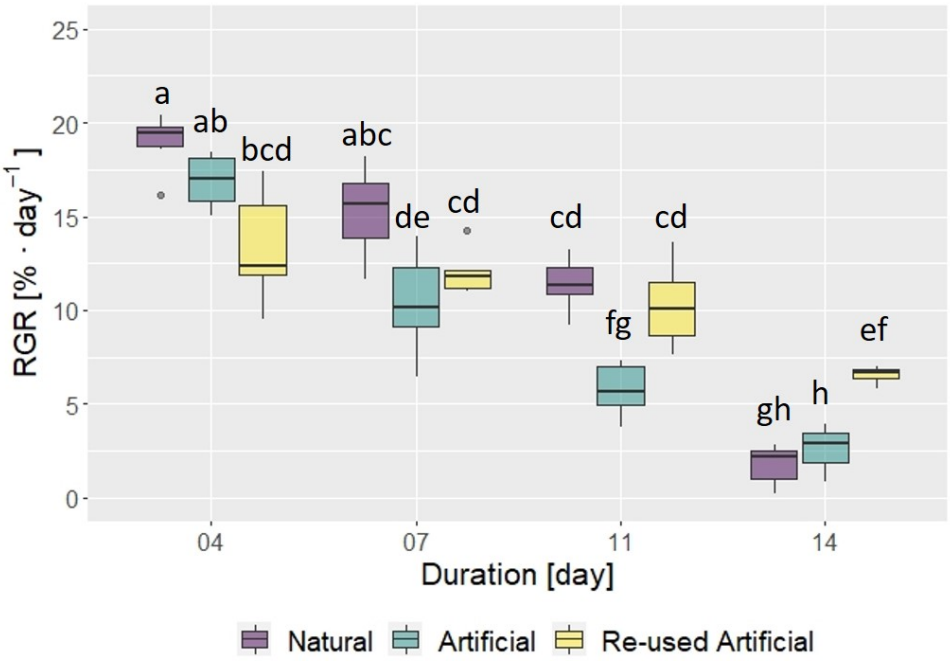

Figure 3.2 Relative growth rate RGR in % growth per day as observed during the seawater type experiment. Significant differences between days and treatments are indicated with letters. Groups that are labeled with differing letter sequences are significantly different from each other.

Stocking density

The stocking density SD_{SA} of *Umbraulva* sp. was significantly influenced by the seawater treatments and duratiion of the experiment. Algae grown in ASW and rASW showed no significantly different SD_{SA} from each other throughout the experiment (Figure 3.3). Algae in NSW experienced the highest stocking density of $3.2 \pm 0.4\, cm^2_{algae} \cdot cm^{-2}_{vessel}$ on days 11 and 14 (Table A.5). A similar stagnation of SD_{SA} after day 11 with values between 2.11-2.65 $cm^2_{algae} \cdot cm^{-2}_{vessel}$ was observed in ASW and rASW, as well.

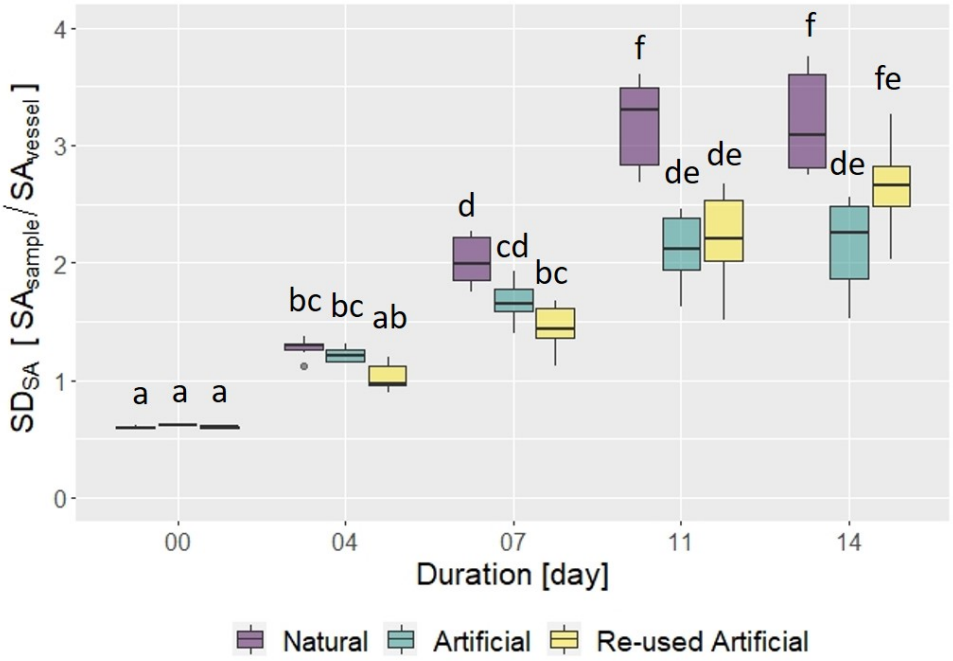

Figure 3.3 Development of *Umbraulva* sp. stocking density in natural, artificial and re-used artificial seawater over a period of 14 days. The values reflect the ratio of algae surface area SA_{algae} to the surface area of the cultivation vessel SA_{vessel}. Groups that are labeled with differing letter sequences are significantly different from each other.

Photosynthetic activity

No significant effect of experiment duration was detected. Photosynthetic activity did, however, vary significantly between treatments on day 7 (Figure 3.4), with *Umbraulva* sp. in rASW exhibiting an F_v/F_m of 0.647±0.04 compared to 0.688±0.05 and 0.68±0.056 in NSW and ASW, respectively (Table A.6). Although the difference is not significant, rASW still exhibited the lowest photosynthetic activity on days 0 and 14, as well. All measured values stayed within a range of 0.639±0.05 (rASW, day 14) to 0.688±0.05 (NSW, day 7).

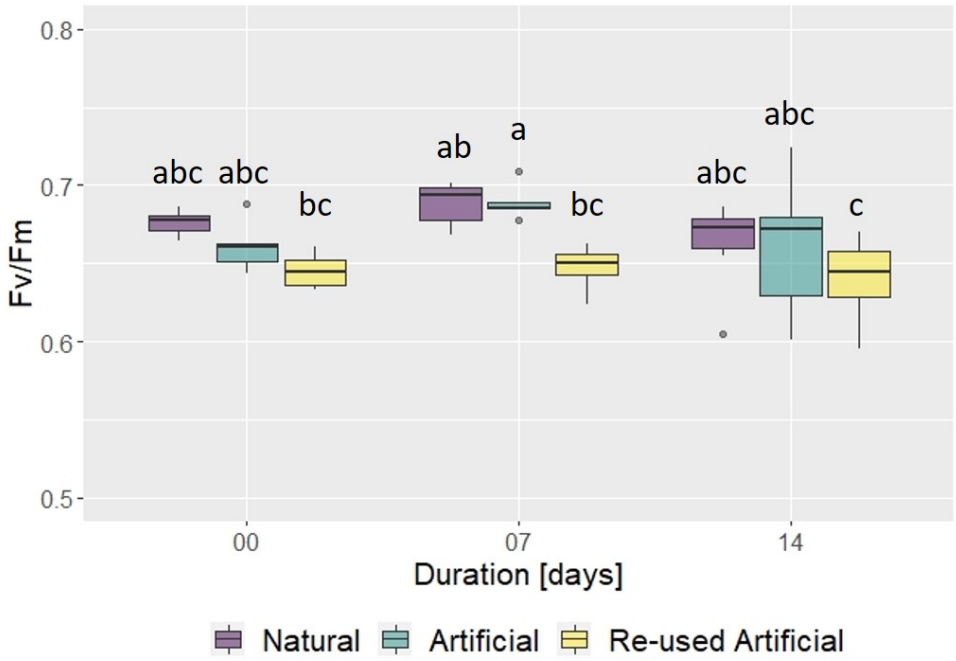

Figure 3.4 Photosynthetic activity was measured as F_v/F_m weekly and compared between seawater treatments. Groups that are labeled with differing letter sequences are significantly different from each other.

3.1.2 Vessel Size

Stocking density

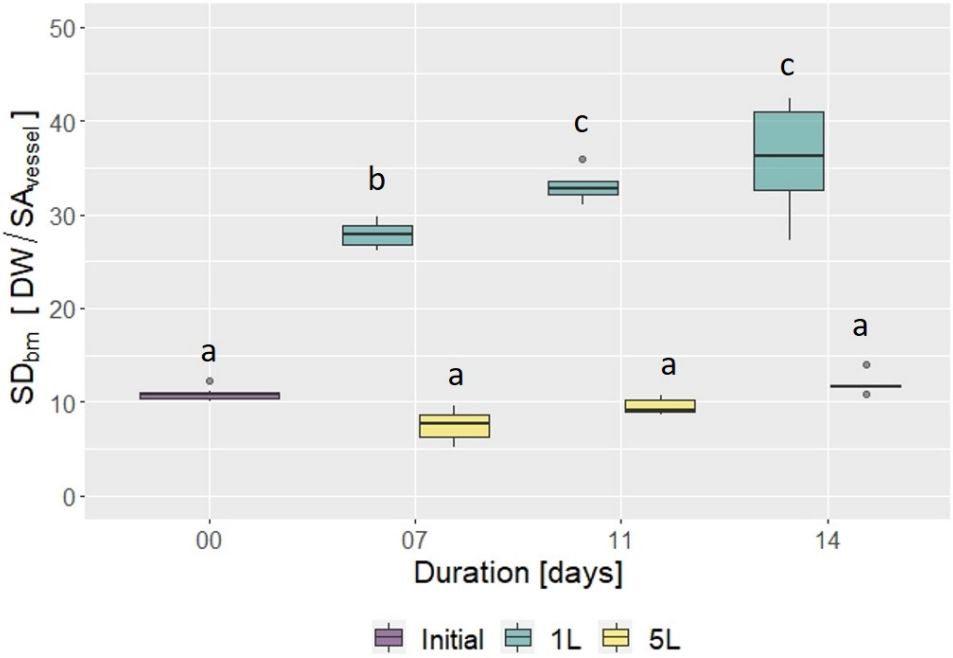

Figure 3.5 Development of *Umbraulva* sp. stocking density in natural, artificial and re-used artificial seawater over a period of 14 days. The values reflect the ratio of algae biomass in dry weight *DW* to the surface area of the cultivation vessel SA_{vessel}. Groups that are labeled with differing letter sequences are significantly different from each other.

The biomass stocking density SD_{bm} was the manipulated variable in this experiment. Figure 3.5 illustrates the significant difference between the 1 L and 5 L cultivation vessels. SD_{bm} remained stable around $10\,g \cdot cm^{-2}$, while it increased to a maximum of $36 \pm 5.96\,g \cdot cm^{-2}$ in the 1 L cultivation vessel on day 14 (Table A.11.

Total surface area

No significant effect of the vessel size on total surface area *tSA* was detected (Figure 3.6. *Umbraulva* sp. did, however, increase in size from initially $88.4 \pm 1.71\,cm^2$ to $476.15 \pm 51.21\,cm^2$ in 1 L and $409.4 \pm 57.44\,cm^2$ in 5 L cultivation vessels (Table A.7.

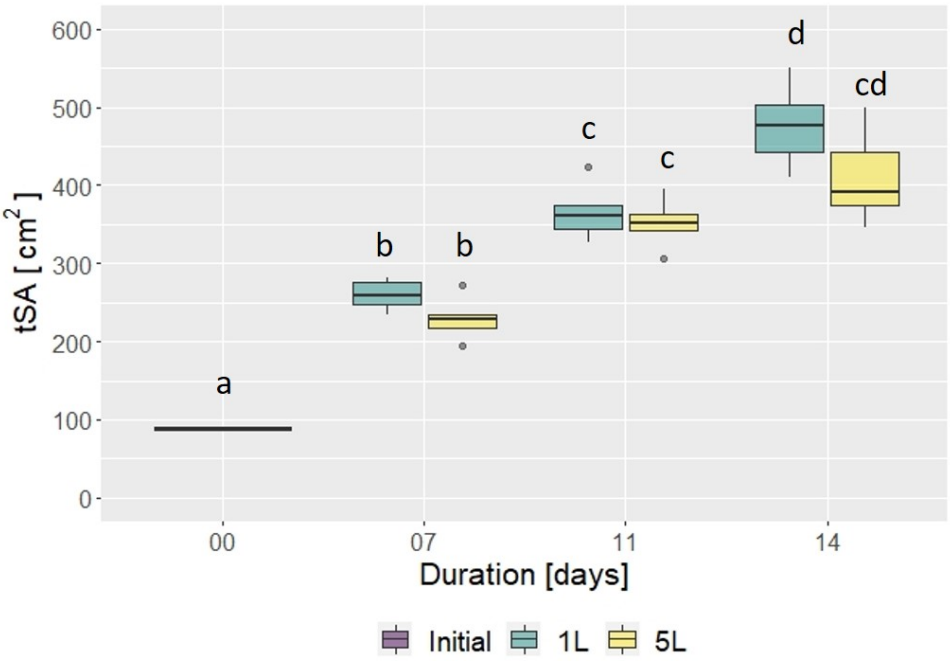

Figure 3.6 Shown is the development of total surface area *tSA* of *Umbraulva* sp. over 14 days in 1 L and 5 L cultivation vessels. Groups that are labeled with differing letter sequences are significantly different from each other.

Dry weight

Dry weight *DW* increased significantly throughout the experiment with no significant effect of vessel size on the outcome (Figure 3.7. *DW* increased from initially 0.08 ± 0.01 *g* to 0.26 ± 0.04 *g* and 0.27 ± 0.03 *g* in 1 L and 5 L vessels, respectively (Table A.8.

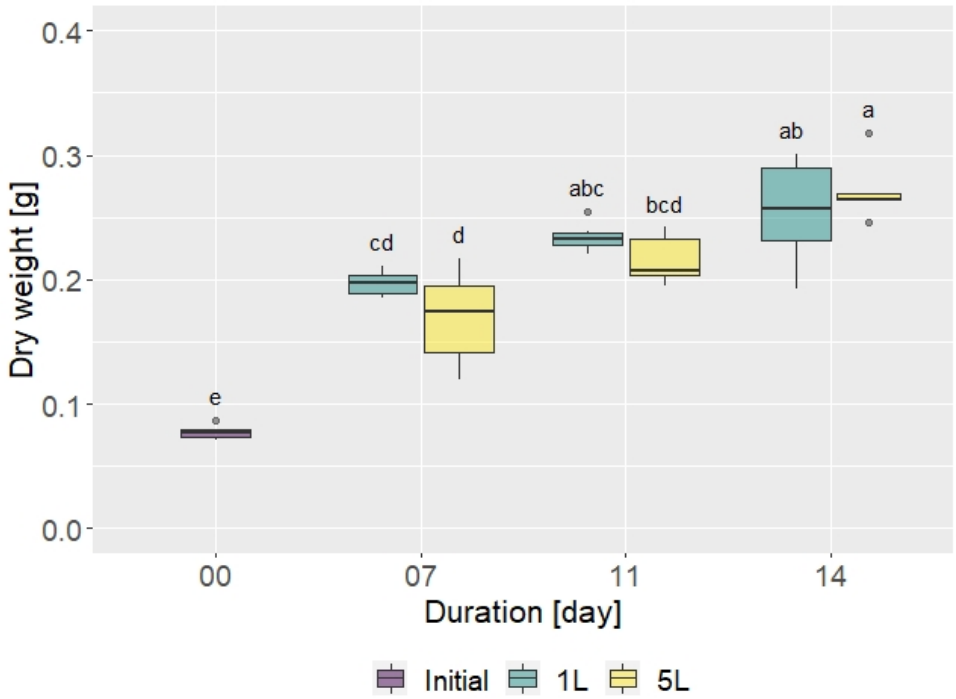

Figure 3.7 Development of dry weight *DW* of *Umbraulva* thalli throughout 14 days in 1 L and 5 L cultivation vessels. Groups that are labeled with differing letter sequences are significantly different from each other.

Growth rate

No significant differences over time or between treatments was recorded for the daily growth rate *dGR*, which ranged between $37.21 \pm 19.99\, cm^2 \cdot d^{-1}$ in 1 L on day 14 and $19.41 \pm 18.61 cm^2 \cdot d^{-1}$ in 5 L seawater on day 14 (Table A.10).

The relative growth rate declined throughout the experiment, but showed no significant reaction to the two treatments (Figure 3.8). *RGR* after 4 days measured at $15.35 \pm 0.93\, \% \cdot d^{-1}$ in the 1 L cultivation vesssels and $13.54 \pm 1.62\, \% \cdot d^{-1}$ in 5,L. It then declined towards $5\, \% \cdot d^{-1}$ on day 14 of the experiment.

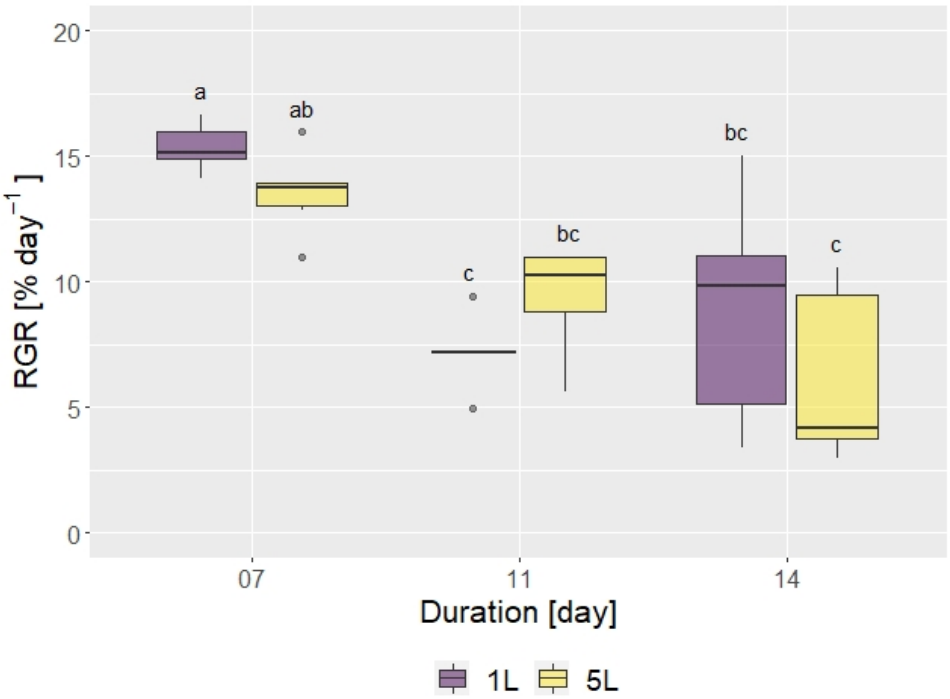

Figure 3.8 Relative growth rate RGR in % growth per day, as observed during the vessel size experiment. Significant differences between days and treatments are indicated with letters. Groups that are labeled with differing letter sequences are significantly different from each other.

3.2 Antioxidant enhancement

3.2.1 Trolox Standard Curve

The Trolox standard curve was initially measured on Trolox concentrations ranging from 0-600$\mu g \cdot mL^{-1}$ (Figure A.1). An analysis of variance between the concentrations revealed, that the ABTS$^{\bullet+}$ de-colourisation assay is only reliable for samples with a TE of 5-100$\mu g \cdot mL$ (Figure 3.9).

This range was ultimately used to create the calibration curve. The formula of the linear regression model (Equation **??**) was used to calculate the Trolox equivalent concentrations of samples later on (Tables A.13, A.15).

$$y = -0.0071821x + 0.7210714, R^2 = 0.97 \tag{3.1}$$

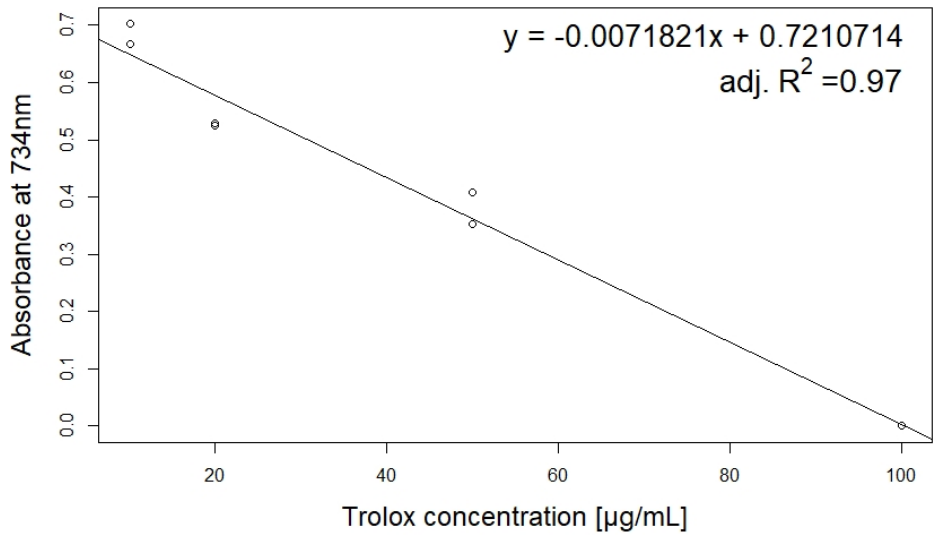

Figure 3.9 Trolox standard calibration curve with a linear regression.

3.2.2 Light quality

Photosynthetic activity

Umbraulva sp. started out with an F_v/F_m of 0.719±0.025 (Table A.12). No significant difference of F_v/F_m was detected between the algae grown under blue or white light for four days (Figure 3.10. The values were, however, significantly lower that the initial one, with 0.65±0.065 under blue light and 0.67±0.046 under white light.

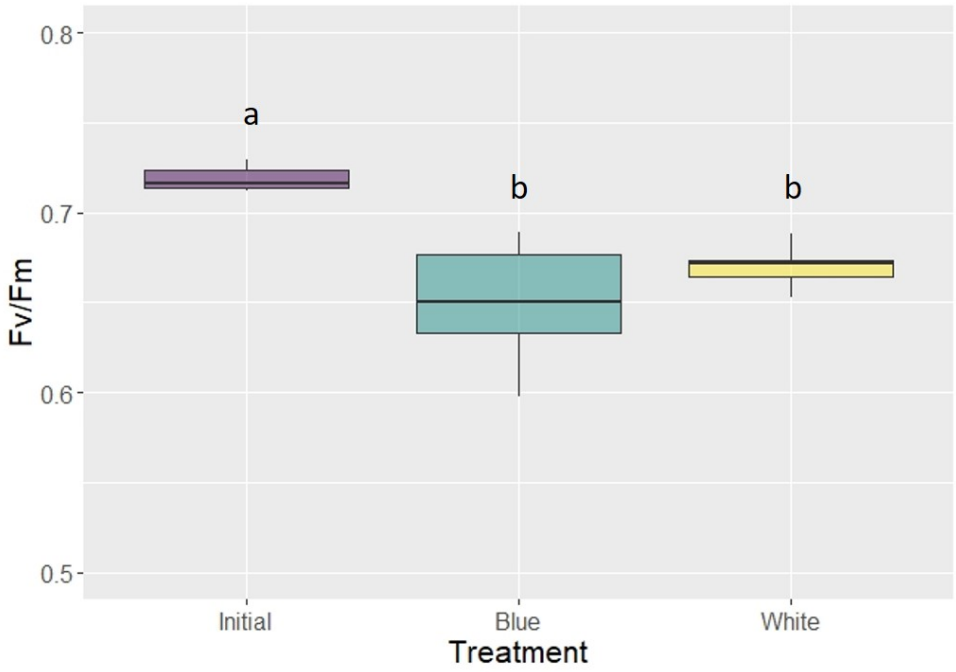

Figure 3.10 Photosynthetic activity measured in F_v/F_m and compared between initial values and those measured after 4 days under blue or white light treatments. Treatments that are labeled with differing letter sequences are significantly different from each other.

Antioxidant activity

The antioxidant activity *AA* of *Umbraulva* sp. starts out at 41.67±5.7 $\mu g \cdot mL^{-1}$ TE (Table A.13. It stays at a similar level after 4 days under blue light, as illustrated in Figure 3.11. Specimen that were subjected to white light for 4 days presented a significantly higher *AA* than the other two treatments with 57.73±4.67 $\mu g \cdot mL^{-1}$ TE.

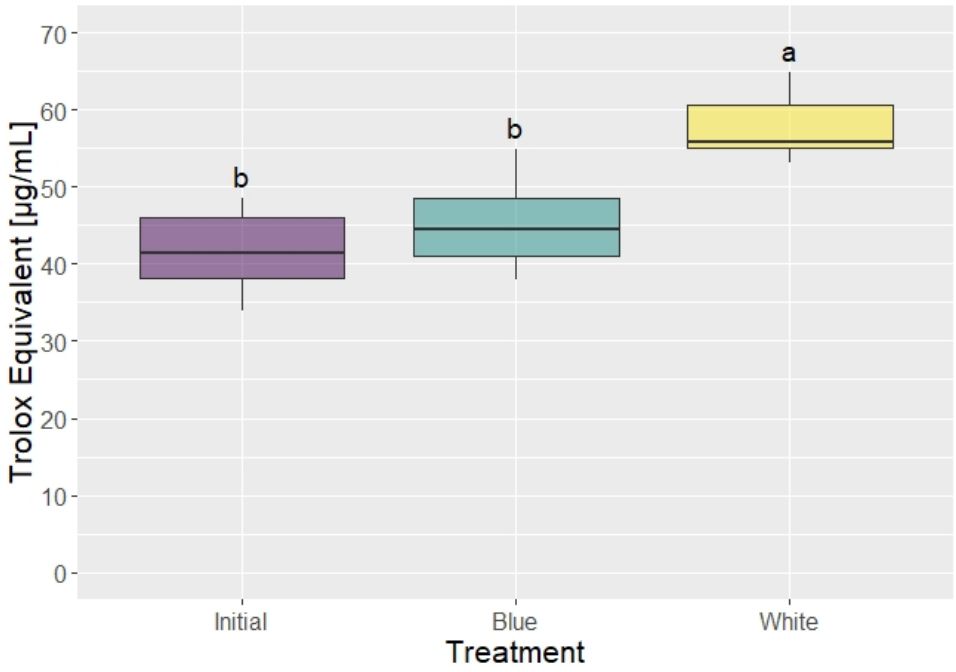

Figure 3.11 Antioxidant activity of *Umbraulva* sp. grown under blue and white light for four days. The activity is measured as Trolox equivalents (TE) in $\mu g \cdot mL^{-1}$. Treatments that are labeled with differing letter sequences are significantly different from each other.

3.2.3 Light quantity

Photosynthetic and antioxidant activity of *Umbraulva* sp. were measured initially and after 4 days under three different treatments. Treatment one (90:5.18) represents standard cultivation conditions with PPFD of 90 $\mu mol photons \cdot m^{-2} \cdot s^{-1}$ and DLI of 5.18 $mol photons \cdot m^{-2} \cdot d^{-1}$. Treatments two (240:5:18) subjected the algae to a higher PPFD of 240 $\mu mol photons \cdot m^{-2} \cdot s^{-1}$ while keeping DLI at 5.18 $mol photons \cdot m^{-2} \cdot d^{-1}$ through a reduction of day-length from 16 h to 6 h. The last treatment (240:13.82) used the high PPFD combined with the longer day-length and resulted in an overall higher DLI of 13.82 $mol photons \cdot m^{-2} \cdot d^{-1}$.

Photosynthetic activity

The initial and standard cultivation (90:5.18) treatments resulted in an F_v/F_m of approximately 0.56±0.06 (Table A.14). After four days under a higher PPFD, F_v/F_m was decreased significantly below 0.5 with 0.456±0.096 in the 240:5.18 treatment and a significantly lower 0.413±0.087 in the 240:13.82 treatment (Figure 3.12.

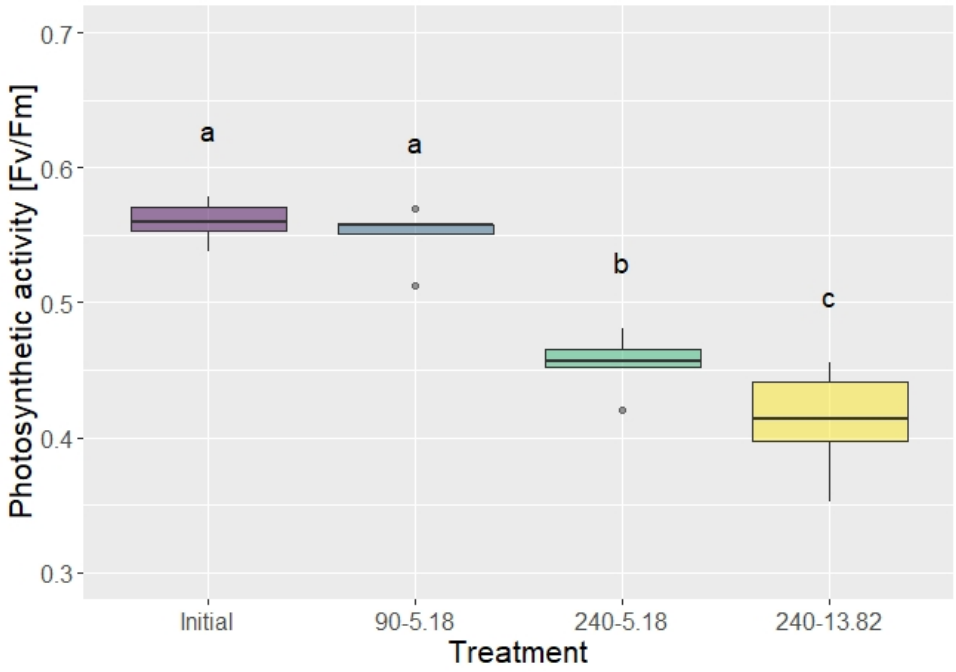

Figure 3.12 Photosynthetic activity was measured in F_v/F_m and compared between initial values and three treatments. Treatment names represent the light settings PPFD:DLI in $\mu mol photons \cdot m^{-2} \cdot s^{-1}$ and $mol photons \cdot m^{-2} \cdot d^{-1}$, respectively. Treatments that are labeled with differing letter sequences are significantly different from each other.

Antioxidant activity

No significant difference in antioxidant activity *AA* was measured between the light quantity treatments, as illustrated in Figure 3.13. Values ranged between 42.46±7.14 $\mu g \cdot mL^{-1}$ TE in treatment 240:13.82 and 47.96±6.28 $\mu g \cdot mL^{-1}$ TE in the 90:5.18 treatment (Table A.15.

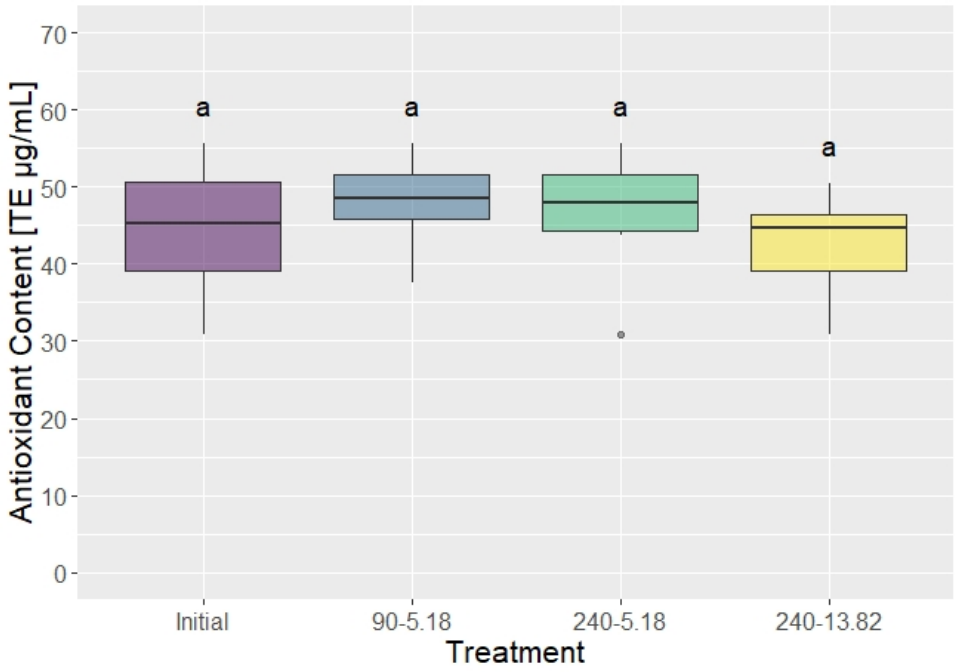

Figure 3.13 Antioxidant activity was measured in $\mu g \cdot mL^{-1}$ Trolox equivalent (TE) and compared between initial values and three treatments. Treatment names represent the light settings PPFD:DLI in $\mu mol photons \cdot m^{-2} \cdot s^{-1}$ and $mol photons \cdot m^{-2} \cdot d^{-1}$, respectively. Treatments that are labeled with differing letter sequences are significantly different from each other.

4 Discussion

Before proceeding to the in-depth discussion of the presented results, a closer look needs to be taken at how they were obtained so that appropriate interpretation can be made possible.
One circumstance that was considered in the design of these experiments was the intended application in industrial scale seaweed cultivation. Some of these experiments were not designed to give provide exact, physiological data of *Umbraulva* sp. They reflect processing practices and are aimed to provide information about the final product instead of the raw material. In the case of antioxidant activity measurements, a drying process was chosen, that does not permit optimal antioxidant retention within the algal material (Amorim et al., 2020). It does, however, present a method that can be applied easily and cheaply in a variety of processing facilities.
Umbraulva is furthermore a relatively little studied genus, compared to its sister genera *Ulva*, *Percursaria* and *Ulvaria* (Wynne and Furnari, 2014; Steinhagen et al., 2019; Kawai et al., 2021). And although it is similar enough to these genera to make presumptions about its characteristics and physiological behaviour under certain conditions, studying it in the laboratory requires a certain degree of trial and error. Most indicative of this is the fact, that culturing conditions, and with it the standard cultivation treatments had to be adjusted between experiments. Due to these changes and additional changes in sampling and measurement schedules, it was not always possible to make comparisons across experiments.

4.1 Cultivation conditions

Two factors of algal cultivation were studied for the identification of suitable long-term culturing conditions. Effects on various dependent variables were measured over the course of 2 weeks.
Filtered natural seawater (NSW), artificial seawater (ASW) and re-used artificial seawater (rASW) had slightly different effects on *Umbraulva* growth. Algae grown in NSW produced the largest thalli, while those in rASW exhibited a more stable growth rate. One possible explanation is, that a stocking density *SD* was reached in NSW and ASW after 11 days, which caused a limitation in space, nutrients or light availability through self-shading (Floreto et al., 1993; Oca et al.,

2019). That *SD* threshold would have been reached at approximately 2.1 $cm^2 \cdot cm^2_{vessel}$ in ASW and at 3.2 $cm^2 \cdot cm^2_{vessel}$ in NSW.
The fact, that rASW ranged between these values and continued to grow at a steady rate indicate, that stocking density and space were not the limiting factor.
Nutrient availability, and more specifically ammonium availability (Floreto et al., 1993; Angelidaki et al., 2011), is more likely to have influenced *Umbraulva* sp. growth. Nutrient addition was increased for the vessel size experiment and the stagnation in surface area *SA* in NSW was not reproduced. A nutrient analysis of NSW under experimental conditions indicated, that nutrient depletion was unlikely with this adjusted setting (see Section A.1).
Initial growth rates ranged between 15-20 % growth per day in the seawater type experiment and around 15 % growth per day in the vessel size experiment. These values are well within the range of 12-24 % increase per day, measured in other Ulvaceae (Fortes and Lüning, 1980; Le et al., 2018).
The decrease in growth rate, both daily and relative, was observed in both cultivation experiments, although a stagnation of *tSA* was not apparent in the vessel size experiment. It was observed for *Ulva ohnoi* that an *SD* threshold of 187 $g_{dw} \cdot cm^{-2}_{vessel}$ needs to be surpassed in order to optimise the growth rate (Oca et al., 2019). *Umbraulva* sp. experienced a *SD* of 36 $g_{dw} \cdot cm^{-2}_{vessel}$ in the 1 L cultivation vessels on day 14 and lies well below the *U. ohnoi* threshold. This higher *SD* causes enough self-shading for the algae to protection themselves against excessive light damage. *Umbraulva* sp., a more subtidal, low light adapted species (Wynne and Furnari, 2014), would presumably benefit from a similarly high *SD*. To date, there is unfortunately no comparable data available for this genus.
A good indicator of occurring light stress in plants and algae is the photosynthetic activity (Büchel and Wilhelm, 1993; Maxwell and Johnson, 2000). Green algae are considered to be healthy and perform photosynthesis efficiently at F_v/F_m levels between 0.5-0.8 (Higo et al., 2017). The photosynthetic activity of *Umbraulva* sp. remained within that range during both cultivation condition experiments. It can therefore be assumed that no excessive light stress or damage occurred during those 14 days.

4.2 Antioxidant enhancement

It was the aim of these two experiments to cause enough light stress on *Umbraulva* sp. for it to induce an elevated antioxidant production. Such conditions prevent optimal growth and are therefore only supposed to be applied within 7 days prior to harvesting (Tretiak et al., 2021). The experiments were initially designed to run for 7 days. This was reduced to a duration of 4 days, after a temporary malfunction of the climate-controlled cultivation room and subsequent

temperature rise.
Blue light quality has proven to induce higher antioxidant activity in several *Ulva* spp. (Le et al., 2018; Schwoerbel, 2019). A contrary result was achieved in *Umbraulva* sp. Algae grown under blue light exhibiting levels of *AA* around 40 $\mu g \cdot mL^{-1}$, similar to the initially measured values. Those under white light did, however, exhibit an increase in *AA* after 4 days.
This reversed result can be traced back to the natural distribution of species belonging to *Ulva* and *Umbraulva*. *Ulva* spp. inhabit intertidal habitats and often float below the water surface. They are therefore adapted white light at high intensities. *Umbraulva* spp. on the other hand are subtidal and live in habitats where blue and green light is more abundant than longer wavelengths (Kageyama et al., 1977; Maggs et al., 2007). Adaptation to these conditions would make them more susceptible to light stress from full-spectrum light. It is unlikely that the standard cultivation white light induced severe light stress, as levels of F_v/F_m remained between 0.5 and 0.8 throughout the entire experiment.
The assumption, that *Umbraulva* sp. would experience light stress when cultivated under high PPFD and high DLI, in the light quantity experiment. Both, the higher light intensity of 240 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the higher daily light integer of 13.82 $mol \cdot m^{-2} \cdot d^{-1}$ caused a severe decline of F_v/F_m below 0.5. It can be assumed, that photosynthesis is not being performed efficiently and cell damage has occurred at these levels (Büchel and Wilhelm, 1993; Higo et al., 2017).
Unfortunately, no increased *AA* was measured as a result of this light stress after 4 days of the experiment. It is possible, that a production of antioxidants was induced in a shorter time frame than 4 days. Another possibility is, that the chosen ABTS $^{\bullet+}$ de-colourisation assay was not the appropriate method to capture the entire response of *Umbraulva* sp. There are a variety of antioxidant quantification methods available, that exhibit a wide range of sensibilities to different types of antioxidants (Raja et al., 2016; Benítez-García et al., 2020).
It is therefore not clear, whether the applied light settings did not induce an increased *AA* or whether such an *AA* was simply not measured with the chosen methods.

5 Conclusion

This thesis intended to answer a series of questions regarding the long-term cultivation conditions and the enhancement of antioxidant activity in *Umbraulva* sp.

> Hypothesis 1: The natural seawater is most likely to promote optimal growth in *Umbraulva* sp., followed by the re-used artificial seawater, as these conditions resemble the natural environment more closely.
>
> Hypothesis 2: It is expected that *Umbraulva* sp. grows better in a smaller cultivation vessel that permits self-shading.
>
> Hypothesis 3: It is expected that an application of blue light will lead to an increased activity of antioxidants, similar to that measured in *Ulva* spp.
>
> Hypothesis 4: Both, increased PPFD and increased PPFD plus increased DLI are expected to cause light stress in *Umbraulva* sp. which will most likely lead to a measurable increase in antioxidant activity.

The comparison of natural, artificial and re-used artificial seawater as cultivation media support Hypothesis 1. The largest surface area was achieved in natural seawater, while the most stable growth rates were observed in re-used artificial seawater. Both seawater types represent a more natural composition with not only nutrients and trace elements, but also living microorganisms. It is almost impossible to imitate such a complex system with artificial seawater.
The algae were already acclimatised to the re-used artificial seawater, making it a suitable environment to grow in. The natural seawater on the other hand originated from Heligoland, same as the *Umbraulva* sp. sampled from the 'Nordsee Aquarium'.
Natural seawater does have one drawback, and that is a lack of consistency. The natural environment experiences fluctuations in salinity, temperature and composition, as well as pollution. The use of artificial seawater in land-based aquaculture systems is therefore more reliable and safer for the production of food products (Tretiak et al., 2021). The experiment demonstrated, that the integration of artificial water from aquaria or fish aquaculture could be beneficial to the *Umbraulva* sp. cultivation. These findings can be communicated with and applied by ROVAL

GbR.
A factor that could not be defined in its entirety was the optimal stocking density. The exact limits of space availability, self-shading and nutrient uptake need to be identified in order to guarantee optimal and efficient *Umbraulva* sp. growth and biomass production. Hypothesis 2 was not be confirmed with the applied experiment and measurements. The analysed vessel size experiment did not provide those answers and further research in this area is recommended, with a focus on longer growth periods, weight-based stocking density measurements and a continuous analysis of nutrient uptake rates.

Both attempts to induce an increase in antioxidant activity through light quality and light quantity variations were unsuccessful. A test of blue against white light confirmed, that *Umbraulva* sp. is better adapted to subtidal environments than its more surface-dwelling relatives in the *Ulva* genus. Hypothesis 3 is therefore not supported by the collected data. It is recommended, to test other light qualities on *Umbraulva* sp., preferably longer wave-length such as red or far-red light that are more common in the upper water column.
The application of high light intensity and high daily photo-dose did result in a reduction of photosynthetic activity, an indicator of occurring light stress. No increased antioxidant activity was related to this development. This does not mean that no increased antioxidant activity occurred. The experiment should be repeated with shorter measurement periods and the application of a combination of antioxidant quantifying methods to test Hypothesis 4 with more conclusive results.

Overall, it can be concluded, that *Umbraulva* sp. is a promising resource. It was able to maintain cultivation over a long time period in the laboratory and measured growth rates were comparable to *Ulva* spp. that are already being applied in the aquaculture and food sector. The suitability for *Umbraulva* sp. as a raw material for the 'Mak-Pak' food packaging needs to be assessed by 'Hochschule Bremerhaven' and the commercial project partners further down the line of production.
But truth be told, a lot more research into the physiology of *Umbraulva* sp. needs to happen before an application of the algae in industrial scale setups becomes realistic. Thresholds and optimum levels of environmental factors, including light, temperature, salinity and nutrient availability need to be assessed. And while this thesis attempted to shed light on some factors separately, it would be more beneficial to assess them as a system and on the larger industrial scale, as well.

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Appendix

A.1 Nutrient Concentration

A small experiment was conducted in order to assess, whether the nutrients were depleted during the experiment. Three replicates of 1 L glass beakers were filled with filtered NSW and 10 *Umbraulva* sp. thallus discs. $100\mu L$ of Blaukorn garden fertiliser were added to each replicate. 10 mL of seawater were sampled at the start and after 1, 2, 4, 24 and 48 hours. The nutrient levels of phosphate P, ammonium NH_4, nitrite NO_2 and nitrate NO_3^- were measured in duplicate by Henrike Hohnholz at AWI.

Mean values of these measurements (Table A.1) show, that phosphate, nitrite and nitrate concentrations oscillated around stable levels. Ammonium was the only nutrient that was reduced in concentration throughout measurement period. This preferential uptake of ammonium over other nitrogen sources is in line with conclusions from other studies (Floreto et al., 1993; Angelidaki et al., 2011). It can be assumed, that no nutrient limitation occurred within the four days between nutrient supplementation, as the decline in ammonium concentration slowed over time.

Table A.1 Concentrations of phosphate, ammonium, nitrite and nitrate in 1 L of filtered natural seawater with 10 *Umbraulva* sp. thallus discs. Samples were taken initially and then after 1, 2, 4, 24 and 48 hours. Each sample was analysed twice and a mean value, standard deviation (sd) and standard error (se) calculated.

Nutrient	**Duration** $[h]$	**Replicates**	c_{mean} $[mg/mL]$	**sd** $[mg/mL]$	**se** $[mg/mL]$
Phosphate (P)	0	6	13	2.7	1.1
	1	6	15.4	0.53	0.22
	2	6	15.05	0.96	0.39
	4	6	13.16	2.28	0.93
	24	6	15.83	1.14	0.46
	48	6	13.81	2.88	1.18
Ammonium (NH_4)	0	6	7.61	1.44	0.59
	1	6	7.99	0.29	0.12
	2	6	8.02	0.18	0.08
	4	6	6.84	1.35	0.55
	24	6	6.25	0.63	0.26
	48	6	4.42	0.5	0.2
Nitrite (NO_2)	0	6	0.01	0	0
	1	6	0.02	0	0
	2	6	0.01	0.01	0
	4	6	0.01	0.01	0
	24	6	0.02	0	0
	48	6	0.02	0	0
Nitrate (NO_3^-)	0	6	9.44	3.53	1.44
	1	6	10.68	1.05	0.43
	2	6	11.29	0.79	0.32
	4	6	9.14	4.09	1.67
	24	6	12.63	1.96	0.8
	48	6	8.93	3.58	1.46

A.2 Summary Tables

Seawater type experiment

Table A.2 Mean values, standard deviation (sd) and standard error (se) of the total surface area (tSA) in cm^2, grouped by day and seawater treatment.

Day	Treatment	N	tSA_{mean}	sd	se
0	Natural	6	84.31	1.27	0.52
0	Artificial	6	87.65	1.51	0.62
0	Re-used Artificial	6	84.84	0.72	0.29
4	Natural	6	180.48	12.1	4.94
4	Artificial	6	172.38	9.21	3.76
4	Re-used Artificial	6	145.56	17.63	7.2
7	Natural	6	286.24	31.83	12.99
7	Artificial	6	236.54	26.22	10.71
7	Re-used Artificial	6	205.24	29.83	12.18
11	Natural	6	452.38	57.07	23.3
11	Artificial	6	299.35	46.15	18.84
11	Re-used Artificial	6	311.77	61.02	24.91
14	Natural	6	453.17	64.69	26.41
14	Artificial	6	304.15	59.71	24.38
14	Re-used Artificial	6	376.19	58.87	24.03

Table A.3 Mean values, standard deviation (sd) and standard error (se) of the relative growth rate (RGR) in % per day, grouped by day and seawater treatment.

Day	Treatment	N	RGR_{mean}	sd	se
4	Natural	6	18.98	1.5	0.61
4	Artificial	6	16.88	1.42	0.58
4	Re-used Artificial	6	13.35	3.05	1.25
7	Natural	6	15.27	2.39	0.97
7	Artificial	6	10.42	2.71	1.11
7	Re-used Artificial	5	12.07	1.31	0.58
11	Natural	6	11.4	1.4	0.57
11	Artificial	6	5.76	1.41	0.58
11	Re-used Artificial	6	10.25	2.27	0.93
14	Natural	5	1.76	1.09	0.49
14	Artificial	6	0.3	2.81	1.15
14	Re-used Artificial	4	6.54	0.51	0.26

Table A.4 Mean values, standard deviation (sd) and standard error (se) of the daily growth rate (dGR) in cm^2 per day, grouped by day and seawater treatment.

Day	Treatment	N	dGR_{mean}	sd	se
4	Natural	6	24.04	2.82	1.15
4	Artificial	6	21.18	2.35	0.96
4	Re-used Artificial	6	15.18	4.47	1.83
7	Natural	6	35.25	7.88	3.22
7	Artificial	6	21.39	6.79	2.77
7	Re-used Artificial	6	19.89	5.04	2.06
11	Natural	6	41.53	7.76	3.17
11	Artificial	6	15.7	5.41	2.21
11	Re-used Artificial	6	26.63	8.9	3.63
14	Natural	5	8.2	5.15	2.3
14	Artificial	6	1.6	8.28	3.38
14	Re-used Artificial	5	23.5	3.03	1.35

Table A.5 Mean values, standard deviation (sd) and standard error (se) of the stocking density (SD) as ratio between surface are of the cultivation vessel and *Umbraulva* sp. surface area, grouped by day and seawater treatment.

Day	Treatment	N	SD_{mean}	sd	se
0	Natural	6	0.6	0.01	0
0	Artificial	6	0.62	0.01	0
0	Re-used Artificial	6	0.6	0.01	0
4	Natural	6	1.27	0.09	0.03
4	Artificial	6	1.21	0.07	0.03
4	Re-used Artificial	6	1.03	0.12	0.05
7	Natural	6	2.02	0.23	0.09
7	Artificial	6	1.67	0.18	0.08
7	Re-used Artificial	6	1.45	0.21	0.09
11	Natural	6	3.19	0.4	0.16
11	Artificial	6	2.11	0.32	0.13
11	Re-used Artificial	6	2.2	0.43	0.18
14	Natural	6	3.2	0.46	0.19
14	Artificial	6	2.15	0.42	0.17
14	Re-used Artificial	6	2.65	0.42	0.17

Table A.6 Mean values, standard deviation (sd) and standard error (se) of the photosynthetic activity in F_v/F_m, grouped by day and seawater treatment.

Day	Treatment	N	Fv/Fm_{mean}	sd	se
0	Natural	60	0.676	0.03	0
0	Artificial	60	0.661	0.04	0.01
0	Re-used Artificial	60	0.645	0.04	0
7	Natural	60	0.688	0.05	0.01
7	Artificial	60	0.68	0.06	0.01
7	Re-used Artificial	60	0.647	0.04	0
14	Natural	60	0.662	0.07	0.01
14	Artificial	60	0.664	0.07	0.01
14	Re-used Artificial	60	0.639	0.05	0.01

Vessel size experiment

Table A.7 Mean values, standard deviation (sd) and standard error (se) of the total surface area (tSA) in cm^2, grouped by day and vessel size treatment.

Day	Treatment	N	tSA_{mean}	sd	se
0	Initial	6	88.4	1.71	0.7
7	1L	6	259.49	18.94	7.73
7	5L	6	229.2	25.73	10.5
11	1L	6	364.52	33.88	13.83
11	5L	6	351.18	29.52	12.05
14	1L	6	476.15	51.21	20.91
14	5L	6	409.4	57.44	23.45

Table A.8 Mean values, standard deviation (sd) and standard error (se) of dry weight *DW* in *g*, grouped by day and vessel size treatment.

Day	Treatment	N	DW_{mean}	sd	se
0	Initial	6	0.08	0.01	0.00
7	1L	6	0.20	0.01	0.00
7	5L	6	0.17	0.04	0.02
11	1L	6	0.23	0.01	0.00
11	5L	6	0.22	0.02	0.01
14	1L	6	0.26	0.04	0.02
14	5L	5	0.27	0.03	0.01

Table A.9 Mean values, standard deviation (sd) and standard error (se) of the relative growth rate (RGR) in % per day, grouped by day and vessel size treatment.

Day	Treatment	N	RGR_{mean}	sd	se
7	1L	6	15.35	0.93	0.38
7	5L	6	13.54	1.62	0.66
11	1L	5	7.2	1.59	0.71
11	5L	5	9.33	2.27	1.02
14	1L	6	8.86	4.52	1.84
14	5L	6	4.95	4.4	1.8

Table A.10 Mean values, standard deviation (sd) and standard error (se) of the daily growth rate (dGR) in cm^2 growth per day, grouped by day and vessel size treatment.

Day	Treatment	N	dGR_{mean}	sd	se
7	1L	6	24.44	2.59	1.06
7	5L	6	20.11	3.69	1.51
11	1L	6	26.25	11.2	4.57
11	5L	6	30.5	11.23	4.59
14	1L	6	37.21	19.99	8.16
14	5L	6	19.41	18.61	7.6

Table A.11 Mean values, standard deviation (sd) and standard error (se) of the stocking density (SD) in biomass algae per vessel surface area ($g_{dryweight} \cdot cm^{-2}$), grouped by day and seawater treatment.

Day	Treatment	N	SD_{mean}	sd	se
0	Initial	6	10.87	0.78	0.32
7	1L	6	27.84	1.39	0.57
7	5L	6	7.46	1.68	0.68
11	1L	6	33.03	1.7	0.69
11	5L	6	9.52	0.89	0.36
14	1L	6	36	5.96	2.43
14	5L	5	11.98	1.19	0.53

Trolox standard curve

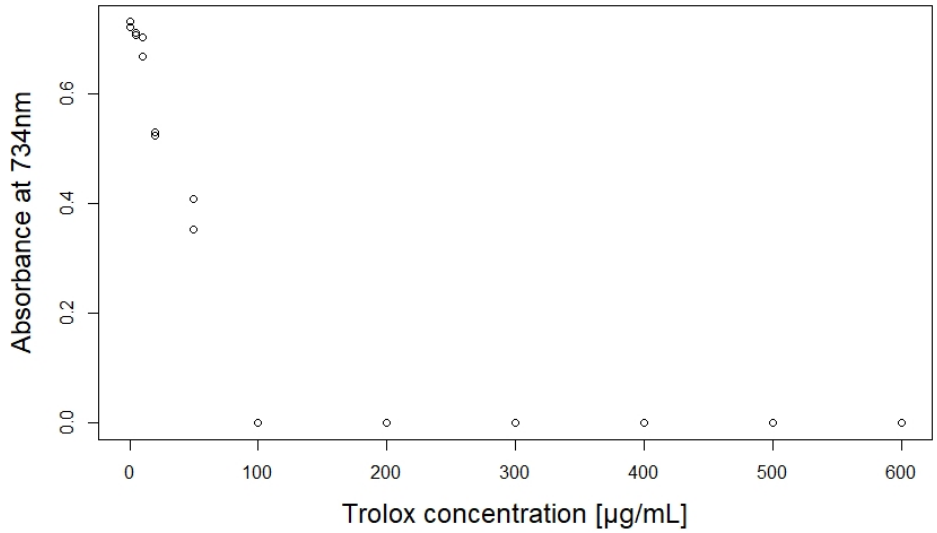

Figure A.1 Absorption measurements of Trolox concentrations ranging from 0-600 $\mu g \cdot mL^{-1}$.

Light quality experiment

Table A.12 Mean values, standard deviation (sd) and standard error (se) of the photosynthetic activity in F_v/F_m, grouped by light quality treatment.Blue and white light measurements were taken after 4 days under experimental conditions.

Treatment	**N**	F_v/F_m	**sd**	**se**
Initial	90	0.719	0.025	0.003
Blue	90	0.65	0.065	0.007
White	90	0.67	0.046	0.005

Table A.13 Mean values, standard deviation (sd) and standard error (se) of the antioxidant activity measured as Trolox equivalent (TE). Measurements were grouped by Light quality treatment, with the blue and white light treatments being measured after 4 days.

Treatment	**N**	$TE[\mu g/mL]$	**sd**	**se**
Initial	6	41.67	5.7	2.33
Blue	6	45.28	6.21	2.54
White	6	57.73	4.67	1.91

Light quantity experiment

Table A.14 Mean values, standard deviation (sd) and standard error (se) of the photosynthetic activity in F_v/F_m, grouped by light quantity treatment. 90:5.18, 240:5.18 and 240:13.82 refer to PPFD:DLI applied during the experiment and were measured after 4 days.

Treatment	N	F_v/F_m	sd	se
Initial	90	0.56	0.068	0.007
90-5.18	90	0.562	0.065	0.007
240-5.18	90	0.456	0.096	0.01
240-13.82	90	0.413	0.087	0.009

Table A.15 Mean values, standard deviation (sd) and standard error (se) of the antioxidant activity measured as Trolox equivalent (TE). 90:5.18, 240:5.18 and 240:13.82 refer to PPFD:DLI applied during the experiment and were measured after 4 days.

Treatment	N	$TE[\mu g/mL]$	sd	se
Initial	6	44.37	9.18	3.75
90-5.18	6	47.96	6.28	2.56
240-5.18	6	46.34	8.76	3.58
240-13.82	6	42.46	7.14	2.91

A.3 Raw Data

The collected raw data can be accessed through this link:
https://drive.google.com/file/d/1Ns8phlR0yQ72N_y3fZchN_WhPxjUxwmk/view?usp=sharing
No data can be taken out of this work without prior approval of the thesis supervisors.

A.4 Statement of authorship

I, Laura Sophie Belter, hereby assure that I have written the presented thesis independently and have not used any sources or tools other than those indicated.
All passages that have been taken literally or analogously from other works, are indicated as such, stating the sources.

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