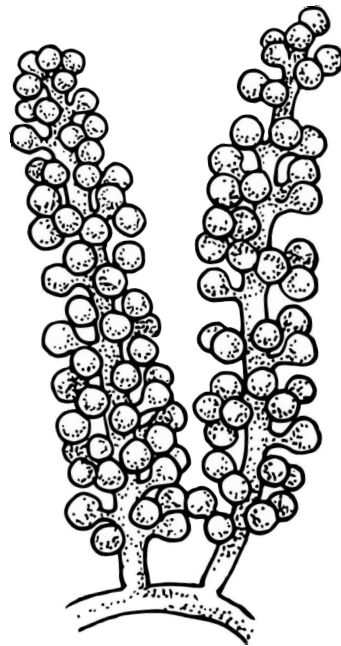# Aquaculture of *Caulerpa lentillifera* (sea grapes, Chlorophyta): Nutritional value, co-cultivation potential and post-harvest procedures of a sea vegetable

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Dissertation

in fulfilment of the requirements for the degree of
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Sketch on title page: *Caulerpa lentillifera* by Mareike Kortmann

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Summary

The global food production is facing challenges due to the natural boundaries of agriculture (shortages of fresh water and arable land) and crises like climate change. The cultivation of edible seaweeds, known as sea vegetables, is discussed as part of a solution to provide healthy, sustainably produced diets for the people. Seaweeds account already for ~50% of the global marine aquaculture production, however the vast majority is accounted for by red and brown seaweeds, with Chlorophyta representing <1%. This leaves a high potential for the cultivation of currently un- or under-utilized species, like the green macroalga *Caulerpa lentillifera*. This sea vegetable is valued for the combination of its high nutritional value and the special texture leading to its reputation as sea grapes or green caviar. However, the global interest in sea grapes is only growing recently and therefore the ecophysiological and biochemical understanding is still limited. With the growing demand, the aquaculture of *C. lentillifera* was introduced in new places, like Van Phong Bay in the Khánh Hòa province of Viet Nam.

The present Thesis investigated the ecophysiology and biochemical composition of *C. lentillifera* along its production cycle at the sea grape farm VIJA in Van Phong Bay in order to identify approaches that could improve the quantity and quality of the harvest, as well as the resource-efficiency of the production. In order to achieve this goal, a variety of complementary physico-chemical, ecophysiological, biochemical and computer-based measurements and methods were applied and the project was conducted in cooperation with researchers of the Institute of Oceanography (IO) in Nha Trang and the sea grape farm VIJA, both in Viet Nam.

In a first step, a structured literature review on *C. lentillifera* was conducted. It emphasized current study topics and applications and identified the state of the art with knowledge gaps regarding sea grape aquaculture (**chapter 2**). In a second step, sea grape's production cycle at the farm VIJA, from the pond cultivation over the harvest to the post-harvest treatment, was documented. Additionally, the study showed that frond weights, lengths and rachis colouration are essential features for determining the product's quality by the farmers (**chapter 3**). In this process, light has been identified as an important abiotic parameter, because high light exposure can lead to photooxidative stress of the shade-adapted *C. lentillifera*. Therefore, the study suggested that light irradiances require precise management during out-door farming, e.g. by application of gauze covers, as well as during post-harvest shelf-life in transparent plastic containers (**chapter 4**). The Thesis demonstrated that the permanent stress-exposure can negatively affect certain quality and growth relevant parameters (e.g. colour, frond length, etc.) of the seaweed. However, *C. lentillifera* produced nutritionally valuable antioxidants as part of the photoprotective response to the high light irradiances. Therefore, targeted light exposure was identified in this Thesis as a low-cost manipulation tool to increase the Antioxidant Activity (AOA) and Total Phenolic Content (TPC) and hence the nutritional value of the sea vegetable (**chapter 5**).

In **chapter 6**, it was attempted to replace the shading gauze cover of sea grapes with the carragenophyte *Kappaphycus alvarezii* in a co-cultivation set-up in an attempt to increase the economic viability of the system. The study showed that the provided shade by the red seaweed was insufficient to avoid photooxidative stress for the sea grapes. However, the cultivation of sea grapes in plastic cages with additional gauze cover below the longlines of *K. alvarezii* was identified as a resource-efficient possibility for a monotrophic *two-layer cultivation* of these seaweeds.

Currently sea grapes are mostly cultivated and consumed in Asia or rather the Indo-Pacific region, however the integration of *C. lentillifera* in the European *Novel Foods Regulation* could open a wider market for the sea vegetable. Therefore, **Chapter 7** focused on the

fertilization of sea grapes with process water of tropical whiteleg shrimp (*Litopenaeus vannamei*) from a German high technology land-based Recirculation Aquaculture System (RAS). The results suggested, that the targeted fertilization with the process water could be used as a tool to manipulate the sea grape's Amino Acid (AA) content and pattern. Additionally, the study introduced this polyculture as an opportunity for the potential cultivation of the tropical species in Germany.

The value-adding and co-cultivation approaches presented in this Thesis were mainly designed with the sea grape's cultivation in Van Phong Bay in mind, but they are adaptable to other scenarios and cultivation locations. However, the approaches were only conducted on an experimental or pilot scale. Therefore, an up-scaling with the involvement of the farmers is absolutely necessary to ensure a successful integration in the production cycle of the species in Van Phong Bay and elsewhere. In conclusion, this Thesis provides an essential basis towards a social-ecologically sustainable sea grape aquaculture oriented at the customers' and farmers' needs.

Zusammenfassung

Die weltweite Nahrungsmittelproduktion steht aufgrund der natürlichen Grenzen der Landwirtschaft (Verknappung von Süßwasser und Anbauflächen) und Krisen wie dem Klimawandel vor großen Herausforderungen. Der Anbau von essbaren Meeresalgen, auch bekannt als Meeresgemüse, wird als Teil einer Lösung diskutiert, um eine gesunde, nachhaltig produzierte Ernährung für die Menschheit sicherzustellen. Meeresalgen machen bereits ∼50% der weltweiten marinen Aquakulturproduktion aus, wobei der größte Teil auf rote und braune Algen entfällt, während Chlorophyta nur <1% ausmachen. Daraus ergibt sich ein großes Potenzial für die Kultivierung derzeit nicht oder nur unzureichend genutzter Arten wie der grünen Makroalge *Caulerpa lentillifera*. Dieses Meeresgemüse wird wegen seines hohen Nährwertes und seiner besonderen Beschaffenheit geschätzt, was ihm den Namen Meerestrauben oder Grüner Kaviar eingebracht hat. Das weltweite Interesse an Meerestrauben wächst jedoch erst seit kurzem, sodass das ökophysiologische und biochemische Verständnis der Alge noch begrenzt ist. Allerdings wird die Aquakultur von *C. lentillifera* mit der wachsenden Nachfrage zunehmend an neuen Orten etabliert, wie zum Beispiel in Van Phong Bay in der Provinz Khánh Hòa in Viet Nam.

Die vorliegende Arbeit untersucht die Ökophysiologie und die biochemische Zusammensetzung von *C. lentillifera* entlang der Produktionskette auf der Meeresfarm VIJA in Van Phong Bay, um Ansätze zu identifizieren, die die Quantität und Qualität der Ernte sowie die Ressourceneffizienz der Produktion verbessern könnten. Um dieses Ziel zu erreichen, wurde eine Vielzahl sich ergänzender physikalisch-chemischer, ökophysiologischer, biochemischer und computergestützter Messungen und Methoden angewandt. Das Projekt wurde in Zusammenarbeit mit Wissenschaftler:innen des Instituts für Ozeanographie (IO) in Nha Trang, und der Meerestraubenfarm VIJA, beide in Viet Nam, durchgeführt.

In einem ersten Schritt wurde eine strukturierte Literaturzusammenfassung über *C. lentillifera* erstellt. Dabei wurden aktuelle Studienthemen und -anwendungen hervorgehoben und der derzeitige Wissensstand sowie Wissenslücken in der Aquakultur von Meerestrauben ermittelt (**Kapitel 2**). In einem zweiten Schritt wurde die Produktionskette der Meerestrauben auf der Farm VIJA von der Teichwirtschaft über die Ernte bis zur Nacherntebehandlung dokumentiert. Darüber hinaus hat die Studie gezeigt, dass das Gewicht und die Länge der *Fronds* sowie die Färbung der *Rachis* für die Züchter:innen wesentliche Merkmale zur Bestimmung der Produktqualität sind. (**Kapitel 3**). In diesem Prozess wurde Licht als wichtiger abiotischer Parameter identifiziert, da eine hohe Lichtexposition zu photooxidativem Stress bei den an Schatten-angepassten *C. lentillifera* führen kann. Daher legt die Studie nahe, dass die Lichteinstrahlung genau gesteuert werden muss, sowohl während des Anbaus im Freien als auch in der Zeit nach der Ernte beim Verkauf in transparenten Kunststoffbehältern, z. B. durch das Anbringen von Gaze-Abdeckungen (**Kapitel 4**). Die Arbeit hat gezeigt, dass sich die permanente Stressbelastung negativ auf bestimmte qualitäts- und wachstumsrelevante Parameter (z.B. Farbe, Frond-Länge, etc.) der Algen auswirken kann. Allerdings produzierte *C. lentillifera* als Teil der photoprotektiven Reaktion auf die hohen Lichtbestrahlungen ernährungsphysiologisch wertvolle Antioxidantien. Daher wurde in dieser Arbeit eine gezielte Lichtexposition als kostengünstiges Manipulationsinstrument identifiziert, um die antioxidative Aktivität, als auch den Gesamtphenolgehalt und damit den Nährwert des Meeresgemüses zu steigern (**Kapitel 5**).

In **Kapitel 6** wurde versucht, die schattenspendende Gazeabdeckung von Meerestrauben durch den Carrageen-Lieferanten *Kappaphycus alvarezii* in einer Co-Kultivierung zu ersetzen, um den wirtschaftlichen Ertrag des Systems zu erhöhen. Die Studie zeigte, dass der Schatten, den der rote Seetang spendete, nicht ausreichend war, um photooxidativen Stress der Meerestrauben zu vermeiden. Die Kultivierung von Meerestrauben in Kunststoffkäfigen mit zusätzlicher Gaze-Abdeckung unter den Langleinen von *K. alvarezii* wurde jedoch als

ressourceneffiziente Möglichkeit für eine monotrophe *Two-layer* Kultivierung dieser Algen identifiziert.

Derzeit werden Meerestrauben hauptsächlich in Asien bzw. im indopazifischen Raum angebaut und verzehrt, doch die Aufnahme von *C. lentillifera* in die europäische *Verordnung über neuartige Lebensmittel* könnte einen neuen Markt für das Meeresgemüse eröffnen. Daher konzentrierte sich **Kapitel 7** auf die Düngung von Meerestrauben mit Prozesswasser von tropischen Weißfuß-Garnelen (*Litopenaeus vannamei*) aus einer deutschen hochtechnologischen, landbasierten Kreislaufanlage. Die Ergebnisse deuten darauf hin, dass die gezielte Düngung mit dem Prozesswasser als Instrument zur Manipulation des Aminosäure Gehaltes und Musters der Meerestrauben genutzt werden kann. Darüber hinaus stellte die Studie diese Polykultur als eine Möglichkeit für den potenziellen Anbau der tropischen Art in Deutschland vor.

Die in dieser Arbeit vorgestellten Wertschöpfungs- und Ko-Kultivierungsansätze wurden hauptsächlich mit Blick auf den Anbau von Meerestrauben in Van Phong Bay entwickelt, sind aber auch auf andere Szenarien und Anbaustandorte übertragbar. Allerdings wurden die Ansätze nur in einem Versuchs- oder Pilotmaßstab durchgeführt. Daher ist ein *Up-scaling* unter Einbeziehung der lokalen Züchter:innen unbedingt erforderlich, um eine erfolgreiche Integration in die Produktionskette der Art in Van Phong Bay und anderswo zu gewährleisten. Zusammenfassend liefert diese Arbeit eine wesentliche Grundlage für eine sozial-ökologisch nachhaltige, an den Bedürfnissen der Konsument:innen und Züchert:innen orientierte Aquakultur von Meerestrauben.

Contents

Chapter 1

Introduction

Sea grapes (*Caulerpa lentillifera*) photographed at the Institute of Oceanography in Nha Trang, Viet Nam.

1.1   Seaweeds in global aquaculture

1.1.1   Seaweeds as part of a solution

In the face of various overlapping crises, including climate change, as well as a growing population, surpassingly reaching >9 billion inhabitants in 2050, society is confronted with various important questions determining our future life. One of them being: "What are we going to eat in the future?".

One third of the global anthropogenic greenhouse gas emissions are accounted for by food systems, especially agriculture and land use/change activities [Crippa et al., 2021]. Shortages of arable land and freshwater are expected to affect terrestrial food production systems [Leng and Hall, 2019] and experts focus on an increasing importance of aquaculture as part of a possible solution [Costello et al., 2020]. In general, a shift from animal- to plant-sourced calories is seen as a path to face high occurrence of diet-related chronic diseases on one hand and sustainable human food production on the other hand [Pörtner et al., 2023]. Cultivated animals need to eat and the downwards shift of the trophic level, especially in marine production, could increase the overall biomass output and decrease the Carbon (C) footprint [Duarte et al., 2009, Pörtner et al., 2023]. Hence, aquaculture of phototrophic seaweeds is recently being re-advertised as part of a solution to tackle complex and multi-dimensional global challenges [Costa-Pierce and Chopin, 2021].

On one hand, seaweeds and their cultivation are providing various ecosystem and social services [Duarte et al., 2022], on the other hand the biomass has various possibilities for different (industrial) applications besides their use as sea vegetable [Farghali et al., 2023]. In general, marine phycoculture requires much less fresh water, fertile land and fertilizer, compared to the cultivation of terrestrial crops [Zheng et al., 2019, Spillias et al., 2023b]. Nonetheless, even though the potential is great and seaweed aquaculture can be part of a solution for current problems, marine phycoculture is not a cure-all and different social and ecological challenges need to be addressed and taken into account, while growing this industry (examples Fig.1.1A). Food systems and related challenges are complex and local solutions are often required. For example, on one hand seaweeds present excellent nutrient bioremediators, e.g. to mitigate coastal eutrophication [Neori et al., 2004, Kang et al., 2021] and they could function as blue C storage [Yong et al., 2022]. On the other hand extensive seaweed aquaculture could lead to decreased nutrient, light and Carbon Dioxide (CO_2) levels in their surroundings, negatively impacting other local communities [Campbell et al., 2019, Costa-Pierce and Chopin, 2021].

1.1.2   Current status of seaweed aquaculture

Seaweed cultivation accounts with >50% for the major harvest of global marine and coastal aquaculture and takes for the utter most part place in Asia [FAO, 2022]. In other parts of the world, like Europe, algae cultivation is still in its infantry and dominated by microalgae and harvest of wild seaweeds [Araújo et al., 2021]. The global seaweed production is dominated by species of the two phyla Ochrophyta (class of Phaeophyta, brown algae) and Rhodophyta (red seaweeds), with only eight species accounting for 93.7% of the algae produc-
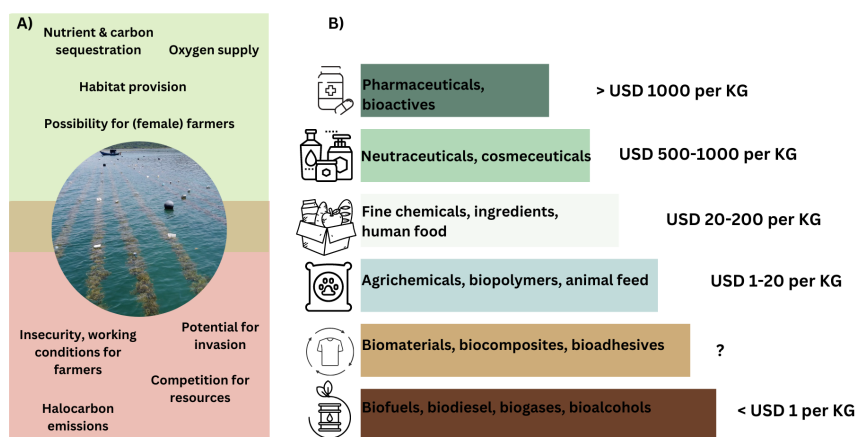
Figure 1.1: **A)** (Dis-)advantages, opportunities and challenges regarding seaweed aquaculture. This list does not claim to be complete, it is rather meant as a collection of points for discussion [Lüning and Pang, 2003, Fröcklin et al., 2012, Campbell et al., 2019, Costa-Pierce and Chopin, 2021, Keng et al., 2021, Duarte et al., 2022, Glasson et al., 2022]. **B)** Simplified value and volume estimation of different uses for seaweed (compounds) [Chopin and Tacon, 2020].

tion (Table 1.1). The biomass of seaweed aquaculture is mostly used for human nutrition as sea vegetables, like kombu, wakame or nori or for the extraction of sulfated polysaccharides (e.g. carrageenan, agar, fucoidan) from the seaweeds cell walls (Table 1.1). These compounds exhibit biological (e.g. antioxidant, anti-viral) and functional (e.g. gelling, thickening, binding) properties that make them suitable for industrial applications e.g. as hydrocolloids and nutraceuticals in the food sector [Muthukumar et al., 2021]. However, even though algae dominate the sector by volume, seaweeds are mostly low-value commodities and accounted for ∼5.4% of the United States Dollar (USD) 275 billions of the world aquaculture production in 2019. The average first sale prices of brown, red and green seaweeds were USD 0.47, 0.39 and 0.79 kg^{-1} (Wet Weight (WW)) in 2019 [Cai et al., 2021a]. On the supply side, the price depends highly on the scale, initial investment, farming technology and labor costs, whereas the application of the biomass is important for the price development, with the use for human nutrition tending to be more valuable (Fig.1.1) [Chopin and Tacon, 2020, Cai et al., 2021a]. Therefore, various seaweed producing countries work on strategies to add value to their product (valorization) and raise the net profit [Soethoudt et al., 2022, Msuya et al., 2022], e.g. by efficient usage of the waste biomass [Zhou et al., 2022].

1.1.3 Diversification of aquaculture and the role of seaweeds

The principle of diversification has been proposed and advertised to mitigate challenges from diverse contexts (e.g. ecological, social, economic) not exclusively, but also, for the aquaculture sector [Di Bene et al., 2022]. Aquaculture can diversify on different scales, including species, technologies, geography and environment and markets and governance, driven by a divers

Table 1.1: Global algae production from 2020 in thousand tonnes live weight and grouped by species or genus. Numbers in brackets are percent of total production. Table design and data are based on [FAO, 2022].

	Common name	Latin name	Production in thousand tonnes	main use / known for
Brown seaweeds	Japanese Kelp	*Laminaria japonica*	12 469.8 (35.5%)	Sea vegetable: Kombu
	Wakame	*Unadaria pinnatifida*	2 810.6 (8.0%)	Sea vegetable: Wakame
	Fusiform sargassum	*Sargassum fusiforme*	292.9 (0.8%)	Sea vegetable: Hijiki
Red seaweeds	Eucheuma seaweeds	*Eucheuma* spp.	8 129.4 (23.2%)	Carrageen
	Gracilaria seaweeds	*Gracilaria* spp.	5 180.4 (14.8%)	Agar agar
	Nori	*Porphyra* spp.	2 220.2 (6.3%)	Sea vegetable: Nori
	Elkhorn sea moss	*Kappaphycus alvarezii*	1 604.1 (4.6%)	Carrageen
	Spiny eucheuma	*Eucheuma denticulatum*	154.1 (0.4%)	Carrageen
	Subtotal of 8 major species		3 2861.5 (93.7%)	

set of drivers, such as market demand, funding opportunities or climate change [Harvey et al., 2017]. The livelihood of various small-scale farmers, often women, depends on seaweed farming and is repeatedly endangered [Msuya and Hurtado, 2017, Mariño et al., 2019]. One example is the increasing problem of seaweed pests and diseases, potentially even amplified by environmental conditions due to climate change. It causes declines in the harvests quantity and commercial value, which is especially challenging for marginal farmers [Ward et al., 2020]. Besides biosecurity measurements [Campbell et al., 2020], increasing genetic diversification, which often suffered from the domestication, might lead to a higher susceptibility against pests and diseases [Harvey et al., 2017, Ward et al., 2020]. Diversification in species' richness and evenness, namely the cultivation of currently un- or under-utilized species, could "decrease risks, capitalize on opportunities and provide resilience" [Harvey et al., 2017] (page 3). Considering that >90% of the global seaweed cultivation is represented by eight groups (Table 1.1), the potential for species diversification is high.

Polyculture, and hence a form of system diversification [Harvey et al., 2017], is a promising alternative to facilitate some of the ecological, social or economic hurdles identified in monocultural aquaculture practices and to move towards a more sustainable aquaculture industry [Thomas et al., 2021]. The species' compatibility and complementarity are essential criteria to design a successful polyculture. Thomas et al. outlined a conceptual framework with four steps to consider for implementing a polyculture, reaching from the selection of (*1*) species combinations, and (*2*) the farming system, over (*3*) the management of the systems complexity until the (*4*) actual implementation for the approach, also with regard to the stakeholders' expectations [Thomas et al., 2021]. Seaweeds play already a crucial role in polyculture systems, like Integrated Multi-Tropic Aquaculture (IMTA), e.g. due to their property for bioremediation of nutrients [Roleda and Hurd, 2019, Kang et al., 2021]. However, the potential for polyculture set-ups with seaweeds is not exhausted, considering the wide variety potential synergies among algae themselves or with other taxa [Roleda and Hurd, 2019, Kang et al., 2021].

1.1.4   The largely untapped potential of Chlorophyta

The phylum of Chlorophyta is diverse and the species' elemental compositions differ in several aspects from that of Rhodophyta and Ochrophyta, due to evolutionary differences [Moreira et al., 2021]. Seaweeds are highly plastic regarding their biochemical composition, often in response to (a)biotic environmental parameters [Stengel et al., 2011]. However, in general the protein content of Chlorophyta is moderate (9±26% of Dry Weight (DW)) with Glutamic Acid (Glu) and Aspartic Acid (Asp) being the most prominent AAs [Fleurence et al., 2018]. The lipid content on the other hand is in general low for all seaweeds [Fleurence, 2016, Garcia-Poza et al., 2020], whereas the content of macro and trace elements can reach values ∼10 times higher than that of terrestrial plants (ash content of 20-50% DW) [Lozano Muñoz and Díaz, 2022]. Various of Chlorophytas' pigments, lipids, polysaccharides and secondary metabolites exhibit strong bioactivities [Kidgell et al., 2019, Cotas et al., 2020, Moreira et al., 2021]. The bioactivity properties, comparable high growth rates and bioremediation capacities recommend Chlorophyta for applications in integrated IMTAs [Kang et al., 2021], as well as in the context of biorefinery [Kostas et al., 2021], bioplastics, the food industry, or pharmaceutical and cosmetical applications [Cotas et al., 2020, Moreira et al., 2021]. However, the research effort regarding natural product isolation from green macroalgae was neglected, compared to red and brown seaweeds [Leal et al., 2013, Moreira et al., 2021]. Hence, the economic potential might actually be even larger than currently expected. Chlorophyta account for <1% of the global algae production, presenting a compelling opportunity for diversification in the algae cultivar [Moreira et al., 2021]. The production is mostly limited to the Chlorophyta taxa *Ulva* spp., *Capsosiphon fulvescens*, *Codium* spp., *Monostroma* spp. and *Caulerpa* spp. [Moreira et al., 2021].

1.2   The organism *Caulerpa lentillifera*

1.2.1   Biology

Caulerpa is the only genus in the family Caulerpaceae of the order Bryopsidales with 104 accepted species and 39 varieties [Guiry and Guiry, 2023]. The genus is distributed from the tropics to the subtropics with some species extending to the Mediterranean (e.g. invasive *C. taxifolia* and *C. racemosa*) and temperate regions of Australia [Zubia et al., 2020, Guiry and Guiry, 2023]. However, some *Caulerpa* are well-known for their high phenotypic plasticity as a reaction to different environmental parameters, which led to confusions regarding the species identification [de Gaillande et al., 2017, Estrada et al., 2020].

   Caulerpa means loosely translated *crawling stem* (Greek *Caulos* – stem and *Erpo* – I crawl) [Silva, 2003, Guiry and Guiry, 2023]. The name alludes to the morphological appearance of the seaweeds (Fig.1.2B): The thallus consists of a horizontal axis (stolon) with colourless rhizospheres and upright photosynthetic assimilators (fronds) with a central rachis, which can be leave-like or ramuli-bearing [Zubia et al., 2020]. The *Caulerpa* species *C. racemosa* and *C. lentillifera* are often referred to as sea grapes or green caviar since they have grape-like ramuli [Zubia et al., 2020]. Sea grapes are usually found in a depth ≤20 m on sand

or hard substrate often around coral reefs [Benzie et al., 1997, Paul and de Nys, 2008, Terada et al., 2012, Guiry and Guiry, 2023].

The genus exhibits a diplontic sexual life-cycle. The sexual reproduction is holocarpic, hence the whole diploid (2n) thallus is transformed into haploid (1n) biflagellate gametes. This process is possibly triggered by environmental factors, like temperature [Ohba et al., 1992, Panayotidis and Žuljevic, 2001, Phillips, 2009, Zubia et al., 2020]. However, the sexual reproduction of *Caulerpa* is understudied [Silva, 2003], because the asexual reproduction via fragmentation or rhizoid extension, resulting in clonal growth, seems to be more common [Zubia et al., 2020].
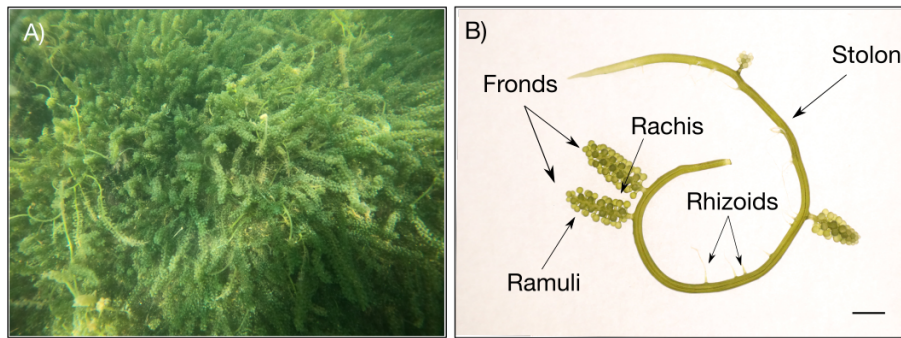
Figure 1.2: **A)** Benthic sea grapes growing in the sand. **B)** Thallus of *Caulerpa lentillifera* with fronds, stolons and rhizoids. Scale bar = 1 cm.

1.2.2 Ecophysiology

1.2.2.1 Physiological stress definition

The term *stress* has been defined as a "disturbance of homeostasis due to the action of a stressor" by Borowitzka [Borowitzka, 2018]. The author argues that cells leave the status of stress, as soon as they "have restored homeostasis by acclimation or adaptation" [Borowitzka, 2018]. The increased formation of Reactive Oxygen Species (ROS) is a common consequence at sub/supra-optimal environmental conditions, termed oxidative stress [Dring, 2005]. The alga's response to a stressor can be manifold and depends on various circumstances, including the exposure time and magnitude, the initial state of the organism and especially the particular optimum condition [Borowitzka, 2018]. The optimum environmental conditions of a species depend again on the prevailing habitat and their respective adaptions.

1.2.2.2 Sea grapes natural environment and abiotic stress

Green seaweeds are in general found in the shallower waters of the intertidal or the upper subtidal zones, since their main photosynthetic pigment, Chlorophyll (Chl) a, absorbs mostly light from the red spectrum, which is the first to be absorbed or scattered at the water surface [Terada et al., 2021]. However, sea grapes were found in a depth of up to $\sim$20 m [Terada et al., 2012]. The success of some green algae, including *C. lentillifera* in these depth with only limited irradiances and certain spectral parts could be associated with their adaption to

low light irradiances [Guo et al., 2015a, Xing et al., 2017, Kang et al., 2020] and the presence of green-light absorbing Carotenoid (Car)s like siphonoxanthin and siphonein [Kageyama et al., 1977, Raniello et al., 2004, Terada et al., 2021, Seki et al., 2022]. However, whereas the lower limit in the bathymetric distribution depends mainly on the algae's effective light harvesting, photoprotective mechanism are required to thrive in the upper sublittoral, where *Caulerpa* are faced with highly varying light environments [Raniello et al., 2004, Raniello et al., 2006]. Besides, seaweeds that are mainly thriving in the intertidal are usually exhibiting a high tolerance to desiccation to be able to sustain longer periods of air exposure [Davison and Pearson, 1996]. The single-celled *C. lentillifera*, however, is susceptible to water loss and associated limitations of their photosynthesis at a humidity of 50% [Terada et al., 2021]. Desiccation is a form of water deprivation, potentially leading to strong cellular dehydration [Holzinger and Karsten, 2013]. The ionic concentrations increase due to the water loss, but the ratios remain constant, which can influence the electron flow at the Photosystem II (PSII) and could lead to an increase in ROS formation [Satoh et al., 1983, Karsten, 2012]. The species is often found adjacent to coral reefs in rather oligotrophic conditions [Paul and de Nys, 2008, Guo et al., 2015b], with the preference of Nitrate (NO_3^-) over Ammonium (NH_4^+) in the presence of both Nitrogen (N) sources [Liu et al., 2016]. Growth rates are, among others, depending on the N and Phosphorus (P) supply, with highest values at 500 µmol L^{-1} NO_3^- and 100 µmol L^{-1} Phosphate (PO_4^{3-}) [Guo et al., 2015b], but in a nonlinear manner [Hsu et al., 2023]. However, a lower N supply and PO_4^{3-} levels of 10 µmol L^{-1} seemed to be limiting the growth of sea grapes [Guo et al., 2015b]. Besides, the (sub-)tropical species growth and physiology is temperature and salinity dependent with optimum conditions around S_A35 and $\geq 27°C$ [Guo et al., 2015b, Tanaka et al., 2020, Cai et al., 2021b, Terada et al., 2021]. Fig.1.3 summarizes the main publications investigating the effect of the respective environmental conditions and interactive effects on the sea grapes physiology, but the data coverage is low and variations between populations are to be expected.

1.2.2.3 Photooxidative stress

Light is the essential energy-source for photoautotrophic organisms, like seaweeds. However, since sea grapes are adapted to low irradiances [Guo et al., 2015a, Xing et al., 2017, Kang et al., 2021], they are in high risk that the incoming light irradiances exceed their need for photosynthesis. PSII is a water-plastoquinone oxireductase protein complex located in the thylakoid membrane of the chloroplasts, where it catalyzes the light-driven oxidation of Water (H_2O) to Diatmoic Oxygen (O_2) and the reduction of plastoquinone to plastoquinol [Pospíšil, 2016]. The energy of an incoming photon is captured by accessory pigments in the PSII's antenna complex and transported to the reaction center by temporal transfer of the molecules to the Singlet Excited State of Chlorophyll ($^1Chl^*$). The excited Chl *a* molecule in the reaction center reduces the plastoquinone through charge separation and thereby starts the electron transport chain, yielding the generation of Adenosine Triphosphate (ATP) and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) [Diner and Babcock, 1996, de Wijn and van Gorkom, 2002].

However, at high light irradiances, when the plastoquinone-pool is highly reduced, the elec-
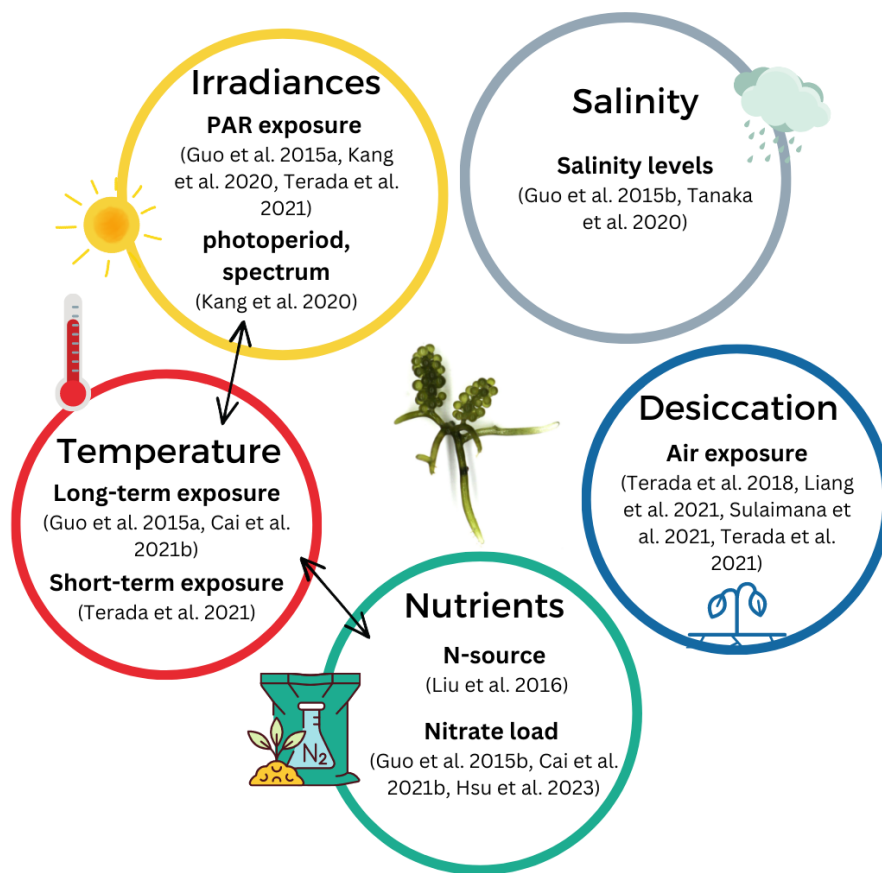
Figure 1.3: Overview of publications regarding effects of abiotic parameters on *Caulerpa lentillifera*'s physiology. Arrows indicate interaction of two parameters. List includes major articles, however it does not claim to be complete.

tron spins of ^{1}Chl* can be rephased and the molecule transfers to a lower energy excited state: Triplet Excited State of Chlorophyll (^{3}Chl*). ^{3}Chl* can transfer energy to O_2 and form the highly reactive Singlet Oxygen ($^1O_2$) [Krieger-Liszkay, 2004], which is seen as a main cause of photoinhibition at high light irradiances [Krieger-Liszkay, 2004, Dring, 2005], often quantified by decreased values of maximum quantum yield of PSII (F_v/F_m). The reduction of O_2 by one, two, or three electrons causes the formation of the Superoxide Anion (O_2^-), Hydrogen Peroxide (H_2O_2) and the Hydroxyl Radical (˙OH), respectively [Mehler, 1951, Asada et al., 1974]. Those highly reactive ROS can damage cellular and molecular components of the seaweed, due to oxidation with different biomolecules (e.g. proteins, like the D1 protein of PSII, pigments, lipids, Deoxyribonucleic Acid (DNA)) [Krieger-Liszkay, 2004, Sharma et al., 2012]. Seaweeds have different mechanism to adjust to changing light conditions and hence to avoid irreversible photodamage [Demming-Adams and Adams, 1992, Aro et al., 1993, Demmig-Adams and Adams, 1996].

Avoidance mechanisms like chloroplast movements can prevent exposure to excess light irradiances in the first place [Kasahara et al., 2002], but excess energy can also be dissipated harmlessly as heat (non-photochemical quenching). The process involves multiple components, like the xanthophyll cycle [Müller et al., 2001], with first findings suggesting a special role of the lutein-siphonaxanthin interconversion for *Caulerpa* [Raniello et al., 2006] and is termed dynamic photoinhibition [Osmond, 1994, Häder et al., 1997, Hanelt et al., 1997]. Additionally, plants possess a complex defense system of enzymatic (e.g. Superoxide Dismutase (SOD), Catalase (CAT)) and non-enzymatic antioxidants to scavenge ROS. Non-enzymatic molecules of the antioxidant defense are Cars, vitamins (e.g. tocopherol/vitamin E, ascorbic acid/vitamin C) or phenolic compounds (e.g. flavonoids) [Sharma et al., 2012]. *C. lentillifera* exhibits various antioxidative compounds resulting in high AOAs [Matanjun et al., 2008, Fakhrulddin et al., 2021]. Some of these bioactive compounds are exclusively synthesized by photoautotrophic organisms [Sharma et al., 2012], but with essential roles in human nutrition [Mohamed, 2014].

An unsuitable light environment is not the only stressor, leading to oxidative stress and often there is a multitude of different environmental stressors at the same time [Davison and Pearson, 1996]. Hence, the threshold for a light induced stress reaction might be even lower, when the seaweed is additionally exposed to other stressors, like desiccation [Demming-Adams and Adams, 1992].

1.2.3   Nutritional value and bioactive compounds

1.2.3.1   Sea grapes as food

Caulerpa are used as food in Japan, Polynesia, The Phillipines and Indonesia since many centuries [Silva, 2003]. Nowadays, approximately 15 *Caulerpa* species are consumed [de Gaillande et al., 2017], with a focus on *C. lentillifera* and *C. racemosa* being known as a delicacy (green caviar) due to the striking texture and the fresh, salty taste. Local names, preparation and integration in dishes differ between locations. However, sea grapes are usually eaten as sea vegetables fresh in salads or with a special sauce [de Gaillande et al., 2017] (Fig.1.4A).

Besides the sensory and visual properties, like the typical green colour, the composition of sea grapes is in general another argument for their consumption [Syakilla et al., 2022].
Sea grapes have generally low lipid contents [Matanjun et al., 2009, Saito et al., 2010, Paul et al., 2014, Zhang et al., 2020], with comparable high load of (essential) AAs [Matanjun et al., 2009, Long et al., 2020, Zhang et al., 2020] and minerals [Ratana-arporn and Chirapart, 2006, Paul et al., 2014, Long et al., 2020]. Besides, the seaweed features high contents of antioxidative compounds, including vitamin C, E [Matanjun et al., 2009], Cars [Paul et al., 2014] and phenolic compounds, like flavonoids [Fakhrulddin et al., 2021]. Several of these metabolites are perceived as important dietary component for counteracting the metabolic syndrome [Mohamed, 2014, Rezayian et al., 2019, Cotas et al., 2020, John et al., 2020]. However, the antioxidant content is only one of the reasons sea grapes are discussed as *functional food* candidates [Tapotubun et al., 2020, Nurkolis et al., 2023]. They exhibit various other activities, including, but not limited to anti-obesity [You et al., 2022b, Nurkolis et al., 2023] and anti-bacterial activities [Nagappan and Vairappan, 2014] with pharmacological potential. Sea grapes' sensory and visual properties, the nutritional benefits as well as the attribute as a delicacy suggest a high economic potential for the seaweeds also outside of Asia. However, the species is not yet covered by the *European Regulation on Novel Foods (2015/2283)*, currently excluding the product from the European market.
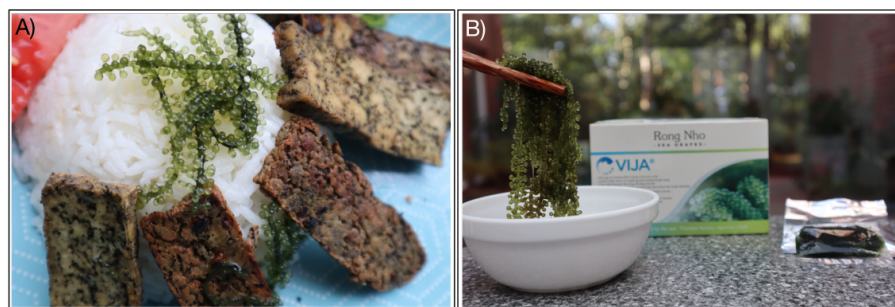
Figure 1.4: **A)** Fresh sea grapes served with tofu and rice and **B)** dehydrated sea grapes in the packaged state and after re-hydration in freshwater.

1.2.3.2   Targeted manipulations of the nutritional value

Sea grapes' biochemical characteristics, including the nutritional and bioactive compounds, can differ strongly along tempo-spatial scales with the prevailing environmental parameters [Stengel et al., 2011, Syakilla et al., 2022]. Even though this poses a challenge for the species aquaculture, it also provides an opportunity to specifically manipulate the cultivation environment and to enhance the accumulation of target compounds. This approach is already established for the cultivation of microalgae. The *two-stage cultivation* describes a process where the conditions are in a first step adjusted to yield a high rate of cell proliferation. In a second step different stressors, such as nutrient deprivation, high light or temperature are applied to trigger the synthesis of the desired metabolites [Liyanaarachchi et al., 2021].

For seaweeds, however, the targeted manipulation of cultivation parameters to enhance the production of certain metabolites was only investigated rarely [Godínez-Ortega et al.,

2008, Angell et al., 2014, Angell et al., 2015, Magnusson et al., 2015, Toth et al., 2020]. Seaweeds are, other than microalgae, often cultivated in less-controllable environments, like the open ocean or out-door tanks, making the practical implementation of manipulations difficult. However, light irradiances and nutrient concentrations are comparably easy to control without technical requirements, e.g. by manipulation of the seaweed density, artificial shading and by using aquaculture effluents for fertilization. Induced photooxidative stress could be used to influence the protective antioxidative response of seaweeds [Magnusson et al., 2015] and the manipulation of nitrate levels could trigger accumulation of proteins and valuable essential AAs [Angell et al., 2014]. Such manipulation techniques could function as toolkits for farmers without a strong financial background to increase their harvests' nutritional qualities. However, the design of the protocols requires a deep understanding of the species' physiology, as well as the specific aquaculture practices.

1.3 Sea grape aquaculture

1.3.1 History of the sea grape aquaculture

Caulerpa were usually harvested by hand from the wild rather than cultivated in aquaculture [Zubia et al., 2020]. The pond aquaculture of *C. lentillifera* started by accident in The Philippines in the early 1950's. A fish farmer threw leftovers of the seaweed in his milkfish pond and discovered a few days later, that the sea grapes grew very well [Yap, 1999, Trono and Largo, 2019]. The industry developed over the years [Estrada et al., 2021] and between 1950 and 2019 an average of >6400 tonnes WW of *Caulerpa* spp. were produced in the country [Cai et al., 2021a]. In the South Pacific region *Caulerpa*, and especially *C. racemosa*, harvest has a long history as well [Chamberlain, 1998, Conte and Payri, 2006] and is nowadays providing the basis for a subsistence fisheries [Morris et al., 2014]. In Japan, the commercial sea grape cultivation started in 1986 in the southern Island of Okinawa [Trono and Toma, 1993]. However, with an increasing economic success and higher demands, their cultivation spread throughout Asia, including to Thailand, India and Viet Nam [Mary et al., 2009, Hong and Ha, 2022, Lewmanomont and Chirapart, 2022].

1.3.2 Cultivation systems and set-ups

After the unintentional, but successful, introduction of *C. lentillifera* in milkfish ponds, the cultivation in tidal ponds was continued [Yap, 1999, Trono and Largo, 2019]. The site selection and earthen pond construction is described in detail by Trono and Toma [Trono and Toma, 1993], but in general clean, salty water is important and the water exchange should be ensured by the tidal cycle to avoid the need of fertilization. The seedstock can be planted by hand in the sediment (sowing method) or between two perforated plastic sheets/net frames (tray or net frame cultivation), or it can be broadcasted directly in the pond [Trono and Toma, 1993, Rabia, 2016, Lewmanomont and Chirapart, 2022]. The crop harvest is handiwork as well, with 20-25% remaining in the pond for the next crop [Trono and Toma, 1993]. Other methods include growth in nets, cages, trays, baskets and rarely even longlines [Al Mamun

Siddiqui et al., 2019, Zubia et al., 2020], which are placed on the bottom of earthen or concrete ponds, as well as hang or swim in the water of the pond or shallow, open lagoons [Trono and Toma, 1993, Lewmanomont and Chirapart, 2022].

Freshwater input in the form of rivers or as rain is appointed as most crucial environmental parameter of sea grape cultivation on the spatial-temporal scale, influencing growth season and cultivation location [Trono and Toma, 1993, Chamberlain, 1998]. In order to surpass heavy dependencies of environmental water parameters, land-based concrete raceways are increasingly used for the cultivation [de Gaillande et al., 2017], whereas the cultivation of land based in high technologized RAS is not yet described in the literature. However, it would provide an opportunity for the species' cultivation also in temperate regions.

1.3.3 Integrated aquaculture

Nutrient effluents of aquaculture can cause coastal eutrophication [Troell et al., 1999, Read and Fernandes, 2003] and hence seaweeds are often proposed to mitigate excessive release of those [Kang et al., 2021], while proving for an additional income-source for farmers [Neori et al., 2004]. Even though sea grapes are a rather oligotrophic species [Paul and de Nys, 2008, Guo et al., 2015b], they were already successfully cultivated in the effluents of the economically important snails *Babylonia areolata* [Chaitanawisuti et al., 2011, Dobson et al., 2020] and whiteleg shrimp *Litopenaeus vannamei* [Anh et al., 2021, Ly et al., 2021]. The focus in those approaches lies often on the seaweeds as bioremediatory species to mitigate negative impacts on the environment. However, an overproduction of extractive species in a certain environment could also have detrimental effects [Costa-Pierce and Chopin, 2021]. Hence, research focused to sustaining a resource-efficient targeted fertilization of seaweeds with locally available aquaculture effluents in the ocean, as well as in in-door set-ups, is also necessary when developing seaweed aquaculture.

Sea grapes renowned reputation as delicacy, as well as the current trend of seaweeds in western countries [Costa-Pierce and Chopin, 2021] suggests a high market potential for *C. lentillifera*, once administrative hurdles, like the *European Regulation on Novel Foods*, are taken. Hence, land-based RAS producing tropical fed seafood, like shrimp *L. vannamei*, could provide an opportunity to integrate the cultivation of the sea vegetables. The effluents provide a NO_3^--rich fertilization, which sea grapes prefer over NH_4^+ [Liu et al., 2016] and the sea vegetables could be retailed as a resource-saving alternative to imports from Asia.
Another problematic aspect of extensive seaweed aquaculture is the competition with other organisms for light [Lüning and Pang, 2003, Campbell et al., 2019, Cai et al., 2021a]. The integrated cultivation with other seaweed species can evoke a more efficient use of space and resources and hence increase farmers yields per area, as well as keeping potential detrimental effects on the environment, such as shading of benthos, as small as possible. The co-cultivation of different seaweed species has been investigated only scarcely for *C. lentillifera* [Paul et al., 2014, Liu et al., 2016]. However, their cultivation practises are quite diverse, as described in section 1.3.2, proving various opportunities for co-cultivation set-ups, like a resource-efficient two-layer cultivation. The low light requirements and the preference of NO_3^- over NH_4^+ suggest a species with complementary cultivation needs, like the carrageno-

phyte *Kappaphycus alvarezii*, which is among others cultivated on longlines at the water surface and could provide natural shade for species kept below [Ask and Azanza, 2002].

Independently from the co-cultivation approach, the advantage of system diversification from one to multiple species is the mitigated risk of failure of one species, as well as the additional income [Neori et al., 2004]. *C. lentillifera* is of special interest, since the retail price is well above the average for seaweeds in general, offering an economic opportunity for farmers [Dobson et al., 2020, Cai et al., 2021a].

1.3.4 Post-harvest treatment and shelf-life

The harvest of sea grapes is usually done manually, by cutting fronds of the target size. The formation of a wound-plug of the injured siphonous algal fronds is supported by storage in clean saltwater. After draining, the modes of preservation vary between regions and families [de Gaillande et al., 2017, Zubia et al., 2020]. However, the common challenge is, that the fresh product of *C. lentillifera* is still photosynthetically active and alive during post-harvest until consumption. In general, the harvested and cleaned sea grapes should be kept in the shade and in a high humidity environment, e.g. wrapped in banana leaves, in coconut baskets or closed plastic packaging of different types with a moisture sheet [Chamberlain, 1998, Tuong et al., 2016, de Gaillande et al., 2017, Terada et al., 2018, Zubia et al., 2020]. Different techniques have been investigated to extend the fresh products shelf-life [Tuong et al., 2016, Sulaimana et al., 2021]. For long-term preservation the sea grapes' dehydration under pressure at a brine solution might be the most reliable method [Tolentino et al., 2021], even though this could affect the nutritional characteristics, like AOA.
In the packaging environment, the fresh sea grapes are air exposed, suffering desiccation. However, the moisture sheets maintain a high humidity $\geq$90% leading to less water loss, compared to desiccation at $\geq$50% [Terada et al., 2018, Terada et al., 2021]. Even though, desiccation during shelf-live still causes oxidative stress [Terada et al., 2018, Liang et al., 2021] and there is a high potential that the impact of additional stressors, like high light, could cause even stronger physiological stress reactions during shelf-life. Preservation, as well as aquaculture techniques vary strongly between different locations.

1.4 Sea grape aquaculture in Van Phong Bay, Viet Nam

1.4.1 Van Phong Bay, Khánh Hòa province, Viet Nam

The country of Viet Nam stretches over ~15 latitudes (~ 8-23°N) with a width of only 50-600 km [ISPONRE, 2009]. The coast line of ~3260 km gives rise to various opportunities for marine and coastal aquaculture. Not surprisingly, Viet Nam ranks within the first places in a global comparison regarding the marine and coastal aquaculture production of finfish (7th rank, 305 thousand tonnes in 2020), crustaceans (2nd rank, 1.1 million tones in 2020), mollusks (5th rank, 211 thousand tonnes in 2020) and algae (11th rank, 14 thousand tonnes) [FAO, 2020].

One of the many bays along the countries coast line is Van Phong Bay. Van Phong Bay is

the largest of four bays in the Central-South of Viet Nam in the Khánh Hòa province (Fig.1.5). It is a semi-open bay encompassing ~510 km^2 with an average depth of ~15 m and various small rivers discharging in the bay [Barthel et al., 2009, Phu et al., 2022]. The province experiences a rainy season from August/September, with a peak in October/November until ~December [ISPONRE, 2009]. During the dry season, the temperatures are increased with March, April and May being the hottest months [ISPONRE, 2009]. Various aquaculture activities are conducted in the bay and on the coastal area, including, but not limited to, lobster [Phu et al., 2022], green mussel (*Perna viridis*), pond cultivation of *B. areolata* and *L. vannamei* [Nghia et al., 2009], as well as of seaweeds *K. alvarezii* on longlines in the northern part of the bay and *C. lentillifera*.
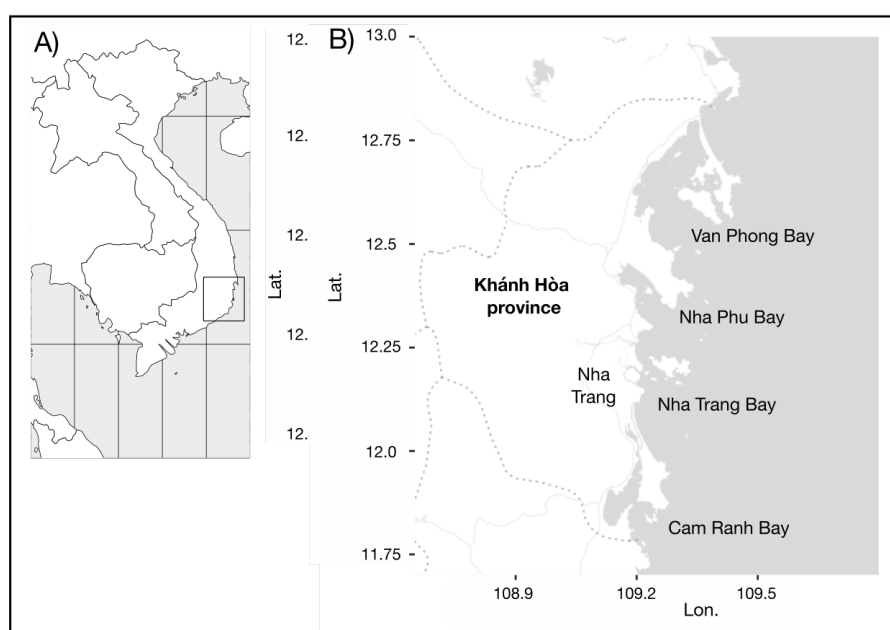
Figure 1.5: **A)** The Khánh Hòa province is located in the Central South of Viet Nam and **B)** Van Phong Bay is the northern most of the four bays in the province.

1.4.2   Dimension of sea grape production in Khánh Hòa province

In the Khánh Hòa province sea grape farming takes place in Van Phong Bay and Cam Ranh Bay. Information on the dimension of sea grape production in Viet Nam and the province are scarce. However, local news webpages provide an idea about the development of this rather new aquaculture crop. A japanese strain of *C. lentillifera*, even though native to Viet Nam, has been introduced for aquaculture purposes to Khánh Hòa in the beginning of the 2000's [Dai et al., 2009, Son, 2022]. This strain was expected to have a higher nutritional value and production yield [Son, 2022]. In the next years several companies started sea grape farming in different areas of the province. Reports on sea grape production vary, with 30 tonnes ha^{-1} year^{-1} in 2008 [Khanh Hoa News, 2008] and in 2020 an estimate of >400 tonnes year^{-1} at an area of ~50 hectares [Son, 2022]. Farm gate prices were reported as USD 4.35 kg^{-1} [Dobson et al., 2020] and a sales price of 8-10 USD kg^{-1} in 2008 [Khanh Hoa News, 2008]. A company

founder reported that the business provides a sustainable livelihood to various local farmers as an alternative to fish and shrimp farming or commercial fisheries [Son, 2022]. Sea grape farming in Van Phong Bay takes especially place in the southern part of the bay (Fig.1.5) with VIJA being one of the local sea grape farming companies operating ponds in this area.

1.4.3 Production cycle of sea grapes

The sea grape farm VIJA was a local partner in the work conducted for this thesis. Hence, reports on the sea grape farming were based especially on the research conducted at ponds of this farm. However, according to conversations with others farmer, most aspects seem to be transferable at least to farms along Van Phong Bay.

In Van Phong Bay, *C. lentillifera* are cultivated in earthen, tidal ponds during the dry season, using the sowing or tray cultivation, depending on the properties of the bottom. The harvest takes places approximately every two weeks, where fronds of the target size are removed and stored for wound healing in land-based tanks. The frond's quality is quantified based on certain visible characteristics and the product is grouped as *export grade* and *local grade* to be used for different purposes, like especially export to Japan or for retail in local restaurants or on the market. Farmers group the fronds based on experience and eye-sight and the exact quality characteristics are not known. However, the fronds characteristics could potentially be specifically maintained or enhanced by adjustment of the cultivation or post-harvest parameters. The sea grapes retail takes place in transparent Polyethylene Terephthalate (PET) plastic containers with moisture sheets or the product is dehydrated for longer durability (Fig.1.4B).

During the whole sea grape production, light management is crucial. Farmers shade the ponds with gauze material and also keep the harvested fronds in the shade. However, during shelf-life in transparent containers where sea grapes suffer desiccation and potentially additional light stress, the proper light management seems to be neglected. On the other hand, the production cycle of sea grapes offers different opportunities to manipulate the sea grapes cultivation or post-harvest environment in order to enhance the nutritional value, taking an example from the *Two-stage cultivation* of microalgae. For optimal growth, the cultivation ponds are shaded with gauze material. However, after the harvest targeted exposure to high light could increase the seaweeds antioxidant content and hence the nutritional value.

The cultivation of *C. lentillifera* in Van Phong Bay is currently only taking place in tidal ponds. However, coastal space is restricted and the diverse cultivation methods of the species allow for different set-ups. One opportunity is the resource-efficient co-cultivation with an other local species, like the red carragenophyte *K. alvarezii*, providing additional income for the farmers and making optimal use of available space and supply chains.

1.5 Aims of the thesis

Seaweeds are discussed as one possibility to face the challenge of future human nutrition also in view of global crisis like climate change and natural boundaries to agriculture. Sea grapes are already successfully implemented as sea vegetables in some parts of the world and their

Figure 1.6: **A)** Illustration of sea grapes production cycle in Van Phong Bay, Khánh Hòa, Viet nam with pictures of the respective stages, including the **B)** cultivation in earthen tidal ponds, **C)** the harvest and the post-harvest with wound healing, **D)** cleaning and sorting, followed by packaging and retail in the **E)** fresh or **F)** dehydrated form.

promising set of properties indicates that their demand will increase. However, since global interest in sea grapes is only rising since recent years, the physiological and biochemical understanding of the species is limited and restricted to a comparable small body of literature and long-established cultivation-hubs. The production in the Khánh Hòa province in Viet Nam was established only recently. Therefore, the overarching aim of this thesis was to gain a deeper understanding of *C. lentillifera*'s physiology and biochemical composition over the different stages of the production cycle with a focus on the cultivation at farm VIJA in Van Phong Bay, Khánh Hòa province, Viet Nam in order to identify possible improvements in the quality and quantity of the farmers production. In the next step, the physiological understanding of the effects of certain environmental parameters on the sea grape's composition can be used as a tool-kit to manipulate cultivation conditions and to trigger the accumulation of valuable target compounds, as well as to design potential co-cultivation set-ups for farmers use. Experiments were conducted at the IO in Nha Trang in cooperation with local researchers and at sea grape farm VIJA.

Facing the potential opening of the European market for *C. lentillifera*, the thesis additionally aimed to make a first approach of integrating sea grapes in the well-established RAS cultivation of tropical *L. vannamei* in Germany as a resource-efficient alternative to Asian imports of the fresh product.

1.6   Thesis objectives

Sea grapes have been identified as shade-adapted species with high light irradiances resulting in photooxidative stress [Guo et al., 2015a, Kang et al., 2021] and potentially triggering the algaes' protective mechanisms, like antioxidant production. In Van Phong Bay the sea grapes are exposed to a variety of light environments over the production cycle, which affects the organisms physiology. The farmers acknowledge the organisms' shade-adapted nature

by artificially shading the cultivation ponds. However, it is still unknown, if the shading is well adapted to the sea grapes and the light management needs further attendance over the species production cycle, including the shelf-life, where sea grapes are at a desiccated state and potentially more prone to additional photooxidative stress [Terada et al., 2018].

Hypothesis I: The light management is an essential tool to consider over the *Caulerpa lentillifera* production cycle.

I.A The gauze shading of sea grape ponds at farm VIJA provides a light environment that avoids photooxidative stress of the sea grapes.

I.B *Caulerpa lentillifera* fronds are prone to photooxidative stress in the transparent PET containers during the state of desiccation.

I.C In a two-layer cultivation of sea grapes, the red seaweed *Kappaphycus alvarezii* can function as a natural shade provider.

The sea grape farmers have certain expectations towards their product and the harvested fronds are grouped in different quality categories according to physical characteristics. Reports from Thailand describe the importance of frond's weight, length, number of branches, ramuli density, as well as colouration [Chaiklahan et al., 2020]. It is still unknown which frond properties are decisive for quality of the harvest. Besides the visual properties of the product, sea grapes are in general valued for their nutritional benefits, including AOA and AA composition [Matanjun et al., 2008, Matanjun et al., 2009, Paul et al., 2014, Syakilla et al., 2022]. However, the biochemical composition and the phenotypical morphological and visual properties of seaweeds are highly dependent on the prevailing parameters in the alga's environment [Stengel et al., 2011]. The understanding on one hand of the farmers and costumers needs and on the other hand of the sea grapes' physiological reaction towards their abiotic environment, could enable the design of manipulation techniques to trigger the accumulation of target metabolites or properties. However, the manipulations should be applicable and financially affordable for farmers. Hence, management of light irradiances through adjustment of shading and nutrient supply, e.g. through locally available aquaculture effluents seem feasible.

Hypothesis II: Targeted manipulations of the sea grapes' cultivation environment can be used to increase the nutritional value of the seaweed.

II.A The sea grape fronds weight, length, ramuli density, and rachis, as well as ramuli colouration are decisive characteristics for their quality at farm VIJA.

II.B Provoked photooxidative stress can be used as a tool to trigger the sea grapes' antioxidant production and increase their nutritional value.

II.C Aquaculture effluents of the whiteleg shrimp *Litopenaeus vannamei* can be used as fertilization of *Caulerpa lentillifera* to increase their AA quantity and quality.

The understanding of the farmers' and costumers' needs is important for achieving improvements in the products quality, therefore *hypothesis II.A* provides an essential basis to work on targeted manipulation techniques for the sea grapes nutritional values. The importance and especially the possibilities of a successful light management (*hypothesis I*) would be additionally enforced, if light stress could be used as an antioxidant manipulation tool (*hypothesis II.B*). The *hypotheses I.C* and *II.C* introduce the opportunity of implementing sea grapes with other seaweeds or fed-aquaculture species for a resource-efficient co-cultivation set-up.

Hypothesis III: Co-cultivation set-ups of *Caulerpa lentillifera* with other economically important seaweeds or fed-aquaculture species can enhance the resource-efficiency of the cultivation.

III.A Sea grapes and *Kappaphycus alvarezii* can be cultivated resource-efficiently in a two-layer cultivation in Van Phong Bay, Viet Nam.
III.B Aquaculture effluents of the whiteleg shrimp *Litopanaeus vannamei* can be used as fertilization to grow *Caulerpa lentillifera*.

In order to answer the stated hypotheses, three different steps were taken: First a comprehensive literature review was conducted to summarize relevant literature and identify research gaps (**chapter 2**). Secondly, the sea grapes physiology and biochemistry over the production cycle in respect to the farmers' quality assessment and in relation to the key environmental parameter of light irradiances were conducted (**chapter 3** and **4**). Ultimately, different management tools aiming to increase the sea grapes quality (nutritional value) and quantity (harvest), as well as resource-efficiency during cultivation were investigated for application in Viet Nam. Namely, the targeted exposure to high light irradiances in order to increase the sea grapes antioxidant content (**chapter 5**) and the two-layer cultivation of *C. lentillifera* with economically important carragenophyte *K. alvarezii* (**chapter 6**). Additionally, the potential implementation of sea grape cultivation in Europe, by implementation in a land-based RAS of whiteleg shrimp (*L. vannamei*) taking advantage of the cultivation infrastructure, as well as targeted fertilization with the process water (**chapter 7**) was investigated. Subsequently, all chapters were be discussed in a synoptic discussion (**chapter 8**).

1.7   Publication outline and declaration of author contributions

The research questions and outlines of **publications I – VII** are presented in the following by chapters, followed by title, the authors, the journal and the current status (published, submitted, in preparation).

<u>Research question I</u>:   What are current knowledge gaps regarding sea grape aquacul-

ture?

In **chapter 2** the journal articles on *Caulerpa lentillifera* were systematically scanned and 130 articles published between 1900 and October 2022 were grouped according to their research topic (e.g. *Biochemical composition*, *Ecophysiology*) and research application (e.g. *Fundamental research*, *Nutritional value*). The in-depth analysis of the literature focusing on sea grapes aquaculture, namely the applications of *Cultivation*, *Nutritional value*, and *Post-harvest* revealed among others knowledge gaps regarding the light management of the species, as well as the potential of their cultivation in co-culture set-ups.

Status of **publication I** presented in this chapter:

Title: Sea grapes (*Caulerpa lentillifera*, Chlorophyta) for human use: Structured review on recent research in cultivation, nutritional value, and post-harvest management
Authors: <u>Lara E. Stuthmann</u>[1], Beatrice Brix da Costa[1], Karin Springer, Andreas Kunzmann; [1] joined first-authorship
Journal: Journal of Applied Phycology, published July 2023, doi: 10.1007/s10811-023-03031-x

Research question II: Which characteristics of sea grape fronds are decisive for their grouping to a certain quality?

In **chapter 3** the production cycle at the sea grape farm VIJA was described in detail with a focus on the cultivation and the post-harvest treatment. The harvested sea grape fronds are grouped according to certain characteristics in two qualities of different value, rising research question II. Physical characteristics of the fronds, including size, weight, colour and ramuli density, as well as physiological and biochemical parameters of F_v/F_m, AOA and (TPC) were monitored over two months in order to identify essential characteristics of fronds of the respective quality. A binominal model was run to identify the decisive physical frond characteristics. The results were expected to function as a basis for potential improvements of the cultivation or post-harvest management to increase their quality.

Status of **publication II** presented in this chapter:

Title: Sea grape (*Caulerpa lentillifera*) aquaculture in Van Phong Bay, Viet Nam: Evaluation of the post-harvest quality
Authors: <u>Lara E. Stuthmann</u>, Hoang Trung Du, Beatrice Brix da Costa, Karin Springer, Andreas Kunzmann
Journal: Applied of Applied Phycology, published June 2023, doi: 10.1007/s10811-023-03030-y

Research question III: What is the (mean) irradiance suitable for cultivation and shelf-life in transparent PET containers of sea grapes in respect to their photosynthesis?

Chapter 4 covers the importance of light management during sea grapes' cultivation and post-harvest treatment. The effect of irradiances of Photosynthetically Active Radiation (PAR) on the sea grapes' physiology during the cultivation and the shelf-live in transparent PET plastic containers were investigated. The laboratory experiment was based on the mean irradiances quantified in the cultivation environment, where farmers shade the ponds with gauze material, as a control treatment (50 µmol photons m^{-2} s^{-1}). Respectively a low (25 µmol photons m^{-2} s^{-1}) and high light treatment (100 µmol photons m^{-2} s^{-1}) were designed and the effect on sea grapes' photosynthetic state (F_v/F_m) and the recover potential at control irradiances were tested over two weeks. In the PET packaging environment, sea grapes are air exposed and hence suffer desiccation. In this scenario, high light irradiances are expected to function as an additional stressor. Therefore, the effect of three irradiance environments (darkness, room irradiances, high light) and the potential of recovery at rehydration and room irradiances were investigated.

Status of **publication III** presented in this chapter:

Title: Cultured and packed sea grapes (*Caulerpa lentillifera*): effect of different irradiances on photosynthesis
Authors: Lara E. Stuthmann, Karin Springer, Andreas Kunzmann
Journal: Journal of Applied Phycology, published December 2020, doi: 10.1007/s10811-020-02322-x

Research question IV:
A) Can exposure of sea grapes to high light irradiances be used as a tool to increase the AOA?
B) Which irradiances and exposure times provide a substantial increase of antioxidants, while maintaining a application-adapted colouration?

In **chapter 5** the understanding of the effects of light irradiances of PAR on sea grapes' physiology were applied to use light stress as a management tool to trigger the seaweeds antioxidant production and hence increase the nutritional value. This chapter encompasses two publications. In **publication IV**, the proof of the concept was given. The AOA was quantified at ascending irradiances and the results were compared to dehydrated, commercially available sea grapes and renowned *superfruits* pomegranate and aronia, which are valued for their high content of antioxidants.
In **publication V**, the successful concept was investigated further to determine the potential of application for farmers. Sea grapes' AOA and TPC, as well as colour and Chl a content were quantified at irradiances of $50 - 600$ µmol photons m^{-2} s^{-1} over 14 days. Additionally, the usability of colour measurements from pictures as a non-invasive and inexpensive estimation of Chl a was tested.

Status of **publication IV** presented in this chapter:

Title: The antioxidative potential of sea grapes (*Caulerpa lentillifera*, Chlorophyta) can be triggered by light to reach comparable values of pomegranate and other highly nutritious fruits
Authors: Jonas Sommer, Andreas Kunzmann, <u>Lara E. Stuthmann</u>, Karin Springer
Journal: Plant Physiology Reports, published January 2022, doi: 10.1007/s40502-021-00637-6

Status of **publication V** presented in this chapter:

Title: Improving the nutritional value of edible *Caulerpa lentillifera* (Chlorophyta) using high light intensities. A realistic tool for sea grape farmers
Authors: <u>Lara E. Stuthmann</u>, Revathi Achuthan, Mia Pribbernow, Hoang Trung Du, Karin Springer[1], Andreas Kunzmann[1]; [1] joined senior-authorship
Journal: Algal Research, published July 2022, doi: 10.1016/j.algal.2022.102785

<u>Research question V</u>: Can seaweeds *Caulerpa lentillifera* and *Kappaphycus alvarezii* be cultivated successfully in a two-layer cultivation?

In **chapter 6** the two-layer cultivation of sea grapes with the economically important carragenophyte *Kappaphycus alvarezii* in Van Phong Bay was investigated. The complementary cultivation requirements of light and N source suggested that *K. alvarezii* could provide shade for shade-adapted *C. lentillifera* and both species could use fertilization with process water of locally available *Babylonia areolata* effectively. The implication of *K. alvarezii* in tidal sea grape ponds was studied unsuccessfully. For the implementation of sea grapes in *K. alvarezii* cultivation, two different methods, namely the growth in plastic cages below *K. alvarezii* longlines, as well as the two-layer cultivation of both seaweeds in net cages, were tested. The field experiment was conducted in Nha Trang Bay close to the research facilities of IO. The potential of using *B. areolata* effluents as fertilizer during land-based off-season maintenance was investigated in a laboratory based experiment. At the end of both experiments, growth data, F_v/F_m, diurnal changes of F_v'/F_m', AOA, TPC, C and N tissue contents, as well as the colouration of *K. alvarezii* and information on the initial investment were used to determine the effect of the different cultivation set-ups on the seaweeds in order to derive the respective suitability.

Status of **publication VI** presented in this chapter:

Title: Potential of resource-efficient two-layer cultivation of carragenophyte *Kappaphycus alvarezii* and sea vegetable *Caulerpa lentillifera*
Authors: <u>Lara E. Stuthmann</u>, Beatrice Brix da Costa, Aaron Johannes Cordes, Hoang Trung Du, Andreas Kunzmann[1], Karin Springer[1]; [1] joined senior-authorship
Journal: Aquaculture (submitted July 2023)

Research question VI: Is the process water of whiteleg shrimp *Litopenaeus vannamei* a suitable fertilizer for *Caulerpa lentillifera*? Which dilution could be used in order to increase the seaweeds' nutritional values?

In **chapter 7** a first step towards examining the integration of sea grape cultivation in land-based RAS for *Litopenaeus vannamei* cultivation is done. The cultivation of the tropical whiteleg shrimp is increasingly practiced in Europe and the integration of the (sub-)tropical sea grapes could enable a resource-efficient alternative for the European import from Asia. The fertilization with different dilutions of the shrimp process water with and without additional PO_4^{3-} fertilization were examined. At the end of the experiment, the growth, the share of economically important fronds, as well as the AOA, TPC and C:N ratio were examined. Additionally, the AA quantity and quality (AA pattern) of sea grapes were tested at different fertilization treatments in order to examine the possibility to use the fertilization as a manipulation tool to trigger production of essential AA, e.g. for use as human nutrition or as feed for aquaculture species.

Status of **publication VII** presented in this chapter:

Title: Potential for resource-efficient co-culture and value-adding manipulation: Fertilization of sea vegetable *Caulerpa lentillifera* with process water of *Litopenaeus vannamei*
Authors: Lara E. Stuthmann, Leona Ritter von Stein, Hoang Trung Du, Andreas Kunzmann[1], Karin Springer[1]; [1] joined senior-authorship
Journal: to be decided, in preparation

Table 1.2: Declaration of author contributions as % of total workload.

% of workload	Publication						
	I	II	III	IV	V	VI	VII
Experimental concept and design	60	90	90	30	90	90	90
Experimental work and acquisition of the data	50	60	100	5	100	80	100
Data analysis and interpretation	50	95	95	45	95	95	95
Preparation of figures and tables	50	100	100	25	100	100	100
Drafting of the manuscript	60	95	95	40	95	95	95

Table 1.2 summarizes the declaration of contribution for the respective publications. Additionally, some chapters contain an authorship statement of contributions, as requested by the respective journals.

Chapter 2

Publication I

Sea grape fronds at farm VIJA in Van Phong Bay, Viet Nam
(Foto: K. Springer).

Sea grapes (*Caulerpa lentillifera* J. Agardh, Chlorophyta) for human use: Structured review on recent research in cultivation, nutritional value, and post-harvest management

Lara Elisabeth Stuthmann[1], Beatrice Brix da Costa[1], Karin Springer, Andreas Kunzmann
[1]shared first authorship

Abstract

Seaweeds are a major contributor to global marine aquaculture production, with the biomass being mainly used among others for human nutrition, pharmaceutics, and cosmetics. However, green seaweeds are severely underrepresented, compared to red and brown macroalgae. *Caulerpa lentillifera* (known as sea grapes or green caviar) is an edible, green seaweed with a distinctive texture and various nutritional benefits. In this review, all articles on sea grapes published between 1900 and October 2022 and found in the scientific citation databases Scopus and Web of Science (search string: *caulerpa* AND *lentillifera*) were grouped by research topic and the intended application following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) approach. 51% of the 130 articles included in the review focused on the topic of *Biochemical composition*, followed by *Water treatment* (18%) and *Ecophysiology* (15%). The most prominent application was *Pharmaceutics*, followed by *Cultivation* and *Fundamental research*. In order to provide a knowledge base to researchers and practitioners of *C. lentillifera* aquaculture, research that was simultaneously grouped under one of the topics *Biochemical composition*, *Water treatment*, or *Ecophysiology* and the applications *Cultivation*, *Nutritional value* or *Post-harvest* was summarized in more detail. Light management of sea grapes, their use as a high-value co-culture species and the capacity to bioremediate nutrients, as well as their short shelf-life were identified as important areas of research interest. The assessment revealed several knowledge gaps, for example the need for intra-species comparisons of *C. lentillifera*s' biochemical composition across spatial and temporal scales.

Keywords: Bioremediation, Co-cultivation, Functional Foods, Green caviar, Nutritional value

2.1 Introduction

Macroalgae represent >50% of global marine and coastal aquaculture products (35 million tonnes in 2020, based on Wet Weight (WW)), mainly due to production for human consumption [FAO, 2020, Chopin and Tacon, 2020]. Eight genera of red and brown macroalgae dominate the production, whereas green macroalgae are underrepresented with <1% [FAO, 2020] (reviewed by [Moreira et al., 2021]). However, *Caulerpa* is one genus that is gaining increasing popularity, with the highest mean contribution to global green seaweed cultivation in the years 1950-2019 (annual average of 6404 tonnes WW), but with declining values until 2019 (1090 tonnes WW, [Cai et al., 2021a]). However, these production values are likely to be underestimated, mainly based on reports from The Philippines.

Caulerpa species, and especially edible *C. racemosa* and *C. lentillifera* (known as sea grapes or green caviar) are particularly popular in the Indo-Pacific region, where they are consumed fresh or salt-preserved in salads or as a snack [Long et al., 2020]. In particular, the striking texture (Fig.2.1A [Zubia et al., 2020]), the nutritional benefits, including e.g. the content of bioactive compounds and Polyunsaturated Fatty Acids (PUFA), and the pleasant taste have led to an increasing demand of sea grapes worldwide [de Gaillande et al., 2017, Chen et al., 2019, Zubia et al., 2020]. Compared to average seaweeds, sea grapes achieve high market prices [Dobson et al., 2020, Cai et al., 2021a] and they are proposed as promising functional food ingredient [Syakilla et al., 2022], or certain compounds are being investigated in a pharmaceutical context, e.g. for their antidiabetic and anticancer activities [Daud et al., 2020, Fajriah et al., 2020, Manoppo et al., 2022]. However, the species is not yet covered by the Novel food Regulation of the European Union (EU), which limits the potential customer base [Barbier et al., 2019].

Historically, sea grape cultivation began in Japan (Okinawa) and The Philippines [Trono and Toma, 1993, Yap et al., 2019]. In The Philippines sea grapes were introduced by accident into fish ponds, but the successful growth of the species ensured its targeted cultivation [Trono and Largo, 2019]. As sea grapes can be propagated by fragmentation, they are easy to cultivate without the need for expensive infrastructure or strong expertise (Fig.2.1B-F, [de Gaillande et al., 2017]). Cultivation methods vary according to country and system [Trono and Largo, 2019]. In The Philippines and Viet Nam the algae are grown in perforated plastic trays or nets (tray method) or are planted directly into the sediment in tidal ponds (sowing method, [Rabia, 2016]), sometimes shaded with e.g. gauze material (Fig.2.1B, C). In Japan and China, land-based raceway cultures are increasingly used to meet the high demand for sea grapes [de Gaillande et al., 2017]. However, sea grapes can also be grown in sheltered coastal areas in nets or trays [Tanduyan et al., 2013]. The rapid growth and relatively low habitat requirements of sea grapes have also led to their increased use in integrated aquaculture systems [Paul and de Nys, 2008], in order to mitigate the potentially problematic nutrient rich effluent of wastewaters and to provide an additional income from the metabolized biomass [Largo et al., 2016, Bambaranda et al., 2019a, Bambaranda et al., 2019b, Dobson et al., 2020]. After harvesting of the edible fronds, they are soaked in tanks with seawater to allow the siphonous alga to heal tissue injuries. Subsequently, the fronds meeting the required quality standards (e.g. bright green colour, size) are stored in plastic
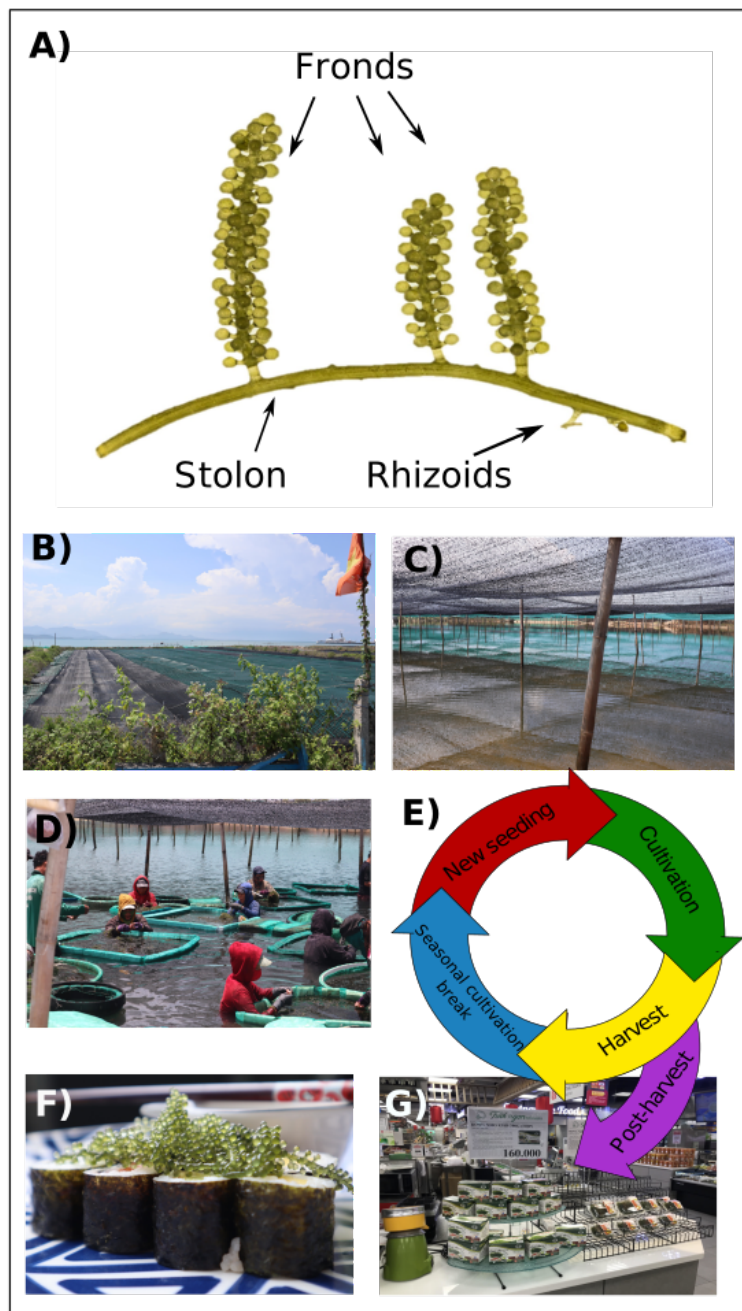
Figure 2.1: **A)** *Caulerpa lentillifera* consists of upright fronds (assimilators) with grape-like, vesiculate ramuli irregularly arranged around a pedicel, which are attached to creeping stolons with rhizoids [Zubia et al., 2020]. The life-cycle of the sea grapes in aquaculture consists of different stages **(E)**. Seedlings are applied to start the cultivation, which can take place in outdoor, tidal ponds **(B)** or in land-based systems. The shade-adapted seaweeds are shaded from the sun, e.g. with gauze material **(C)**. Sea grape fronds reaching harvestable size are continuously harvested during the cultivation season **(D)** and the harvest is collected at a collection point for cleaning and sorting of the product before retail of the fresh or dehydrated sea grapes **(G)**. *C. lentillifera* fronds are e.g. served with sushi or as a salad **(F)**. Pictures were taken at a sea grape farming facility in Viet Nam, Khanh Hoa province.

containers with moisture sheets for shipment or retail as a fresh product, or for preservation (dehydrated or brine-cured, [de Gaillande et al., 2017, Terada et al., 2018, Chaiklahan et al., 2020]). Biomass that does not meet food quality standards (60–70%) is discarded as waste, but there is potential for its further use [Chaiklahan et al., 2020].

Along with the economic interest, the number of scientific publications seems to be increasing. Recent review articles and book chapters focused on the consumption, nutritional value and farming of the genus *Caulerpa* [Chaiklahan et al., 2020], as well as the biology and its use [Zubia et al., 2020] and the nutraceutical and pharmaceutical potential [Darmawan et al., 2020]. To our knowledge one review article from 2019 sums up the research on the species *C. lentillifera* [Chen et al., 2019] and one review summarizes the nutrients, phytochemicals and health benefits [Syakilla et al., 2022].

The present review article aims to (*1*) conduct a scientometric analysis of the published literature to identify trends of the different research topics and applications, in order to reveal knowledge gaps and identify future research directions. To achieve this goal, seven research topics (e.g. *Biochemical composition*, *Genetics*, *Water treatment*) and nine research applications (e.g. *Pharmaceutics*, *Fundamental research*, *Cultivation*) were formulated and the articles were grouped in the respective topic and application. In a next step (*2*), the literature focusing on the aquaculture of *C. lentillifera*, namely the topics of *Cultivation parameters*, *Nutritional value*, and *Post-harvest* applications were summarized in concise manner to provide a structured overview for practitioners in the field and researchers working with this species.

2.2 Material and methods

2.2.1 Literature review

We conducted a systematic literature search using two popular scientific citation databases, namely Web of Science (WoS) and Scopus. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement was applied [Liberati et al., 2009]. In both databases the search string *caulerpa* AND *lentillifera* for the period 1900 to 2022 was used to search within title, keywords and abstract. The search took place on 29.10.2022 and resulted in a total of n=192 studies (after removal of duplicates, Fig.2.2).

2.2.2 Selection criteria

In order to check for eligibility of the studies the following selection criteria were used (*a*) *Caulerpa lentillifera* is a main topic of the article and (*b*) language of the article was English, (*c*) the article was not a review article, (*d*) scientific accuracy was given. This was evaluated by screening the titles and abstracts of the documents. In case the information provided was not sufficient to determine the question, the complete document was screened.

Identification of studies via databases and registers

Identification

Records identified from:
    Scopus (n =155)
    Web of Science (n= 149)

Records removed *before screening*:
    Duplicate records removed (n = 107)

Screening

Records screened
(n = 192)

Records excluded
(n = 0)

Reports sought for retrieval
(n = 192)

Reports not retrieved
(n = 7)

Reports assessed for eligibility
(n = 185)

Reports excluded:
    Reviews (n = 8)
    Wrong topic (n = 38)
    Language (not English) (n = 7)
    Lack of scientific accuracy: (n = 2)

Studies included in review
(n = 130)

Figure 2.2: Flow diagram for the systematic literature review on *Caulerpa lentillifera*. The different stages for the identification, screening, and inclusion of relevant articles are shown. A template of the flow diagram was downloaded from www.prisma-statement.org.

2.2.3 Data extraction

From the studies declared *eligible* according to the criteria (n=130, Fig.2.2), the following information was extracted: (*a*) publication year, (*b*) study location, (*c*) affiliation of the main author, (*d*) location of the affiliation of the main author, *(e)* type of article (journal article, review article, book chapter, book, technical report), (*f*)topic of research (definitions in Appendix A.1) and (*g*) application of research (definitions in Appendix A.1). The topics and applications were defined by the authors after reviewing the existing literature. The search and extraction criteria were tested by a pilot classification, where two authors (BBC and LES) categorized 30 studies and discussed and cross-checked their choices in order to ensure a reliable and homogenized coding. Two papers, namely [Stuthmann et al., 2020] and [Paul et al., 2014] have been sorted in two categories since they dealt with various topics and/or applications (*Ecophysiology – Post-harvest* & *Ecophysiology – Cultivation* and *Biochemical composition – Nutritional value* & *Water treatment – Cultivation*, respectively) within each respective article.

This review was set-up on one hand as a scientometric analysis of the existing literature in order to identify research trends and knowledge gaps, and on the other hand as a contextual synopsis of sea grape aquaculture for practitioners and field-researchers. Hence, the review investigated certain combinations of topics and applications in more contextual detail, namely the topics of *Ecophysiology*, *Biochemical composition* and *Water treatment* with the respective applications of *Cultivation*, *Nutritional value* or *Post-harvest*. In a subsequent discussion, first the results of the scientometric analysis were considered and secondly the contextual summary was analysed across topics and applications with a focus on the peculiarities and knowledge gaps identified. The salinity units were reported as they appeared in the respective papers.

2.3 Results

2.3.1 Scientometric analysis: Number of publications, research topics and applications

Since 1990, eight review articles on the topic of *Caulerpa lentillifera* have been published, but all of them in the period 2019-2022, and six of them focused only on sea grapes. Until the search for this review (October 2022) 130 research articles were published (Fig.2.3A). However, since 2018, the annual number of published journal articles about sea grapes was $\geq$10, and in total added up to 86 (66% of total published journal articles).

The majority of articles were on the topics of *Biochemical composition* (51%), followed by *Water treatment*, *Ecophysiology* and *Genetics*, whereas only $\leq$5 articles researched *Microbiome*, *Distribution* and *Ethnophycology*, respectively (Fig. 2.3B). In contrast, the application of the research articles was more distributed, with *Pharmaceutics* having the highest and *Feedstock* and *Cosmetics* the lowest count, focusing on the use for bio-oil production [Ong et al., 2019, Wuttilerts et al., 2019] or in creams ([Thu et al., 2018, Chang et al., 2021], Fig.2.3C). The application as *Animal feed* was also underrepresented with only three stud-
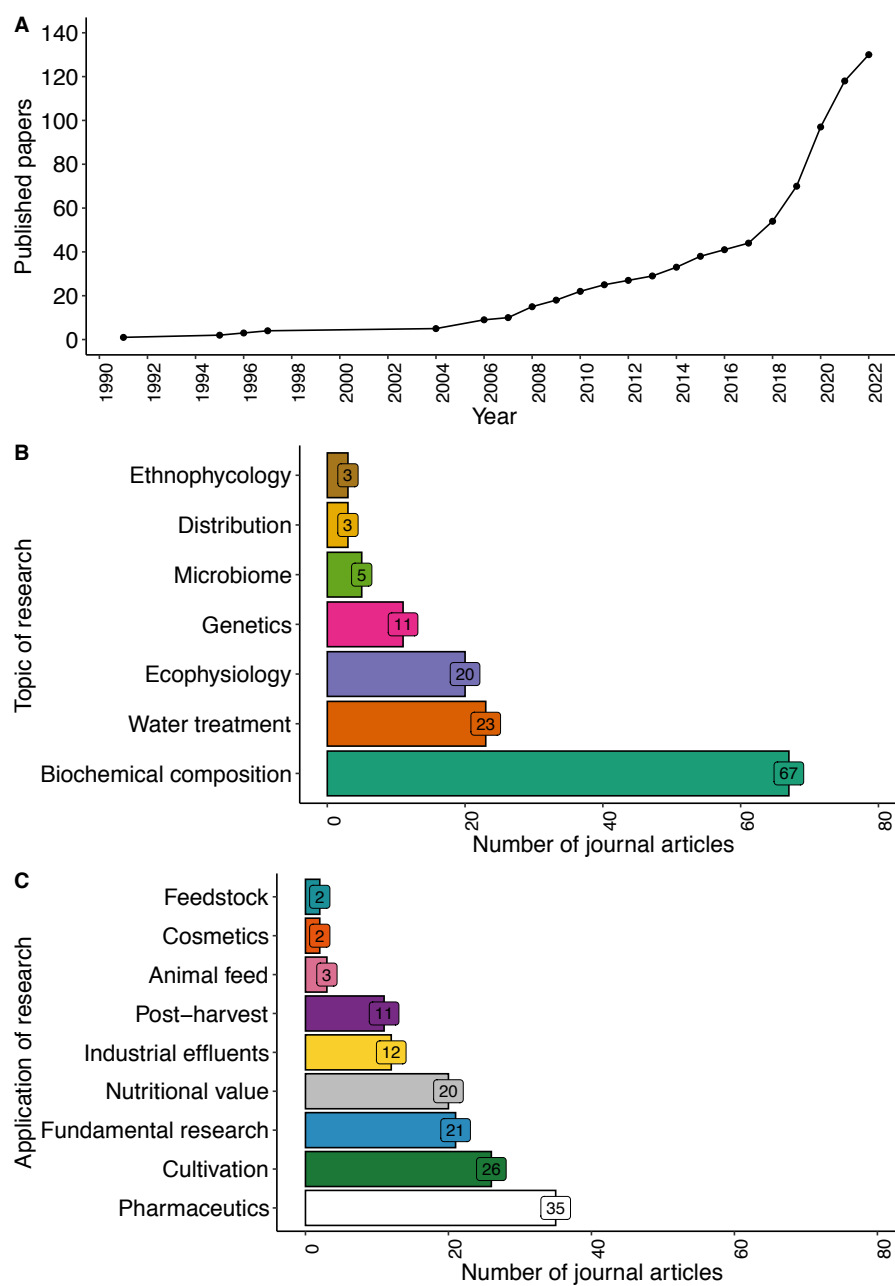
Figure 2.3: **A**) Cumulative plot of published papers on *Caulerpa lentillifera* (journal articles only) and the respective **B**) topics, as well as **C**) applications of the research presented in the studies.

ies, using sea grapes as fish [Ilias et al., 2015, Arisa et al., 2020] and shrimp [Putra et al., 2019] feed. The application of *Pharmaceutics* was made up almost exclusively by articles from the research topic *Biochemical composition*, majorly contributing to the high frequency of this topic (Fig.2.4) and focusing on various bioactivities of the seaweeds' metabolites, including anti-inflammatory [Yoojam et al., 2021], anti-diabetic [Khairuddin et al., 2020] and anti-viral [You et al., 2022a]. *Industrial effluences* encompassed mainly articles, where *C. lentillifera* biomass was used as a bio-adsorbent for basic dyes [Marungrueng and Pavasant, 2006, Marungrueng and Pavasant, 2007, Pimol et al., 2008] and heavy metals [Apiratikul and Pavasant, 2006, Pavasant et al., 2006, Apiratikul and Pavasant, 2008, Apiratikul et al., 2011, Zakeri and Bakar, 2013, Apiratikul, 2017, Apiratikul, 2020, Li et al., 2021]. The application of *Fundamental research* encompassed all studies of the topics *Genetics* and *Distribution*, as well as a few on *Biochemical composition*, *Ecophysiology* and *Ethnophycology* (Fig.2.4). Studies within the topic of *Genetics* focused e.g. on the alga's chloroplast [Gao et al., 2018], mitochondrial [Zheng et al., 2018, Jia et al., 2019] and complete genome [Arimoto et al., 2019a] and Deoxyribonucleic Acid (DNA) in their pyrenoid core [Miyamura and Hori, 1991, Miyamura and Hori, 1995], as well as on population genetics [Benzie et al., 1997, Kazi et al., 2013]. It accompanied research from the topic *Distribution*, reporting on (re)discoveries of *C. lentillifera* in the Gulf of Mannar [Mary et al., 2009] and Gulf of Kutch [Mantri, 2004], India and Hainan Island, China [Gao et al., 2020]. The topic *Microbiome* encompassed four studies, of which half were on the application of *Post-harvest*, namely the effect of season, washing [Pang et al., 2022] and petrifilm aerobic count plate [Kudaka et al., 2010] and the other half on *Cultivation*, dealing with the microbiome of healthy and diseased *C. lentillifera* [Liang et al., 2019, Kopprio et al., 2021].

2.3.2 Scientometric analysis: Research networks

The majority of journal articles were published by first authors who were affiliated with institutions in Asia (Fig.2.5), particularly in China (n=27, 20.8%), Thailand (n=23; 17.7%), Malaysia (n=18; 13.8%), Indonesia (n=12, 9.2%), and Japan (n=11; 8.5%). Besides, outside of Asia authors with affiliations in Australia (n=6, 4.6%) and Germany (n=4; 3.1%) were majorly present. The seven papers that were not included in the study, because they were not written in English were published in Japanese (n=4), Chinese (n=2) and Bahasa Indonesia (n=1).

In the following, the main output articles grouped in the topics of *Ecophysiology*, *Biochemical composition* and *Water treatment* with the respective applications of *Cultivation*, *Nutritional value* or *Post-harvest* were summarized by topics.

2.3.3 Ecophysiology

A total of 20 papers were grouped under the topic *Ecophysiology* with 17 articles conducting research on the topic of *Ecophysiology* and the application of *Cultivation* (n=9), followed by *Post-harvest* (n=6) and *Nutritional value* (n=2, Fig.2.4, Fig.2.6). The studies focused on the effect of one or more abiotic parameters on the physiology of the alga, and light was the major parameter studied (n=8, Fig.2.6A). The response variables seemed to depend on the appli-
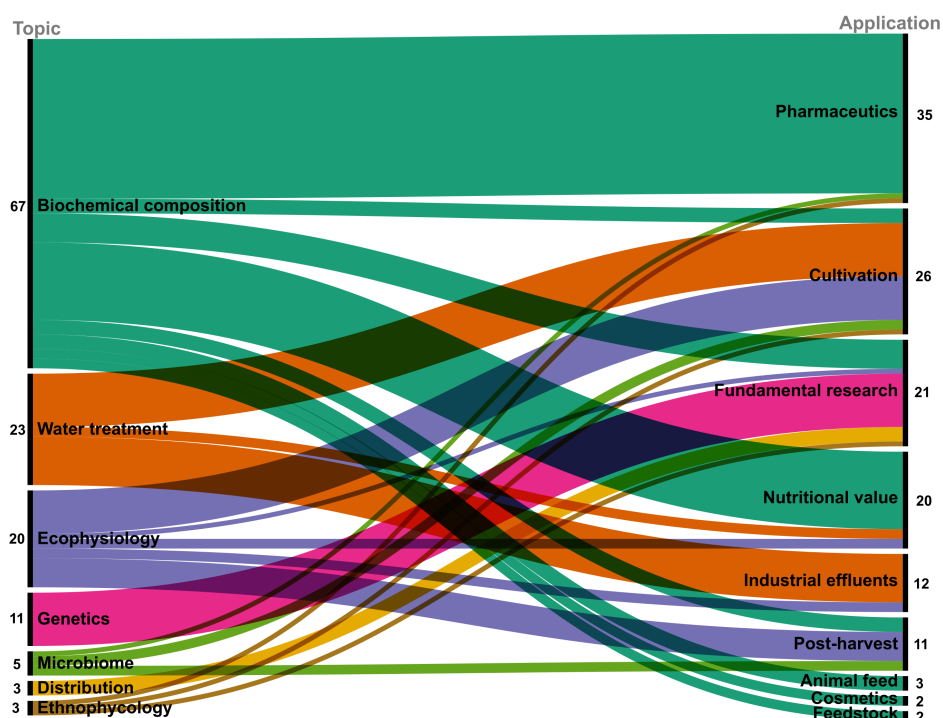
Figure 2.4: Sankey-plot visualizing the distribution of *Caulerpa lentillifera* related articles by topics (*left*) and applications (*right*). The numbers represent the articles included in the respective category. In total, 130 articles were included. Two papers were sorted in two categories since the articles dealt with various topics and/or applications.
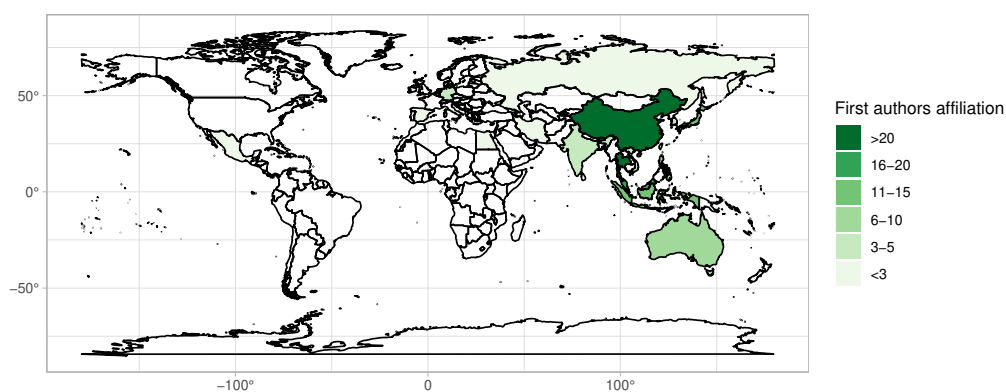
Figure 2.5: Affiliations of first authors, who have published *Caulerpa lentillifera* related articles. The countries were grouped by the number of appearances.

cation (Fig.2.6B), with biomass production, biochemical composition and Chlorophyll (Chl) *a* fluorescence being most commonly used for research in the topic of *Cultivation*, whereas studies focusing on *Post-harvest* mainly quantified water content, biochemical composition and colour/ pictures (Fig.2.6B). Most studies were designed to test the effect of a single factor (n=13), rather than running a crossed design experiment (n=4, Fig.2.6C).

2.3.3.1 Cultivation

Biomass production (n=6) and the biochemical composition (n=6), including especially pigment [Guo et al., 2015a, Guo et al., 2015b, Kang et al., 2021, Cai et al., 2021b], protein [Long et al., 2020, Cai et al., 2021b] or fatty acid content and pattern [Long et al., 2020], were the most frequently used response variables (Fig.2.6B, Appendix A.2), whereas light was majorly used as an experimental parameter (n=5, Fig.2.6A). The shade-adapted sea grapes showed highest biomass productions at Photosynthetically Active Radiation (PAR) of 40 and 100 µmol photons m^{-2} s^{-1}, compared to 20 and 100 µmol photons m^{-2} s^{-1} [Guo et al., 2015a] and 50 and 150 µmol photons m^{-2} s^{-1} (1Blue, 5Red Light Emitting Diode (LED), [Kang et al., 2020]), respectively. However, irradiances of $\geq$100 µmol photons m^{-2} s^{-1} were reported to cause physiological stress [Guo et al., 2015a, Kang et al., 2020, Stuthmann et al., 2020]. Besides the level of irradiances, which also impacted sea grapes' morphology [Guo et al., 2015a, Fakhrulddin et al., 2021], the photoperiod and the light spectrum had profound effects on the physiology of sea grapes, resulting in different biomass productivities, pigment contents and bioactivities [Kang et al., 2020]. Blue light triggered Phytoene Desaturase (PDS) expression and Antioxidant Activity (AOA), whereas red light rather enhanced biomass production. Hence, authors recommended a spectrum of 1B5R (16.7% blue + 83.3% red) and a photoperiod of 12 h light and 12 h darkness for the indoor cultivation of sea grapes [Kang et al., 2020]. Ultraviolet (UV) light is known to induce oxidative stress in seaweeds [Dring, 2005] and a reaction of *C. lentillifera* to different exposure scenarios is to be expected. However, only low absorbance in the UV-spectrum was recorded for sea grapes [Tanaka et al., 2020] and the effect of UV light on the ecophysiology of *C. lentillifera* has not yet been investigated. Despite light being the main experimental stressor, the studies were only running for 7 days until 4 weeks and only one article focused on shorter exposure times (<72 h, Appendix A.2, [Terada et al., 2021]).

Three studies focused on the effects of temperature, salinity, and nutrients on the physiology of *C. lentillifera*, respectively (Fig.2.6A, Table 2.1). Temperature and salinity had profound effects on the biomass production of sea grapes, with highest growth rates at 27°C and 27.5°C (at 60 and 40 µmol photons m^{-2} s^{-1}, respectively [Guo et al., 2015a, Cai et al., 2021b]) and 35 Practical Salinity Units (PSU) [Guo et al., 2015b, Tanaka et al., 2020]. Temperatures and salinities outside of the optimal conditions caused not only a decrease in biomass production, but also changes in the photosynthetic efficiency F_v/F_m, photosynthesis vs. irradiance curve parameters, enzymatic antioxidant expression (Catalase (CAT), Superoxide Dismutase (SOD)) and pigment content (Table 2.1).

With regards to nutrients, the effect of Nitrate (NO_3^-) [Guo et al., 2015b, Cai et al., 2021b] and Phosphate (PO_4^{3-}) levels [Guo et al., 2015b], as well as different fertilizers [Fakhrulddin et al., 2021] on the physiology and growth of sea grapes were tested. NO_3^- levels did not
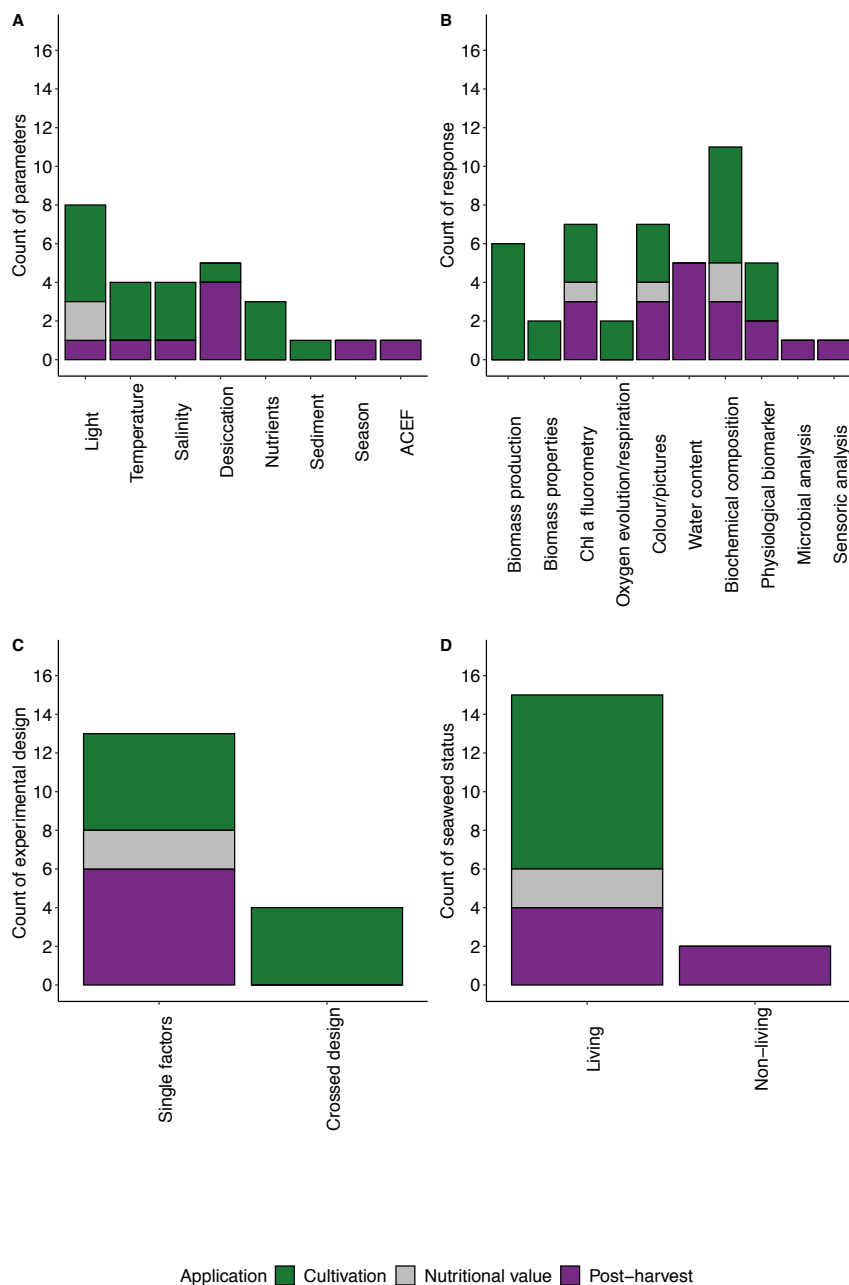
Figure 2.6: Count of (**A**) experimental parameters tested, with alternating current electric field abbreviated as ACEF, (**B**) response variables quantified, (**C**) experimental design and (**D**) seaweed status performed in papers in the topic of *Ecophysiology*, *Nutritional value* and *Post-harvest*, grouped by the different applications (Count of papers: *Cultivation* (n=9), *Nutritional value* (n=2), *Post-harvest* (n=6)).

affect the growth rates of sea grapes at PO_4^{3-} levels of 10, 29, and 400 µmol L^{-1} [Guo et al., 2015a, Cai et al., 2021b], respectively; whereas an effect was reported at 100 µmol L^{-1} of PO_4^{3-} [Guo et al., 2015b]. Consequently, the presence of commercial fertilizers did result in higher biomass production, compared to the control with natural sea water [Fakhrulddin et al., 2021]. Increasing Nitrogen (N) levels lead to ascending Chl *a*, Carotenoid (Car) and soluble protein concentrations [Guo et al., 2015b, Cai et al., 2021b], which might however also depend on the prevailing PO_4^{3-} concentrations [Guo et al., 2015b]. Nutrient accumulation of *C. lentillifera* seemed also influenced by the presence of bottom sediment, which caused an increase in ash, mineral elements and heavy metals, and changes in the Amino Acid (AA) composition; but decreased the content of PUFAs and carbohydrates [Long et al., 2020].

Five studies investigated the effect of a single parameter, while four quantified cross effects (Fig.2.6C, Table 2.1). Interactive effects of light and temperature [Guo et al., 2015a, Terada et al., 2021], Phosphorus (P) and N concentrations [Guo et al., 2015b], and N levels and temperature [Cai et al., 2021b] were studied. For instance, effects on photosynthesis and respiration of *C. lentillifera* caused by temperature were reversed by increases in the NO_3^- level, implicating that eutrophication and climate change could have interactive effects on sea grapes during cultivation [Cai et al., 2021b].

2.3.3.2 Post-harvest

Six papers investigated the ecophysiology of *C. lentillifera* with a focus on their post-harvest. Four of these focused on the post-harvest storage of the fresh seaweed product in different packaging environments, where desiccation was the main stressor. Therefore, the authors most frequently chose water content as the essential response variable (Fig.2.6A, B, [Terada et al., 2018, Stuthmann et al., 2020, Liang et al., 2021, Sulaimana et al., 2021].

Desiccation of *C. lentillifera* fronds, independent of the different packaging materials and experimental set-ups, resulted in varying degrees of water loss over different experimental runs, from ∼5% after 5 days [Liang et al., 2021], to ∼25-40% after 9 days [Sulaimana et al., 2021] and ∼9 to 72% water loss after 12 days (Table 2.1, [Terada et al., 2018, Stuthmann et al., 2020]). Desiccation induced oxidative stress, quantified by decreasing F_v/F_m values [Terada et al., 2018, Stuthmann et al., 2020, Liang et al., 2021], increasing levels of stress biomarkers, including Malondialdehyde (MDA), Superoxide Anion (O_2^-), Hydrogen Peroxide (H_2O_2), peroxidase, proline and antioxidant enzymes (CAT, SOD, [Liang et al., 2021, Sulaimana et al., 2021]), as well as decreases in Chl *a*, *b* and soluble protein content [Liang et al., 2021, Sulaimana et al., 2021]. However, when sea grapes were rehydrated after desiccation, a recovery, e.g. by increasing photosynthetic efficiency values (F_v/F_m) was documented [Terada et al., 2018, Stuthmann et al., 2020, Liang et al., 2021]. Supra-optimal light irradiances of PAR induced additional stress on the physiology of sea grapes, resulting in higher F_v/F_m decreases and the trend of colour loss [Stuthmann et al., 2020]. Hence, room irradiances (3 µmol photons m^{-2} s^{-1}) were suggested during sea grape storage [Stuthmann et al., 2020].

Sea grapes are commonly stored for transport or retail in plastic containers, however, the plastic material differs and seemed to affect the physiological response of the seaweed

[Stuthmann et al., 2020, Terada et al., 2018]. Additionally, the initial constitution of the sea grapes, e.g. influenced by the harvesting season, had an effect on the physiological response during desiccation, with slower decomposition rates at better initial physiochemical constitutions [Sulaimana et al., 2021]. Additionally, applying alternating current electric field on *C. lentillifera* to suppress Reactive Oxygen Species (ROS) accumulation during storage resulted in reduced water loss, Chl and phenol degradation, as well as MDA production and thus provides a post-harvest treatment method with potential that should be further investigated [Sulaimana et al., 2021].

Two studies investigated the ecophysiology of sea grapes which were not alive (Fig.2.6D), but cured in a brine solution and oven-dried [Anantpinijwatna et al., 2018] during post-harvest to extend the shelf-life [Tolentino et al., 2021]. Storage in brine solutions $\leq$5% exceeded the bacterial limit and was rated less acceptable in sensory testing, whereas storage in brine solutions of 10, and 15% for ten days had acceptable bacterial counts and better sensory evaluations, regarding e.g. colour, odour and texture, especially after re-hydration [Tolentino et al., 2021]. However, total Chl and Car content decreased significantly more at higher salinity concentrations [Tolentino et al., 2021].

Table 2.1: Compilation of the different environmental cultivation parameters and their ecophysiological effects on sea grapes (*Caulerpa lentillifera*) during cultivation, post-harvest and in co-culture set-ups.

Environmental parameter	Application	Effect	Studied interactions with...	Source
Light	Irradiances (PAR[1])	Cultivation	higher growth rate at 40 µmol photons m^{-2} s^{-1} (27.5°C), than at 20 and 100 µmol photons m^{-2} s^{-1}, physiological stress at $\geq$ 100 µmol photons m^{-2} s^{-1}	temperature	[Guo et al., 2015a, Stuthmann et al., 2020, Fakhrulddin et al., 2021, Kang et al., 2020, Terada et al., 2021]
		Post-harvest	room irradiances (3 µmol photons m^{-2} s^{-1}) evoked best F_v/F_m values and least water losses, compared to high irradiances (70 µmol photons m^{-2} s^{-1}) and dark treatment	desiccation	[Stuthmann et al., 2020]
		Nutritional value	antioxidant activity was triggered on level of pomegranate by light stress (300 µmol photons m^{-2} s^{-1}, 14 days); antioxidant activity and total phenolic content more than doubled during irradiance exposure (50-600 µmol photons m^{-2} s^{-1}, 3-14 days), but targeted increase in antioxidant activity and total phenolic content negatively correlated to F_v/F_m, bleaching was caused with decrease in green colouration		[Sommer et al., 2022, Stuthmann et al., 2022]
	Photoperiod	Cultivation	12:12 light:dark had best weight gain percentage, compared to 8:16 and 16:8		[Kang et al., 2020]
	Spectrum	Cultivation	1:5 ratio of blue:red light (at 100 µmol photons m^{-2} s^{-1}, 12:12h photoperiod) resulted in good growth and development		[Kang et al., 2020]
Temperature		Cultivation	higher growth rates at ∼27°C, than at 20-25 and 30°C, temperatures of 25-30°C induced the formation of branches, enzymatic antioxidants (superoxide dismutase, catalase), chlorophyll *a* and carotenoid trend of being decreased at 27°C temperatures, compared to 22°C, photosynthetic parameters depended on nitrogen levels	nutrients	[Guo et al., 2015b, Cai et al., 2021b, Terada et al., 2021]

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[1]Photosynthetically active radiations

	Post-harvest	oven-drying temperatures (50-80°C) affected colour of sea grapes and energy consumption of the process over different drying times		[Anantpinijwatna et al., 2018])
Salinity	Cultivation	survival between 20 – 50 PSU, development at 30 – 40 PSU, maximum growth rate at 35 PSU, considering a range from 15 – 55 PSU, negative growth at salinities < 20 PSU, decrease of growth with salinities from 35 (5.62% day^{-1}), 30, 25 (2.54% day^{-1})		[Guo et al., 2015b, Tanaka et al., 2020, Fakhrulddin et al., 2021]
	Post-harvest	concentration of brine solutions of 10, 15% resulted in sensory evaluation and acceptable bacterial count, but total chlorophyll and carotenoid content decreased, compared to lower brine solution concentrations (0, 5%), where bacterial counts exceeded the acceptable limit		[Tolentino et al., 2021]
Desiccation	Cultivation	continuous desiccation at humidity of 50% for $\geq$ 60 min. did not recover F_v/F_m after rehydration, F_v/F_m drop, when absolute water content was $\leq$90%	temperature	[Terada et al., 2021]
	Post-harvest	progressing desiccation resulted in decreasing water content, F_v/F_m, and increasing content of oxidative stress biomarkers, re-hydration lead partly to recovery reactions	light	[Terada et al., 2018, Stuthmann et al., 2020, Liang et al., 2021, Sulaimana et al., 2021]
Nutrients	Cultivation	increased nitrate levels (48, 188, 750 µmol L^{-1}) caused increased soluble protein, chlorophyll a, carotenoid contents, photosynthetic parameters depended on temperature, interaction between phosphor and nitrogen contents in surrounding water	temperature	[Guo et al., 2015b, Cai et al., 2021b, Fakhrulddin et al., 2021]
Bottom sediment	Cultivation	presence increased ash, mineral elements, heavy metals		[Long et al., 2020]

2.3.4 Nutritional value

The nutritional value was the focus of two studies from the topic *Ecophysiology*. The AOA was triggered by exposure of sea grapes to light-stress and resulted in higher AOA values of light-stressed algae (300 µmol photons m^{-2} s^{-1}, 14 days), compared to the dehydrated product and similar values to that of the renowned *super fruit* pomegranate [Sommer et al., 2022]. However, the Chl content decreased during light-stress exposure, causing a bleaching of the alga and potentially decreasing consumer acceptance [Stuthmann et al., 2022]. Therefore, the duration and intensity of the light treatment should be applied to the intended usage of the biomass as a fresh (e.g. food product) or dry (e.g. cosmetic) product. Medium irradiances (200-600 µmol photons m^{-2} s^{-1}) and shorter exposure periods (3-7 days) resulted in significantly enriched antioxidants, but without strong bleaching of the sea grapes, whereas high irradiances and longer exposure periods (200-600 µmol photons m^{-2} s^{-1}, up to 14 days) increased antioxidants even more, but with significant loss of Chl and colour [Stuthmann et al., 2022].
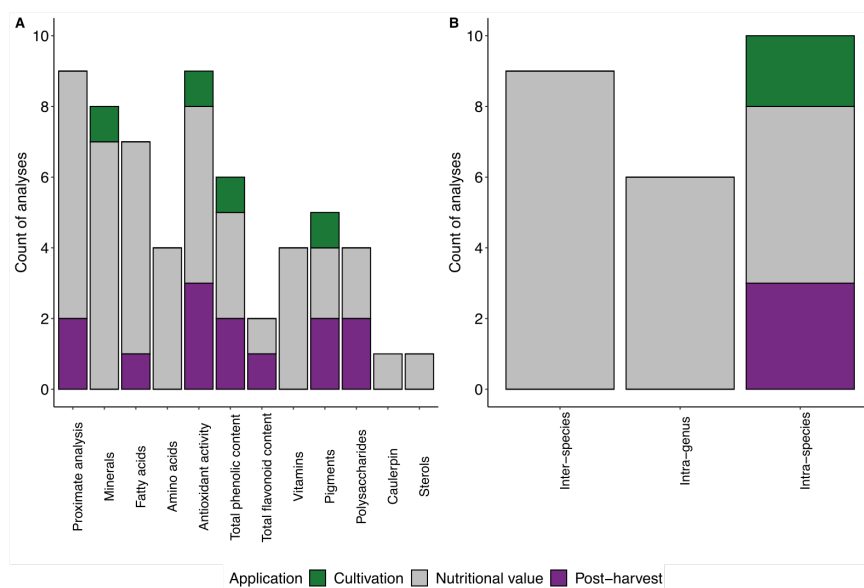
Figure 2.7: Count of (**A**) analyses and (**B**) comparison with species of other genus (interspecies), other species from the genus *Caulerpa* (intra-genus) and comparisons of *Caulerpa lentillifera* (intra-species) performed in papers in the topic *Biochemical composition*, grouped by the different applications (Count of papers: Cultivation (n=2), Nutritional value (n=16), Post-harvest (n=3))

2.3.4.1 Biochemical composition

The topic *Biochemical composition* comprised the overall highest number of papers, compared to the other topics (Fig.2.3A), with the applications of *Nutritional value* (n=16), *Post-harvest* (n=3) and *Cultivation* (n=3) accounting for a total of 23 (Fig.2.4). The authors focused on different biochemical compounds and hence conducted various analyses (Fig.2.7, Appendix

A.3, TableA.2). Considering all three applications, proximate analysis (n=9) and AOA (n=9) were analysed most frequently, followed by mineral (n=8) and fatty acid analysis (n=7) and the Total Phenolic Content (TPC) (n=6, Fig.2.7A). Most of the studies compared *Caulerpa lentillifera* organisms (intra-species comparisons, n=10) from different regions [Zhang et al., 2020], cultivation seasons [Wichachucherd et al., 2019] or set-ups [Syamsuddin et al., 2019], whereas nine and six studies compared *C. lentillifera* with species from a different (inter-species comparison) or the same genus (intra-genus comparison, Fig.2.7B).

2.3.4.2 Cultivation

Two intra-species comparisons quantified seasonal [Wichachucherd et al., 2019] and cultivation method [Syamsuddin et al., 2019] related effects on the biochemical composition of sea grapes in the frame of their *Cultivation* (Table 2.2, Fig.2.4). Indoor and outdoor cultivation of *C. lentillifera* resulted in biochemical differences in sea grape tissue, possibly due to sediment type and light irradiances. *C. lentillifera* showed differences in mineral (indoor: 49.92-52.79% ash, outdoor: 32.04–36.60% ash), Car (indoor: 1.32-2.11 ppm, outdoor: 1.71-2.29 ppm) and fibre (indoor: 5.03-5.56%, outdoors: 7.64-8.65%) content, as well as weight increase (indoor: 1.17–80.12 g, outdoor: 1.66–25.15 g, [Syamsuddin et al., 2019]). However, substrate mixture, culture depth [Syamsuddin et al., 2019] and temporal changes in salinity and NO_3^- content [Wichachucherd et al., 2019] also caused differences in specific target substances.

2.3.4.3 Nutritional value

The edibility of sea grapes was the most common reason given for their research by scientists investigating the *Nutritional value* (Table 2.2). The components examined seemed to follow a pattern. Analysis of proximate composition was often conducted in combination with the fatty acid and AA content, vitamins and minerals [Ratana-arporn and Chirapart, 2006, Salleh and Wakid, 2008, Matanjun et al., 2009, Zhang et al., 2020]. Researchers also focused on the antioxidant composition, namely the AOA, TPC and/or Total Flavonoid Content (TFC) [Matanjun et al., 2008] in combination with minerals [Nufus et al., 2019, Ismail et al., 2020], pigments [Balasubramaniam et al., 2020], or the proximate composition ([Nguyen et al., 2011], Table 2.2). The authors quantified mean water contents ranging from 87.05–95.95% Fresh Weight (FW). Sea grapes were rather high in carbohydrates (27.19-72.90% Dry Weight (DW)) and crude proteins (9.26-19.38% DW), but lower in crude lipids (0.70-2.87% DW) and fibre (1.91-12.98% DW). The mean ash content ranged widely (2.10-47.80% DW), which might have influenced the equally wide range of minerals (Appendix A.3, TableA.3). Overall, highest concentrations of macro- and microminerals were found for Sodium (Na) (1229.7-16050 mg 100 g^{-1} DW) and Iron (Fe) (9.3-1972.9 mg 100 g^{-1} DW), respectively (Appendix A.3, TableA.3). Regarding the fatty acid composition, *C. lentillifera* contained mostly saturated fatty acids (40.7-82.69% of total fatty acids) and approximately similar amounts of Monounsaturated Fatty Acids (MUFA)s (8.43-36.83% of total fatty acids) and PUFAs (9.49-38.07% of total fatty acids). Palmitic acid (C16:0, 8.74–49.46% of total fatty acids), omega-6 PUFA Linoleic acid (C18:2N6C, 4.26-11.85% of total fatty acids) and omega-3 PUFA α-Linolenic (C18:3N3, 2.73-13.42% of total fatty acids) were most abundant (Appendix A.1, TableA.3). The total AA

(101.63-147, with for human essential 44.02-57.01 and non-essential shares 54.08-89.99 mg g^{-1} DW) were mainly represented by the Essential Amino Acid (EAA) Glutamic Acid (Glu) (13.47-17.8 mg g^{-1} DW), Aspartic Acid (Asp) (8.33-14.89 mg g^{-1} DW) and Glycine (GLY) (5.14-19.23 mg g^{-1} DW) and the non Essential Amino Acid (non-EAA) Valine (Val) (6.18-11.16 mg g^{-1} DW), Leucine (Leu) (7.79-12.86 mg g^{-1} DW) and Phenylalanine (Phe) (4.81-19.95 mg g^{-1} DW, Appendix A.3, TableA.3). Lysine (Lys) (1.22-8.2 mg g^{-1} DW) was reported to be the most limiting AA in *C. lentillifera* [Matanjun et al., 2008, Terriente-palacios and Castellari, 2022].

The TPC of *C. lentillifera* was quantified using the Folin-Ciocalteu (FC) assay and ranged from 1.30 to 57.97 mg gallic acid as Gallic Acid Equivalents (GAE) g^{-1} DW (Appendix A.3, TableA.3, [Matanjun et al., 2008, Nguyen et al., 2011, Ismail et al., 2020]). The TFC was only determined once (1506.41 mg Quercetin Equivalents (QE) 100 g^{-1}, [Ismail et al., 2020]). Different assays with individual sets of (dis)advantages [Karadag et al., 2009] were often used supplementary and correlations between the results were common [Matanjun et al., 2008].

Pigment composition was quantified by two studies, whereas one only focused on Chl *a* and *b* (258±25 and 147±14 mg 100 g^{-1} DW) and the other one on a variety of others, including canthaxanthin, and astaxanthin. Both studies also determined β-carotene (15±1.0 and 19.5±0.0 mg 100 g^{-1} DW) content (Appendix A.3, Table A.8).

The vitamin contents of vitamins A, B1 (thiamine), B2 (riboflavin), B3 (niacin), C (ascorbic acid), and E (α-tocopherol) were investigated in four studies. Vitamin C (1-50.33 mg 100g^{-1} WW) was the most prominent vitamin, followed by E (2.22-8.41 mg 100g^{-1} WW). However, B vitamins 1, 2, 3 were present in concentrations <1.1 mg 100g^{-1} WW (Appendix A.3, Table A.9). Vitamin D content has not yet been quantified, even though the vitamin was found in other (green) seaweeds [Debbarma et al., 2016].

The majority (n=9) of studies conducted an inter-species comparison of *C. lentillifera* with seaweeds from genera outside of *Caulerpa*, followed by six studies comparing the alga with other *Caulerpa* species and five studies investigating only *C. lentillifera* (Fig.2.7B). Authors stated that they chose the seaweeds based on their presence at the study location, and often also due to their role in human nutrition. Hence, *Sargassum* and *Eucheuma* were most prominent, besides *C. lentillifera* (Table 2.2, [Salleh and Wakid, 2008, Matanjun et al., 2008, Matanjun et al., 2009, Balasubramaniam et al., 2020]. In the direct comparison with these seaweeds, *C. lentillifera* showed significantly enriched carbohydrate, Na, Magnesium (Mg), and total AA contents, but was significantly depleted e.g. in ash, crude fibre, and Iodine (I) [Matanjun et al., 2009]. On the other hand, *C. lentillifera* had by far the highest ash content when compared to *Chaetomorpha*, *Gracilaria* and *Ulva* [Setthamongkol et al., 2015]. The vitamin contents were in a similar range with *Sargassum* and *Eucheuma* species [Salleh and Wakid, 2008, Matanjun et al., 2009], but seemed to be higher compared to *Ulva reticulata* [Ratana-arporn and Chirapart, 2006]. Besides, the PUFA content of *C. lentillifera* was significantly lower compared to *E. cottonii* and *S. polycystum* [Matanjun et al., 2009]. Homotaurine and hypotaurine contents of *C. lentillifera* (0.60±0.05, 0.14±0.02 mg 100 g^{-1} DW), as well as the EAA to non-EAA ratio (0.51±0.02) were compared to a variety of other commercial seaweed products [Terriente-palacios and Castellari, 2022]. The TPCs and AOAs (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, Ferric Reducing Antioxidant

Power (FRAP) assay) were enriched compared to *E. cottonii* and *E. spinosum* (22.50±2.78, 15.82±1.24 mg Phloroglucinol Equivalents (PGE) g^{-1} DW) and other red and brown seaweeds of the genera *Dictyota*, *Padina* and *Halymenia* [Matanjun et al., 2008]. On the other hand, radical scavenging activity (2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay) and AOA (Oxygen Radical Absorbance Capacity (ORAC)) of sea grapes were lower than in *E. denticulatum* [Balasubramaniam et al., 2020].

In the intra-genera comparison, *C. lentillifera* was mostly investigated alongside *C. racemosa* (Table 2.3, [Matanjun et al., 2008, Salleh and Wakid, 2008, Nagappan and Vairappan, 2014, Paul et al., 2014, Setthamongkol et al., 2015, Ismail et al., 2020]), but the results did not show a clear trend and seem to be highly influenced by local factors. Thus, *C. lentillifera* was reported to have overall lower nutritional values than *C. racemosa* regarding the PUFA and pigment content [Paul et al., 2014], but also with higher PUFA content [Nagappan and Vairappan, 2014]. The TPC values of both *Caulerpa* species were similar (*C. lentillifera* 42.85±1.22 vs. *C. racemosa* 40.36±1.05 mg PGE g^{-1} DW, [Matanjun et al., 2009]), however, the growth rates were unanimously reported significantly higher for *C. lentillifera* [Paul et al., 2014, Setthamongkol et al., 2015].

Intra-species comparisons were conducted in order to test for the effect of growth region [Zhang et al., 2020], cultivation set-up (laboratory, the wild, or mariculture, [Shevchenko et al., 2009, Saito et al., 2010]) or different extraction and analytical methodologies [Nguyen et al., 2011, Long et al., 2020]. Zhang et al. found among others significant differences in the proximate composition, and vitamin C content in *C. lentillifera* from China's Hainan and Shandong province [Zhang et al., 2020]. However, also the cultivation set-up [Shevchenko et al., 2009, Saito et al., 2010], and the analytical methodology had an effect on the nutritional composition. Thermal drying yielded significantly lower phenolic contents, compared to freeze drying (1.30±0.02 vs. 2.04±0.03 mg GAE g^{-1} DW; [Nguyen et al., 2011]).

2.3.4.4 Post-harvest

Three studies investigated the *Biochemical composition* of sea grapes within the application of *Post-harvest* (Fig.2.4), all aiming to contribute to a circular economy approach by valorising waste biomass of *C. lentillifera* generated during the aquaculture. The nutritional value was reported to be not different from that of food-grade products [Chaiklahan et al., 2020]. The studies focused on the polysaccharide [Chaiklahan et al., 2020, Honwichit et al., 2022], as well as the lipid [Srinorasing et al., 2021] fraction. In intra-species comparisons (Table 2.2) different extraction methods, namely varying algae-to-ethanol ratios, extraction times, stages and purifications were tested to obtain the highest yield of the respective target metabolites [Chaiklahan et al., 2020, Srinorasing et al., 2021]. Regarding polysaccharide extraction, two-stage extraction with 60 min/stage, a solid-to-liquid ratio of 1:15 (Weight per Volume (w/v)), extraction temperature of 90°C, and two time precipitation by a concentration of 75% ethanol was reported to obtain highest polysaccharide yields of around 25% of DW [Chaiklahan et al., 2020] and the hot water extraction (pH 6, 90°C for 20 min) was the most cost-effective [Honwichit et al., 2022]. For lipid extracts, optimum extraction conditions were three-stage extraction with 15 min/stage, solid-to-liquid ratio of 1:10 (w/v) at room temperature for 30 min.

At these conditions, crude lipids yields of around 28% DW were obtained [Srinorasing et al., 2021]. One study conducted an economic evaluation for the production of polysaccharide in Thailand [Chaiklahan et al., 2020]. Based on their estimation the polysaccharide extract could be profitable for the farmers.

Table 2.2: Overview of studies and absence or presence (+) of respective measurements (proximate analysis/PA, mineral content/M., fatty acid analysis/FA, amino acid analysis/AA, antioxidants/AO, total phenolic content/TPC, vitamin content/Vit, pigments/Pig, polysaccharides/Pol, caulerpin/Cau, sterols/St) for articles in the topic *Biochemical composition*, as well as the purpose the study. The articles are grouped by the application.

Reference	Purpose	Regions	PA	M.	FA	AA	AO	TPC	TFC	Vit	Pig	Pol	Cau	St	Application
[Syamsuddin et al., 2019]	Intra-species comparison (indoors vs outdoors), including growth data	Sulawesi, Indonesia		+						+					Cultivation
[Wichachucherd et al., 2019]	Intra-species comparison (timeseries over three month in pond culture)	Phetchaburi Province, Thailand					+	+							
[Balasubramaniam et al., 2020]	Inter-species comparison with *Eucheuma denticulatum*, *Sargassum polycystum* (edible algae)	Malaysia					+			+					
[Ismail et al., 2020]	Intra-genus comparison from two locations in Malaysia, Johor: *C. racemosa*, *C. manorensis*; Port Dickens: *C. sertularioides*, *C. racemosa* var *lamourouxii*, *C. lentillifera*, *C. racemosa* var *cylindracea* and *C. racemosa* (edible algae)	Port Dickens, Malaysia, Johor, Malaysia		+			+	+	+						Nutritional value
[Long et al., 2020]	Assessment with one species	China										+			
[Matanjun et al., 2008]	Inter-species and Intra-genus comparison with *Eucheuma cottonii*, *E. spinosum*, *Halymenia durvillei*, *C. racemosa*, *Dictyota dichotoma*, *Sargassum polycystum*, *Padina* sp. (edible algae)	Borneo, Malaysia					+	+							
[Matanjun et al., 2009]	Inter-species nutritional comparison with *Eucheuma cottonii*, *Sargassum polycystrum* (edible algae)	North Borneo, Malaysia	+	+	+	+				+					
[Nagappan and Vairappan, 2014]	Intra-genus comparison with *C. racemosa* var. *clavifera f. macrophysa*, *C. racemosa* var. *laetevirens* (edible algae)	Sabah, Malaysia	+		+								+		

Reference	Description	Location								
[Nguyen et al., 2011]	Intra-species, Analytical assessment and antioxidants (freeze-drying vs thermal drying, oolong tea)	Penghu, Taiwan	+				+	+		
[Nufus et al., 2019]	Inter-species comparison with *Halimeda opuntia*	Pramuka island, Indonesia		+		+				
[Paul et al., 2014]	Intra-genus comparison with *C. racemosa* var. *laetevirens* Including biomass production and properties (Frond/ stolon) (edible algae)	Townsville, Australia		+	+				+	
[Ratana-arporn and Chirapart, 2006]	Inter-species comparison with *Ulva reticulata* (edible algae)	Phetchaburi, Thailand	+		+	+			+	
[Saito et al., 2010]	Intra-species comparison (cultured vs wild) and inter-species with *Cladosiphon okamuranus*	Okinawa, Japan			+					
[Salleh and Wakid, 2008]	Inter-species and intra-genus nutritional comparison with brown and green seaweeds, *Padina gymnospora*, *Sargassum baccularia*, *Sargassum binderi*, *Turbinaria conoides*, *Caulerpa racemosa*	Port Dickson, Malaysia	+						+	
[Setthamongkol et al., 2015]	Inter-species and intra-genus comparison with red and green seaweeds *Chaetomorpha crassa*, *Chaetomorpha linum*, *Ulva rigida*, *Caulerpa racemosa*, *Caulerpa brachypus*, *Caulerpa taxifolia*, *Gracilaria tenuistipitata* and *Gracilaria fisheri* Additional growth data of three weeks culture Development of novel seaweed products	Thailand	+							
[Shevchenko et al., 2009]	Intra-specific comparison (laboratory vs mariculture grown)	Khanh Hoa Province, Vietnam							+	+

Reference	Description	Location									
[Terriente-palacios and Castellari, 2022]	Inter-species comparison with various other commercial macro- and microalgae (products, including fresh and dried) and alga-enriched products	Commercial products bought at			+						
[Zhang et al., 2020]	Intra-species nutritional comparison between regions in China and lit. values	Hainan, China; Shandong, China	+	+	+				+		
[Chaiklahan et al., 2020]	Sea grape waste (different extraction methods), increase value by usage of waste	Phetchaburi Province, Thailand				+	+			+	+
[Srinorasing et al., 2021]	Sea grape waste (different extraction methods), increase value by usage of waste	Phetchaburi Province, Thailand		+		+	+	+		+	
[Honwichit et al., 2022]	Sea grape waste (different extraction methods), increase value by usage of waste	Phetchaburi Province, Thailand	+			+				+	

2.3.5 Water treatment

Within the topic of *Water treatment*, papers were grouped into the applications *Industrial effluents* (n=10), *Cultivation* (n=11), and *Nutritional value* (n=2), of which only the latter two were evaluated here. Snails, fish, and shrimp were most often co-cultivated with *C. lentillifera*, followed by other seaweeds (Fig.2.8A). The most prominent treatment and response variable quantified were the mono- vs co-culture applied by seven studies (Fig.2.8B) and the nutrient removal/uptake rate (n=9, Fig.2.8C).
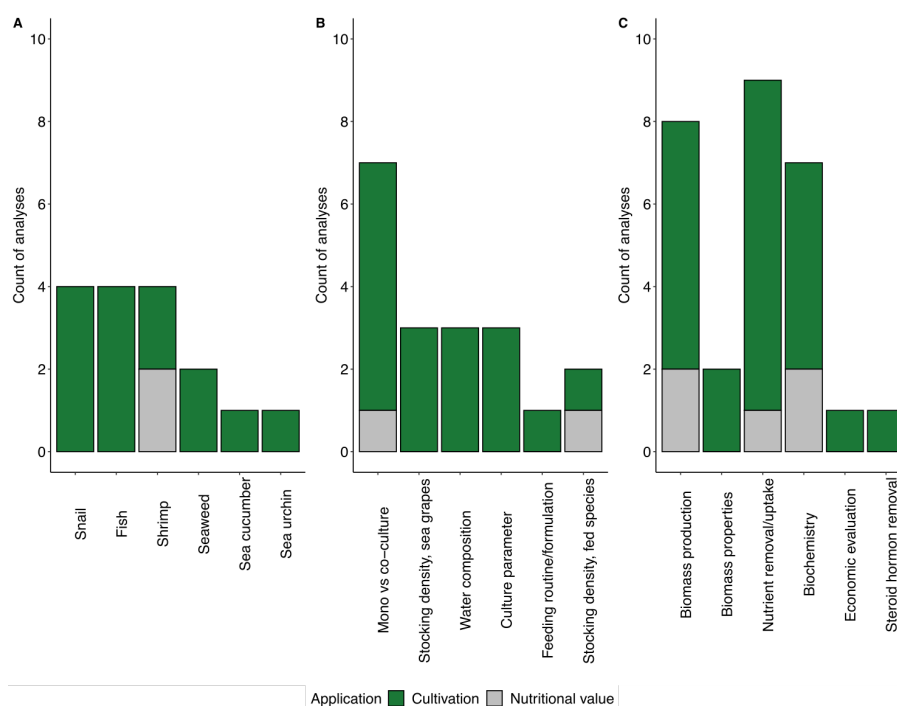
Figure 2.8: Count of analyses of (**A**) organisms, (**B**) treatment during the study and (**C**) response parameter quantified in a co-culture set-up with *Caulerpa lentillifera* in the topic of *Water treatment*, grouped by the different applications (Count of papers: Cultivation (n=11), Nutritional value (n=2))

2.3.5.1 Cultivation

A total of eleven studies focused on the application of *Cultivation* within the topic of *Water treatment*. The majority were conducted in pilot aquaculture systems (n=8), including e.g. experimental Recirculation Aquaculture System (RAS)s, open water Integrated Multi-Tropic Aquaculture (IMTA)s, or larger scale same tank cultures, whereas three studies were conducted at laboratory scale (Table 2.3.5.2). The experiments conducted in larger-scale systems had considerably longer experimental runs (19–120 days, mean: 58 days), compared to the laboratory-based studies (10h, 24h and 15 days, Table 2.3, Appendix A.10).

The majority of studies focused on *C. lentillifera* as a bioremediator of nutrients in aquaculture effluents from different organisms (Table 2.3), whereas one study investigated the ability to remove sterol hormones [Lu et al., 2021]. *C. lentillifera* was mostly integrated with

one other species. Snails (*Babylonia areolata*, abalone) and fish (*Poecilia latipinna*, *Lates calcarifer* and grouper) were the most prominent organisms co-cultured with sea grapes, followed by shrimp (*Litopenaeus vannamei*), sea cucumber (*Holothuria scabra*) and sea urchin (Table 2.3, Fig.2.8A). However, even though abalone were investigated in two studies (*Haliotis asinine*: [Largo et al., 2016], species unknown: [Paul et al., 2014]), reports on its compatibility for co-culture with *C. lentillifera* are still missing. On the one hand, *C. lentillifera* was reported to be too fragile for the culture in baskets as part of an open water IMTA system with abalone, and therefore had to be replaced with a more robust species [Largo et al., 2016]. On the other hand, although Paul et al. used abalone and sea urchins as cultivation medium, the authors focused on the co-cultivation of two *Caulerpa* species and did not further report on the fed-species [Paul et al., 2014]. The biomass production, usually expressed as growth rate, ranged from 0.46–4% day^{-1} (Appendix A.10). On the one hand, *C. lentillifera* showed higher growth rates when compared to *C. racemosa* [Paul et al., 2014] and *Gracilaria salicornia* [Chaitanawisuti et al., 2011]. On the other hand, similar and lower values were obtained when compared to *G. lichenoides* [Liu et al., 2016] and other *Caulerpa* species [Paul and de Nys, 2008]. Besides biomass production, the texture of sea grapes is a unique selling point [de Gaillande et al., 2017] and therefore, biomass properties, including the respective frond to stolon ratio, the harvestable biomass, as well as the ramuli density are important parameters to consider, so far reported by two studies [Paul et al., 2014, Dobson et al., 2020].

Most studies experimentally compared the mono-culture of fed-species with the integration of *C. lentillifera* (mono- vs. co-culture, Fig.2.8B), reporting positive effects on the water quality and the fed-species (Table 2.3). The nutrient removal/uptake was the most prominent response variable (n=8, Fig.2.8C), highlighting the function of the seaweed in the co-culture set-ups as a bioremediator. Various studies reported that *C. lentillifera* efficiently removed nutrients from aquaculture effluents, leading to decreased N (total Ammonium (NH_4^+), Nitrite (NO_2^-), NO_3^-, [Dobson et al., 2020]) and P levels [Chaitanawisuti et al., 2011, Bambaranda et al., 2019a, Bambaranda et al., 2019b, Anh et al., 2021, Ly et al., 2021], also recognizable by negatively correlated nutrient loads with sea grape densities [Anh et al., 2021, Ly et al., 2021, Margono et al., 2021] and N-enrichment in sea grape tissue [Paul and de Nys, 2008, Liu et al., 2016, Bambaranda et al., 2019b]. Sea grape tissue-N was one of the quantified response parameters summarized as biochemical composition, along with tissue-Carbon (C) content [Paul and de Nys, 2008, Liu et al., 2016], Chl [Lu et al., 2021], and heavy metal content [Bambaranda et al., 2019b].

The growth of *C. lentillifera* was higher in a low N environment (0.017 mg L^{-1}, ∼3% day^{-1}), compared to a high N environment (1.4 mg L^{-1}, ∼4.2% day^{-1}, [Paul and de Nys, 2008]) and at NH_4^+:NO_3^- ratios of around 1:5, as the species seemed to prefer NO_3^- over NH_4^+ as a N source, in the presence of both [Liu et al., 2016]. As the nutrient load entering an aquaculture system mainly depends on the fed-species, it is not surprising that the growth rate of sea grapes was also affected by the feeding rate and density of *L. vannamei* in the same tank (Fig.2.8B, [Anh et al., 2021]). The growth rates increased from 0.46 to 1.05% day^{-1} with increasing feeding rates, but decreased (0.45–0.82% day^{-1}) with increasing shrimp densities (1000-3000 ind. m^{-3}), possibly influenced by shrimp grazing on *C. lentillifera* [Anh et al., 2021]. However, in other cases, the growth performance and biomass properties of sea grapes

were independent of the presence of the co-cultured species, such as for snails and sea cucumbers (growth rate: $1.86\pm0.12\%$ day^{-1}, [Dobson et al., 2020]). These results indicated that the relation between nutrient input (feeding, stocking densities), water volume and algal biomass is essential, which can also be altered by adapting the initial seaweed stocking density in the system, as reported in three studies (Fig.2.8B, [Chaitanawisuti et al., 2011, Bambaranda et al., 2019a, Ly et al., 2021]. Interestingly, the positive effect of the presence of *C. lentillifera* on growth rate, survival, and production of *L. vannamei* shrimp [Ly et al., 2021], as well as slight improvement of the yield and survival rate of snail *B. areolata* did not or only minimally depend on the initial *C. lentillifera* stocking densities (investigated ranges of 0.5–2 kg m^{-3}, [Ly et al., 2021] and 0.280–0.840 kg m^{-3}, [Chaitanawisuti et al., 2011]. However, *C. lentillifera* growth rates significantly decreased with increasing initial biomass [Chaitanawisuti et al., 2011, Ly et al., 2021], e.g. from $2.58\pm0.09\%$ day^{-1} at a density of 390 g m^{-2}, to 1.92-1.70% day^{-1} at higher initial densities (790 g m^{-2}, 1170 g m^{-2}, [Chaitanawisuti et al., 2011]). Additionally, the growth tended to be highest in the first 14–20 days of the longer experimental runs, compared to the subsequent periods [Bambaranda et al., 2019b, Ly et al., 2021]. This is most likely caused by changes in the light environment, due to increasing mutual shading of the algae [Bambaranda et al., 2019b] or loss of water transparency [Ly et al., 2021]. Besides, feeding on *C. lentillifera* by *L. vannamei* may have led to improved Food Conversion Ratio (FCR) of the shrimp, but also to decreases in sea grape biomass [Anh et al., 2021]. Same tray co-culture with *C. racemosa* resulted in lower biomass productivities regardless of the initial stocking densities, potentially due to a delayed establishment, suggesting rather a mono-culture of the species [Paul et al., 2014]. On the other hand, the presence of sea grape trays was assumed to result in a nearly 50% decreased yield of sea cucumber *Holothuria scabra*, compared to the set-up without the seaweed. Arguably because the shading provided by the trays inhibited the growth of microalgae in the sediment, which are an essential food source of sea cucumbers [Dobson et al., 2020].

Only one study conducted an economic assessment [Dobson et al., 2020], reporting that the integration of *C. lentillifera* could increase the gross yield value substantially (United States Dollar (USD) 44.27 m^{-2}), compared to a sandfish mono-culture (USD 3.80 per m^{-2}) and a sea cucumber – *Babylonia* (USD 21.53 per m^{-2}) system. The monetary yields were only based on the farm-gate prices and neglect the initial investment, as well as work-force [Dobson et al., 2020].

Apart from nutrients, *C. lentillifera* can also be used to effectively remove steroid hormones from grouper aquaculture effluents [Lu et al., 2021], which was investigated by changing the sterol content in the water and quantify the sterol uptake rates (Fig.2.8B, C). Of the four investigated seaweeds (*U. pwetusa*, *G. lemaneiformis*, and *Codium fragile*), *C. lentillifera* was most efficient in removing steroid hormones 17 β-estradiol and 17α-ethinylestradiol (EE2) within 12 h (4 g L^{-1} seaweed, more than 90% removal). The sterol removal rates were also affected by temperature and salinity [Lu et al., 2021]. Salinity and aeration affected nutrient uptake rates of *C. lentillifera* from effluents of a saline molly (*P. latipinna*) in a 24h laboratory study, and the authors identified optimal salinity levels (29-30 PSU) and aeration regime (to be present) using (non)linear regression [Bambaranda et al., 2019a], before testing the set-up (30 g L^{-1} , 30 PSU, aeration) in a scaled-up system [Bambaranda et al., 2019b]. Due

to substantial losses in an *in-sit*u settlement pond experiment of a commercial barramundi (*L. calcarifer*) aquaculture, possibly induced by epiphytic filamentous algae, the influence of fragment size and culture depth was tested. Depth did not affect *C. lentillifera* growth, but larger fragments (60.3±10.6 g) seemed to induce higher losses compared to fragments one decimal smaller (6.4 ± 1.3 g, [Paul and de Nys, 2008]).

2.3.5.2 Nutritional value

Two studies in the topic of *Water treatment* focused on the nutritional value of *C. lentillifera*. Both studies evaluated the co-cultivation of sea grapes and *L. vannamei* in the same culture unit with capacities of 50 L [Omont et al., 2022] and 500 L [Anh et al., 2022], respectively (Fig.2.8A). The studies tested the effect of a shrimp and/or sea grapes mono- vs. co-culture (eight shrimp and 15.23±0.02 g sea grapes, respectively, [Omont et al., 2022]) and different *L. vannamei* densities (100- 500 ind. m^{-3} and 1 kg m^{-3} sea grapes, [Anh et al., 2022]) on sea grapes biomass production, biochemical composition, including proximate composition [Anh et al., 2022, Omont et al., 2022] and mineral content [Omont et al., 2022], as well as nutrient removal efficiency (Fig.2.8C, [Omont et al., 2022]). The presence of shrimp significantly increased the percentage (DW) content of protein, lipids and ash, while decreasing the carbohydrates, compared to the initial biomass retrieved from pond cultivation.

However, increasing shrimp densities significantly increased the protein content and decreased the ash content of sea grapes, with fibre, moisture, carbohydrates and lipids being similar among the density treatments [Anh et al., 2022]. On the other hand, Omont et al. also reported an increased percentage (DW) of protein for sea grape tissue in co-cultivation, but relatively lower ash contents [Omont et al., 2022]. However, the total content of trace elements increased significantly (Na by 12.5%, Molybdenum by 78.0%, Boron by 50.8%), whereas the content of Cobalt decreased [Omont et al., 2022]. The growth of sea grapes was highly negatively affected by the presence of shrimp (40.6±9.8 vs. 2.6±0.4% day^{-1}, [Omont et al., 2022]) and tended to decrease with increasing shrimp densities, with significant depletion at the highest density treatment (500 ind. m^{-3}, 1.30±0.11% day^{-1}, [Anh et al., 2022]). Grazing might have been a reason for this pattern, but it resulted in increased levels of Fe and Zinc (Zn), total body cholesterol and muscle lipid content in the shrimp [Omont et al., 2022].

Table 2.3: *Caulerpa lentillifera* in integrated aquaculture, compilation of basic data, as well as success stories and pitfalls. In the column *Success ?* the sign + indicates that authors refer to the integration of *C. lentillifera* in the respective integrated system as an overall success, whereas the sign - indicates that authors report of profound problems.

Source	Location	Co-cultured organism	Culture system	Success?	Lesson learned	Treatment	Result parameter	Experimental run	Application
(Anh et al. 2021)	Vietnam	Whiteleg shrimp (Litopenaeus vannamei)	Experimental, 120 L tanks	+	Pilot, significant reduction of nitrogen and phosphorous and improvement of shrimp post larvae growth, survival, yield, compared to mono-culture; RGR[2] of *C. lentillifera* increased with feeding rate of shrimp; *C. lentillifera* as complementary food source for shrimp	Mono vs co-culture, feeding rate/ratio formulation, stocking density of fed species	Biomass production, water quality, nutrient removal capacity	45 days	Cultivation
[Bambaranda et al., 2019b]	Thailand	Saline molly (*Poecilia latipinna*)	Experimental RAS[3]	+	Significant reduction of inorganic nutrients of aquaculture effluents by *C. lentillifera*	Proof of concept: *C. lentillifera* as bioremediatory species	Biomass production, water quality, nutrient removal capacity	60 days	
[Chaitanawisuti et al., 2011]	Thailand	Juvenile spotted babylon snail (*Babylonia areolata*)	Hatchery scale low-technology RAS	+	Pilot, highest growth rate at algal density of 280 g m^{-3}, increased survival rate of snails with *C. lentillifera*	Mono vs. co-culture, stocking density *C. lentillifera*	Biomass production, water quality, nutrient removal capacity	120 days	

[2]Relative growth rate
[3]Recirculating aquaculture system

[Dobson et al., 2020]	Vietnam	Spotted babylon snail (*B. areolata*), sea cucumber (*Holothuria scabra*)	Experimental, 500 L tanks	+	Pilot, underestimation of *C. lentillifera* biomass, total ammonia reduced with *C. lentillifera*, growth unaffected by co-culture, sea cucumber weight gain decreased when *C. lentillifera* present	Mono vs co-culture	Biomass production, survival and production of fed species, water and sediment quality, nutrient removal capacity, economic evaluation	84 days
[Largo et al., 2016]	Philippines	Abalone (*H. asinina*)	Open water IMTA[4] system, cage culture	-	Fragile nature of the seaweed thalli did not allow culture in strong currents	Proof of concept	-	-
(Ly et al. 2021)	Vietnam	Whiteleg shrimp (L. vannamei)	Experimental, 500 L tanks	+	Pilot, significant reduction of inorganic nutrients and improvement of shrimp growth, survival, and production. Algal density of 1 kg m^{-3} shows best trend, but 0.5 - 2 kg m^{-3} possible	Mono vs co-culture, stocking density *C. lentillifera*	Biomass production, water quality, nutrient removal capacity, survival, production, and feed conversion ratio of fed species	56 days

[4]Integrated multi-trophic aquaculture

Reference	Location	Focus/Species	System	+/-	Findings	Variables	Measured parameters	Duration
[Paul and de Nys, 2008]	Australia	Barramundi (*Lates calcarifer*)	Settlement pond	-	Growth restricted due to uninitiated growth of green filamentous tide alga	Culture method, seedling size, culture depth	Biomass production, water quality, nutrient removal capacity, culture parameter	6 weeks (pond)/19 days (RAS)
[Paul et al., 2014]	Australia	Focus on *C. racemosa* co-culture, but wastewater from abalone and sea urchin	Experimental RAS	+	Co-culture with *C. racemosa* in the same tray possible, but *C. lentillifera* has higher biomass production	Mono vs co-culture	Biomass production, biomass properties, biochemical properties	6 weeks
[Bambaranda et al., 2019a]	Laboratory	Sailfin molly (*Poecilia latipinna*)	Plastic containers filled with 300 mL filtered aquaculture effluents from *P. latipinna* culture	+	Pilot, best uptake rates modelled at Salinities between 29.1-30.7 ppt. and algal densities of 20, ∼30,50 g L-1	Stocking density of *C. lentillifera*, culture parameters (Salinity, Aeration)	Nutrient uptake rates	24 h
(Liu et al. 2016)	Laboratory	Comparative study with *Gracilaria lichenoides*	1 L Erlenmeyer flasks		C. lentillifera selectively takes up nitrate prior to ammonia and the nitrate uptake rate was 7.43-50.43 µmol g^{-1} (dw) h^{-1}	Artificial changes (nutrient concentrations/ ratios)	Biomass production, nutrient uptake rates, biochemical properties	15 days (growth experiment) 10 h (nutrient uptake experiment)

[Lu et al., 2021]	Laboratory	Grouper	500 mL beaker, 50 L tanks containing effluents of grouper culture		Efficient removal of steroid hormones 17β-estradiol and 17α-ethinylestradiol (90% of 10g L^{-1} within 12h) from mariculture effluets	Mono vs co-culture, artificial changes (nutrients, sterols), culture parameter	Biochemistry, steroid removal	14 days (tank experiment)/24 h (beaker experiment)
[Anh et al., 2022]	Vietnam	Whiteleg shrimp (*L. vannamei*)	Experimental 500 L tanks	+	Co-culture with shrimp lead to higher contents of protein, lipids, and ash (compared to initial) of *C. lentillifera*, maintenance of appropriate water quality even at high shrimp densities, improvement of production efficiency with shrimp densities of up to 400 ind. m^{-3} with 1kg m^{-3} sea grapes	Stocking density of *L. vannamei*	Biomass production, water quality, biochemical composition of cultured organisms, survival, and feed conversion ratio of fed species	56 days

2.4 Discussion

2.4.1 Main research topics, applications, and author affiliation

Sea grape aquaculture has its roots in The Philippines and Japan (Okinawa) in the 1980s [Trono and Toma, 1993, Estrada et al., 2021]. Reliable, global production statistics for *Caulerpa* seaweeds are missing and local data are scarce, as they are not listed in national aquaculture statics [Moreira et al., 2021]. However, it is generally accepted that the production and demand for this species is rising since approximately a decade ago. This might explain the elevated number of articles since 2018 revealed in the scientometric analysis, with >60% of all publications (section 2.3.1). First authors were mainly from Asian countries (section 2.3.2) where the majority of sea grape cultivation takes place [Chen et al., 2019].

The dominance of the research topic *Biochemical composition* and the application in *Pharmaceutics* highlights the interest in *C. lentillifera* for its bioactive compounds (section 2.3.1). Considering that marine natural products isolated from Chlorophyta are still underrepresented in the database MarineLit with 8% (1965-2012) compared to red (53%) and brown (39%) algae [Leal et al., 2013, Moreira et al., 2021], a further growth of interest in this topic and application is to be expected. This trend is possibly also driven by the comparatively higher values of these bioactive compounds in pharmaceuticals or cosmetics, compared to biomass e.g. for animal feed and biopolymers of feedstock [Chopin and Tacon, 2020].

2.4.2 Sea grapes and their (a)biotic environment

Several environmental parameters are important for the cultivation of *C. lentillifera*. They can be adjusted precisely to the seaweeds' needs during indoor cultivation, which is, however, associated with higher effort compared to outdoor cultivation. For the outdoor cultivation, crossed and interactive effects of different environmental factors are particularly important, considering daily, seasonal, or long-term changes and shifts. In the Northwestern Pacific, temperature and irradiance were major factors limiting *C. lentillifera* cultivation to certain seasons [Terada et al., 2021], whereas in The Philippines and other South-East Asian regions, temperature and salinity, caused by precipitation during the rainy season, restrict the cultivation to the dry season [Estrada et al., 2021]. The cross-effects of many of these factors on the seaweeds' physiology and biochemical composition, like salinity and temperature, have not yet been tested in experimental set-ups, leaving room for further studies. Chemical diversity within a single seaweed species is not uncommon and spatial as well as temporal variability of environmental parameters are often cited as causes [Stengel et al., 2011]. Different compounds are generally the result of specific responses to environmental parameters [Stengel et al., 2011].

However, many of the studies evaluated in section 2.3.4.3 conducted inter-species or intra-genus comparisons of sea grapes' nutritional value with other, often edible or economically interesting, seaweeds from the same location. In contrast, only a few studies intra-specifically compared biochemical composition of *C. lentillifera* across spatial regions, temporal scales, and culture methods (section 2.3.4.2, 2.3.4.3). However, the reported intra-specific variability enforced the importance of understanding the effects of single and crossed (a)biotic cul-

ture factors on the physiology (section 2.3.3.1), microbial community (Topic: *Microbiome*, Application: *Cultivation*, [Pang et al., 2022]), and biochemical composition (section 2.3.4.3, [Wichachucherd et al., 2019]) of the sea grapes observed in the pond environment. Besides, chemical variability between thallus parts is common within seaweeds [Stengel et al., 2011], and should be investigated for *C. lentillifera*, especially since differential gene expression in the thallus parts have been reported [Arimoto et al., 2019b].

2.4.3   The special role of light

Light has been identified as a major stressor for sea grapes, due to their unusually low irradiance saturations, also compared to other green seaweeds (e.g. *Codium* spp., *Ulva* spp., [Nakamura et al., 2020, Marques et al., 2021]). Hence, supra-optimal irradiances induced oxidative stress during cultivation (section 2.3.3.1) and post-harvest (section 2.3.3.2), but they were also reported as an opportunity to increase the nutritional quality of *C. lentillifera*, by triggering its antioxidant production (section 2.3.4). Sub-optimal irradiances, on the other hand, might have caused decreases in growth rates after a certain cultivation period, as reported from pilot co-culture systems (section 2.3.5.1). Consequently, management of initial biomass or harvest periods could ensure continuously optimal light conditions or purposefully increase sea grapes' quality already in the culture set-up [Magnusson et al., 2015]. Most studies focused on the continuous exposure to PAR with consistent photoperiods (section 2.3.5) and only individual studies included changes in absorption spectra for photosynthesis, photoperiod, and short or extended exposure times [Kang et al., 2020, Terada et al., 2021].

This leaves various knowledge gaps for further studies, such as considering variations on the temporal continuums, from high frequencies (e.g. evoked by high turbidity, movement of the cultivation covers, passing of co-cultured species), medium frequencies (daily solar circle) to low frequencies (seasons, [Comerford et al., 2021]). Additionally, even though *C. lentillifera* seemed to contain only minor quantities of UV-absorbing compounds [Tanaka et al., 2020], the exposure to this stressor could impact the physiology of the alga and alter the secondary metabolite composition, as observed for other seaweeds [Polo and Chow, 2022].

2.4.4   Sea grapes (not only) as bioremediators in co-culture approaches

The nutrient acquisition of seaweeds is complex and depends on various parameters [Roleda and Hurd, 2019]. The N and P loads in the application of cultivation water were reported to affect biomass production and composition of sea grapes in both mono-, and co-culture (sections 2.3.3.1 and 2.3.5.1). Sea grapes bioremediated nutrients from the water, as indicated by reduced water nutrient levels (section 2.3.5.1). However, the actual nutrient acquisition rate was only examined once in the context of preferred N-sources [Liu et al., 2016]. The comparison between studies focusing on the nutrient uptake was difficult, as different thallus parts of *C. lentillifera* were used. Fronds and the below ground parts (stolon with rhizoids) are expected to have different N and P acquisition rates, as reported for *C. prolifera* [Alexandre and Santos, 2020]. This might explain the differences in composition, caused by the presence

of bottom sediments and potentially higher nutrient loads in the pore water, compared to the water column [Long et al., 2020]. These information have important implications for the choice of cultivation method (trays, sowing method or the open water cage cultivation, [Syamsuddin et al., 2019]), especially when sea grapes are exposed to unusually high (e.g. for nutrient bioremediation in aquaculture, section 2.3.5.1, or eutrophication, [Cai et al., 2021b]) or rather low (oligotrophic waters) nutrient loads. Besides, salinity [Bambaranda et al., 2019a], N-sources [Liu et al., 2016] and potentially various other parameters [Roleda and Hurd, 2019] affected the nutrient uptake rates.

Higher growth rates than other *Caulerpa* species and the preference of NO_3^- over NH_4^+ makes *C. lentillifera* a promising candidate for co-cultures (section 2.3.5.1). Regarding the application of sea grapes as a bioremediator in aquaculture systems with fed-species, it might be beneficial to implement polyculture of different seaweeds in order to remove different nitrogen compounds more efficiently. Commonly used species for biofiltration include *Undaria lactuca* and *U. pinnatifida* [Cahill et al., 2010], *Gracilaria birdiae* and *G. vermiculophylla* [Marinho-Soriano et al., 2009, Abreu et al., 2011], and *Porphyra leucosticta* which take up mainly NH_4^+ [Chung et al., 2002]. Since aquaculture effluents are usually higher in NO_3^- than NH_4^+ content, implementing *C. lentillifera* together with commonly used species can enhance the bioremediation of the effluents [Neori et al., 2004]. This has only been studied once [Paul et al., 2014].

In general, *C. lentillifera* is a promising candidate for the use as a biofilter in integrated tank-based aquaculture systems, rather than in open water systems, at least when exposed to high water movements (section 2.3.5). However, when sea grapes were integrated in the same unit with *L. vannamei*, grazing of the shrimp on the seaweed was reported (section 2.3.5), leading on one hand to a loss of biomass, but on the other hand to reduced FCRs and a beneficial change in nutritional composition of the shrimp [Ly et al., 2021, Omont et al., 2022]. Similarly, the integration of *C. lentillifera* powder in the feed (30 g kg^{-1}) of the black tiger shrimp (*Penaeus monodon*) significantly increased growth rate and FCR of the post larvae (Topic: *Biochemical composition*, Application: *Animal feed*, [Putra et al., 2019]). However, a spatial segregation of the species could allow for a targeted feeding with sea grape biomass, e.g. the lower quality waste, integrated in the feed or provided fresh, and still allow for the seaweeds to bioremediate nutrients. On the other hand, this would require more space which could negatively impact the costs for the farmers [Dobson et al., 2020], reinforcing the importance of an economical assessment as basis for farmers decision making.

2.4.5 Economic assessment

Sea grape farming has been described as a lucrative business in The Philippines, among others, with the potential for global upscaling, but limited awareness has been identified as a hurdle [Dumilag et al., 2019, Estrada et al., 2021]. Ethnophycological studies are least represented in this literature review (section 2.3.1), even though the sea grape farmers are an essential part of the value chain of *C. lentillifera*, their knowledge, needs and access to scientific findings are of great interest. An essential part of such applied research could be the integration of an economic analysis of new co-culture approaches or cultivation and post-

harvest methods, which has only been done scarcely [Chaiklahan et al., 2020, Dobson et al., 2020]. The farm-gate price, likely for the use in human nutrition, of sea grapes reported from Viet Nam (USD 4.35 kg, [Dobson et al., 2020]) lies clearly above the rather low average value of brown, red and green seaweed biomass (USD 0.47, 0.39, 0.79 kg^{-1} WW, respectively, [Cai et al., 2021a]). Hence, sea grape farming could provide a good source of income, especially as initial investments, e.g. in the tidal pond cultivation, are rather low. Considering that parts of the harvest do not meet the required quality standards [Chaiklahan et al., 2020], the waste valorisation should be brought into focus (section 2.3.4.4).

2.4.6   Sea grapes as human food

Red and brown algae dominate the commercial seaweed production and the share of green macroalgae is vanishingly low [FAO, 2020, Moreira et al., 2021]. *C. lentillifera* can compete with commercial seaweeds regarding their nutritional value, exhibiting similar or even higher amounts of for example minerals, vitamins, and antioxidative properties (section 2.3.4.2). The biochemical composition, including protein, lipid and carbohydrate content and quality, as well as bioactive compounds over vitamins and pigments varied considerably between studies (section 2.3.4.1, Appendix A.3), supported by a recent review on health benefits and nutrients of *C. lentillifera* [Syakilla et al., 2022].

Since the amounts of different biochemical compounds showed a large variability, it is important to understand the factors leading to these differences in order to improve their concentrations in the framework of *C. lentillifera* as functional food ingredient. The cultivation conditions or set-ups could even be managed to increase certain target compounds, like antioxidants (section 2.3.4) or proteins through co-cultivation (section 2.3.5.2). One delicate part in the life-cycle of *C. lentillifera* is the post-harvest handling, as the product is still alive and photosynthetically active. The shelf-life is therefore considerably short and quick transportation and retail are required. The packaging and storage materials differ locally, as well as the form of retail [de Gaillande et al., 2017], ranging from natural materials up to plastic [Terada et al., 2018, Stuthmann et al., 2020]. The packaging materials [Terada et al., 2018, Stuthmann et al., 2020], in addition to the environmental conditions during previous cultivation [Minh et al., 2019] and during storage, influenced the quality of the sea grapes (section 2.3.4, [Stuthmann et al., 2020]). However, considering studies on *C. lentillifera* during cultivation, it is expected that temperature [Terada et al., 2018], as well as the microbiome (Topic: *Microbiome*, Application: *Cultivation*, [Liang et al., 2019, Kopprio et al., 2021]) also have a major impact on the quality of the sea grapes during storage.

The main costumer base for *C. lentillifera* is currently in Asia. However, the interest in this food product might be growing in Europe as well, especially since the demand for vegetarian/vegan food products [Lusk, 2017] and the awareness of health and environmental issues related to food choices [de Boer et al., 2007, Wendin and Undeland, 2020] is increasing. Sea grapes, with their unique texture and nutritional components, are an interesting candidate to contribute to human nutrition outside the current market in Asia. However, this requires advances in the land-based cultivation of this tropical species or in the improvement of the shelf-life. Furthermore, *C. lentillifera* is not yet considered by the European Novel food law

[Barbier et al., 2019, Mouritsen et al., 2019]. While single brown and red seaweeds (e.g. representatives of the orders Laminariales, Fucales and of the genera *Porphyra/Neopyropia*) are included in the Novel Food Catalogue, edible green macroalgae were rather neglected [Lähteenmäki-Uutela et al., 2021]).

2.5 Conclusions

C. lentillifera is a promising candidate for aquaculture in general and for co-cultivation, especially since the value of the product is higher compared to other seaweeds, among others due to its striking texture (green caviar). The present review highlighted the interest in the alga's *Biochemical composition* with the application for *Pharmaceutical* and *Nutritional value*, likely due to the various bioactive compounds of the sea grapes and the nutritional benefits for the human nutrition. However, more research is needed to understand the complex interactions between environmental parameters, which vary over regional and temporal scales, and the biochemical composition of the species, in order to potentially increase the production of target-compounds. Additionally, the comparable short shelf-life of the fresh product and the main restriction to the Asian market were identified as bottlenecks for global retail. In the future, sea grapes could contribute to strengthen the role of green algae in the global seaweed aquaculture sector.

Data availability The data supporting the conclusions of this manuscript have been made openly available at https://doi. org/10.6084/m9.figshare.21953276 and https://doi.org/10.6084/m9.figshare.21953267.

Chapter 3

Publication II

Post-harvest sorting of sea grapes at farm VIJA in Van Phong Bay, Viet Nam.

This chapter is published as

Sea grape (*Caulerpa lentillifera*) aquaculture in Van Phong Bay, Viet Nam: Evaluation of the post-harvest quality

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Abstract

Caulerpa lentillifera, known as sea grapes or green caviar is increasingly in demand as a sea vegetable for human consumption. The seaweed is cultivated in ponds in the Khánh Hòa province in Van Phong Bay, Viet Nam, during the dry season (March-October). The harvested sea grape fronds are graded into different qualities based on their physical characteristics for retail on the local market or for export. Based on systematic observations of sea grape fronds of two different qualities, the frond weight, frond length and rachis colouration were identified as physical characteristics important for grading. Fronds of the best quality had significantly longer (12.59±2.89 vs. 10.01±2.51 cm) and heavier (2.37±0.59 vs. 1.60±0.5 g) fronds with darker rachis than the other quality group. However, a logistic regression model revealed that frond weight was the best predictor of frond quality. The physiological parameter of F_v/F_m was slightly different between the qualities, but always with means >0.7, whereas the Antioxidant Activity (AOA) and the Total Phenolic Content (TPC) were similar (98.34±19.22 vs. 95.96±24.98 mmol Trolox Equivalents (TE) 100 g^{-1} Dry Weight (DW) and 163.8 ±20.14 vs. 149.85±15.44 mg Gallic Acid Equivalents (GAE) 100 g^{-1} DW). To the best of our knowledge, this study took a first approach to identify quality characteristics of sea grape fronds from Van Phong Bay, Viet Nam, which can serve as a basis for adjusting cultivation parameters to improve the harvest quality by developing cultivation and post-harvest protocols. However, further research is needed to investigate the effect of certain cultivation parameters on the specific frond characteristics.

Keywords: Colouration, Macroalga, Phycoculture, Sustainable Food Production, Post-Harvest Protocol

3.1 Introduction

Caulerpa lentillifera J.Agardh is a green seaweed of the order Bryopsidales [Guiry and Guiry, 2023] and known as a delicacy by the names sea grapes or green caviar. Sea grapes have traditionally been harvested, cultivated, and eaten in The Philippines and Okinawa in Japan [Trono and Toma, 1993, Yap, 1999]. However, the interest in this sea vegetable has increased due to, among other things, its nutritional benefits [Syakilla et al., 2022] and the special texture of the fronds, which consist of small grape-like ramuli arranged around a central axis, called rachis [de Gaillande et al., 2017, Zubia et al., 2020].

Aquaculture of *C. lentillifera* has also taken off in other countries in the Indo-Pacific region, including Viet Nam. Here, a long coastline with bays and lagoons provides various opportunities for aquaculture. It is therefore not surprising that Viet Nam ranks among the top five marine aquaculture producers for molluscs and crustaceans, and among the top seven for finfish [FAO, 2022]. The Khánh Hòa province is located in the Central South of the country and a recent hot spot for sea grape cultivation [So, 2022]. However, global production estimates of *C. lentillifera* are likely underestimated, and reports are limited to The Philippines [Cai et al., 2021a]. Local news media from the Khánh Hòa province report production estimates of >400 tons year^{-1} at an area of ∼50 hectares for 2020 [Son, 2022]. The sea grapes are cultivated in tidal ponds [Stuthmann et al., 2020] using the sowing or tray method [Rabia, 2016], especially for export to countries such as Japan [Terada et al., 2018]. However, sea grapes are also sold on local markets, supermarkets and served in restaurants. Biomass below the quality requirements for food use is discarded, which has been reported to be up to ∼60-70% of the total biomass in Thailand [Chaiklahan et al., 2020]. The farmers grade the fronds' quality based on physical characteristics, including weight, length, number of branches, ramuli density, as well as colouration [Chaiklahan et al., 2020].

Caulerpa is a genus known for its morphological plasticity as response to environmental changes [Estrada et al., 2020]. Changes of environmental parameters over seasons are common in the Indo-Pacific region and they are known to restrict or impact sea grape cultivation [Wichachucherd et al., 2019, Terada et al., 2021]. The seaweed cultivation in the Khánh Hòa province is restricted to the dry season, since *C. lentillifera* is particularly sensitive to decreasing salinity [Guo et al., 2015b]. Exposure of sea grapes to different stressors, including temperature, salinity, nutrient concentrations or Photosynthetically Active Radiation (PAR) irradiances leads to changes in Chlorophyll (Chl) *a*, *b* and Carotenoid (Car) composition, colour, and stolon:frond composition of the thallus [Guo et al., 2015a, Guo et al., 2015b, Cai et al., 2021b, Stuthmann et al., 2022]. Biochemical parameters, including the Antioxidant Activity (AOA) or Total Phenolic Content (TPC) and Chl *a* fluorescence parameters are also expected to change in response to environmental parameters [Wichachucherd et al., 2019, Zhang et al., 2020, Cai et al., 2021b, Stuthmann et al., 2022], although this may not be visible. Hence, the quality of the sea grapes might vary between harvests during the season.

The average weight and length of preserved sea grape fronds from a Vietnamese company were reported to be 0.73±0.18 g and 7.27±1.59 cm, with a significant positive correlation between both parameters [Lapong et al., 2019]. The sea grape fronds are usually graded directly on site based on experience of the workers. There has been an attempt to automatize

the frond grading based on photographs and using a deep learning model. The authors of the study used a circular high transform method to detect the shape of the ramuli and the appearance was grouped into feature, shape, colour, and compactness [Chinnasarn et al., 2022]. The model estimations for each quality were considerably high with an accuracy of >0.9 (relates to 90%). However, the study did not account for potential colour differences between pictures taken with different cameras, nor did it determine the importance of each attribute in the grading. Knowledge of the priority of frond characteristics and interactive effect of cultivation stressors with *C. lentilliferas*' nutritional quality and the physical appearance of the species could enable farmers to estimate and manipulate their quality of harvest.

Therefore, this study aimed to (*1*) report on the *C. lentillifera* cultivation cycle and environmental parameters at the VIJA farm in Van Phong Bay, Khánh Hòa province and to (*2*) quantify the sea grape fronds of two different qualities over three sampling points in May and June 2022. (*3*) A binominal model was used to estimate the most important physical characteristics of sea grape fronds graded as different qualities. Based on observations at the sea grape farm, we hypothesize that the grading is influenced by the physical characteristics of frond lengths, weights, ramuli density, and colour of the ramuli, as well as rachis.

3.2 Material and methods

3.2.1 Experimental location

The Central South of Viet Nam, including the Khánh Hòa province, is characterized by a monsoon weather regime with a wet (October-January) and a dry (January/February-October) season [Lam et al., 2002, Ilyash and Matorin, 2007]. The highest precipitation is usually recorded during the Northeast Monsoon (October – November, >500 mm month^{-1}), with less rain from December onwards (Northwest Monsoon,<200 mm month^{-1}) [Lam et al., 2002]. The Khánh Hòa province comprises of four bays, with Van Phong Bay being the northernmost and the largest bay of the province ($\sim$510 km^2). The bay has an average depth of 15 m with a maximum of 34 m [Barthel et al., 2009, Phu et al., 2022]. Several aquaculture activities are taking place in the bay, including lobster cage farming, longline cultivation of the red alga *Kappaphycus alvarezii*, pond cultivation of the shrimp *Litopenaeus vannamei*, the snail *Babylonia areolata* and the seaweed *C. lentillifera* (own observation, [Phu et al., 2022]). Sea grape cultivation was introduced to Viet Nam about a decade ago [So, 2022] and since then several companies started commercial farming of this species, mainly for the export to Japan [Terada et al., 2018, So, 2022]. The present study was conducted at one of these sea grape farms, called VIJA (12°35'17.9"N 109°13'38.5"E), located in the southern part of Van Phong Bay.

3.2.2 Sea grape production cycle

The cultivation season of *C. lentillifera* lasts about eight months, from March to October. The farm VIJA consists of several ponds of different sizes, which are connected through adjustable channels to the nearby coast. The water exchanges are conducted approximately every two

days in accordance with the tidal range. The ponds are shaded with black gauze material to provide a light environment of ~50 μmol photons m^{-2} s^{-1} on average, but with high diurnal fluctuations [Stuthmann et al., 2022].

The sea grapes are cultivated using trays (Fig.3.1A) or the sowing method (Fig.3.1B), depending on the nature of the material at the ground of the pond. In the case of tray cultivation, the farmers lift or float the trays at the surface for harvesting. The trays are being thinned by picking the healthy fronds (7-10 cm) in an interval of 7-15 days. In the case of the sowing method and sometimes also during tray cultivation, farmers use floating devices to swim on the surface of the ponds and pick the good-sized fronds (Fig.3.1C). Fronds of the target size are harvested every ~15 days, yielding approximately 1000 kg Wet Weight (WW) in a 5000 m^{-2} pond. However, the yield tends to decrease towards the end of the harvest season due to increasing rainfall and decreasing temperatures. After harvesting, the sea grape fronds are kept in clean sea water to ensure wound plug formation.

Tidal pond cultivation

~ March till October

Tray or Sowing method

Harvest

~ all 15 days, depending on weather

Post-Harvest

collection and wound healing of fronds, potentially cleaning

grading of fronds based on physical characteristics

discard of unsatisfactory biomass

Retail

export e.g. to Japan (Quality 1)

local market or restaurants (Quality 2)

fresh or dehydrated product

Figure 3.1: Production cycle of *Caulerpa lentillifera* in Van Phong Bay, including the **A**) sowing cultivation and the **B**) trays from the tray cultivation. **C**) The process of sea grape frond harvest and **D**) place of collection and **E**) sorting. Sea grapes being sold fresh or **F**) dehydrated.

The harvested fronds from the different ponds were collected in a central location (Fig.3.1D) and sorted into two groups according to their appearance (Fig.3.1E), which de-

termines the fate of the product. Fronds of the highest quality are mainly used for the export to Japan, while fronds of the lesser quality are used for retail in local restaurants and markets. The two are referred to in the following as *quality 1* (*export grade*) and *quality 2* (*local grade*). Sea grape products are sold fresh or as dehydrated product (Fig.3.1F). Sea grapes that do not reach the minimum requirements for sale ($\sim$30%, including stolons) are mostly discarded. A small amount is kept for other applications, such as cosmetics, especially if they do not meet the length requirements. The seedlings for the next season are kept in the cultivation ponds if the environmental parameters allow it. When salinities are too low, the seaweeds are kept in cultivation tanks on land (personal and written communication with two farmers from different sea grape farms).

3.2.3   Environmental parameters

The environmental parameters salinity (Absolute Salinity (S_A)), temperature (°C), pH and irradiance of PAR (µmol photons m^{-2} s^{-1}) were monitored during several field trips to the VIJA farm in the years 2019, 2020, and 2022. The parameters were quantified using different methods. In the years 2019 (May – August) and 2020 (February–June) data loggers for light (MX2202, HOBO, USA) and salinity/temperature (U24-002-C, HOBO, USA) were installed in the ponds with a logging interval of 30 minutes. Data were recorded for the whole month or only for some days. An overview of the exact measurement days is presented in Appendix B.1. The pH measurements and the measurements in June 2022 were carried out using a multiparameter probe (Manta2, Eureka, USA). Measurements were conducted for several minutes and at different locations in the pond (information on dates are presented in Appendix B.1). All data are presented as mean±Standard Deviation (SD) for each month. In June 2022, measurements were taken on two different days (09.06.22 and 23.06.22) and the data were averaged. Light irradiance data were quantified on the same farm and have already been published [Stuthmann et al., 2022].

3.2.4   Study design and data collection

The sea grape fronds were graded at the collection facilities by workers and stored in tanks with natural sea water. The fronds at the collection facility were harvested from different ponds. Based on observations of the researchers and conversations with the farmers, the following physical characteristics of the fronds were identified as potentially important: Frond weight (g), frond length (cm), ramuli density (number of ramuli per cm frond), colour of ramuli, and colour of rachis (Fig.3.2).

On each of the three sampling days (26.05.22, 09.06.22, 23.06.22), 100 fronds were randomly selected for each of the two qualities, respectively (n total=600). The weight was quantified for each frond separately, before taking a photograph of ten fronds collectively (Fig.3.2). The pictures were taken using a Canon EOS M50 camera (Canon Zoom Lens EF-M 14–45 mm), and for uniform illumination of the photographs a styrofoam box was equipped with two lamps. A grey reference scale, including a reference bar (B.I.G., photo equipment – Brenner Import and Handels GmbH, Weiden i.d. OPf., Germany) was placed next to the fronds in

each picture. The length was quantified using the software ImageJ [Schneider et al., 2012] and the respective reference bar in the picture. The ramuli of each frond were counted in a row along the right side of the respective frond and expressed as the number of ramuli per cm frond (ramuli density) according to the following formula: (W_{part}) and the total weight (W_{total}), following the formula

$$Ramuli\ density = \frac{\#\ of\ ramuli}{frond\ length\ (cm)}, \tag{3.1}$$

with # of ramuli denoting the count of ramuli along the right side and frond length (cm) being the length measurement of the frond, derived from Dobson et al. [Dobson et al., 2020]. The colour of the rachis and ramuli of the sea grapes was analyzed following the description of Winters et al. [Winters et al., 2009], adapted by Stuthmann et al. [Stuthmann et al., 2022] using the software octave [Eaton et al., 2021]). The colour was expressed as value between 0 (black) and 255 (white) in the Red Green Blue (RGB) colour space. On each frond, five measurement points (25 pixels) were randomly chosen along the rachis and from different ramuli, respectively. The mean value of these measurement points was used as respective measure for each individual frond. Following the procedure of Stuthmann et al. [Stuthmann et al., 2022], solely the Red (R) colour channel (R value) was used as a measure for the Chl content and colouration.

To quantify the physiological state of the fronds, the maximum quantum efficiency of Photosystem II (PSII) (F_v/F_m) was determined using a portable Diving-Pulse-Amplitude Modulated (PAM) chlorophyll fluorometer (Walz, Effeltrich, Germany) after 7 min of dark adaptation. However, the parameters length, F_v/F_m, R values, and ramuli density were quantified for at least 50 of the 100 samples taken for each sampling day (n=50), respectively. On each sampling day and for each quality, four replicates of frond biomass were collected for the antioxidant analysis (n=4).

3.2.5 Antioxidant analysis

The biomass was stored under exclusion of light at -80°C before freeze drying the samples (Beta 1-8 LSCbasic, Christ GmbH, Germany). The biomass was pulverized using a FastPrep-24 (MP Biochemicals, Germany) for 20 s. For the extraction, approximately 40 mg of powder was weighed in and 1 mL of Ethanol (70%) was added. The samples were kept in a water bath (47°C) for four hours and vortexed hourly. After centrifuging the samples (2500 rpm; 20°C, 10 min) the supernatant was transferred to an Eppendorf tube and frozen at -80°C until analysis within the next two days.

For analysis of the AOA, an 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)·+ assay) was carried out following a modified method of Re et al. [Re et al., 1999]. The ABTS·+ stock solution (7 mM) was prepared at least 16 hours before by oxidation with potassium disulfate (2.45 mM) in order to prepare the working solution. On the day of measurement, the stock was diluted with absolute ethanol until an absorption of 0.7±0.02 was reached. The absorption was measured with a UV/VIS-Spectrophotometer (Thermo Scientific Genesys 140/150, Fisher Scientific GmbH, Germany). For the measurement, 10 µl of sample
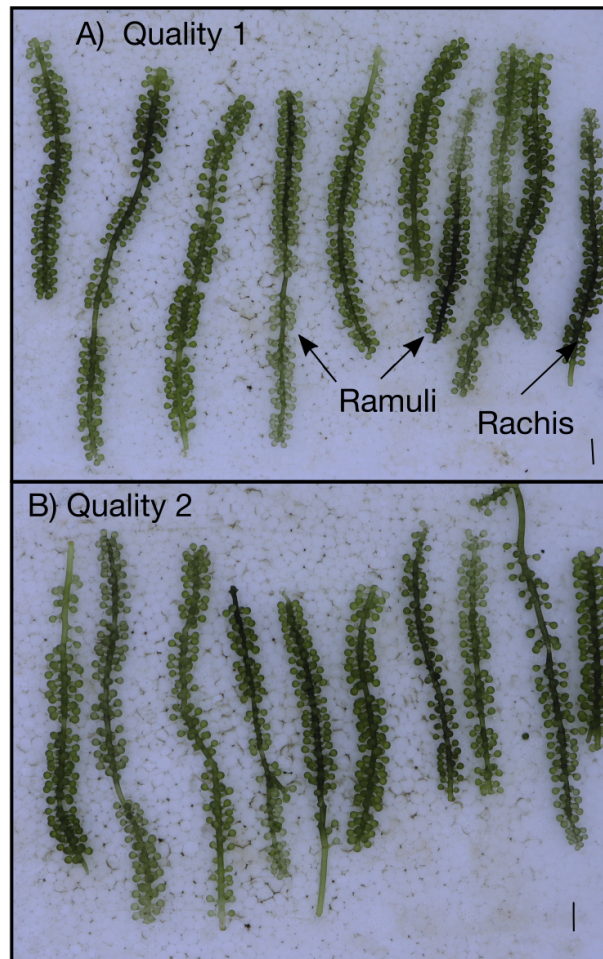
Figure 3.2: Sea grape (*Caulerpa lentillifera*) fronds of **A)** *grade 1* and **B)** *grade 2*. The pictures represent ten fronds of each grade from the sampling on the 09.06.2022 with ramuli and rachis indicated. Scale bar=1 cm.

extract were mixed with 1 mL ABTS$^{.+}$ working solution and absorption was quantified after 6 min reaction time (734 nm). The AOA was expressed as Trolox Equivalents (TE).

For analysis of the TPC, the Folin-Ciocalteu (FC) method was used with modifications [Ainsworth and Gillespie, 2007]. Sample extract (150 μL) and 300 μL 10% FC reagent (Volume Fraction (v/v)) were vortexed thoroughly before adding 1200 μL sodium carbonate (700 mM). After 45 min incubation at room temperature and subsequent centrifugation (5000 rpm, 20°C, 3 min), 1 mL was transferred to a cuvette and absorption was read at 765 nm. The TPC was expressed as Gallic Acid Equivalents (GAE).

3.2.6 Data analysis

Five different physical characteristics of the fronds, namely frond weight (g), frond length (cm), R value rachis (0-255), R value ramuli (0-255), and ramuli density (ramuli/ cm frond) were used to test the effect on the assigned quality. F_v/F_m values, AOA, and TPC were used to quantify the physiological state and the antioxidant content of the fronds.

The Levene's test (homogeneity of variance, p>0.05) and the Shapiro-Wilk test (normal distribution, p>0.05) were used to assess each data set. A one-way Analysis of Variance (ANOVA) with a Tukey's Honest Significant Difference (HSD) post-hoc test was conducted to explain the effect of quality on the respective quantified variable. In case the requirements for an ANOVA were not met, a Kruskal-Wallis test followed by a Dunn-Bonferroni post-hoc test was applied. Correlations between variables weight (g) and length (cm) and AOA and TPC were conducted using Spearman's rank correlations, because data were non-parametric (Shapiro-Wilk test, p>0.05) and Pearson correlation test, respectively.

A logistic regression model was used to estimate the influence of the different physical characteristics as explanatory variables on the binary outcome (*quality 1* or *2*). A univariant analysis (generalized linear model, glm with family *binomial*) was run for each explanatory variable separately. Subsequently, a multivariate model was designed including all explanatory variables with a significant effect on the response. However, since weight (g) and length (cm) were highly correlated and their effect in the univariant models was highly significant as well, two models with either weight (g, glm (quality ∼(weight (g), R value rachis (0-255))) or length (cm, glm (quality ∼(length (cm), R value rachis (0-255))) were constructed. The uni- and multivariate models were evaluated based on the Akaike Information Criterion (AIC), the Accuracy and the Area Under the Curve (AUC) received from conducting a Receiver Operating Characteristic (ROC) curve. The Accuracy was calculated from a confusion matrix comparing the predicted vs. observed values.

The univariate logistic model of weight (g) reached a comparable high accuracy, AUC and a low AIC, compared to the multivariate models and simultaneously the quantification of frond weight (g) required comparable little effort for the farmers, compared e.g. to length to colour measurements. Therefore, this model was tested to predict the quality based on the frond weight (g) of the 300 samples which were not included in the analysis. All analyses were conducted using R Studio [R Core Team, 2019] with the meta package tidyverse [Wickham et al., 2019] and pROC [Robin et al., 2011]. The level of significance was set to $\alpha = 0.05$. The results of all statistical tests are included in the Appendix B.2.

Table 3.1: Environmental data derived from data loggers (HOBO, USA) and multiparameter (Manta2, Eureka, USA) measurements from a sea grape farm (VIJA) in Van Phong Bay, derived over the years 2019 - 2022. Letters give details about measurements; **A**: monthly mean $\pm$ standard deviation (SD) values calculated from logger measurements in 30 minutes intervals; FRAP ferric reducing antioxidant power: mean $\pm$ SD of point measurements over shorter periods (< 10 minutes) on one or two (June 2022, 09.06.22 and 23.06.22) single days within the month; **C**: only a few days of the month considered; Pond 1, 2, 3 are different ponds operated by VIJA. Pond 1 and 2 were demolished/not used anymore in the second and third year of fieldwork due to constructions in the area, therefore, field measurements were moved accordingly. Ponds were located in the immediate vicinity of each other. Data on irradiances of photosynthetically active radiation (PAR) were sourced from [Stuthmann et al., 2022]. For specific information on dates and sampling frequencies refer to Appendix B.1. The raw data of the salinity, temperature and MANTA data can be accessed online.

Month, Year	Temperature °C	Absolute Salinity SA	pH	Irradiance µmol photons m^{-2} s^{-1}	Pond
February 2020	26.8 $\pm$ 0.5 ^{A,C}	32.4 $\pm$ 1.4 ^{A,C}	9.0 $\pm$ 0.05 ^{B}	-	2
March 2020	28.9 $\pm$ 0.8 ^{A}	32.5 $\pm$ 0.3 ^{A}	-	-	2
April 2020	29.4 $\pm$ 0.9 ^{A}	32.5 $\pm$ 0.5 ^{A}	-	-	2
May 2019	30.7 $\pm$ 0.7 ^{A}	30.1 $\pm$ 0.6 ^{A}	-		1
May 2020	30.9 $\pm$ 0.8 ^{A}	31.9 $\pm$ 0.4 ^{A}	-	-	2
June 2019	29.8 $\pm$ 0.9 ^{A}	31.5 $\pm$ 1.1 ^{A}	8.4 $\pm$ 0.02 ^{B}	71.0 $\pm$ 62.9 ^{A}	1
June 2020	30.9 $\pm$ 0.6 ^{A}	31.2 $\pm$ 0.2 ^{A}	-	-	2
June 2022	30.5 $\pm$ 0.1 ^{B}	33.8 $\pm$ 0.4 ^{B}	8.6 $\pm$ 0.05 ^{B}		3
July 2019	29.0 $\pm$ 1.1 ^{A}	34.2 $\pm$ 0.6 ^{A}	8.4 $\pm$ 0.1 ^{B}	56.9 $\pm$ 52.8 ^{A}	1
August 2019	27.9 $\pm$ 1.0 ^{A}	33.5 $\pm$ 0.3 ^{A}	8.3 $\pm$ 0.1 ^{B}	69.4 $\pm$ 74.2 ^{A}	1

3.3 Results

3.3.1 Environmental parameters

Data on salinity, temperature and pH are presented for the months of February to August (Table 3.1). Overall, the temperature measured in the sea grape ponds ranged between mean values of 26.8$\pm$0.5 and 30.9$\pm$0.6°C. The temperature increased from February to April, with highest values of ~30-31°C in May and June (Table 3.2), and a trend of decrease can be seen in July and August. The salinity values ranged between S_A 30.1$\pm$0.6 and 34.2$\pm$0.6, with lowest values in May (Table 3.2). The pH (8.3$\pm$0.1–9.0$\pm$0.05) was rather similar between the measurements, with the highest mean quantified in February 2020 (Table 3.2).

3.3.2 Sea grape quality parameters

The fronds assigned to *quality 1* (*very good*) were significantly heavier (2.37$\pm$0.59 vs. 1.60$\pm$0.5 g, Chi-Square (1)=103.71, p<0.001, Fig.3.3A) and longer (12.59$\pm$2.89 vs. 10.01$\pm$2.51 cm, Chi-square (1)=61.37, p<0.001, Fig.3.3B), compared to *quality 2*. Both parameters were significantly positively correlated (r_S=0.818, p<0.001). The R value (0-255) of the ramuli was similar between the different qualities (p=0.232, Fig.3.3D), whereas the rachis of fronds of *quality 1* had significantly lower R values (hence darker), compared to *quality 2* (43.48$\pm$10.98 vs. 54.13$\pm$9.32, Chi-square (1)=46.94, p<0.001, Fig.3.3C). The ramuli density of the fronds

Table 3.2: Results (p-values) of three different logistic regression models and model evaluation criteria Akaike Information Criterion (AIC), the Accuracy score and the area under the curve (AUC) received from conducting a receiver operating characteristic curve (ROC curve). The asterisks ***, **, * represent different significance levels 0.001, 0.01, 0.05, respectively.

Variable	Univariate Frond weight (g)	Multivariate Frond weight (g)	Multivariate frond length (cm)
Frond weight (g)	5.83e^{-16} ***	4.91e^{-15} ***	-
Frond length (cm)	-	-	3.85^{-11} ***
R value rachis (0 - 255)	-	2.16e^{-09} ***	2.47e^{-10} ***
ACI	290.1	242.12	293.45
Accuracy score	0.79	0.79	0.76
AUC	0.8451	0.892	0.8374
Accuracy score based on test data-set	0.77334		

was similar between the qualities (p=0.303, Fig.3.3E).

3.3.3   Logistic Model estimation

The multivariate logistic regression model including R values of rachis (0-255) and frond length (cm) or frond weight (g), respectively, as well as the univariate model of frond weight (g) performed best among all tested models (Table 3.2, Appendix B.2). The multivariate model incorporating weight, as well as the univariate frond weight model outperformed the multivariate length model. Using the test data of 150 frond weights for each quality, the univariate frond weight model reached an accuracy score of 0.77 (meaning 77% of fronds were correctly assigned to a *quality 1* or *2* group), comparable to the score based on the data used for the model construction (Table 3.2).

3.3.4   Physiological and biochemical parameters

The F_v/F_m values were lower for sea grape fronds of *quality 2* (0.71±0.03), compared to those of *quality 1* (0.71±0.02, Chi-Squared (1)=9.60, p<0.01, Fig.3.4A). The AOA and TPC on the other hand were not significantly different between fronds of both qualities with means and SDs of 98.34±19.22 mmol TE 100 g^{-1} Dry Weight (DW) and 163.8 ±20.14 mg GAE 100 g^{-1} DW for *quality 1* and 95.96±24.98 mmol TE 100 g^{-1} DW and 149.85±15.44 mg GAE 100 g^{-1} DW for *quality 2*, respectively (Fig.3.4B, C). However, there was a trend towards lower values for fronds of *quality 2*, compared to *quality 1* (Fig.3.4B, C). AOA and TPC were moderately positively correlated (r_p=0.66, p<0.001).

3.4   Discussion

The environmental parameters were almost exclusively quantified during the cultivation period of the sea grapes from ∼March to October, and they reflected the reported trend of lower temperatures towards the beginning and end of the cultivation season. Growth rates, as well as photosynthetic performance of *C. lentillifera*, are temperature dependent [Guo et al., 2015a, Cai et al., 2021b, Terada et al., 2021], with higher growth rates at 27.5, compared
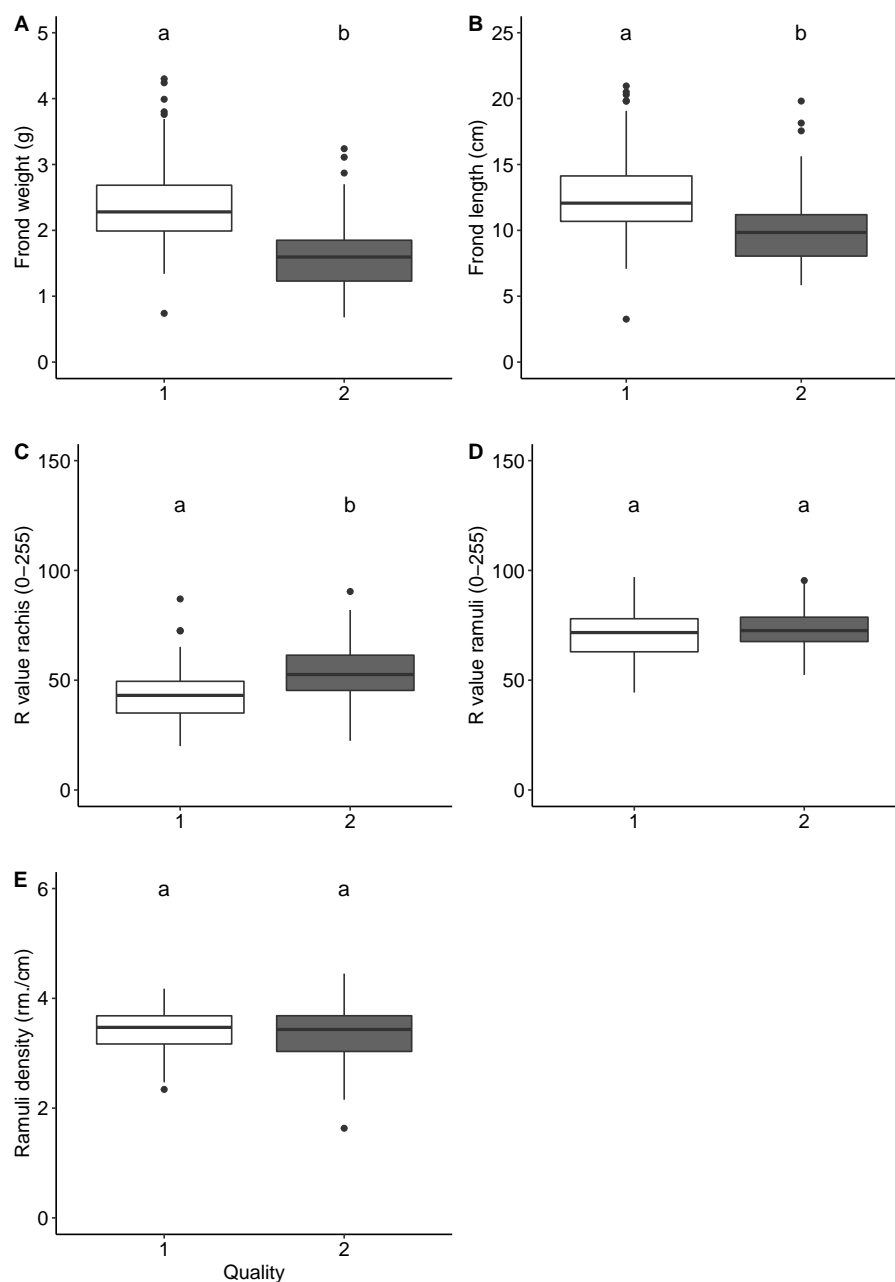
Figure 3.3: Explanatory variables **A**) Frond weight (g), **B**) Frond length (cm), **C**) Red (R) value from the Red Green Blue color space of the **C**) rachis and the **D**) ramuli, as well as the **E**) ramuli density (Ramuli/ cm frond) of *Caulerpa lentillifera* fronds from two different quality standards (*1=very good/Export grade*, *2=ok/local grade*). Data are presented as median with the box drawn from the first to the third quantile and the whiskers presenting the 1.5 interquartile range (n=45 for A-F and n=11-12 for G-H). Different letters represent significant differences of the variables between the different qualities, analyzed using a one-way analysis of variance (ANOVA) or a Kruskal-Wallis test (significance level $\alpha = 0.05$).
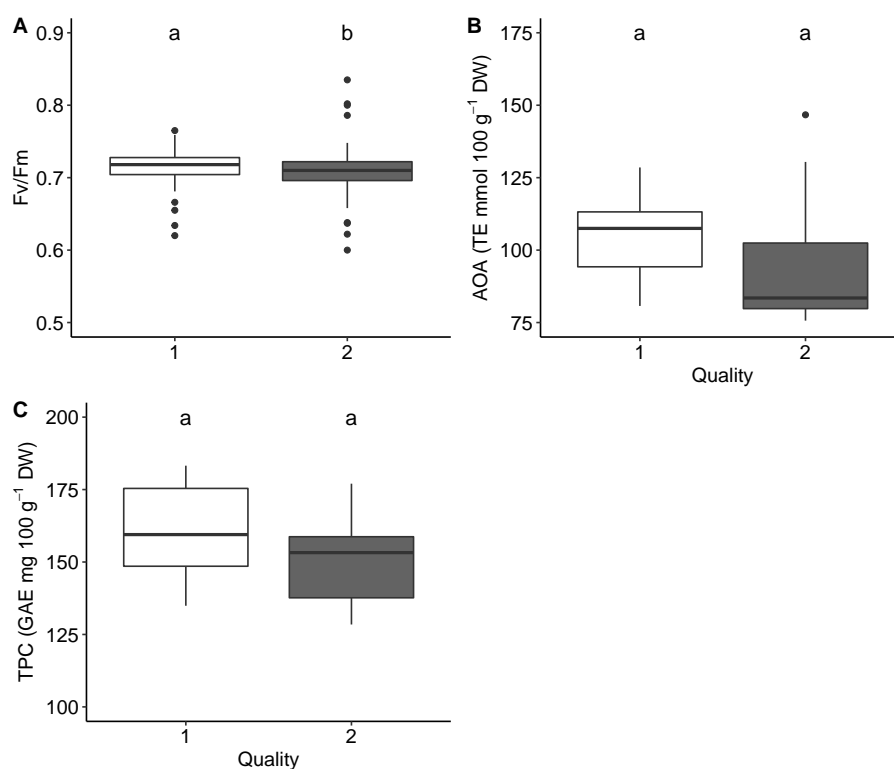
Figure 3.4: Physiological and biochemical parameters **A)** F_v/F_m value, antioxidant activity (AOA, mmol Trolox equivalents, TE 100 g^{-1} dry weight, DW and **C)** total phenolic content (TPC, mg gallic acid equivalents, GAE 100 g^{-1} DW) of *Caulerpa lentillifera* fronds from two different quality standards (*1=very good/export grade, 2=ok/local grade*). Data are presented as median with the box drawn from the first to the third quantile and the whiskers presenting the 1.5 interquartile range (n=45 for A-F and n=11-12 for G-H). Different letters represent significant differences of the variables between the different qualities, analyzed using a one-way analysis of variance (ANOVA) or a Kruskal-Wallis test (significance level $\alpha = 0.05$).

to 30°C [Guo et al., 2015a] and a maximum gross photosynthetic rate at 30.7°C [Terada et al., 2021]. However, the salinity was rather stable without decreases towards the off-season months and still within the reported window of growth [Guo et al., 2015b, Tanaka et al., 2020]. Nonetheless, salinities could spontaneously decrease due to heavy rainfall, which might be indicated by the trend of higher SDs of logger values quantified in February and March 2019. Regenerated sea grape stolons showed lower Chl a and b contents within one week at salinities of S_A $\geq$30 compared to 35, as well as lower growth rates [Guo et al., 2015b, Tanaka et al., 2020]. However, as the highest rainfall is expected during the Northeast monsoon from October-November, the values might decrease towards the end of the respective year [Lam et al., 2002].

The frond length of *C. lentillifera* is highly variable, potentially due to the high phenotypic plasticity of the species [Estrada et al., 2020], as well as their growth cycle. Frond lengths between ∼3 cm and ∼13 cm have been reported in the literature [Paul et al., 2014, Lapong et al., 2019, Estrada et al., 2020, Thi et al., 2020] and the quantified sea grape fronds at the VIJA farm were in the upper end of this range (*quality 1*: 12.59±2.89 cm; *quality 2*: 10.01±2.51 cm). Additionally, they met or exceeded the length guidelines reported by farmers (7-10 cm). The biochemical composition of sea grape fronds might change during their growth cycle, as a reported negative correlation of frond length with nutritionally interesting compounds β-carotene and Eicosatetraenoic Acid (EPA) suggests [Paul et al., 2014]. Hence, even though shorter fronds seem to be perceived as less valuable by farmers, their nutritional composition might be an argument to enhance their market value.

The strong correlation between frond length and weight was not surprising and similarly reported by [Lapong et al., 2019]. Based on literature reports [Chaiklahan et al., 2020] and observations at the sea grape farm, we hypothesized that the ramuli density would also be a quality characteristic. Thi et al. reported similar frond morphometrics between treatments of water levels and exchange rates [Thi et al., 2020]. But the mean values of frond length, ramuli density and diameter over time suggested a higher ramuli density (∼13-14 vs. ∼11 ramuli/cm) and diameter (∼2.2 vs. ∼2.0 mm) of shorter fronds (∼8 cm), compared to longer fronds (∼10-11 cm). Hence, the frond morphometrics could change with the growth cycle of the algae.

However, in contrast to the ramuli density, the ramuli diameter might have differed between the grades, creating the visual impression of a higher ramuli density. The colour of the sea grapes' rachis had an essential effect on the grading, in contrast to the colour of the ramuli. The R value is likely to be highly correlated with the Chl a content of the biomass [Stuthmann et al., 2022]. Colour plays a crucial role in costumers decision making [Pathare et al., 2013] and a colour change of green vegetables has been found to be unacceptable by consumers [Shewfelt, 2002]. Hence, a dark rachis colour induced by high Chl content could be valued by costumers. The Chl distribution in the single-celled *Caulerpa* [Zubia et al., 2020] arguably changes through chloroplast migration and degradation as a result of excess irradiances [Stuthmann et al., 2022] or according to surrounding nitrate composition, temperature or salinity [Guo et al., 2015b, Cai et al., 2021b].

The logistic regression models confirmed that frond length, weight, and rachis R value were crucial characteristics for quality grading of sea grape fronds. However, both univari-

ate and multivariate models including frond weight outperformed models based on frond length (univariate length model evaluation shown in Appendix B). The deep learning model constructed by Chinnasarn et al. successfully extracted the round-shaped ramuli from photographs and graded them based on their features, which enforces the potential role of ramuli in the grading of the fronds [Chinnasarn et al., 2022]. The frond weight variable might contain information about the arguably important trait of ramuli diameter, compared to frond length, potentially resulting in a better prediction of quality. Additionally, frond weight can be easily and quickly quantified with a balance, whereas measuring colour, ramuli morphometrics or length is more time-consuming and costly for farmers. The univariate frond weight model grouped >77% of the test fronds in the right quality grade and hence frond weight seemed like the major predictor of the sea grape quality.

F_v/F_m indicated a good physiological state of all fronds with values $\geq$0.7 [Stuthmann et al., 2020]. However, *quality 2* sea grape fronds seemed to contain more individuals showing signs of photoinhibition compared to *quality 1*, as revealed by lower F_v/F_m values [Goh et al., 2012]. Different environmental stressors such as salinity, temperature or light can enhance photoinhibition in plants [Takahashi and Murata, 2008]. Oxidative stress, induced by exposure to the respective stressors could have resulted in decreased Chl contents and hence colour, in addition to reduced F_v/F_m values [Guo et al., 2015a, Guo et al., 2015b, Stuthmann et al., 2020, Cai et al., 2021b], causing the lower grading of fronds into *quality 2*. The AOA and TPC values were within the range of values reported for *C. lentillifera* [Stuthmann et al., 2022]. *Caulerpa* species contain a variety of secondary metabolites acting as antioxidants, including carotenoids, ascorbic acid (vitamin C), tocopherols (vitamin E), as well as polyphenols like flavonoids [Matanjun et al., 2008, Tanna et al., 2018, Tanna et al., 2019]. Hence, the positive correlation between AOA and TPC was in line with previous reports, suggesting that phenolic compounds contributed essentially to the overall AOA of *C. lentillifera* [Nguyen et al., 2011]. Antioxidants are involved in the scavenging of Reactive Oxygen Species (ROS) on one hand as part of the physiological oxidative stress reaction of seaweeds and on the other hand as essential feature in the human diet [Young and Woodside, 2001, Dring, 2005]. AOA and TPC were not significantly different between qualities, but fronds of *quality 2* tended to have lower values. The chemo-diversity of the algae could be caused by natural variability of abiotic conditions between pond microhabitats, as well as by differences between developmental stages [Stengel et al., 2011]. Additionally, the pre-harvest conditions of the algae are likely to influence the physiology during post-harvest storage similar to other plant products [Sams, 1999], and especially when sea grapes are deprived of water [Stuthmann et al., 2020]. Therefore, the physiological development of different quality fronds should also be observed from harvest until retail.

Sea grape farmers harvest sea grape fronds in tray or sowing cultivation directly in the pond environment, based on judgement and experience, when they expect them to have reached the harvestable size. Grading of frond qualities is performed on land, and weight, as well as rachis colouration seemed to be import. Frond weight was the best predictor of quality grading of sea grapes. The nutritional value of sea grapes, including the antioxidative capacities, could be an interesting additional marketing instrument for the species, which was already denoted as *functional food candidate* [Nurkolis et al., 2023]. Identification of poten-

tial biochemical fluctuation patterns of the sea grapes' developmental phase could increase the value of smaller fronds [Paul et al., 2014].

The fronds grading and the associated differences in value enforce the importance of such intraspecific physiological, morphometric and biochemical differences of the seaweed cultivar for aquaculture [Demes and Pruitt, 2019]. Modern technologies, including the high computational power and various algorithms enable on one hand the quick quantification of information, e.g. through pictures and on the other hand the rapid analysis of large data sets. In agriculture, different methods are used for crop phenotyping, namely the *in-situ* estimation of plant traits, which can be set in relation to their environment and to determine genotypes for plant breeding [Yang et al., 2017, Araus et al., 2021]. Seaweeds, like sea grapes, often reproduce through fragmentation, but the interplay of different traits with their environment could be a valuable tool for aquaculture to increase the harvests' quality and quantity [Demes and Pruitt, 2019]. Shalev et al. use field spectroscopic data and a machine learning algorithm to assess the protein content of *Gracilaria* sp. providing the opportunity for farmers to make *in-situ* decisions for the seaweeds' use [Tadmor Shalev et al., 2022].

This study has only taken a first step to quantify farmers frond grading criteria of importance and further research should target the interlink of those frond properties with different environmental parameters over the production chain. By coupling phenotyping techniques with the farmers' experience plus time-efficient and accurate cultivation and post-harvest protocols could be developed for seaweed cultivation.

3.5   Conclusions

This study demonstrated that frond weight, length, and rachis colour are important quality criteria for grading of sea grapes. However, from an economic perspective, quantification of frond weight is expected to be less time consuming and costly for farmers compared to rachis colour or length. Sea grapes of the better quality are longer, heavier and have darker rachis, compared to the other quality group. However, the antioxidant activity and the total phenolic content were similar.

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Chapter 4

Publication III

Shaded sea grape pond at farm VIJA in Van Phong Bay, Viet Nam.

Cultured and packed sea grapes (*Caulerpa lentillifera*): effect of different irradiances on photosynthesis

Lara Elisabeth Stuthmann, Karin Springer, Andreas Kunzmann

Abstract

The green macroalga *Caulerpa lentillifera* (sea grapes, green caviar) is a promising source for future nutrition due to its beneficial composition for human consumption. It is cultured in tidal ponds, mainly in Viet Nam and The Philippines, and stored for shipment and retail in plastic containers, like Polystyrene (PS) and Polyethylene Terephthalate (PET), exhibiting different properties. This study investigates the influence of irradiances on the physiology of sea grapes under culture and packaging ambience in PET using Pulse-Amplitude Modulated (PAM) fluorometry. F_v/F_m values of *C. lentillifera* significantly decreased <0.54 ± 0.06 Standard Deviation (SD) after 7 days of culture under 100 µmol photons m^{-2} s^{-1}, but with the potential of recovery. In packaging ambience in the state of desiccation, sea grapes exposed to room irradiances (3 µmol photons m^{-2} s^{-1}) for 12 days were still physiologically in a good condition (F_v/F_m=0.70 ± 0.06). However, 12 days under irradiances of 70 µmol photons m^{-2} s^{-1} leads to decreased F_v/F_m (0.42 ± 0.11) and a moisture content of $88.2\pm3.3\%$ of initial. After re-immersion in sea water under room irradiances, F_v/F_m values recovered to a certain degree. In darkness, desiccation was followed by a decrease of F_v/F_m to 0.09 ± 0.19 and moisture content of $49.3\pm20.2\%$ of initial with no recovery after re-immersion under room irradiances. Results suggest shading of *C. lentillifera* in pond culture and PET containers as suitable packaging for sea grapes, but a dimlight source should be provided during storage.

Keywords: Aquaculture, Food, Green caviar, Packaging, Photosynthetic efficiency

4.1 Introduction

Seaweeds as a nutritious and abundant food product are one answer to an explosively growing and hungry world population [Pereira, 2020]. Many macroalgae naturally inhibit coastal zones, where they are exposed to fluctuations in physio-chemical environmental conditions, which influence their physiology such as intensities of Photosynthetically Active Radiation (PAR) and desiccation [Davison and Pearson, 1996].

Other than in the natural habitat, in aquaculture settings, environmental parameters can be partially adapted to the needs of the organism as long as these conditions are known. Sea grapes (*Caulerpa lentillifera*, J. Agardh; Caulerpaceae, Bryopsidales) are green, siphonous macroalgae with a special texture and thallus structure. The species is distributed in the Indo-Pacific region, where it is consumed as a food product eaten fresh in salads, as snack, as sushi, or in a salt preserved form [Long et al., 2020]. The high nutritional composition consisting of Polyunsaturated Fatty Acids (PUFA), Antioxidant Activity (AOA), vitamins, minerals, and bioactive compounds makes sea grapes a nutritious food source and a good candidate to contribute to food security for the rising population, especially in coastal tropical areas [Saito et al., 2010, Nguyen et al., 2011, Paul et al., 2014, FAO et al., 2019].

C. lentillifera are easily and sustainably culturable due to their propagation via fragmentation and the low need for expensive infrastructure or expertise [de Gaillande et al., 2017]. Sea grapes are in particular cultured in open-tidal ponds as in the Philippines and Viet Nam [de Gaillande et al., 2017, Zubia et al., 2020], and in the latter, pond culture is increasingly implemented at the coasts of the Central South in the Khánh Hòa province. In Japan and China, where the demand for sea grapes is especially high, land-based raceway culture is already practiced to some extent [Long et al., 2020, Zubia et al., 2020].

A major factor during sea grape culture is solar radiation, which can be partially controlled through artificial shading of ponds. Although light is essential for seaweeds to maintain their metabolism, an excess of absorbed PAR can oversaturate the electron transport chain capacity without driving the biochemical process of photosynthesis [Franklin and Forster, 1997]. This energy has to be emitted, e.g., through dynamic photoinhibition, a mosaic of photoprotective processes resulting in a declined transfer of excitation energy to the reaction centers in the antenna (non-photochemical quenching) [Osmond, 1994, Häder et al., 1997, Hanelt et al., 1997]. Otherwise, excess excitation energy can lead to irreversible photodamage or photooxidation with a loss of Photosystem II (PSII) reaction centers [Demmig-Adams and Adams, 1992, Aro et al., 1993, Demmig-Adams and Adams, 1996]. However, plants are able to respond to changing light regimes within hours to days by adjusting morphologically and physiologically (photoacclimation, e.g., [Raniello et al., 2004, Marquardt et al., 2010, Aguilera and Rautenberger, 2011]). A common tool to quantify photosynthetic responses of seaweeds to different light conditions is the measurement of Chlorophyll (Chl) a fluorescence using Pulse-Amplitude Modulated (PAM) fluorometry [Maxwell and Johnson, 2000]. Chl fluorescence is mostly produced by PSII, and the fluorescence pattern can be traced back to changes in the transfer of excitation energy to photochemistry (photochemical quenching) and energy dissipation (non-photochemical quenching). The Chl fluorescence parameter maximum quantum yield of PSII (F_v/F_m) is widely used to assess the photosynthetic efficiency of PSII

in dark-adapted leaves, and a decrease of which can be characterized as a result of photoin-hibition [Demmig-Adams and Adams, 1992, Maxwell and Johnson, 2000]. Multiple studies investigated the effect of different irradiances on the photosynthesis of macroalgae and the potential of recovery after light stress exposure [García-Sánchez et al., 2012, Flores-Molina et al., 2014, Giovagnetti et al., 2018, Quintano et al., 2019].

As benthic macroalgae, members of the genus *Caulerpa* are generally sensitive to high light radiation [Horstmann, 1983, Ukabi et al., 2013, de Gaillande et al., 2017]. Consistently, *C. lentillifera* has been found to thrive best under relatively low irradiances (10 to 100 μmol photons m^{-2} s^{-1}) of PAR and to show signs of photooxidation and photodamage under irra-diances of 360 μmol photons m^{-2} s^{-1} [Guo et al., 2015a, Su et al., 2017, Kang et al., 2020]. However, the physiological response of *C. lentillifera* to light irradiances over time spans >1 week and the potential of recovery after light-induced stress exposure is still unknown, but crucial for farmers to adapt culture conditions accordingly.

For sea grape trade, the place of production and retail often differs from each other such as most of the fresh harvested seaweeds in Viet Nam are exported to Japan via air freight [de Gaillande et al., 2017, Terada et al., 2018]. During transport and retail, *C. lentillifera* is stored in a variety of different plastic materials. Due to the thermo-isolating properties of Polystyrene (PS) [Aditya et al., 2017], containers of this material with moisture sheets to counteract desiccation, are commonly used to pack sea grapes for shipment [Terada et al., 2018]. However, for retailing to the end consumer, packaging in different plastic materials is common and the plastic properties can strongly influence the physiology of packed sea grapes [Tuong et al., 2016]. In Viet Nam, sea grapes are frequently stored in Polyethylene Terephthalate (PET) containers, having the advantage that costumers can see the product through the transparent material. PS and PET do differ not only in their transparency and thermal isolation [Aditya et al., 2017], but also in their properties regarding oxygen perme-ability [Zeman and Kubík, 2007]. During storage, algae are in danger of desiccation, leading to dehydration and consequently a loss of weight [Holzinger and Karsten, 2013].

Desiccation stress is in this effect comparable to salinity stress, because both result in a decrease of the alga's water potential [Kirst, 1990]. However in contrast to salinity stress, during desiccation, cellular ion ratios remain constant, while ion concentrations increase [Kirst, 1990, Holzinger and Karsten, 2013]. Therefore, desiccation can result in osmotic and ionic stress, which might ultimately lead to an inhibition of the electron flow at different sites at the photosynthetic apparatus [Wiltens et al., 1978, Satoh et al., 1983, Xia et al., 2004, Gao et al., 2011]. Inhibitions may lead to accumulation of Reactive Oxygen Species (ROS) (oxida-tive stress, [Kumar et al., 2014]) and potentially photodamage [Kirst, 1990]. Multiple studies showed the loss of water is negatively correlated with maximum quantum yield of PSII, but partly, the potential for recovery of F_v/F_m after re-hydration can be observed [Gao et al., 2011, Flores-Molina et al., 2014, Holzinger et al., 2015, Xu et al., 2016]. In nature, intertidal seaweeds are exposed to air, e.g., during low tide, where the common strategy is to reduce the metabolic activities and cope with the desiccation stress. However, packed sea grapes have desiccation times of ~1 week. In the airfreight packaging environment (PS), F_v/F_m values of *C. lentillifera* were found to decline from values of >0.7 to 0.60±0.22 and 0.47±0.26 after 4 and 8 days of desiccation, respectively. After packaging over 12 days, algae were consid-

ered dead with F_v/F_m values of 0.10±0.10 and a water loss of 72% [Terada et al., 2018]. In Nha Trang, Viet Nam, common practice is packaging in transparent PET containers, where algae are, additionally to desiccation stress, exposed to surrounding irradiances, in contrast to light impermeable PS packages. Therefore, light and desiccation are mutually influencing sea grape physiology.

In this study, we investigate the influence of irradiances on sea grapes in the culture and packing environment. We hypothesized that photosynthesis of *C. lentillifera* is best under pond irradiance conditions of 50 µmol photons m^{-2} s^{-1} and would be negatively influenced by irradiances above 100 µmol photons m^{-2} s^{-1} but could be maintained by irradiances around 25 µmol photons m^{-2} s^{-1}. Additionally, we indented to answer the question, whether sea grapes can recover from the potential stress after being transferred back to more suitable light conditions. For the packaging experiment, we hypothesized that sea grapes transported under dark conditions would physiologically suffer, because the non-cyclic photophosphorylation process of photosynthesis requires light in addition to a constant supply of water molecules. Furthermore, we expect that higher irradiances will cause physiological stress reactions, because desiccation might lead to a lack of water essential for photosynthesis. We are making a first attempt in defining the optimal irradiances for sea grapes in the packaging environment.

4.2 Material and methods

4.2.1 Sample collection

The experiments presented in this study were carried out during July to August 2019 and February to March 2020 at the laboratory facilities of the Institute of Oceanography (IO) in Nha Trang ($12°14'25.2''$N; $109°11'55.6''$E), located in the Central South coast of Viet Nam (Fig. 4.2). The experiments are referred to as *culture* and *packaging* experiment, as the influences of different PARs on sea grapes during culture and under the packaging environment were investigated. For the culture experiment, sea grapes were collected at a sea grape farm (VIJA) at Van Phong Bay ($12∘35'11.8''$ N; $109°13'26.7''$ E) in the Khánh Hòa province. *Caulerpa lentillifera* samples for the packaging experiment were purchased from a local market in northern Nha Trang.

4.2.2 Chlorophyll *a* variable fluorescence measurements

Photosynthetic performance was determined in vivo by measuring variable Chl *a* fluorescence using a portable Diving-PAM Chl fluorometer (Walz, Germany). F_v/F_m was measured in 7 min dark-adapted sea grape fronds [Schreiber et al., 1995, Maxwell and Johnson, 2000]. Sea grapes were considered unstressed when F_v/F_m values were ≥ 0.7.
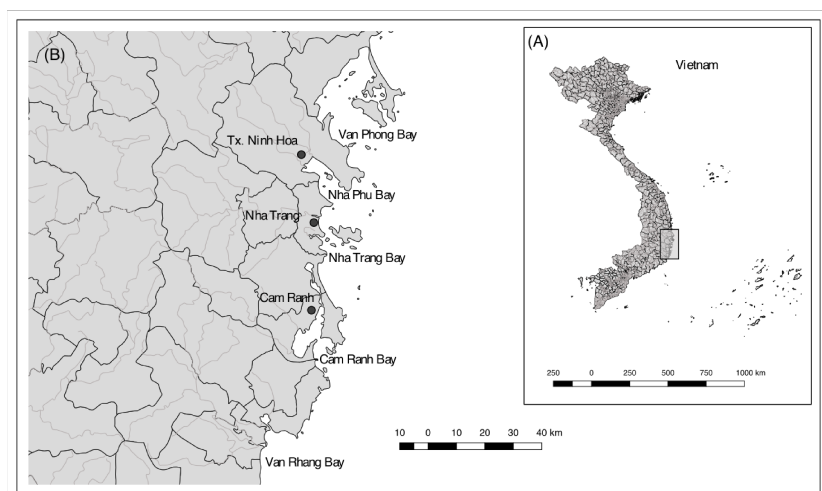
Figure 4.1: **A**) Coast around the city of Nha Trang and Van Phong Bay, where the VIJA sea grape farm is located. Each map has a scale bar at the bottom. **B**) Map of Vietnam.

4.2.3   Culture experiment: Experimental set-up, measurements, and data analysis

Based on the measured sea grape pond conditions of 50 µmol photons m^{-2} s^{-1}, two additional irradiance treatments were designed (25 and 100 µmol photons m^{-2} s^{-1}). Following common practice at sea grape farms, the algae were cultured in tray culture, where sea grapes are placed between plastic meshes. Trays (18.5×9.5 cm) were stocked with an initial of 35.0±1.0 g fresh sea grapes and grown out in natural seawater in an outdoor tank under natural solar irradiances for approximately 1 month prior the start of the experiment. Three aquaria (59×25×25 cm; 37 L, fitting 9 algae trays) for the three treatments and two aquaria (30×20×20 cm, 12 L, fitting 3 algae trays) for the recovery were set up with T5 High Output Fluorescence lights (2×39 W; 10,000 Kelvin (K)) for illumination in a 12:12-h light:dark rhythm.

The different irradiances were adjusted by adapting the height of lamps over the aquaria and monitored using a LI-1400 datalogger (LICOR Biosciences, USA). Each treatment had a variation of ±5 µmol photons m^{-2} s^{-1} within the aquaria. For the 25 µmol photons m^{-2} s^{-1} light treatment, gauze was additionally used for shading between the light source and the water surface. All aquaria were equipped with a constant air supply. Seawater from the adjacent coast was stored in a tank for water exchanges (every 2 days) in the experimental aquaria to ensure constant nutrient levels and water quality over the course of the experiment. Temperature, salinity, and pH were monitored to ensure constant conditions between and within aquaria. Prior to the start of the experiment, algae were acclimatized for 2 days (50±5 µmol photons m^{-2} s^{-1}, 27.2±0.4 °C, Absolute Salinity (S$_A$) 34.6±0.5, pH 8.5±0.3).

During the experiment, changes in F$_v$/F$_m$ were measured using a Diving-PAM fluorometer. F$_v$/F$_m$ values were taken for each tray on the initial day of the experiment, as well as on days 1, 7, 14, and 21. In order to examine the potential of recovery after potential light

induced physiological stress, three replicates per treatment were transferred to the additional recovery aquaria (50 µmol photons m^{-2} s^{-1}) on days 7 and 14. F$_v$/F$_m$ was monitored right before transfer and after 1 and 7 days under recovery irradiances (50 µmol photons m^{-2} s^{-1}).

For statistical analysis, F$_v$/F$_m$ of sea grape trays were averaged as mean and Standard Deviation (SD) per treatment (n=3). Statistical differences between the treatments were analyzed using one-factor Analysis of Variance (ANOVA) (followed by Tukey's Honest Significant Difference (HSD) test) with the fixed factor *treatment* (levels 50, 100, 25 µmol photons m^{-2} s^{-1}) which was conducted for each day of measurement over the experimental course between the alga groups without transfer to recovery, with recovery after 7 and 14 days, respectively. Analyses were conducted with a significance level of p<0.05. All statistical tests were conducted in R Core Team [R Core Team, 2019]), and graphics were produced using ggplot2 [Wickham, 2016].

4.2.4 Packaging experiment: experimental set-up, measurements, and data analysis

The purchased sea grape fronds were already cut from the stolon, as common practice for consumption and retail of the fresh product. Sea grapes were acclimated in sea water (28.2°C, S$_A$ 34.2, pH 8.5) under room irradiances for 3 days prior start of the experiment. Four sea grape fronds were placed on the long side of PET containers (9×9×15 cm, capacity of 500 g) not attached to each other. A moisture sheet in each container kept the humidity constant at 100%.

Initial F$_v$/F$_m$ were measured for 50 randomly chosen fronds from the batch and initial biomass as Wet Weight (WW) for sea grapes of each container was taken. WW and F$_v$/F$_m$ values of the stored sea grapes were quantified after storage of 2, 4, 8, and 12 days under three different irradiances (darkness 0, room irradiance 3±5, and high irradiance 70±5 µmol photons m^{-2} s^{-1}). Five replicates per irradiance treatment for each time period were prepared. The containers for the dark treatment were wrapped in aluminum foil, and the caps were coloured with black spray. A T5 High Output Fluorescence light (2×39 W; 10,000 K) was placed over the containers of the high and medium light treatment, and adjustments of the heights of the lamp ensured an irradiance of 70±5 µmol photons m^{-2} s^{-1} of PAR in a 12:12-h light:dark rhythm. Temperature loggers (HOBO, USA) were placed in one container of each treatment to monitor the temperature over the course of the experiments in 30-min intervals. In order to determine the potential of recovery, the sea grapes were re-immersed in seawater under room irradiances of 3±5 µmol photons m^{-2} s^{-1} after the desiccation period and F$_v$/F$_m$ values were quantified 10 min, 3 h, 6 h, and 24 h after re-immersion. Percentage of difference in F$_v$/F$_m$ over recovery period was calculated following the formula:

$$Percent\ of\ initial\ after\ desiccation\ (\%) = F_v/F_{m\,t} \times (\frac{100}{F_v/F_{m\,i}}) - 100, \qquad (4.1)$$

with F$_v$/F$_{m\,t}$ being measured after time t of desiccation and subsequent 24 h of re-immersion in seawater and F$_v$/F$_{m\,i}$ being the value measured directly after desiccation. Mois-

ture content after each desiccation period (M_t %) was calculated following the formula:

$$M_t(\%) = (\frac{(W_i - W_t)}{W_i}) \times 100, \hspace{2cm} (4.2)$$

with W_i as the initial WW of sea grapes after moisture removal at start of the experiment, and W_t as the WW after desiccation period t in days [Seremet et al., 2016, Terada et al., 2018]. The additional irradiance treatment of 20 μmol photons m^{-2} s^{-1} was quantified following the same protocol described above. However, physiological response was only quantified by F_v/F_m values and recovery potential and moisture content were not conducted. The results are therefore presented separately as comparison between the three light treatments (3, 20, and 70 μmol photons m^{-2} s^{-1}).

For statistical purposes, F_v/F_m of sea grapes were averaged per container and mean and SD were calculated (n=3–5). Outliers were identified using Grubbs' test. Differences in F_v/F_m and moisture content of sea grapes measured after the desiccation period were compared between treatments on each day with a one-factor ANOVA (followed by Tukey's HSD test) and the fixed factor *treatment* (levels 0, 3, 70 μmol photons m^{-2} s^{-1}). In order to test for differences in F_v/F_m of sea grapes over the desiccation and recovery period, a one-factor ANOVA (followed by Tukey's HSD test) with the fixed factor *period* (levels *initial*, *after desiccation period*, *after 24 h recovery*) was conducted and differences between the three light treatments were tested using a fixed term *treatment* (levels 0, 3, 70 μmol photons m^{-2} s^{-1}). In all cases, Levene and Shapiro-Wilk tests were carried out, and if requirements for ANOVA were not met, a Kruskal-Wallis test (followed by pairwise Dunn test with Bonferroni correction) was conducted. Analyses were conducted with a significance level of p<0.05. All statistical tests were conducted in R Core Team [R Core Team, 2019], and graphics were produced using ggplot2 [Wickham, 2016].

4.3 Results

4.3.1 Culture experiment

At the farm facility in Van Phong Bay, algae were maintained in shaded tidal ponds (∼50 μmol photons m^{-2} s^{-1}), with F_v/F_m values indicating a good physiological state (≥0.7, unpublished data). Temperature in the experimental aquaria showed a mean of 28.4±1.2 °C. Salinity and pH values ranged from 34.5 to 37.5 and 8.4 to 9.0, respectively. Initial F_v/F_m of all three treatments (25, 50, and 100 μmol photons m^{-2} s^{-1}) were similar, with values between 0.67±0.02 and 0.7±0.02 (Fig.4.2).

F_v/F_m of sea grapes cultured under 25 and 50 μmol photons m^{-2} s^{-1} did not change significantly from each over the 21 experimental days (P>0.05). However, F_v/F_m of sea grapes exposed to 100 μmol photons m^{-2} s^{-1} was significantly lower after 7 (0.54±0.06), 14 (0.54±0.08), and 21 days (0.63±0.03) than that of sea grapes under 25 and 50 μmol photons m^{-2} s^{-1} (≥ 0.70±0.03), respectively (Fig.4.2). However, algae cultured under 100 μmol photons m^{-2} s^{-1} showed a trend of increase in F_v/F_m values from day 14 to 21 of 0.09.

After sea grapes were transferred from 100 μmol photons m^{-2} s^{-1} to recovery conditions
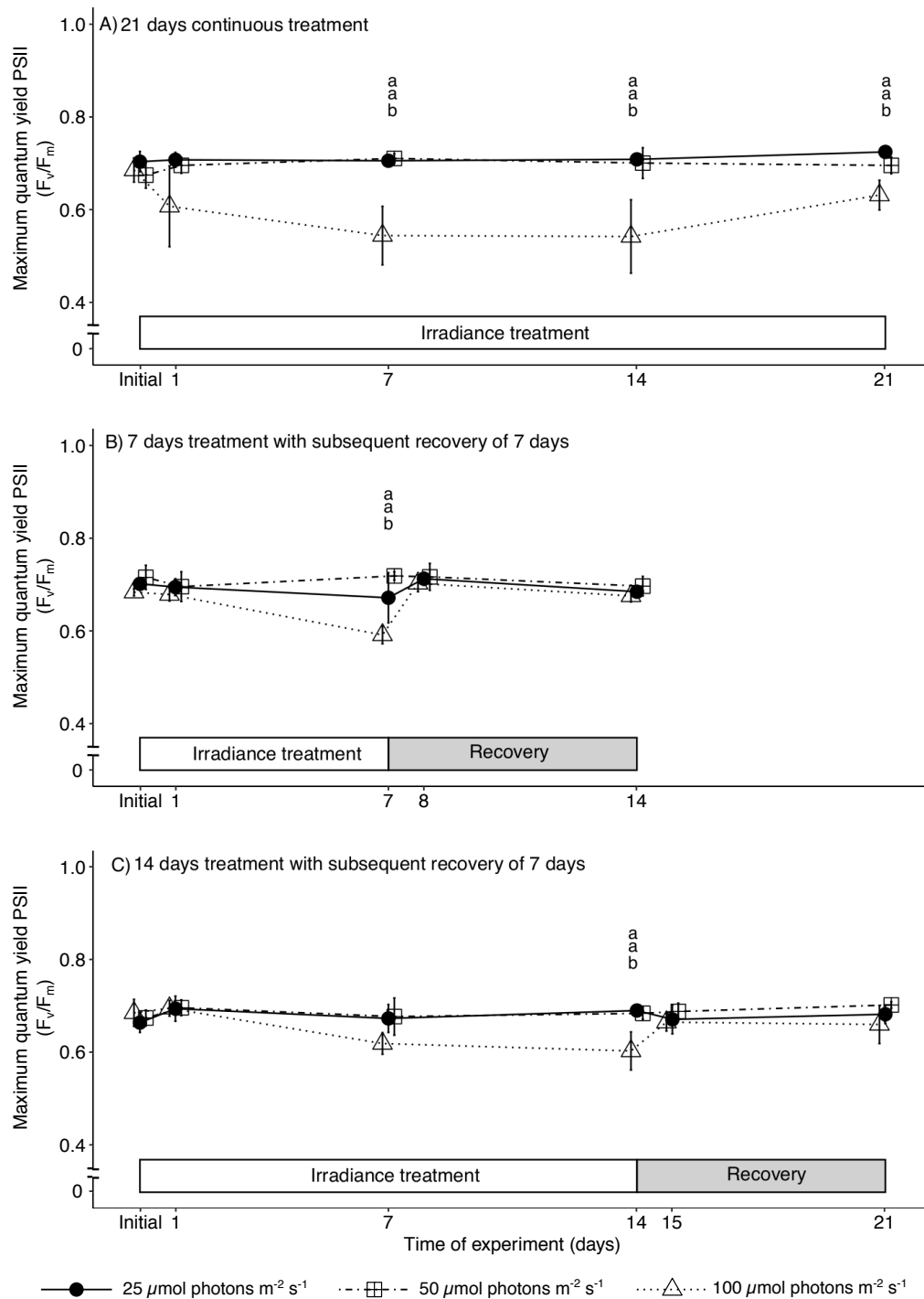
Figure 4.2: Change of F_v/F_m of *Caulerpa lentillifera* under three different treatment irradiances (25, 50, 100 µmol photons m^{-2} s^{-1}, 12:12 light:dark photoperiod) over 21 days continuously (**A**) and with transfer to recovery conditions (50 µmol photons m^{-2} s^{-1}, 12:12 light:dark photoperiod) after 7 (**B**) and 14 days (**C**). White and grey bars indicate exposure to the different treatment irradiances or recovery conditions, respectively. Data are mean values~standard deviation (n=3). Letters indicate significant differences between treatments at p<0.05. Letters are assigned to treatments top down according to order in graph.

Treatment irradiances (μmol photons m^{-2} s^{-1})
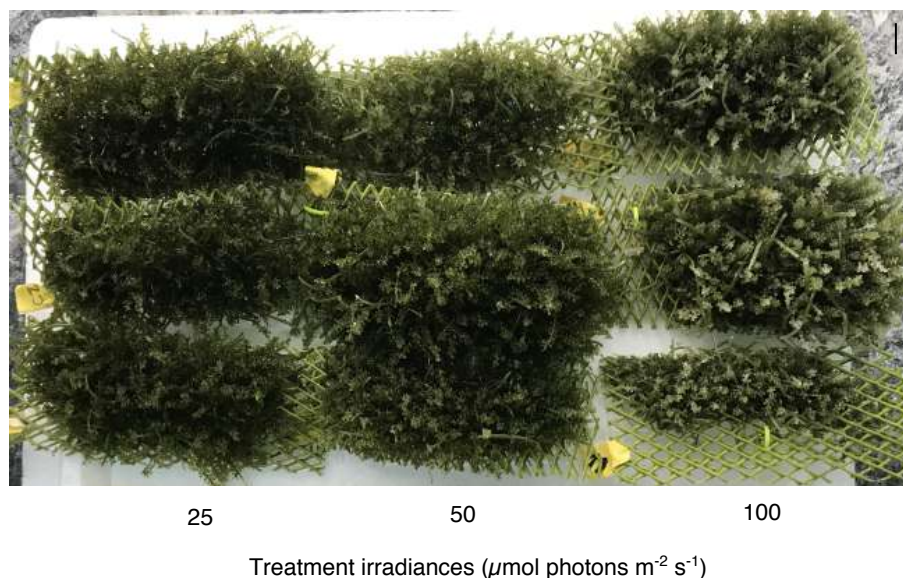
Figure 4.3: Photograph of sea grape (*Caulerpa lentillifera*) trays (n=3) after exposure to irradiances of 25, 50 and 100 µmol photons m^{-2} s^{-1} for 21 days. Black scale bar in the right top corner represents 2 cm.

(50 µmol photons m^{-2} s^{-1}) after 7 and 14 days of exposure, F_v/F_m increased instantaneously by 0.11 and 0.06 over 1 day and no significant difference between all three treatments was observed. Sea grapes under high irradiances (100 µmol photons m^{-2} s^{-1}) showed a fading of colour after 21 days of culture (Fig.4.3).

4.3.2 Packaging experiment

The temperature measured by HOBO loggers in the packaging containers did not vary between the three treatments (25.8$\pm$0.5°C, 25.7$\pm$0.4°C, and 26.8$\pm$0.8°C for 0, 3, and 70 µmol photons m^{-2} s^{-1}, respectively). Sea grapes were in a good physiological state at the start of the experiment (0.74$\pm$0.03, n=50). F_v/F_m developed differently between treatments over the desiccation period (Fig.4.4A). Sea grapes under room irradiance (3 µmol photons m^{-2} s^{-1}) showed only a slight decrease of F_v/F_m to 0.70$\pm$0.06 after 12 days of desiccation with moisture content not dropping below 91.0$\pm$7.0% (Fig.4.4B). However, desiccation over 2 days under an irradiance of 70 µmol photons m^{-2} s^{-1} leads to significantly decreased F_v/F_m of 0.59$\pm$0.07 compared to room irradiances. The decrease continued to a value of 0.42$\pm$0.11 after 12 days. However, F_v/F_m values showed a trend of recovery after re-hydration under room irradiances.

The moisture content after 12 days under 70 µmol photons m^{-2} s^{-1} was with 88.2$\pm$3.3%, only slightly lower than in the treatment of irradiance of 3 µmol photons m^{-2} s^{-1}. Under exclusion of light, F_v/F_m values remained stable over the first 4 days (0.74$\pm$0.02) but decreased rapidly after 8 and 12 days of packaging to significantly lower values compared to other two treatments (0.16$\pm$0.22 and 0.10$\pm$0.19, respectively). Exemplary pictures of sea grape fronds depict strong differences in thallus structure when packed under darkness; therefore, two pictures were provided for desiccation period of 8 and 12 days (Fig.4.5). No recovery
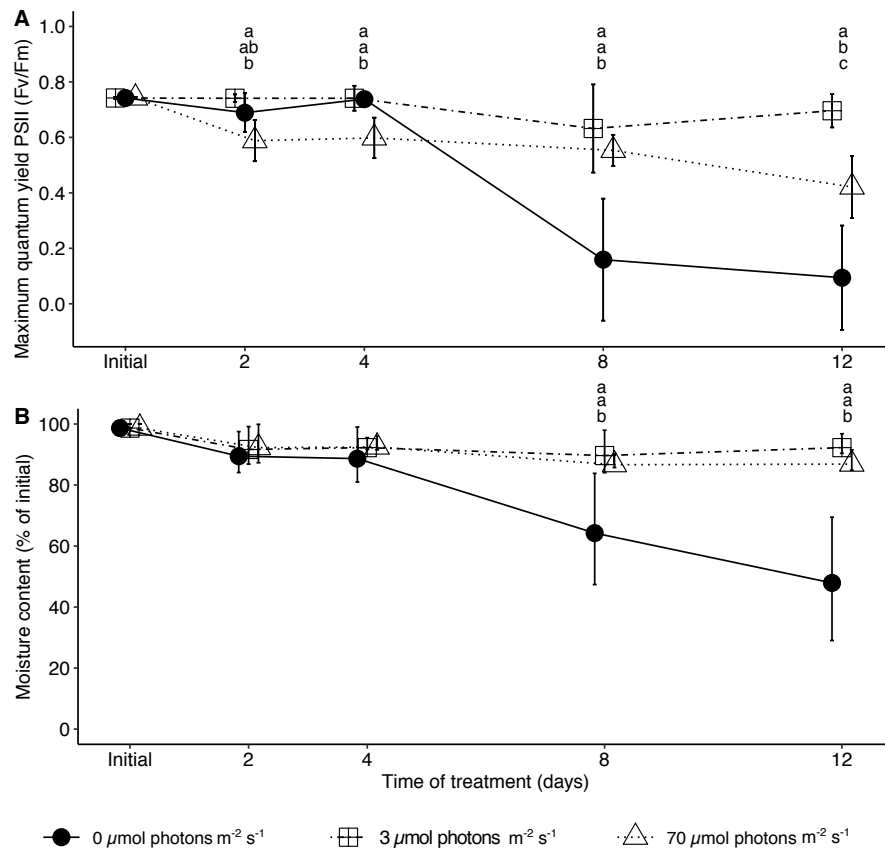
Figure 4.4: (**A**) Maximum quantum yield of photosystem II (PSII, F_v/F_m) and (**B**) moisture content (% of initial) of *Caulerpa lentillifera* packed in transparent polyethylene terephthalate containers exposed to three different irradiances (0, 3, 70 µmol photons m^{-2} s^{-1}) over a period of 12 days, respectively. Data represent mean values∼SD (n=4-5). Letters indicate significant differences between treatments (one-factor ANOVA followed by Tukey's HSD or Kruskal Wallis test followed by pairwise Dunn test with Bonferroni correction, p<0.05) and are assigned to treatments top down according to order in graph,

of F_v/F_m was observed, but rather a further decrease of the values (Fig.4.6, absolute values see Appendix C). Moisture content decreased strongly from 90±9.0% (4 days) to 49.25±20% (12 days) (Fig.4.4B). Sea grapes under 20 µmol photons m^{-2} s^{-1} had constantly slightly lower F_v/F_m values than algae under room irradiances (Fig.4.7). This difference was significantly lower 4 days under packaging ambience with 0.61±0.04. However, F_v/F_m values were consistently higher than of sea grapes under 70 µmol photons m^{-2} s^{-1}.
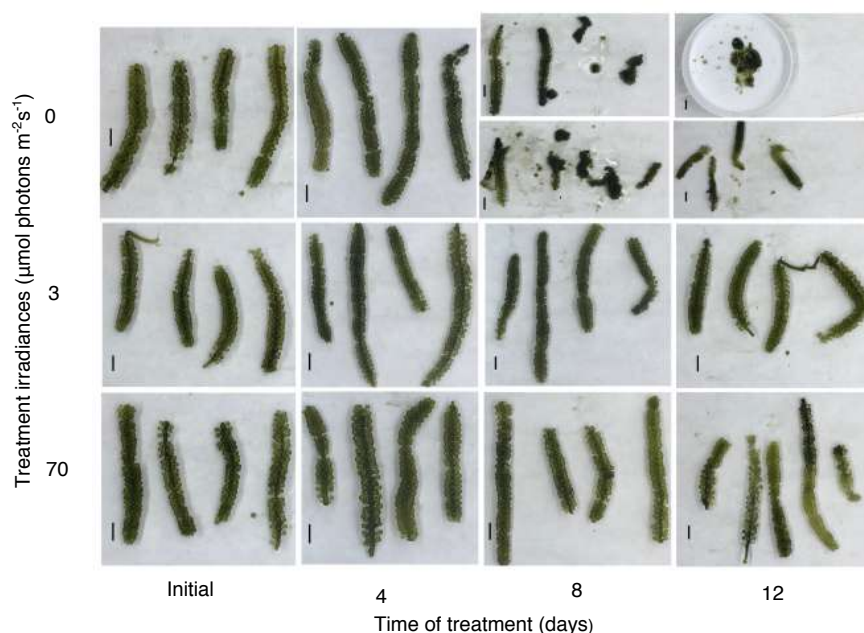
Figure 4.5: Exemplary pictures of *Caulerpa lentillifera* packed in polyethylene terephthalate containers from initial state, and after 4, 8 and 12 days under irradiance treatments 0, 3 and 70 µmol photons m^{-2} s^{-1}. After day 8 and 12 under packaging ambience in darkness, sea grapes have very different thallus structures, therefore two pictures are presented in order to demonstrate the pigmentation ranges of different desiccation stages of algae. Black scale bar in the left corner of each picture represents 1 cm.

4.4 Discussion

In this study, we found that light irradiances have a considerable impact on sea grapes' physiological constitution, both in the culture as well as in the packaging environment. Inappropriate irradiances seem to adversely affect the alga's physiology. However, in some cases, the sea grapes have the potential to recover. We used PAM fluorometry with F_v/F_m and can confirm that this tool is suitable to quantify the physiological state of *C. lentillifera* [Guo et al., 2015a, Guo et al., 2015b, Terada et al., 2018].
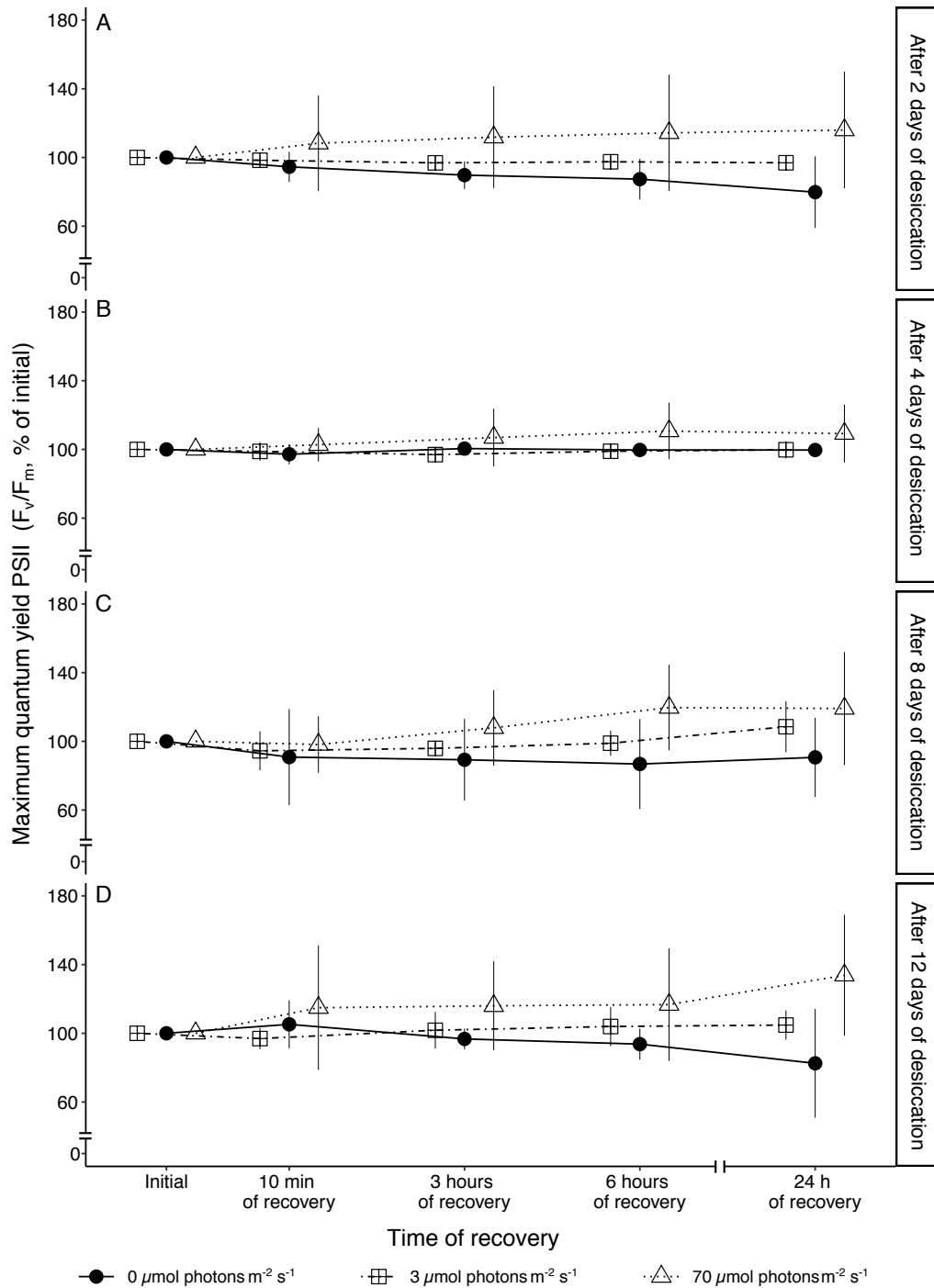
Figure 4.6: Changes of maximum quantum yield (F_v/F_m) (% of initial) of *Caulerpa lentillifera* under recovery conditions (re-hydration at an irradiance of 3 µmol photons m^{-2} s^{-1}, 12:12 light:dark photoperiod) over 24 hours. Experiment was carried out after a desiccation period under packaging ambience (polyethylene terephthalate container) of 2 (**A**), 4 (**B**), 8 (**C**) and 12 (**d**) days. % of initial relates to F_v/F_m values measured at the end of the desiccation and the start of the recovery experiment. Dashed lines denote 100% of initial. Data represent mean values~standard deviation (n=4-5).

4.4.1   Culture experiment

Based on the results of the culture experiment, we can confirm our hypothesis that sea grapes thrive best under irradiances of 25 and 50 µmol photons m^{-2} s^{-1} by maintaining their F_v/F_m values over the course of 21 days, indicating that they were in a good physiological state and not negatively impacted by the irradiances they were exposed to. These results are in line with studies identifying *C. lentillifera* and other representatives of the genus *Caulerpa* (e.g., *C. racemosa*) as shade-adapted low light plants, which is evident for some benthic seaweed [Horstmann, 1983, Ukabi et al., 2013, de Gaillande et al., 2017]. Furthermore, the decline in F_v/F_m under 100 µmol photons m^{-2} s^{-1} accompanied by the observed bleaching of the fronds is in line with observations by Guo et al. [Guo et al., 2015a]. The authors observed a decline in F_v/F_m of 0.16 in sea grape fronds over 7 days exposure to 100 µmol photons m^{-2} s^{-1} along with a significant decrease in Chl *a* content. The abrupt decrease in F_v/F_m as a consequence of high irradiances is a characteristic sign of photoinhibition [Goh et al., 2012] and has been observed widely in different temperate species of the genus *Caulerpa* [Ukabi et al., 2013] and also in *C. lentillifera* [Guo et al., 2015a].

However, the immediate and full recovery of F_v/F_m values of *C. lentillifera* within 24h after transfer to recovery conditions demonstrates the ability of the sea grapes to rapidly restore previous photosynthetic efficiency after certain stress exposure [Osmond, 1994, Häder et al., 1997, Hanelt et al., 1997]. This process of recovery from high irradiances was also observed in other green macroalgae (e.g., *Ulva rotunda*, [Franklin et al., 1992]). Han et al. found *U. pertusa* and *Umbraulva japonica* showing a decline of F_v/F_m values with exposure to increasing doses of PAR [Han et al., 2007]. Subsequent recovery under dim light increased F_v/F_m within 24 h completely and partially in connection with the habitat-related sensitivity, respectively. *U. pertusa* thrives in the intertidal, comparable with *C. lentillifera* [Norashikin et al., 2013].

However, intertidal algae are exposed to highly fluctuating environmental conditions [Davison and Pearson, 1996] and an elasticity of light requirements for photosynthesis might therefore be a coping mechanism of the seaweed survival, potentially related to their xanthophyll cycle or AOA [Han et al., 2003]. The increase of F_v/F_m within the third week under 100 µmol photons m^{-2} s^{-1} might potentially be due to a long-term acclimation of *C. lentillifera* to the changed irradiance environment. Longterm photoacclimation as an answer to changes in photo-regime, e.g., through morphological and physiological alternations, has been observed in several *Caulerpa* species (e.g., [Horstmann, 1983, Riechert and Dawes, 1986, Raniello et al., 2004, Malta et al., 2005, Raniello et al., 2006, Marquardt et al., 2010]. Raniello et al. describe the capacity of *C. racemosa* to reorganize the photosynthetic apparatus, change pigment composition, and eventually display different photosynthetic traits in relation to light availability over seasons and in the canopy [Raniello et al., 2004]. The observed trends are particularly interesting taking into account the economic value of sea grapes. Photoinhibition can decrease productivity and growth and therefore critically impact the harvest of *C. lentillifera* [Goh et al., 2012]. However, if sea grapes have the capacity to acclimate to higher irradiances, farmers could use the opportunity to their benefits. Therefore, this potential capacity should be explored further.

4.4.2 Packaging experiment

We attempted to contribute in defining suitable storage irradiances for sea grapes. The stable F_v/F_m values with only minimal loss of moisture content of *C. lentillifera* stored under room irradiances (3 µmol photons m^{-2} s^{-1}) in PET containers suggest a good physiological state of the alga and thus a sufficient quality of the product for the end consumer even after 12 days of storage.

However, Terada et al. found F_v/F_m of *C. lentillifera* packed in PS containers (irradiances of 3 µmol photons m^{-2} s^{-1}) declining to 0.10±0.10 along with 72% critical water loss and absence of recovery after re-immersion in sea water [Terada et al., 2018]. The authors suggest this might be caused by cellular alterations resulting in dysfunctional algae. These results imply potentially more favorable conditions of storage in PET than PS containers. However, potential explanations for the strong deviation between the results are the properties of packaging materials (PET vs. PS) and different storage temperatures (∼26 °C vs. 20 °C). Polymer type of containers has been found to influence the amount of total aerobic bacteria on packed sea grapes [Tuong et al., 2016], possibly due to differences in gas and especially Diatmoic Oxygen (O_2) permeability [Zeman and Kubík, 2007, Siracusa, 2012]. Accordingly, the microbial community was also found to influence the postharvest physiology of seaweeds [Liot et al., 1993].

Our hypothesis that packaging of sea grapes under dark (0 µmol photons m^{-2} s^{-1}) as well as high light (70 µmol photons m^{-2} s^{-1}) environments negatively influences the physiological status of *C. lentillifera* was supported by the results. Absence of light clearly constitutes a source of limitation stress for the seaweeds. Insufficient irradiance leads to a lack of carbon assimilation by plants, and under carbohydrate starvation, plants have to substitute sugar with protein and lipids before running out of energy to sustain metabolism [Brouquisse et al., 1998, Lavaud et al., 2020]. However, some polar seaweeds have been found to be adapted to survival under extended periods of darkness, e.g., through substantial starch storage [Gómez et al., 1997, Weykam et al., 1997, Wiencke et al., 2007]. Other plants are physiologically not that well equipped for extended dark or even light limiting periods, as studies on, for example, *Laminaria*, sea grasses, and microalgae show [Smayda and Mitchell-Innes, 1974, Dieck, 1993, Silva et al., 2013].

C. paspaloides was found to have significant lower starch concentrations following overwintering, along with stolons forming a higher percentage of the whole thalli biomass compared to the algas' fronds [O´Neal and Prince, 1988]. Thus, cutting of the sea grapes' stolons before packaging might even decrease carbon storage of the algae and therefore adversely affect survival. Over 4 days of packaging in darkness, sea grapes were still active with minor loss of water, indicating sufficient storage of essential nutrients. But the rapid decrease of moisture content over 8 and 12 days of packaging with simultaneously declining F_v/F_m values and without potential of recovery indicates an irreversible damage of the photosystem. However, a high variability in moisture contents, F_v/F_m values, and thallus structure (soft vs. intact) after 8 and 12 days of desiccation under dark conditions might be traced back to unequal nutrient storage of the organisms. Interestingly, the decreased photosynthetic performance provoked by high light stress was reversible under recovery conditions, whereas
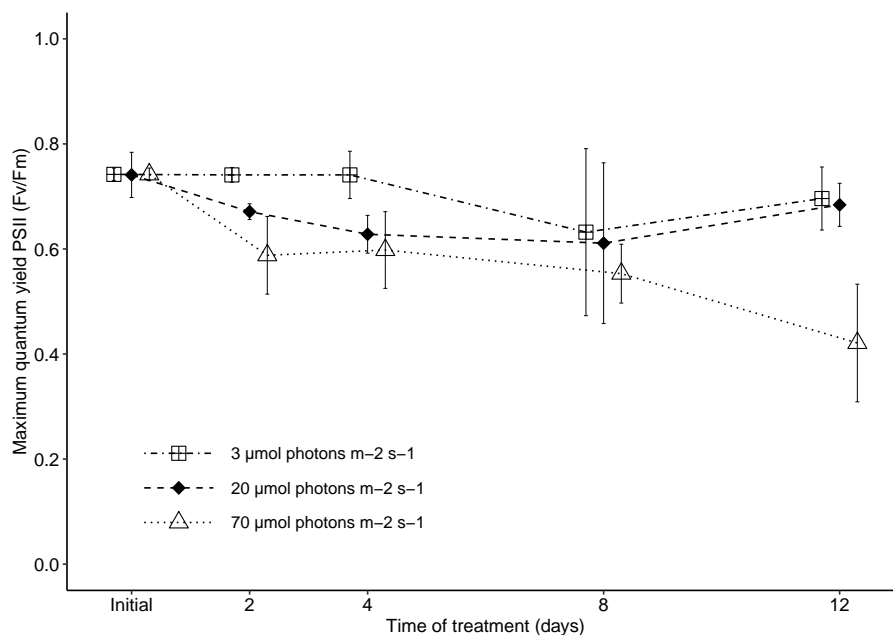
Figure 4.7: Maximum quantum yield of photosystemII (PSII, F_v/F_m) of *Caulerpa lentillifera* packed in polyethylene terephthalate containers under three different irradiances (3, 20, 70 µmol photons m^{-2} s^{-1}). Data are mean values~standard deviation (n= 3-5). Letters indicate significant differences between treatments at p<0.05. Letters are assigned to treatments top down according to order in graph

induced by darkness, a further decrease of F_v/F_m was observed.

This indicates that desiccation under darkness distinctively affected the ultrastructure of the sea grape's membrane [Davison and Pearson, 1996, Holzinger and Karsten, 2013, Flores-Molina et al., 2014], whereas the thalli under light stress were still intact but showed a faster decrease of F_v/F_m. Desiccation and the resulting hypersalinity in the cells seem to affect the process of photosynthesis at different steps. It might have restricted the inflow of Water (H_2O) molecules as essential electron donor at the water splitting side of PSII, as well as interrupting the electron transport from PSII to Photosystem I (PSI) and energy transfer between pigments [Satoh et al., 1983, Gao et al., 2011]. The reduced ability to use absorbed light energy requires a corresponding increase in processes that dissipate excess solar energy to avoid damage [Davison and Pearson, 1996]. Consequently, desiccation stress seems to lower the threshold of increased non-photochemical quenching occurrence caused by high irradiances. This observation could explain the successive decrease of F_v/F_m with increasing irradiances (3, 20, 70 µmol photons m^{-2} s^{-1}), which was observed under desiccation conditions. The decreased F_v/F_m under 20 µmol photons m^{-2} s^{-1} compared to room irradiances in the packaging ambience on one hand and stable photosynthesis activity under similar irradiances under immersed conditions suggests that energy absorption exceeded the limit to be used in photochemical quenching under the desiccation packaging conditions. The potential of recovery and the apparently intact thallus structure, however, imply that no lasting photodamage appeared, but that protective mechanisms were still intact.

4.5 Conclusions

Our objective to investigate suitable irradiances for sea grapes in culture and packaging conditions resulted in certain recommendations for sea grape farmers and retailers. For outdoor sea grape culture, our results suggest that shading of sea grapes is beneficial. Additionally, PET containers equipped with moisture sheets seem to be a suitable opportunity for the product's storage over at last 12 days, but the additional provision of a dim light environment is essential to maintain a good physiological state of *Caulerpa lentillifera* and therefore offer a fresh product of high quality to the end consumer.

Chapter 5

Publication IV & V

Pomegranates prepared to eat.

5.1 Publication IV

The antioxidative potential of sea grapes (*Caulerpa lentillifera*, Chlorophyta) can be triggered by light to reach comparable values of pomegranate and other highly nutritious fruits

Jonas Sommer, Andreas Kunzmann, Lara Elisabeth Stuthmann, Karin Springer

Abstract

The interest in edible sea grapes (*Caulerpa lentillifera*) is increasing due to their potentially beneficial effect on human health. This macroalga, already used for direct and indirect human consumption, is grown in aquacultures in Vietnam and The Philippines. Here, the edible fronds of sea grapes were examined for their Antioxidant Activity (AOA) at light intensities from 140 to 300 µmol photons m^{-2} s^{-1} and compared to commercially dehydrated *C. lentillifera* and the renowned highly antioxidative fruits Pomegranates (*Punica granatum*), Goji (*Lycium barbarum* and *L. chinense*) and Aronia (*Aronia melanocarpa*) berries, using an 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)$^{.+}$-assay for all samples. AOA of fronds exposed to 300 µmol photons m^{-2} s^{-1} for 14 days increased by about 320% from the initial value of 72.2±5.6 to 232.2±34.2 Trolox Equivalents (TE) mmol $100g^{-1}$ Dry Weight (DW) onto the level of Pomegranates (272.8±23.0 TE mmol $100g^{-1}$ DW. This application could be used as a post-cultivation treatment in sea grape cultures to increase the quality and nutritional value of the product.

Keywords: ABTS$^{.+}$ assay, Antioxidant activity, Aronia, Goji, High light intensities, Post-harvest

5.1.1 Introduction

While algae are traditionally consumed as sea vegetables in Asian countries [Fleurence, 2016], the rising global interest in a healthy diet has increased the popularity of seaweeds. Especially the edible green macroalga *Caulerpa lentillifera*, also known as sea grapes or green caviar, is commercially cultured in several South East Asian countries (especially Vietnam and The Philippines), due to the ease of propagation, its high growth rate and potential health benefits [Paul et al., 2014]. The thallus consists of a stolon with rhizoids and the edible fronds with vesiculate ramuli, evoking the association with caviar (Fig.5.1) [Zubia et al., 2020].
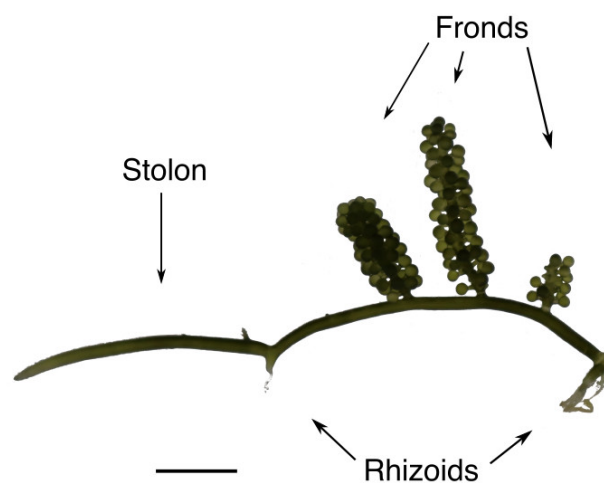
Figure 5.1: *Caulerpa lentillifera* consists of edible fronds, which are connected by stolons with rhizoids. Scale bar, 1 cm.

The aquaculture of the benthic seaweed takes place in tidal ponds and after harvest, the fronds are cleaned and packed for transport or direct retail while still alive and photosynthetically active. During culture and storage of *C. lentillifera*, suitable light conditions have been shown to be a crucial factor for the shade-adapted light-sensitive seaweed, with irradiances higher than 100 µmol photons m^{-2} s^{-1} causing physiological stress reactions, reflected in e.g. lower maximum quantum yields of Photosystem II (PSII) (F_v/F_m) [Guo et al., 2015a, Stuthmann et al., 2020].

A smaller portion of the harvest is dehydrated with brine cure or salt for preservation before retail [Zubia et al., 2020]. The special texture of sea grapes in combination with low levels of lipids [Niwano et al., 2009], multiple Essential Amino Acid (EAA)s, Polyunsaturated Fatty Acids (PUFA)s [Saito et al., 2010] and diverse minerals have increased their popularity, even though nutritional studies on this organism are still rare. Furthermore, different preliminary studies have attributed a naturally high non-enzymatic Antioxidant Activity (AOA) to this species [Matanjun et al., 2009, Nguyen et al., 2011, Paul et al., 2014, Yap et al., 2019]. *C. lentillifera* is rich in ascorbic acid (vitamin C), α-tocopherol (vitamin E) [Matanjun et al.,

2009] and also its polyphenolic content is decisively correlating with their AOA [Nguyen et al., 2011]. The essential importance of antioxidants is based on their ability to defuse Reactive Oxygen Species (ROS), which are related to the pathogenesis of several human diseases such as diabetes mellitus, neurodegenerative disorders, cardiovascular diseases and cancer [Metodiewa and Kośka, 1999, Halliwell, 2000, Zampelas and Micha, 2001].

In photosynthetically active organisms, the probability of ROS production in chloroplasts is increased during high light stress in the saturation region of photosynthesis. As a physiological response, the production of antioxidative compounds is expected to increase under stress conditions [Ito and Hori, 1989]. The variety of assays and extraction methods to measure activity of all antioxidants present within cells of an organism is high and therefore direct comparisons between studies are difficult. One method commonly used in scientific studies to investigate and quantify the total antioxidant capacity of food sources is the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay [Gülçin, 2012].

Humans can obtain antioxidants through their diet, and some food products are especially known for their rich proportion. Pomegranate (*Punica granatum*), Goji or Wolfberry (*Lycium barbarum* and *L. chinense*) and Aronia (*Aronia melanocarpa*) berries are among those repeatedly reported as *superfruit* as a tool to highlight and promote their bioactive compounds and nutritional qualities, including their AOA [Sidhu and Zafar, 2012]. For fruits the term *fruit-quality* is used mainly for the appearance and the taste, however, there has been an association with health benefits when consumed, mainly linked to antioxidative compounds like ascorbic acid [Atkinson et al., 2005].

The opportunity to use physiological stress treatments as an opportunity for manipulation of the antioxidant potential of fruits and an inherent increase of the *fruit-quality* has been proposed and discussed [Atkinson et al., 2005]. However, a successful manipulation requires a fundamental understanding of the organism's physiology.

The present study was designed to apply this concept to the sea grape *C. lentillifera*, making a first attempt to increase the alga's quality as food product by triggering AOA, using irradiances of up to 300 µmol photons m^{-2} s^{-1} over a period of 14 days. Furthermore, the AOA of experimentally treated sea grapes was compared with the AOA of commercially cultured, purchasable preserved dehydrated fronds of *C. lentillifera*, as well as renowned highly antioxidative fruits like *P. granatum*, *L. barbarum*/*L. chinense*, and *A. melanocarpa*.

5.1.2 Material and methods

5.1.2.1 Sample material

C. lentillifera organisms used in this experiment were harvested in the Vietnamese sea grape-farm VIJA (Van Phong Bay, Vietnam) in June 2019 and were transported to the Marine Experimental Ecology (MAREE) aquaculture facility of the Leibniz Centre for Tropical Marine Research (ZMT) in Bremen, Germany. The algae were cultured in aquaria (130cmx36cmx80cm) filled with artificial sea water at constant temperature (25.6±1.3°C), absolute salinity (Absolute Salinity (S_A) 34.5±0.4), pH (8.1±0.1) and irradiance (50 µmol photons m^{-2} s^{-1}).

For comparison of the AOA, different commercial products were acquired in German supermarkets, namely *P. granatum*, dried *L. barbarum/L. chinense* and dried *A. melanocarpa* berries. Also, three commercially cultured and dehydrated types of sea grapes were tested for their AOA (SeA-VIET, Vietnam, VIJA, Vietnam, UMI, Korea). The dehydrated sea grape samples were re-hydrated in freshwater prior to the biochemical analysis, following the recommendations of the retailers.

5.1.2.2 Experimental set-up

For testing the antioxidant potential in respect to different light intensities, five levels of irradiances of Photosynthetically Active Radiation (PAR) were chosen: 140, 180, 220, 260 and 300 µmol photons m^{-2} s^{-1}. The sea grapes were held in 1 L glass beakers at constant temperature (24.0 ±0.5°C). For each treatment, five beakers containing four *C. lentillifera* organisms with approximately 2-4 fronds attached to the stolon were placed underneath a Light Emitting Diode (LED) bar (SolarStinger Sunstrip 800mm, ECONLUX GmbH, Cologne, Germany) radiating white light in a 12:12 light:dark cycle using the standard settings. To adjust the different light intensities the irradiances were measured directly at the water surface using a LI-COR data logger (LI-189, Lincoln, USA). The seawater was stirred twice a day to evade gradient formation regarding nutrients and also to maintain a balanced exposure to the light. The seawater was exchanged after 7 days.

5.1.2.3 Sample preparations and extraction

Fresh and rehydrated *C. lentillifera* biomass was frozen (-80°C) directly after sampling and freeze-dried for 24 hours at 1 mbar (ALPHA 1-4 LD plus, Christ GmbH, Osterode am Harz, Germany). The freeze-dried samples were ground to a powder for 20 sec using a benchtop homogenizer (FastPrep-24, MP Biomedicals, Germany). The fruits were crushed in liquid nitrogen. Sea grapes *C. lentillifera* and *P. granatum* (0.05 g Dry Weight (DW)), as well as *L. barbarum/L. chinense* and *A. melanocarpa* (0.035 g DW) were dissolved in 1 mL ethanol (70%) and extracted in a water bath (47°C) for 4 hours, being vortexed hourly. Prior to analysis, samples were centrifuged (2500 g, 20°C) for 5 minutes.

5.1.2.4 Analysis of antioxidant activity

The AOA was determined after a modified ABTS$^{.+}$ assay [Re et al., 1999], also known as Trolox Equivalent Antioxidant Capacity (TEAC) assay. A stock solution of 2.45 mM ABTS$^{.+}$ was obtained by oxidising 7.0 mM ABTS (Sherman Chemicals, Dorset, UK) with potassium disulfate for 16h. By dilution with ethanol (absolute) a working solution with a consistent photometrically measured (Thermo Scientific Genesys 140/150, Fisher Scientific GmbH, Schwerte, Germany) absorption of 0.7±0.02 at a wavelength of 734 nm was obtained. For analysis, 1 mL ABTS$^{.+}$ working solution was added to 10 µL sample extract and the deradicalization was measured after 6 minutes. AOA of the samples was expressed as Trolox Equivalents (TE) (TE mmol 100g^{-1} DW).

5.1.2.5   Statistical analysis

All statistical analyses and the creation of graphics were conducted using the statistic-software Red (R) (Version i386 4.0.2) combined with RStudio (Version 8.3) [RStudio Team, 2018]. For determination of significant differences, one-way Analysis of Variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) tests were conducted. Quantitative data are presented as mean values with the respective standard deviation.

5.1.3   Results and discussion

5.1.3.1   Antioxidant activity of sea grapes

Following exposure of *C. lentillifera* fronds to treatment irradiances (140–300 µmol photons m^{-2} s^{-1}), an enrichment of antioxidants was observed. The rise of the AOA was significantly dependent on the applied light intensity (F=19.93, p<0.001) and also on time (F=24.08, p<0.001, Fig. 5.2). *C. lentillifera* showed an initial value of 72.2±5.6 TE mmol 100g^{-1} DW after a cultivation at a light intensity of 50 µmol photons m^{-2} s^{-1}). The maximum AOAs were detected in sea grapes exposed to 300 µmol photons m^{-2} s^{-1} with 169.9±24.5 TE mmol 100g^{-1} DW after 7 and 232.2±34.2 TE mmol 100g^{-1} DW after 14 days of exposure, translating to an increase of about 320% compared to initial AOA values.
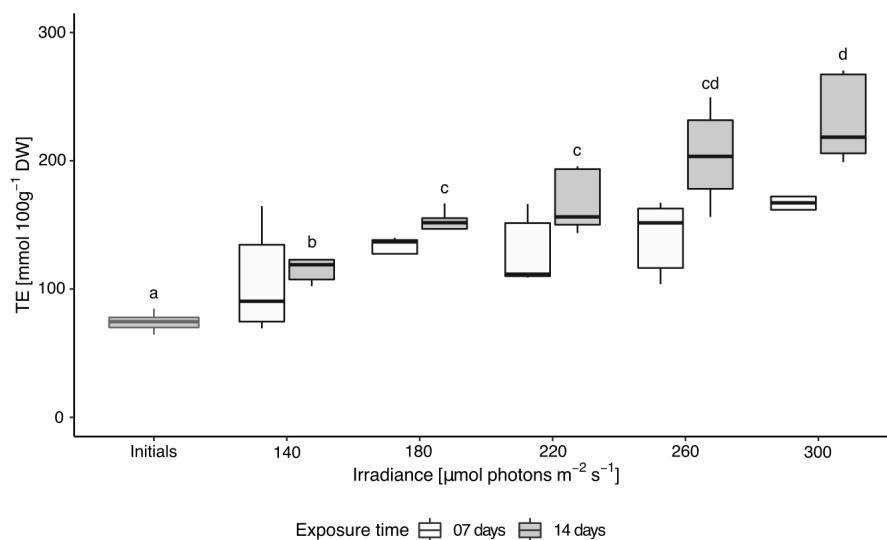
Figure 5.2: Effect of different light intensities on the antioxidant activity (AOA) of *Caulerpa lentillifera* fronds expressed as Trolox Equivalents (TE, mmol 100g^{-1} dry weight (DW) applied for 7 and 14 days, respectively (n=5-6). Letters indicate significant differences between initials and associated 14 days treatments (one-way ANOVA followed by Tukey's HSD, p<0.05)

The ABTS assay does not indicate specifically which compounds are responsible for the AOA, but sea grapes cultured under 50 µmol photons m^{-2} s^{-1} showed values of Total Phenolic Content (TPC) of 124.5±25.5 Gallic Acid Equivalents (GAE) mg 100g^{-1} DW. The TPC values increased under 14 days exposure to irradiances of 100, 200 and 400 µmol photons m^{-2} s^{-1}) to 152.6±23.2, 221.9±25.7 and 241.7±85.2 GAE mg 100g^{-1} DW, respectively [Stuthmann et al.,

2022]. The simultaneous increase of AOA and TPC indicates that polyphenolics are one major group of antioxidative compounds of sea grapes quantified in this study.

The measured AOA was expected to increase as a reaction of the exposure to irradiances above the photon saturation limit of photosynthesis and therefore as a protection against produced ROS [Hajiboland, 2014]. The saturation irradiance of shade-adapted seaweeds, like *Caulerpa* is in general lower than for other seaweeds [García-Sánchez et al., 2012]. The results suggest that the increase in AOA can be triggered by irradiances of 140 µmol photons m^{-2} s^{-1} and higher. However, Kang et al. cultured *C. lentillifera* under ascending irradiances (50, 100, 150 µmol photons m^{-2} s^{-1}, 16.7% blue+83.3% red spectral distribution) over 12 days and found similar AOAs for all treatments, with significantly higher reducing power for *C. lentillifera* at 150 µmol photons m^{-2} s^{-1} [Kang et al., 2020]. This indicates that an irradiance of approximately 150 µmol photons m^{-2} s^{-1}) might be a tipping point.

However, despite the steady increase of AOA with light treatment and exposure time, no saturation region of the AOA was detectable in this study and therefore it is assumed that the antioxidant potential of *C. lentillifera* fronds could be triggered even higher (Fig.5.2).

5.1.3.2   AOA of dehydrated sea grapes and fruits

The AOAs of the freshly harvested organisms and the purchased dehydrated sea grapes were similar, indicating that the post-harvest processing and dehydrating of the sea grapes did not negatively affect the antioxidative potential (Fig.5.3). However, the light triggered fronds exposed to 300 µmol photons m^{-2} s^{-1} for 14 days were significantly enriched in antioxidants compared to the other sea grape categories (p<0.05, Fig.5.3). These results introduce the possibility for farmers to expose sea grapes before retail or dehydration to higher light irradiances if an enrichment in AOA is desired.

Overall, the light triggered sea grapes showed similar antioxidative levels compared to *P. granatum* (272.8±23.0 TE mmol 100g^{-1} DW, p>0.05). *L. barbarum*/*L. chinense* and *A. melanocarpa* exhibited a significantly higher AOA compared to all measured sea grape fronds (p<0.001), with 408.5±27.6 and 435.9±46.1 TE mmol 100g^{-1} DW, respectively. Intra-study comparisons like this are important, since methodological parameters, like assay and extraction method, are varying widely among studies investigating AOA of *C. lentillifera* [Matanjun et al., 2008, Nguyen et al., 2011, Yap et al., 2019] and other food products [Gülçin, 2012] and direct comparisons between results are therefore hardly possible.

Therefore, this study provides a unique comparison of *C. lentillifera* with several *superfruits*. The competitiveness regarding AOA of *C. lentillifera* with renowned *superfruits* in combination with all the other specific health beneficial compounds (such as PUFAs, proteins etc.) makes this macroalga an exceptional food, also aiming at a commercial application in both human (as nutraceuticals for novel food) and animal health (feed additives for e.g. shrimps and fish in aquaculture approaches). The demonstrated potential of post-harvest manipulations needs closer investigations, but might be a useful and comparatively easy tool for farmers and retailers to further increase the value of this seaweed.
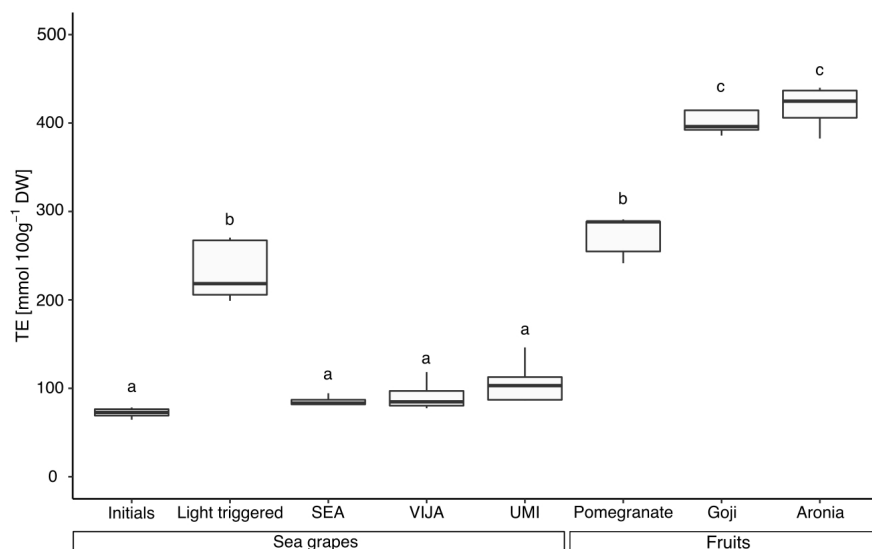
Figure 5.3: Levels of antioxidant activities of *Caulerpa lentillifera* initials and after exposure to 300 µmol photons m^{-2} s^{-1} for 14 days in comparison to commercial dehydrated sea grapes from Vietnam (SEA, VIJA) and Korea (UMI) and Pomegranate, dried Goji and Aronia berries (n=4-6). Values are expressed as Trolox Equivalents (TE, mmol 100g^{-1} dry weight (DW). Letters indicate significant differences between associated categories (one-way ANOVA followed by Tukey's HSD, p<0.05)

5.1.4   Conclusions

The successful increase of sea grapes AOA by exposure to increased irradiances on the level of *superfruit* Pomegranate (*P. granatum*), introduces the possibility to establish light treatments as post-harvest processing before sea grape dehydration or fresh retail, for example during cleaning process or transport. To determine the saturation region of AOA in *C. lentillifera*, even higher irradiances need to be applied in further studies.

5.2 Publication V

Improving the nutritional value of edible *Caulerpa lentillifera* (Chlorophyta) using high light intensities. A realistic tool for sea grape farmers.

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Abstract

Edible sea grapes (*Caulerpa lentillifera*) are produced in shaded ponds ($\sim$50 µmol photons m^{-2} s^{-1}) and are of increasing demand for direct human consumption. The controlled exposure to light-stress after harvest could increase algae's content of nutritionally valuable antioxidants, including phenols. In order to define a light-stress tool for farmers to use, we investigated the effect of five irradiances (50-600 µmol photons m^{-2} s^{-1}) for an exposure time of up to 14 days. Antioxidant Activity (AOA) (initial of 150.93$\pm$25.50 mmol Trolox Equivalents (TE) 100 g^{-1} Dry Weight (DW)) and Total Phenolic Content (TPC) (initial of 124.45$\pm$10.07 mg Gallic Acid Equivalents (GAE) 100 g^{-1} DW) significantly increased to values of up to 228.8$\pm$12.4 and 222.2$\pm$22.7% of initial, respectively. However, targeted increases in antioxidant parameters correlated with decreased values of F_v/F_m (p<0.001) and the Chlorophyll (Chl) *a* content in the edible frond was significantly lower at irradiances $\geq$400 µmol photons m^{-2} s^{-1} compared to the control (50 µmol photons m^{-2} s^{-1}), causing bleaching. As the physiological response of the alga (F_v/F_m, AOA, TPC, Chl *a*) depended on exposure time and irradiance treatment, both parameters could be adjusted to define treatments according to the consumer's needs. Light irradiances measured at a sea grape farm revealed that farmers could integrate such treatments labour- and cost-effectively by removing layers of plastic-meshes off their culture or post-harvest units. The pattern of Chl *a* loss and Red (R) colour channel values in sea grapes thallus parts implied, that a combination of degradation and chloroplast migration was responsible for bleaching at light-stress. Additionally, R channel values extracted from pictures could be used for Chl *a* estimation, due to strong correlation (r_S=-0.786, p<0.001). In conclusion farmers can use high light as post-harvest treatment in order to increase valuable antioxidants.

Keywords: Antioxidant content manipulation, Functional food, Green caviar, Post-harvest treatment

5.2.1 Background

Seaweeds currently represent $> 50\%$ of the total marine and coastal aquaculture production in terms of live weight equivalents [FAO, 2020]. The harvested biomass is increasingly used as sea-vegetable for direct human consumption [Chopin and Tacon, 2020]. However, the major part of seaweed production is limited to only eight genera of seaweeds [Chopin and Tacon, 2020], neglecting for example the potential of green algae [Moreira et al., 2021].

Sea grapes (*Caulerpa lentillifera*) are green sea-vegetables, known as *green caviar* (Europe), *umibudo* (Japan) or *rong nho* (Vietnam), and especially in Asian countries of high demand [de Gaillande et al., 2017]. This siphonous alga is appreciated for its special texture, consisting of horizontal stolons with rhizomes and edible fronds bearing vesiculate ramuli [Zubia et al., 2020], in combination with various nutritional benefits [Matanjun et al., 2009]. Sea grapes, like other intertidal, benthic algae, are adapted to habitats with highly fluctuating abiotic stressors over short temporal and local scales [Davison and Pearson, 1996]. In order to cope with these changing environmental conditions, seaweeds produce a large range of natural secondary metabolites, including carotenoids, tocopherols (vitamin E), ascorbic acid (vitamin C), and polyphenols often resulting in high Antioxidant Activity (AOA)s [Davison and Pearson, 1996, Cotas et al., 2020]. Many of the natural metabolites are recognized as essential components in human diets to counteract the metabolic syndrome and vitamin deficiencies [Rezayian et al., 2019, Cotas et al., 2020, John et al., 2020]. Consequently, these physiological responses of seaweeds could be manipulated in a controlled aquaculture environment in order to enhance biosynthesis of specific target-metabolites [Magnusson et al., 2015, Toth et al., 2020].

Sea grapes are currently cultured especially in tidal ponds in Vietnam and the Philippines, mainly for export to Japan [de Gaillande et al., 2017]. The alga is a shade-adapted plant and sensitive to high Photosynthetically Active Radiation (PAR), which is considered by farmers and retailers over the complete life-cycle of the organism, e.g. by shading the ponds with plastic meshes or keeping packed algae at low indoor irradiances [Guo et al., 2015a, Stuthmann et al., 2020]. Light is essential for plants to photosynthesize, but when the incoming light exceeds the limit needed for Carbon Dioxide (CO_2)-assimilation, photoprotective mechanisms are used to avoid severe photoinhibition and photodamage [Murchie and Niyogi, 2011]. Sea grapes showed signs of photoinhibition, like decreases in F_v/F_m values, at ≥ 100 µmol photons m^{-2} s^{-1} [Guo et al., 2015a, Kang et al., 2020], but with potential for recovery [Stuthmann et al., 2020].

Photosynthetic active organisms, including sea grapes, developed mechanisms to avoid the absorption of excess irradiances in the first place, i.e. with chloroplast movement [Haupt, 1982, Kasahara et al., 2002, Suetsugu et al., 2010] or by adjusting the antenna pigments within the Light-Harvesting Complex (LHC) in the photosystems [Ruban et al., 2012]. Chloroplasts of *Caulerpa* can be transported between different thallus parts, due to the siphonous structure of the seaweed [Williams et al., 1985], possibly resulting in different Chlorophyll (Chl) and consequently colour levels in sea grapes thallus parts (stolons, fronds). However, when light photons are absorbed, but energy is not used to drive photochemistry, highly destructive Singlet Oxygen ($^1O_2$) can form and trigger a cascade of (Reactive Oxygen

Species (ROS)) [Krieger-Liszkay, 2004, Triantaphylidès et al., 2008, Sharma et al., 2012]. Even though ROS act as important messengers in low concentrations [Reczek and Chandel, 2015], seaweeds need a complex protective system of (non-)enzymatic antioxidants to equilibrate the ROS production by continuously scavenging them [Sharma et al., 2012]. Consequently, sea grapes have been identified as a rich source of natural antioxidants [Nguyen et al., 2011, Paul et al., 2014, Yap et al., 2019], showing high AOA in inter- [Matanjun et al., 2008] and intra-family comparisons [Ismail et al., 2020] and have been suggested as promising functional food ingredients [Tanna et al., 2018]. Some of the underlaying contributing antioxidant compounds of sea grapes are specifically vitamins C, E [Matanjun et al., 2009], and β-carotene [McDermid and Stuercke, 2003]. Additionally, polyphenols (i.e. flavonoids) play an important role in sea grapes' bioactivity [Nguyen et al., 2011, Tanna et al., 2018, Tanna et al., 2019, Cotas et al., 2020].

In this study, sea grapes were deliberately exposed to light-stress, due to the nutritional beneficial character of their antioxidants. Following this reasoning, Magnusson et al. increased the antioxidant content by up to 88% in the green seaweed *Derbesia tenuissima* by adapting the management of stocking densities in a land based system [Magnusson et al., 2015]. Additionally, sea grapes' AOA was already successfully raised to 320% of the unstressed initial. However, the saturation point for AOA was not identified [Sommer et al., 2022]. In (sub-)tropical regions, like the Khánh Hòa province in Central-South Vietnam, where sea grapes are produced in shaded ponds ($\sim$50 µmol photons m^{-2} s^{-1}), solar irradiances are basically an inexhaustible resource. Therefore, this triggering method would be cost- and labour-effective for sea grape farmers. However, other physiological responses of sea grapes to light-stress, like bleaching [Horstmann, 1983, Kang et al., 2020, Stuthmann et al., 2020] need to be considered when designing such a tool.

Therefore, this study aims to determine the effect of five light irradiances (50–600 µmol photons m^{-2} s^{-1}) during an experimental run of two weeks on AOA, Total Phenolic Content (TPC), the photosynthetic efficiency, Chl *a* content and colour, quantified by image analysis of *C. lentillifera* pictures. We hypothesize, that the applied excess-irradiances will induce photo-oxidative stress and therefore trigger the production of antioxidant compounds, benefiting the alga's nutritional quality. Additionally, it might cause a bleaching, due to partial degradation of Chl *a*, and trigger relocation movement of chloroplasts from the fronds towards the stolons. We aim to quantify the physiological stress reactions and discuss the feasibility of implementing the tool in the production-cycle of sea grapes in Vietnam, considering local data on light availability.

5.2.2 Material and Methods

5.2.2.1 Field measurements of light irradiances

PAR measurements were conducted at the sea grape farm VIJA located in the Central-South of Vietnam in the Khánh Hòa province at Van Phong Bay (12° 35' 11.8" N, 109° 13' 26.7" E) from May–July 2019 in the growth season of the algae. Data loggers (MX2202, HOBO, USA) were deployed at bamboo sticks in the tidal culture ponds in the shaded and sun-

exposed part of a representative culture pond (Fig.5.4A). Light irradiances were logged in 30 minutes intervals in lumen feet^{-2}. The conversion from lumen feet^{-2} was conducted based on a linear regression formula (f(x)=0.2657) obtained through counter measurements with the data logger and a LI-1400 datalogger with a 2-π flathead sensor (LI-COR Biosciences, USA). In the post-harvest tanks, where sea grapes were washed and kept prior retail and over the winter season (Fig.5.4B), irradiances were measured occasionally using a LI-1400 datalogger (LI-COR Biosciences, USA). Data are presented as mean±Standard Deviation (SD) per month and over the diurnal cycle.

Figure 5.4: Pictures of the shaded (**A**) tidal culture ponds and (**B**) post-harvest tanks at the farm VIJA in Van Phong Bay, Khánh Hòa province, Central South Vietnam.

5.2.2.2   Biomass sampling for laboratory experiments

Sea grapes were cultivated for two years at the experimental facilities (called MAREE) of Leibniz Centre for Tropical Marine Research (ZMT) in Bremen, Germany. Biomass originated from sea grapes from the farm VIJA in 2019.

5.2.2.3   Experimental set-up

The experiment was carried out in 2 L glass beakers filled with artificial seawater and covered with a transparent plastic film. The artificial seawater was prepared using RedSea salt (RedSea, Verneuil dÁvre et d'Iton, France) according to the companies manual. Beakers were placed in a water bath consisting of a flow-through system with different aquaria in or-

Table 5.1: Irradiance treatments of photosynthetically active radiation (PAR) expressed in µmol photons m^{-2} s^{-1} for five treatments as mean±SD (standard deviation) between all measured values in the beakers used as experimental units.

	µmol photons m^{-2} s^{-1}		
Targeted irradiance	mean±SD	minimum	maximum
50	49.4 ±2.1	47.32	51.5
100	102.6±4.1	98.4	106.8
200	195.0±14.6	180.8	210.0
400	396.2±16.8	379.4	413.0
600	583.0±15.7	567.3	598.7

der to keep the temperature stagnant at 23.0±0.5°C. Water in the beakers was stirred daily. The irradiance treatments were chosen to represent approximately the range present in the pond environment, ranging from 50 to 600 µmol photons m^{-2} s^{-1} with a photoperiod of 12:12 light:dark. 50 µmol photons m^{-2} s^{-1} is the light irradiance prevailing in average in the pond environment and sea grapes were cultured at this irradiance prior the start of the experiment for at least six months.

For the five experimental light treatments (Table 5.1), Light Emitting Diode (LED) (Aquaillumination, Hydra, Germany) were adjusted to 12 Kelvin (K) throughout the whole experiment and treatments of PAR were K adapted by altering the percentage of K. For the treatment of 600 µmol photons m^{-2} s^{-1} two lamps with the same set-up were used to reach the respective irradiance treatment. For the adjustment of experimental irradiance a LI-COR with a 2-π flathead sensor (LI-COR Biosciences, USA) was used. The light spectrum (280–720 nm) was quantified when the LED light was adjusted to 50 µmol photons m^{-2} s^{-1} using an integrated hyperspectral radiometer Ramses ACC UV/ VIS (Trios, Rastede, Germany, Appendix D.2).

For the different analysis, the biomass was collected from the beakers, washed with distilled water and carefully dried. The sampling for the different analyses, including which part of the thallus, is described in sections 5.2.2.4 and 5.2.2.7.

5.2.2.4   Chlorophyll *a* fluorescence measurements and analysis

Photosynthetic performance was determined in *vivo by* measuring Chl fluorescence using a portable Diving-Pulse-Amplitude Modulated (PAM) Chl fluorometer (Walz, Effeltrich, Germany). Photosynthetic efficiency, F_v/F_m [Schreiber et al., 1995, Maxwell and Johnson, 2000] and Rapid Light Curve (RLC) [Silsbe and Kromkamp, 2012] were measured in 7 min dark-adapted sea grape fronds. For RLCs sea grapes were exposed to stepwise increasing irradiances of actinic white light ranging from 8 to 349 µmol photons m^{-2} s^{-1} at intervals of 30 s (factor 0.64, ind 2). Electron Transport Rate (ETR) was calculated following the formula

$$ETR = yield \times PAR \times 0.5 \times ABS \tag{5.1}$$

with yield as the effective quantum yield of Photosystem II (PSII) measured after the respective irradiance exposure, and *PAR* being the irradiance that the sample was exposed to

prior the beam. Absorbance (ABS) was determined for sea grapes using a 2-π flathead sensor with a LI-COR (LI-COR Biosciences, USA), where the ABS of a fresh sea grape thallus was measured as difference between irradiance without the sample (PAR$_0$) and with the sample on the sensor (PAR$_t$), with the formula

$$ABS = 1 - \left(\frac{PAR_t}{PAR_0}\right). \tag{5.2}$$

Data were analyzed using the R package *phytotools* [Silsbe and Malkin, 2015] with the model of Platt et al. and the RLC parameters of initial slope of the curve (α), maximum Electron Transport Rate (ETR$_{max}$) and Saturation Irradiance (E$_k$) were extracted [Platt et al., 1980].

5.2.2.5 Pictures and colour analysis

Pictures were taken with a Canon EOS M50 (Canon Zoom Lens EF-M 14-45 mm, 1/200, F6.3, ISO100) in a photo tent with 192 dimmable LED lights arranged in two light bars attached in a 90° angle and 36 cm above the background. A grey reference scale (B.I.G, photo equipment – brenner Import and Handels GmbH, Weiden i.d. OPf., Germany) was placed next to the sea grapes. Pictures were analyzed using the open source software GNU Octave version 6.2.0 [Eaton et al., 2021] and custom-made macros originally developed for the photographic assessment of coral Chl content [Winters et al., 2009].

Colour was quantified in the Red (R), Green (G), Blue (B) (Red Green Blue (RGB)) colour model, where the intensity of each colour is defined as a compilation of a value between 0 (the darkest) and 255 (the brightest). Following the protocol of Winters et al., image colour was normalized using the macro *CalibrateImageA* utility 1.0. RGB components in the picture were adjusted based on the RGB profile of the grey scale (20 grays, from #1 *white* to #20 *black*) using a rectangle width in pixels of 25 (default option) [Winters et al., 2009]. Subsequently, the RGB colour intensities of each normalized image were measured for three different areas of sea grape thallus defined as *Frond tip*, *Frond base* and *Stolon* (Fig.5.5) using the macro *AnalyzeIntensity* [Winters et al., 2009]. Ten squares with a rectangle width of 25 pixels were randomly distributed in the respective thallus area. The intensities of RGB were averaged separately for each colour channel and the values are presented as mean±SD between the replicated images for each treatment.

5.2.2.6 Chemicals and reagents

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Sherman Chemicals (Dorset, UK). Potassium peroxidisulfate, 6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), FFolin-Ciocalteu (FC) reagent and Gallic acid were obtained from Sigma Aldrich/Merck (Darmstadt, Germany).

5.2.2.7 Preparation of sample extract

For AOA and TPC analysis sea grape fronds were frozen, stored at -80°C and freeze dried (CHRIST, Alpha 1-4 LD plus, Germany) at -80°C and one bar (VACUUBRAND GMBH &
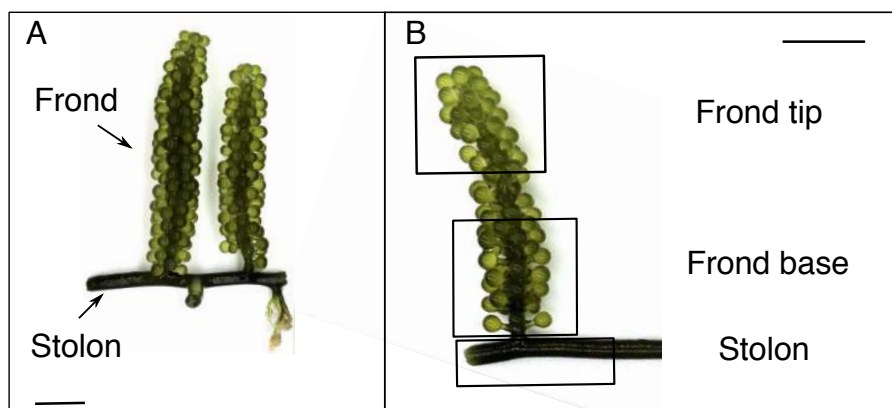
Figure 5.5: (**A**) Thallus of sea grapes (*Caulerpa lentillifera*) with fronds and stolon, and (**B**) divided into areas *Frond tip*, *Frond base* and *Stolon* in order to track potential chloroplast movements or changes in colour. Scale bar = 1 cm

CO KG, Germany) before pulverization with a FastPrep-24 (MP Biochemicals, Germany) for 20 seconds. Approximately 0.05 g Dry Weight (DW) sea grape powder was extracted in 1 mL Ethanol (70%) for 4 hours in a water bath (47°C) and vortexed hourly. Subsequently, samples were centrifuged (2,500 g, 20°C, 10 min) and the supernatant was extracted. For Chl quantification, biomass samples of 0.1 g fresh weight (FW) of different thallus parts (*Frond tip*, *Frond base* and *Stolon*) were cut with a blade and weighted in Eppendorf tubes, frozen in liquid nitrogen (-196°C) and stored at -80°C. Biomass was freeze-dried similarly to AOA and TPC samples and subsequently crushed in the FastPrep for 20 seconds. Sea grape powder was extracted in 1 mL 90% Acetone, vortexed two times for about 5 seconds and stored in a fridge (∼°C) in the dark for 24 hours. After centrifugation the supernatant was extracted.

5.2.2.8 Measurement of antioxidant analysis / ABTS assay

The ABTS$^{.+}$ assay was modified after Re et al. [Re et al., 1999]. A 2.45 mM ABTS$^{.+}$ stock solution was prepared with oxidation of 7.0 mM ABTS with potassium disulfate for 16 hours. An ABTS$^{.+}$ working solution was prepared for each measurement day freshly, by dilution with absolute ethanol until an absorption of 0.70±0.02 at 734 nm was reached, measured with a UV/VIS-Spectophotometer (Thermo Scientific Genesys 140/150, Fisher Scientific GmbH, Germany). Then, 1 mL of ABTS$^{.+}$ working solution was added to 10 µL of sample extract and after 6 minutes reaction time the absorbance was measured (734 nm). AOA was expressed as Trolox Equivalents (TE) (mmol TE 100 g^{-1} DW).

5.2.2.9 Measurement of total phenolic content / Folin-assay

TPC was determined using the FC method described by [Ainsworth and Gillespie, 2007] with slight modifications. A 10% (v/v) FC solution was mixed with 150 µL of sample using a vortex mixer. 1200 µL Na$_2$CO$_3$ solution (700 mM) were added and the mixture was left for 45 minutes in the dark at room temperature. Subsequently, samples were centrifuged (3 min, 5000 rpm, 20°C) and the absorbance was read at 765 nm in the same UV/VIS-Spectrophotometer

as in section 5.2.2.8. As a standard gallic acid was used and results were expressed as 100 mg Gallic Acid Equivalents (GAE) g^{-1} DW.

5.2.2.10   Measurement of chlorophyll *a* and *b*

1 mL of the supernatant was transferred to a quartz cuvette and absorptions were measured at the wavelengths of 647 nm and 664 nm using the same UV/VIS-Spectrophotometer as in section 2.6. Based on the methods of Jeffrey and Humphrey Chl *a* and *b* contents were calculated [Jeffrey and Humphrey, 1975]. To further analyze concentrations of Chl *a* or *b*, the results were offset with the respective dilution volumes (Acetone 90%) and the Fresh Weight (FW) data to express the concentrations as mg g^{-1} FW.

5.2.2.11   Statistical analysis

All statistical analysis and graphical outputs were conducted using R in combination with RStudio [R Core Team, 2019] and packages of the meta package tidyverse [Wickham et al., 2019]. Outliers were excluded from further analyses using Grubbs' test through the webpage GraphPad (https://www.graphpad.com/quickcalcs/Grubbs1.cfm, accessed on 20.01.2022, $p < 0.05$). For each dataset Levene's test (homogeneity of variance, $p > 0.05$) and a Shapiro-Wilk test (normal distribution, $p > 0.05$) were run. A two-way Analysis of Variance (ANOVA) was conducted to explain the effect of main factors light irradiance and exposure time on different response variables (F_v/F_m, TPC, AOA), including Chl *a* for different thallus parts. For between-subject effects (between the treatments on each experimental day) a one-way ANOVA with light treatment as independent variable was run with a Tukey's Honest Significant Difference (HSD) post-hoc test. In case the requirements were not met, a Kruskal-Wallis test followed by a Dunn-Bonferroni post-hoc test was applied.

For Chl *a* (*Frond tip*, day 7, 200 µmol photons m^{-2} s^{-1}) only two observations were available, therefore these data were excluded from statistical tests. However, data were included in the respective plots for completeness. This test was also applied to quantify, if logger position had a significant effect on light irradiance (measured from 6 a.m. to 6 p.m.) in ponds of the sea grape farm and if AOAs from this study were significantly different to AOAs of fruits from another study. Comparisons between AOA values have to be taken with care, however as the data were quantified in the same laboratory and with the same protocol, this comparison seems valid. Correlations between TPC and AOA as well as between each of the variables and F_v/F_m, colour channels and Chl *a* content were conducted using Spearman's rank correlation, because data were non-parametric (Shapiro-Wilk test, $p < 0.05$). The level of significance was set to $\alpha = 0.05$. The results of all statistical tests are included in the Appendix D.1.

5.2.3   Results and discussion

5.2.3.1   Light irradiances during sea grape culture at Van Phong Bay

Irradiances of PAR in the culture ponds of sea grapes at farm VIJA followed typical diurnal cycles (Fig.5.6), similarly observed in the area before [Terada et al., 2016]. Irradiances in the ponds were significantly influenced by the position of the logger ($F_{(1, 3658)} = 37.96$, $p < 0.001$).

Table 5.2: Light irradiances of photosynthetically active radiation quantified with HOBO loggers deployed in the culture ponds at VIJA in a depth of $\sim$70 cm under the surface in the shaded area (shaded) and in the non-shaded area (exposed) as mean±standard deviation for values measured between 6 a.m. and 6 p.m. for the months May, June and July, as well as irradiance measured in the air around midday between 11 a.m. and 1 p.m.. Light irradiances quantified with LI-COR in the post-harvest tanks for three different positions in the tanks (surface, middle, ground), here data are presented as range.

Month	µmol photons m^{-2} s^{-1}					
	Pond		Air	Post-harvest tanks		
	exposed	shaded	exposed	surface	middle	bottom
May	148.5±169.3	71.0±62.9	$\sim$ 1870			
June	93.9±129.1	56.9±52.8	$\sim$ 1500	180-250	15 - 40	<10
July	81.4±101.7	69.4±74.2	$\sim$ 1900			

The sudden decrease in slope around noon arguably originates from a shading of the logger by the bamboo sticks they were attached to. Irradiances in the shaded area of the pond were in the mean <100 µmol photons m^{-2} s^{-1}, suggesting a suitable light regime for sea grapes [Guo et al., 2015a, Stuthmann et al., 2020]. However, the large diurnal and seasonal fluctuations between months, as well as variations between days (expressed as SD in shaded area in Fig.5.6) suggest that *C. lentillifera* possesses biochemical and physiological tools to acclimate to the different irradiances [Cavas and Yurdakoc, 2005, Robledo and Freile-Pelegrín, 2005, García-Sánchez et al., 2012]. Irradiances in the sun exposed area of the pond were still lower during noon, compared to air measured values (Table 5.2). This might be an effect of the particulate matter in the water column, obstructing the light from reaching deeper areas in the pond. Particulate matter has also been observed to cover sea grapes in the pond, possibly providing an additional protection from the light [Horstmann, 1983].

In the post-harvest tanks the irradiances were highly affected by the position in the water column, with highest values directly at the surface (Table 5.2). However, considering the irradiances quantified in the air without shade, irradiances could be increased considerably, by removing the gauze material from above the tanks.

5.2.3.2 Photosynthetic parameters

Photoinhibition, noticeable i.e. as a decrease in photosynthetic efficiency F_v/F_m, is typically the first response when algae are exposed to excess irradiances [Goh et al., 2012]. As hypothesized, F_v/F_m values were significantly affected by exposure time (F(4,90)=48.62, p<0.001) and irradiance treatment (F(4,90)=89.30, p<0.001), as well as by the interaction of both factors (F(16,90)=4.36, p<0.001). Initial F_v/F_m of sea grapes (0.74±0.04) decreased with increasing treatment irradiances (Fig.5.7). Photosynthetic efficiency of sea grapes at treatments of 50 and 100 µmol photons m^{-2} s^{-1} decreased slightly within the first experimental day, but remained >88% of initial.

However, for the high-light treatments 400 and 600 µmol photons m^{-2} s^{-1} values dropped quickly to $\sim$50% of the initial. The abrupt drop of F_v/F_m is a characteristic sign of photoinhibition as a consequence of high-light exposure [Goh et al., 2012]. In order to avoid non re-
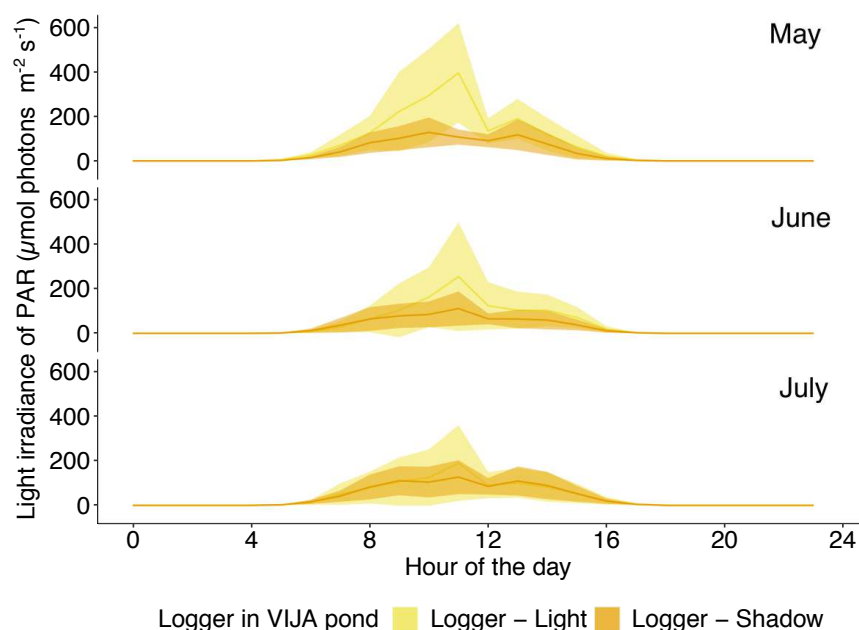
Figure 5.6: Light irradiances of photosynthetically active radiation (PAR) quantified with HOBO loggers deployed in the culture ponds at farm VIJA in the shaded area (Logger-Shadow) and in the non-shaded area (Logger-Light) as mean±standard deviation, SD (bold line is the mean and range is the SD) for the month May, June and July and the respective hours of the day.

versible photodamage, the incoming excess energy has to emitted, e.g. through non-chemical quenching [Osmond, 1994]. These results are in line with other studies, reporting signs of photoinhibition in sea grapes at irradiances of ≥ 100 µmol photons m^{-2} s^{-1} within the first hours after exposure [Guo et al., 2015a, Kang et al., 2020, Stuthmann et al., 2020]. After the first drop, F_v/F_m in *C. lentillifera* remained relatively stable during the first week, with a decrease in the second experimental week to values as low as 0.2±0.24 for the high-light treatment of 600 µmol photons m^{-2} s^{-1}. However, light-stress might have negative effects on the sea grapes growth performance [Guo et al., 2015a, Kang et al., 2020].

RLC's quantify the photosynthetic performance as a function of the irradiance and provide information about the plants light-acclimation state [Ralph and Gademann, 2005]. The RLC parameters α, ETR_{max} and E_k were in the range of values reported for other *Caulerpa* species [Raniello et al., 2004, Robledo and Freile-Pelegrín, 2005, Raniello et al., 2006, Bernardeau-Esteller et al., 2015]. α and ETR_{max} of sea grapes exhibited an overall decrease with ascending irradiances, whereas E_k showed a trend of increase, though not significant (Table 5.3). The angle of increase in the light limiting region, namely α, is proportional to the efficiency of light capture [Ralph and Gademann, 2005], suggesting that the light capture efficiency decreases with ascending irradiances, potentially as a measure of acclimation. Additionally, E_k is related to quenching processes and the trend of increase at higher irradiances, might suggest that non-chemical quenching dominates starting from higher irradiances [Ralph and Gademann, 2005]. This pattern, as well as F_v/F_m values support the hypothesis that *C.*

lentillifera is a shade-adapted species [Guo et al., 2015a, Terada et al., 2021]. The fluorescence measurements suggest that sea grapes physiologically responded to medium (200 μmol photons m^{-2} s^{-1}) and high (400, 600 μmol photons m^{-2} s^{-1}) irradiances, by adjusting the photosynthetic apparatus, including LHC antenna pigments as a measure of photoprotection [Demmig-Adams and Adams, 1992]. This response was likewise observed for *C. racemosa* along different irradiance environments at a depth gradient [Raniello et al., 2006].
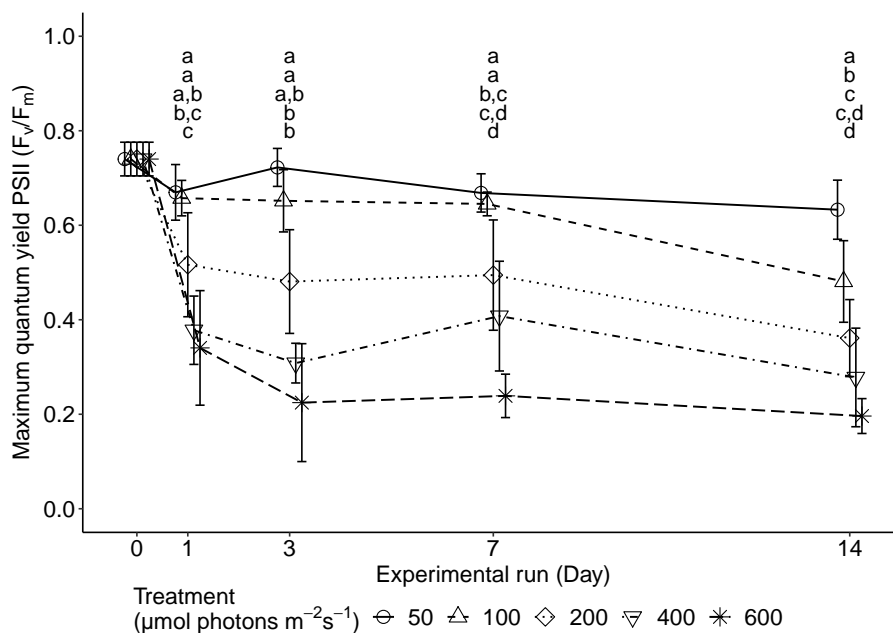
Figure 5.7: Maximum quantum yield values of photosystem II, PSII (F_v/F_m) of sea grape (*Caulerpa lentillifera*) fronds under five different irradiances (50, 100, 200, 400, 600 μmol photons m^{-2} s^{-1}) over an experimental run of 14 days. Values are expressed as mean±standard deviation, n=4-5. Letters indicate significant differences between irradiance treatments per day (One-way ANOVA with post-hoc test, p<0.05).

5.2.3.3 Biochemical parameters

AOA and TPC of sea grapes were triggered via exposure to light-stress, as hypothesized. Both parameters were significantly influenced by the experimental run (AOA: $F_{(4,76)}=34.99$, p<0.001, TPC: $F_{(20,91)}=72.26$, p<0.001), as well as the irradiance treatment (AOA: $F_{(20,60)}=15.35$, p<0.001, TPC: $F_{(4,75)}=36.85$, p<0.001) and the interaction of both factors (AOA: $F_{(16,76)}=2.83$, p<0.05, TPC: $F_{(16,91)}=8.65$, p<0.001). Initial AOAs (150.93±25.50 mmol TE 100 g^{-1} DW, Fig.5.8A) and TPC (124.45±10.07 mg GAE 100 g^{-1} DW, Fig.5.8B) were in a similar order of magnitude with values quantified for sea grapes in other studies [Matanjun et al., 2008, Ismail et al., 2020], however, direct comparisons would be misleading, due to lack of method standardization [Karadag et al., 2009].

Overall, AOAs and TPCs of sea grapes increased with ascending irradiances. Values were significantly higher after one and two weeks at ≥200 μmol photons m^{-2} s^{-1} compared to the control (50 μmol photons m^{-2} s^{-1}, Fig.5.8A,B). Additionally, AOA and TPC were positively

Table 5.3: Photosynthesis parameters of rapid light curves quantified on day 14 of the experimental run, namely α (µmol electrons m^{-2} s^{-1} / µmol photons m^{-2} s^{-1}), maximum electron transport rate (ETR$_{max}$) expressed as µmol electrons m^{-2} s^{-1} and saturation irradiance (saturation irradiance, E$_k$, µmol photons m^{-2} s^{-1}). Values are expressed as mean±standard deviation, n=3-5. Letters indicate significant differences between irradiance treatments per day and parameter (One-factor ANOVA with post-hoc test, p<0.05).

Light Irradiance (µmol photons m^{-2} s^{-1})	α (µmol electrons m^{-2} s^{-1}/ µmol photons m^{-2} s^{-1})	ETRmax (µmol electrons m^{-2} s^{-1})	Ek (µmol photons m^{-2} s^{-1})
50	0.220±0.012[a]	8.23 ± 0.46[a]	37.45 ± 2.76[a]
100	0.197±0.035[a]	6.59±2.65[a,b]	35.47±19.61[a]
200	0.133 ±0.026[b]	6.27±1.78[b]	47.07±9.10[a]
400	0.068±0.018[c]	3.42±1.77[b]	49.23±16.37[a]
600	0.075±0.016[c]	4.72±2.58[a,b]	60.51±24.31[a]

correlated (r_S=0.599, p<0.001, Fig.5.9A), which is in line with the suggestion, that phenolic compounds contribute a substantial part to the antioxidant potential of sea grapes [Nguyen et al., 2011].

The importance of antioxidants for the photoprotection of plants is already established [Sharma et al., 2012], hence the significant negative correlation of AOA and TPC with the photosynthetic stress parameter F_v/F_m (AOA: r_S=-0.573, p<0.001, TPC): r_S=-0.543, p<0.001, Fig.5.9B) is not surprising. It indicates a link between the antioxidant accumulation and light-stress of the algae, as similarly reported by Magnusson et al. [Magnusson et al., 2015]. The excess irradiances have likely accelerated the production of ROS, namely e.g. Hydrogen Peroxide (H_2O_2) and $^1O_2$, at Photosystem I (PSI) and PSII in the chloroplasts [Takahashi and Badger, 2011]. Antioxidants can scavenge the ROS effectively to avoid oxidative stress [Goh et al., 2012]. Consequently, the organisms experiencing higher levels of light-stress (i.e. lower F_v/F_m values, section 5.2.3.2), showed higher AOA and TPC values. However, the increase of AOA after light-stress exposure was quick (e.g. reaching an increase of 162-203% of initial at 200-600 µmol photons m^{-2} s^{-1} after three experimental days). AOA of sea grapes at 600 µmol photons m^{-2} s^{-1} saturated after one week (229% of initial), whereas AOA at 200 and 400 µmol photons m^{-2} s^{-1} still showed a trend of increase in the second experimental week (Fig.5.8A). Sommer et al. did not find a saturation of AOA when exposing sea grapes to light-stress of up to 300 µmol photons m^{-2} s^{-1} for 14 days [Sommer et al., 2022].

The results of this study confirmed the authors' assumption, that a saturation of AOA might be reached at higher irradiances. TPC, on the other hand, increased slower within the first experimental days, but consistently over the two weeks, reaching values of 180–198% of initial (200–600 µmol photons m^{-2} s^{-1}, Fig.5.8B). These differences indicate a contrasting role of the antioxidative compounds in the physiological response of the light-stressed sea grapes. Vitamin C contributes substantially to sea grapes AOA [Matanjun et al., 2009] and its role in photoprotection in chloroplasts of higher plants [Smirnoff, 2000, Paciolla et al., 2019], as an antioxidant and as cofactor for several enzymes, including violaxanthin de-epoxidase [Smirnoff, 2000] is already well established. Accumulation of vitamin C might be part of the immediate physiological reaction of sea grapes to light-stress. In lettuce, accumulation of vitamin C has been observed at short time intervals of ≤3 days [Zhou et al., 2012, Zha et al.,

2019]. On the other hand, polyphenols, like flavonoids, act as antioxidants in chloroplasts as well as integral parts of bio membranes [Hernández et al., 2009]. The biosynthesis of phenolic compounds might be a long-term strategy to acclimate to the excess irradiances, e.g. by integrating polyphenolic compounds in the bio membranes in order to mitigate membrane lipid peroxidation [Caturla, 2003].

5.2.3.4 Chlorophyll concentrations

Chl a and b were present in a ratio of 1.49 ± 0.33 (SD) and strongly correlated ($r_S = 0.98$, $p < 0.001$). Therefore, only Chl a is considered for the further analysis. Chl a content in the stolons significantly depended on exposure time ($F_{(3,91)} = 36.04$, $p < 0.001$), but irradiance treatments did not have any effect ($p = 0.42$). Chl a concentration in frond bases and tips were significantly depending on exposure time (*Frond base*: $F_{(3,75)} = 105.73$, $p < 0.001$ and *Frond tip*: $F_{(3,72)} = 48,40$ $p < 0.001$), irradiance treatment (*Frond base*: $F_{(4,75)} = 9.87$, $p < 0.001$ and *Frond tip*: $F_{(4,72)} = 5.22$, $p < 0.001$), and the interaction of both factors (*Frond base*: $F_{(12,75)} = 7.12$, $p < 0.001$ and *Frond tip*: $F_{(11,73)} = 2.52$, $p < 0.001$).

The quantified Chl a values were in the range of results of other studies [Guo et al., 2015a, Cai et al., 2021b]. Overall, the Chl a content decreased over the experimental run in all treatments, including the control (50 µmol photons m^{-2} s^{-1}, Fig.5.10A), suggesting a degradation of Chl a independently of the applied irradiance, probably due to nutrient-limitation of the algae during the experiment [Pinchetti et al., 1998]. However, sea grapes Chl a concentration showed a trend of overall higher decrease in medium and high, compared to lower (50 and 100 µmol photons m^{-2} s^{-1}) irradiances (Fig.5.10A). This result is in line with studies, which quantified a decreasing Chl content for *Caulerpa* with increasing irradiances from 20 to 100 µmol photons m^{-2} s^{-1} [Guo et al., 2015a] and 50 to 150 µmol photons m^{-2} s^{-1} [Murchie and Niyogi, 2011].

Chls are among the predominant plastid pigments in higher plants, with a central role in photosynthetic light harvesting and their levels typically decrease at light-stress [Adams et al., 2001]. Chl degradation is a multi-step process involving various enzymes, like chlorophyllase, magnesium dechelatase and pheophytinase, as well as intermediate green and colourless catabolites [Hörtensteiner and Kräutler, 2011]. Sulaimana et al. describe a Chl and colour loss in stored sea grapes as a response to oxidative stress, hypothesizing that cholorphyll dephytylation yields the green catabolite chlorophyllide, followed by swampgreen catabolite pheophytin and colourless C13-hydroxychlorophyll [Sulaimana et al., 2021]. In contrast to the frond tips and bases (Fig.5.10B, C), the Chl a content in the stolons did not reveal significant differences between light treatments over the experimental run ($p > 0.05$). Sea grapes exposed to 600 µmol photons m^{-2} s^{-1} even showed a trend of having highest values after 14 days, though with large SD's (Fig.5.10D).

In the frond tip and base, the Chl a content was significantly lower at high-light treatments (400, 600 µmol photons m^{-2} s^{-1}), compared to sea grapes exposed to lower irradiances (Fig.5.10B, C). This pattern could indicate, that the chloroplasts migrated from the tip of the fronds over the base towards the stolon. The pattern becomes more evident, when data are presented as % of sum of Chl a and the general decrease of Chl a content is neglected (Fig.

A

B
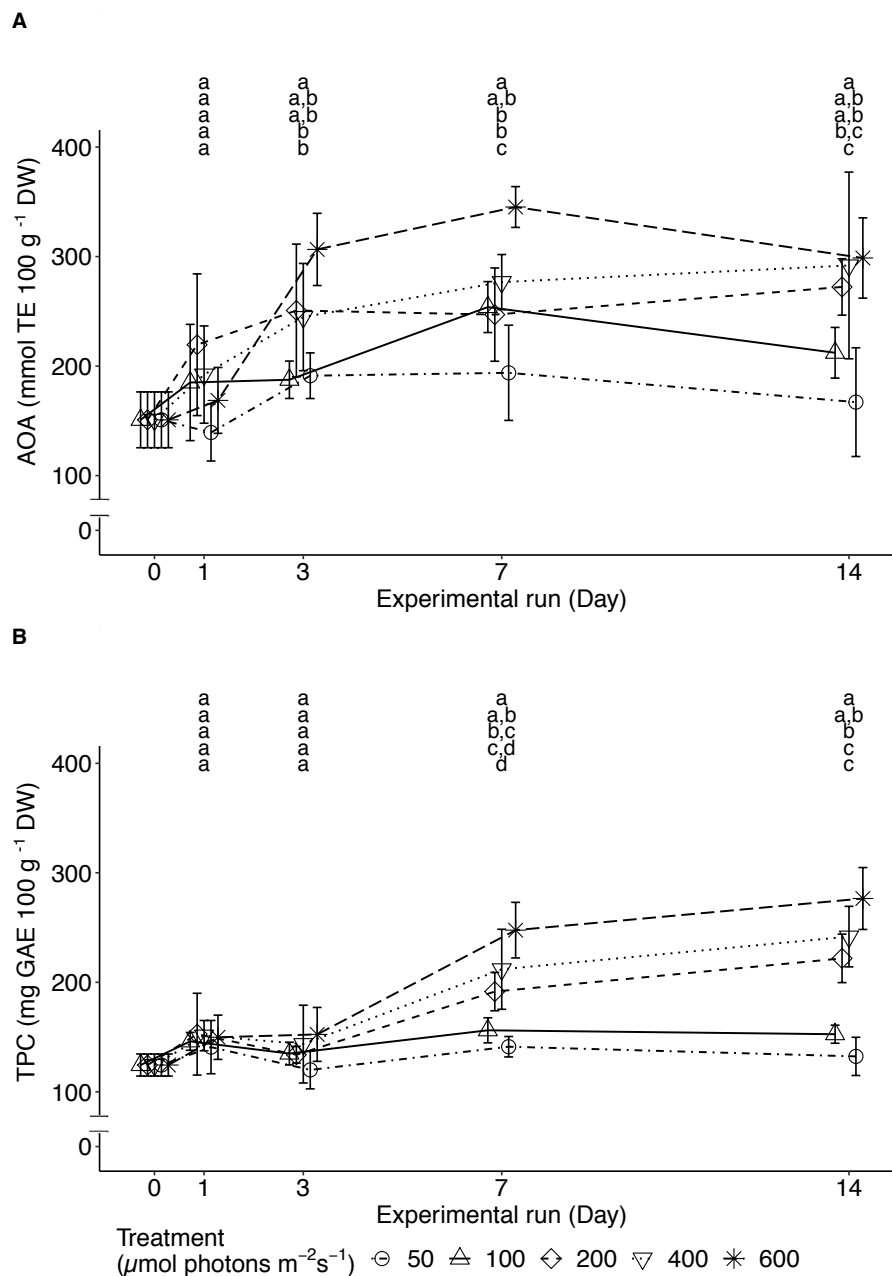
Figure 5.8: **A)** Antioxidant activity (AOA, mmol Trolox equivalents, TE 100 g^{-1} dry weight, DW) and **B)** total phenolic content (TPC, mg Gallic acid equivalents, GAE 100 g^{-1} DW) of *Caulerpa lentillifera* over an experimental run of 14 days and with exposure to five different irradiances of 50, 100, 200, 400, 600 µmol photons m^{-2} s^{-1}. Data are mean±standard deviation, n=3-5 and letters indicate significant differences between irradiance treatments per day (One-way ANOVA with post-hoc test, $p<0.05$).
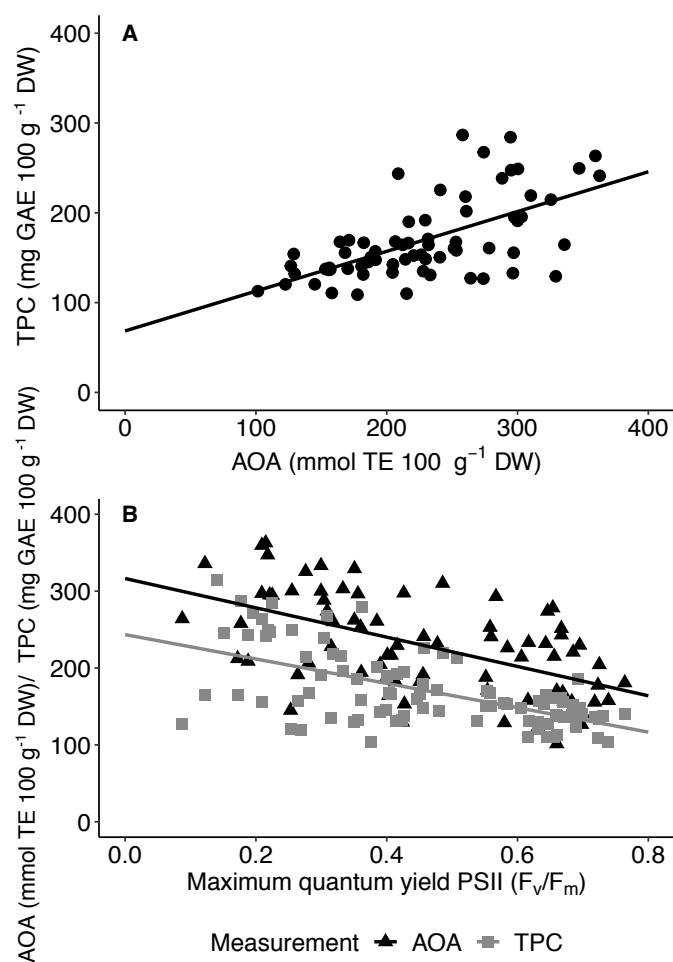
Figure 5.9: **A)** Correlation of antioxidant activity (AOA, mmol Trolox equivalents, TE 100 g^{-1} dry weight, DW) and total phenolic content (TPC), mg Gallic acid equivalents, GAE 100 g^{-1} DW, r_S=0.599, p<0.001). **B)** Correlation of maximum quantum yield of photosystemII, PSII (F$_v$/F$_m$) and AOA (mmol TE 100 g^{-1} DW), triangle, black, r_S=-0.573, p<0.001) and TPC (mg GAE 100 g^{-1} DW), square, grey, r_S=-0.543, p<0.001). Data are raw data of replicates with n=3-5 per treatment group and correlations were done with Spearman's rank correlation test.
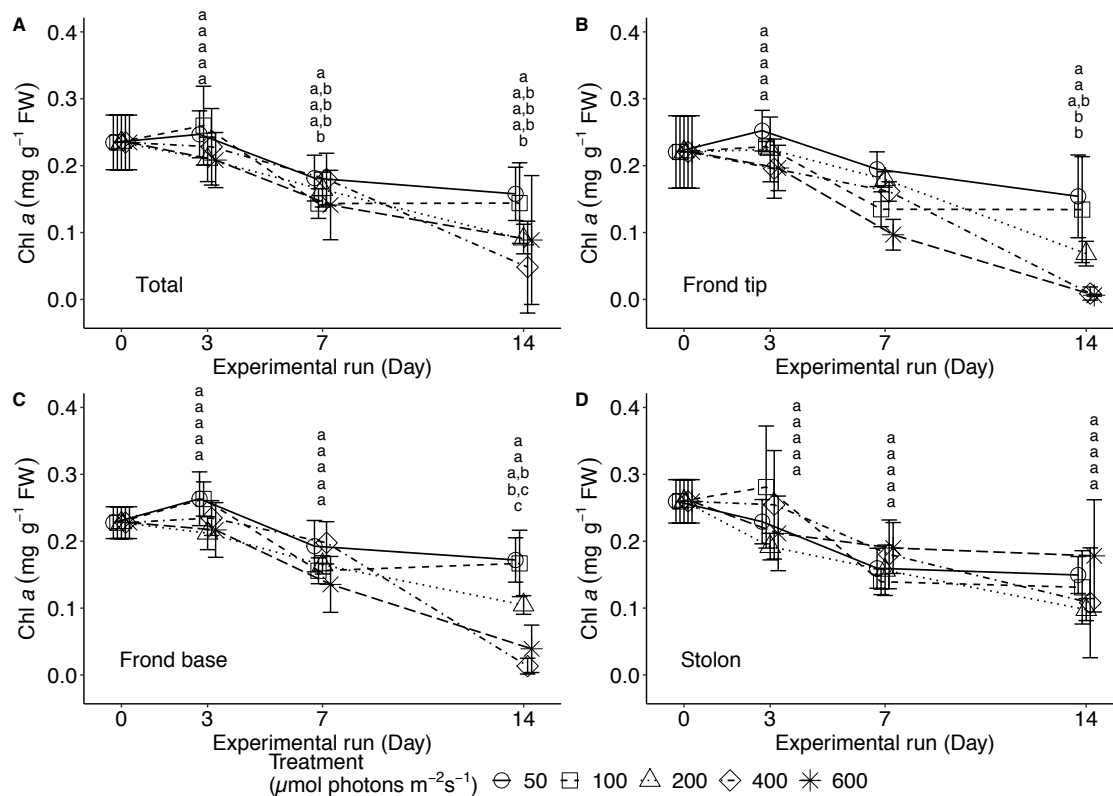
Figure 5.10: Chlorophyll *a*, Chl *a* mg g^{-1} fresh weight (FW) content of *Caulerpa lentillifera* exposed to five different irradiances (50, 100, 200, 400, 600 μmol photons m^{-2} s^{-1}) over 14 days. The different plots consider the Chl *a* content of different parts of the thallus, namely **A**) the overall thallus (Total), **B**) Frond tip, **C**) Frond base, **D**) Stolon. All values are expressed as mean±standard deviation, n=3-5. Different letters indicate significant differences between the light treatments (One-way ANOVA with post-hoc test). For **B**) at day seven and for 200 μmol photons m^{-2} s^{-1} only two replicates were available, therefore no statistical test was performed.

8). Chloroplasts are usually immotile in the thallus of multinucleated *Caulerpa* cells [Menzel and Elsner-Menzel, 1989]. However, as a result of an external stimulus, like excessive irradiances, chloroplasts can be translocated along a network of actin filaments [Menzel and Elsner-Menzel, 1989, Suetsugu and Wada, 2007] or cytoplasmic strands containing microtubule bundles [Haupt, 1982, Menzel and Elsner-Menzel, 1989].

The data suggest that the chloroplast migration would have the direction from the frond tip, over the base towards the stolon, matching the observation of other studies, that especially the fronds bleach at high-light [Horstmann, 1983, Kang et al., 2020]. The stolon acts as a creeping, anchoring structure and is usually rather protected from the light. Additionally, gene expression differs between stolon and frond, showing a slightly higher amount of expressed genes in the stolon with functions generally related to translation and Deoxyribonucleic Acid (DNA) replication [Arimoto et al., 2019b]. Besides, the elongation of stolons could be a strategy to slowly change the alga's position, as observed by for *C. prolifera* under nutrient-limited conditions [Malta et al., 2005]. The differences in gene expression and the ecological function of stolon growth as a response to physiological stress, as well as the purely mechanistical position of stolons at the rather sun-protected bottom could explain the advantage of a chloroplast migration towards the stolon.

5.2.3.5 Colour

The use of digital colour features to quantify the colour of terrestrial plants and to estimate their Chl content becomes increasingly popular, as it presents a cost-effective and non-destructive tool, for example in comparison to application of a Chl Meter or photo spectrometric Chl measurements [Agarwal et al., 2021].

Studies using the RGB colour space, found unequivocally a negative relation of Chl content with R and/or G indices for leaves [Yadav et al., 2010, Rigon et al., 2016] and in microalgae [Su et al., 2008]. In line with this, Chl *a* content of sea grapes was strongly negatively correlated with all three colour channels (p<0.001, Fig.5.12). However, correlation of the R (r_S=-0.786) and G (r_S=-0.780) channel with Chl *a* was stronger than with the B (r_S=-0.737) channel (Fig.5.12). Winters et al. quantified the Chl *a* content from pictures of corals as a measure of bleaching and suggested the use of the R channel, based on the strongest correlation [Winters et al., 2009], which were also found in this study. Therefore, only R channel values were considered for further analysis.

The values of the R channel show in all treatments an overall slight increase (i.e. brightening), following the trend of the Chl *a* data described in section 5.2.3.4 (Fig.5.13A-E). The R values of all three thallus parts were not significantly different at irradiances 50–200 µmol photons m^{-2} s^{-1}. However, at high irradiances (400, 600 µmol photons m^{-2} s^{-1}) significantly lower values were quantified for the frond compared to the stolon, indicating a darker colouration of the stolon.

Colour of seaweeds and other plants and fruits is derived from natural pigments, many of which change over growth period and during post-harvest processes [Pathare et al., 2013]. For example, colour of wakame alga changed as a result of blanching from brown to green, as quantified by image analysis and a panel list [Hamid et al., 2020]. Customers use food
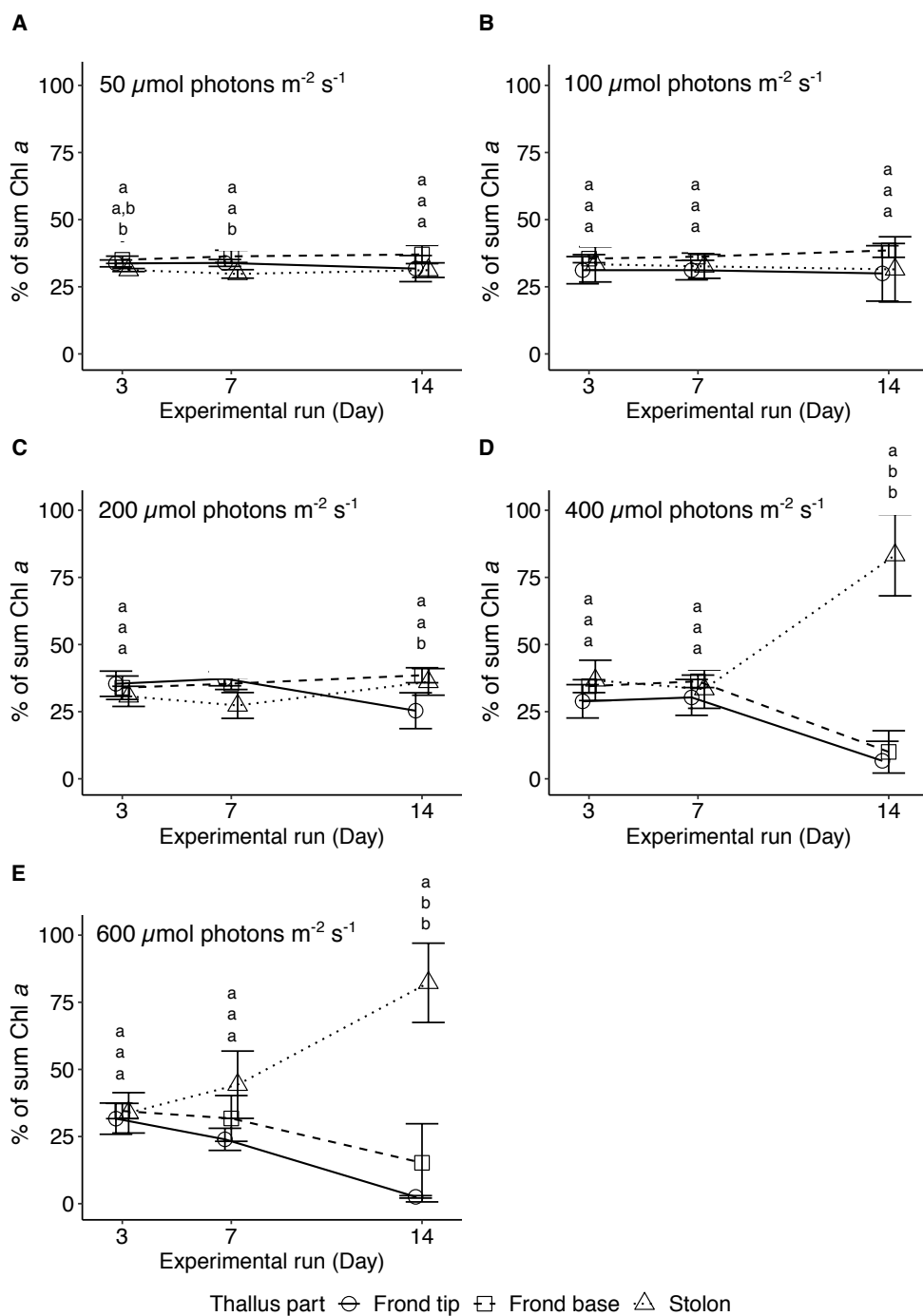
Figure 5.11: Chlorophyll *a*, Chl *a* mg g^{-1} fresh weight (FW) expressed as *% of sum Chl a* in Frond tip, Frond base and Stolon of *Caulerpa lentillifera* over 14 days. The different plots consider the *% sum of Chl a* of *C. lentillifera* exposed to **A**) 50, **B**) 100, **C**) 200, **D**) 400 and 600 µmol photons m^{-2} s^{-1}. All values are expressed as mean±standard deviation, n=3-5. Different letters indicate significant differences between the light treatments (One-way ANOVA with post hoc test). For **C**) at day seven for *Frond tip* only two replicates were available, therefore no statistical test was performed.
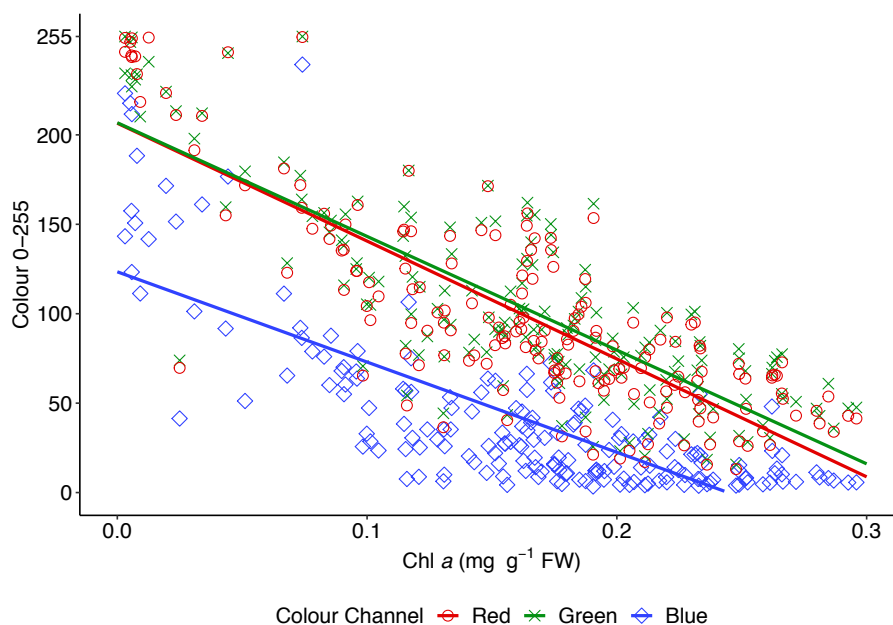
Figure 5.12: Correlation of chlorophyll a concentration (Chl a, mg g^{-1} fresh weight, FW) with colours of channel R, Blue (B) and Green (G) quantified for *Caulerpa lentillifera*.

appearance factors at the point of purchase to indicate freshness and quality of the product, and colour is considered a highly important attribute of food's appearance [Lee et al., 2013, Pathare et al., 2013]. A loss of Chl, indicated by a yellowing in green vegetables was for example considered unacceptable [Shewfelt, 2002].

5.2.3.6 Light stress as post-harvest treatment

Antioxidative compounds, including phenolics accumulated during light-stress of $\geq$200 µmol photons m^{-2} s^{-1} (section 5.2.3.3) and simultaneously a decrease of Chl a content (section 5.2.3.4) and bleaching of the colour overall and especially in the fronds was observed (section 5.2.3.5).

At the sea grape farm in Vietnam, farmers could cost- and labour-efficiently remove layers of gauze to expose algae to controlled light-stress in the pond or tank environment (section 5.2.3.1). However, light-stress might have negative effects on the sea grapes growth performance [Guo et al., 2015a, Kang et al., 2020], therefore treatments could be applied after harvest in the post-harvest tanks. Light-triggered *C. lentillifera* (300 µmol photons m^{-2} s^{-1}, 14 days) can reach similar levels of AOA than pomegranates, which are renowned as *functional food* [Sommer et al., 2022] and in this study sea grapes' AOA even significantly exceeded those values partly (Table 5.4). The direct comparison between the AOA values of berries and sea grapes should, however, be taken with care as all values were produced in the same laboratory and with identical protocols, the comparison seems viable here. Other terrestrial crops, like Aronia and Goji berries still exhibited higher AOA compared to sea grapes (Table 5.4).
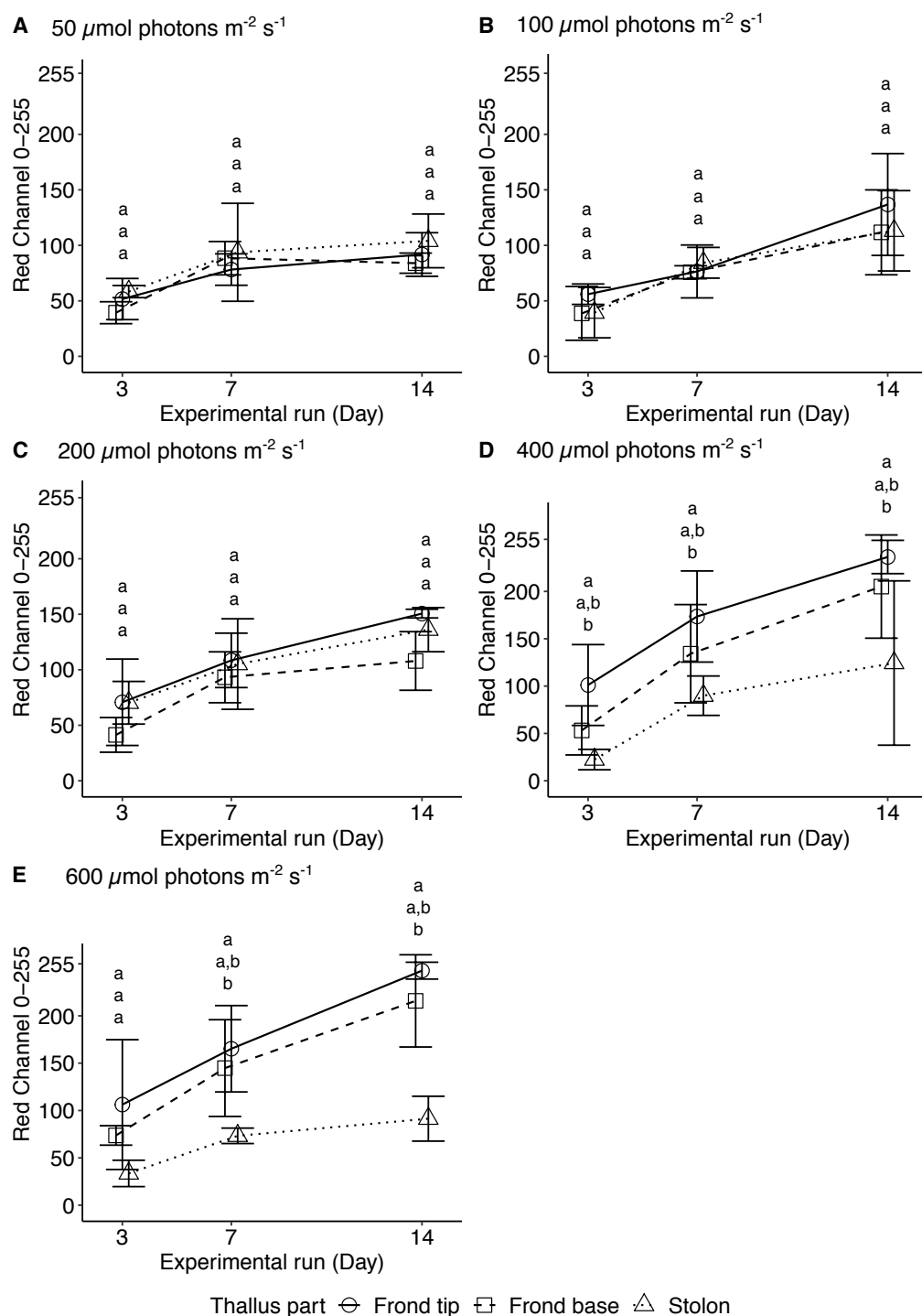
Figure 5.13: Red (R) Channel expressed as value between 0-255 in *Frond tip*, *Frond base* and *Stolon* of *Caulerpa lentillifera* over 14 days under irradiances of **A)** 50, **B)** 100, **C)** 200, **D)** 400 and 600 µmol photons m^{-2} s^{-1}. All values are expressed as mean±standard deviation, n=3-5. Different letters indicate significant differences between the light treatments (One-way ANOVA with post-hoc test).

Table 5.4: Potential scenarios for light-stress treatments to trigger antioxidant production of sea grapes and fruits known for their high antioxidant activity (AOA , mmol Trolox Equivalents, TE 100 g^{-1} dry weight, DW), and total phenolic content (TPC, mg Gallic acid equivalents, GAE 100 g^{-1} DW), Red (R) channel values, chlorophyll *a* (Chl *a*, mg g^{-1} fresh weight, FW) as well as the respective % of initial are expressed as mean±standard deviation, n=3-5. Chl *a* data and Colour for *Frond Tip* are shown, respectively. Different letters for AOA indicate significant differences between the sea grapes exposed to different light treatments and fruits (One-way ANOVA with post-hoc test).

Light treatment		AOA		TPC		Colour	Chl *a*		Source
µmol photons m^{-2} s^{-1}	days	mmol TE 100 g^{-1} DW	% of initial	mg GAE 100 g^{-1} DW	% of initial	R	mg g^{-1} FW	% of initial	
600	3	306.56±33.0^{a,b}	203.1±21.9	152.4±24.63	122.5±19.8	106.24±68.5	0.20±0.03	89.3±15.4	
200	7	247.02±42.60^{a}	163.7±28.2	191.5±17.5	153.9±14.1	108.5±24.5	0.18±0.01	81.5±5.3	This study
200	14	272.3±25.7^{a}	180.4±17.1	221.9±22.1	178.3±17.8	150.6±3.9	0.07±0.02	31.1±8.4	
600	7	345.3±18.6^{b}	228.8±12.4	247.6±25.4	199.0±20.4	165.2±45.6	0.1±0.02	44.0±10.4	
600	14	289.7±36.6^{a,b}	197.9±24.3	276.5±28.7	222.2±22.7	247.7±8.9	0.006±0.001	2.9±0.6	
Pomegranate		272.8±23.0^{a}							[Sommer et al., 2022]
Goji		408.5±27.6^{c}							
Aronia Berry		435.9±46.1^{c}							

Magnusson et al. compared the green alga *D. tenuissima* and other seaweeds with terrestrial crops, like apples and berries regarding their AOA and TPC. In the direct comparison seaweeds mostly underperformed, compared to the fruits and berries. However, when considering the areal productivity, *D. tenuissima* outperformed the terrestrial crops in their AOA and TPC production [Magnusson et al., 2015]. Therefore, macroalgae, like sea grapes, are promising candidates to provide natural antioxidants for different applications, e.g. for the functional food industry [Magnusson et al., 2015, Kumar et al., 2018]. Especially when seaweed cultivation would have at least similar or in the long term even better life cycle demands, compared to land plants [Taelman et al., 2015].

The application of light-stress as a tool could help to ensure the constant provision of the bioactive compounds. However, negative implications need to be considered. Sea grape fronds are mostly sold fresh or dehydrated for the direct consumption [Chen et al., 2019]. Considering results of studies on vegetables, bleaching of sea grape fronds will have negative implications on consumers' appreciation of the product [Shewfelt, 2002]. Therefore, the increase of antioxidants and the decrease of pigments and colour should be balanced, when designing a tool for farmers. Potential scenarios of light-stress could be shorter in order to sustain the colour or longer to maximize the antioxidant potential for direct retail of the product (Table 5.4). An application of light-stress of 200 or 600 µmol photons m^{-2} s^{-1} for respective shorter periods of 7 or 3 days, could still achieve values of 164 or 203% of initial AOA and 123 or 154% of initial TPC with Chl *a* values of >80% of initial (Table 5.12).

On the other hand, sea grapes could also be used as supplement in foods [Kumar et al., 2018], cosmetics [Susilowati et al., 2019, Zubia et al., 2020] or as fish feed [Putri et al., 2017], where high AOAs could be a valuable property, even when the original structure is no longer recognizable and colour might be irrelevant. In these cases, light-stress for longer periods of up to 14 days could be applied and result in high AOA (up to 228% of initial) and TPC (up to 229% of initial) values with respective low Chl *a* contents of ~3-44% of initial and high R values of up to 245 (Table 5.4). Farmers could adapt the treatment to the needs of the retailer.

5.2.4 Conclusions

This study demonstrated that sea grapes antioxidant production can be triggered by exposure to high-light intensities and that this tool could be implemented in the production cycle of sea grapes to improve their nutritional quality. However, sea grapes responded to light-stress with other acclimation strategies as well, including photoinhibition and potentially chloroplast re-location and chloroplast degradation. This could lead to bleaching of the edible sea grape fronds, which could impact the consumers appreciation of the product negatively. Therefore, the light-treatment should be adapted to the intended purpose of the sea grape biomass to minimize negative impacts. Additionally, this study showed that the analysis of the red channel from sea grape pictures could be implemented as a cost-effective and non-invasive tool to estimate Chl a content of sea grapes. However, for a potential application more research is needed. It is recommended that farmers use high light as post-harvest treatment.

Chapter 6

Publication VI

Kappaphycus alvarezii longline cultivation in Van Phong Bay, Viet Nam (Foto:A. Cordes).

Potential of resource-efficient two-layer cultivation of carragenophyte *Kappaphycus alvarezii* and sea vegetable *Caulerpa lentillifera*

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Abstract

Economically important carragenophyte *Kappaphycus alvarezii* and sea vegetable *Caulerpa lentillifera* are cultivated in-shore on longlines and in tidal ponds in Van Phong Bay, Viet Nam, respectively. The complementary light and Nitrogen (N) requirements of the light sensitive and Nitrate (NO_3^-) preferring *Caulerpa lentillifera* and *K. alvarezii* seaweeds introduce the opportunity for a resource-efficient two-layer cultivation. Three different set-ups were tested in a field experiment, namely the integration of *K. alvarezii* in sea grape ponds, the integration of sea grape plastic cages on longlines and the co-cultivation of both species in net cages. Here we show, that the *K. alvarezii* cultivation on longlines (Relative Growth Rate (RGR): $4.42\pm0.84\%$ day^{-1}) with *C. lentillifera* integrated below in inexpensive, self-made, customizable plastic cages with additional gauze protection is the most promising set-up from a physiological and economic point of view. The RGRs of *K. alvarezii* were highest on longlines, compared to net cages in mono- and co-cultivation ($4.42\pm0.84\%$ day^{-1} vs. 2.12 ± 0.64 and $0.62\pm0.54\%$ day^{-1}), whereas they died due to warm temperatures and absence of water movement in ponds. However, strong recurring water movements at the experimental site caused high losses of *K. alvarezii* fragments (39% of initial) and impaired growth of the delicate, siphonous *C. lentillifera* causing negative RGRs in all treatments. Gauze wrapping of plastic cages provided protection against the water movement with only minimal biomass loss, compared to net cages and plastic cages without gauze (RGR: -1.29 ± 0.78 vs. -6.37 ± 0.78 vs. $-9.76\pm0.56\%$ day^{-1}). However, the water flow at the target location of the two-layer set-up in Van Phong Bay, Viet Nam is considerably lower than at the experimental site. *K. alvarezii* on longlines shaded the sea grapes, but additional gauze wrapping was necessary to avoid signs of photooxidative stress for *C. lentillifera* (significantly decreased F_v/F_m, increased antioxidant production). For fertilization in land-based cultivation during off-season, locally available, inexpensive, diluted effluents from *Babylonia areolata* snails can be used, resulting in increased RGRs of *C. lentillifera*, a darker thallus colour of *K. alvarezii* and decreased Carbon (C):N ratio for both species, compared to the nutrient-low control (natural seawater). Hence, the two-layer cultivation is a promising way to increase farmers income, while keeping additional investments and resources low.

Keywords: Babylon snail, Green caviar, Macroalga, Phycoculture, Sea grapes, Seaweed, Sustainable Food Production

6.1 Introduction

Seaweeds are an essential part of global aquaculture production [FAO, 2022] and the biomass is used as sea vegetables or food additives, as well as in pharmaceutical and industrial contexts [Duarte et al., 2022]. Seaweeds' low trophic position and their bioremediation properties allow for a sustainable cultivation and their aquaculture is seen as an essential part to cover the shortage of resources under the increasing world population [Chung et al., 2017]. The vast majority of seaweeds is sourced from aquaculture in Asia [FAO, 2022] and often from systems with only one seaweed species [Neori et al., 2004].

However, in their natural habitat, seaweeds occur often in dense, multi-layered mats [Lüning and Pang, 2003]. Larger seaweeds, like kelp, can act as *light umbrellas* by shading seaweeds in the lower layers of the community and hence e.g. avoiding epiphyte growth [Lüning and Pang, 2003]. In horticulture, this cultivation concept is known as multi-storey or multi-layer cropping [John and Nair, 2000]. Crop plants are assembled by their height to make efficient use of the 3-dimensional space and the natural resources on the arable land [John and Nair, 2000], yielding higher production per area, compared to mono-cultivations [Nimbolkar et al., 2016, Sharma et al., 2020]. In the context of phycoculture, seaweed multi-layer systems could enhance the farmers yield per area, especially when species exhibit complementary resource uses, like Nitrogen (N) preferences [Liu et al., 2016, Roleda and Hurd, 2019, Kang et al., 2021] or light environments [Lüning and Pang, 2003].

Kappaphycus alvarezii is the 6th most cultivated macroalgae globally, being mainly used as a raw material for carrageenan extraction [FAO, 2022] with applications e.g. as stabilizer, gallic agent or emulsifier for different products in the cosmetic and food industry [Pong-Masak and Sarira, 2020]. The tropical species is often cultivated using the *tie-tie technique*, where single fragments are attached to a main rope as fixed off-bottom, longline or a raft system. The technique is labor-intensive, however it is simple, inexpensive and the seaweeds grow well [Ask and Azanza, 2002]. Bags and tubular nets are also used on a smaller scale. And even though the technique is less labor intensive, the capital costs are usually higher and the seaweeds grow slower, compared to the tie-tie technique [Ask and Azanza, 2002].

Caulerpa is a highly diverse genus of green, siphonous macroalgae [Zubia et al., 2020]. The species of *C. racemosa* and *C. lentillifera* are consumed under the name green caviar or sea grapes mainly in Asia and Oceania, but with increasing interest also in western countries [Zubia et al., 2020]. The sea grape thallus is characterized by assimilators consisting of vesiculate ramuli attached to creeping stolons. This special texture in combination with an overall beneficial nutritional composition [Saito et al., 2010, Paul et al., 2014, Syakilla et al., 2022] distinguishes sea grapes as a good candidate for future human nutrition [Zubia et al., 2020]. The benthic seaweed is usually cultivated in raceways or ponds using the tray or sowing technique [Rabia, 2016, Zubia et al., 2020], however in-shore cultivation in nets or trays has been reported as well [Zubia et al., 2020].

In Van Phong Bay, in the Central South of Viet Nam, *Kappaphycus* and sea grape cultivation take place in relatively close proximity. *C. lentillifera* is grown in land-based tidal ponds, whereas *K. alvarezii* is farmed on longlines in the northern inshore area of the bay. Even though cultivation and environment of both species seem incoherent at first sight, especially

complementary light and nutrient requirements, as well as growth seasons introduce the opportunity of a *two-layer cultivation* of both species. *C. lentillifera* is a light-sensitive shade-adapted benthic alga and farmers cover the ponds with gauze material to provide an average low-light environment of 10–150 µmol photons m^{-2} s^{-1} of Photosynthetically Active Radiation (PAR), which sea grapes were found to prefer [Guo et al., 2015a, Xing et al., 2017, Kang et al., 2020, Stuthmann et al., 2020]. *K. alvarezii* was suggested to be a low-light adapted species, but with regulative mechanism to cope with excessive as well as deficient light conditions typical for the intertidal [Hurtado et al., 2006, Guan et al., 2013]. Hence, in a *two-layer cultivation K. alvarezii* could shade *C. lentillifera*, potentially replacing or reducing the gauze material. Besides, *K. alvarezii* and *C. lentillifera*, respectively prefer Ammonium (NH_4^+) and Nitrate (NO_3^-) in the presence of both N forms [Qian et al., 1996, Hayashi et al., 2008, Liu et al., 2016], with *K. alvarezii* conducting surge uptake [Dy and Yap, 2001, Paul and de Nys, 2008]. Therefore, the species' complementary N-uptake strategies could enhance the common bioremediation capacities, that both species already exhibited independently from each other [Rodrigueza and Montaño, 2007, Hayashi et al., 2008, Dobson et al., 2020, Anh et al., 2022].

Aquaculture effluents could be used as a cheap and locally available source of fertilization for seaweeds in the in-door cultivation, e.g. when maintaining the biomass during off-season [Martino et al., 2021]. *K. alvarezii* showed highest growth rates in the Central South of Viet Nam during the colder months ($\sim$ October - March), whereas warmer temperatures ($\sim$April – September) could trigger diseases, like *ice-ice* [Diem Hong et al., 2010]. The growth season of sea grapes is restricted by decreased salinities during the colder rainy season, with highest growth rates during the dry season [Barthel et al., 2009, ISPONRE, 2009, Stuthmann et al., 2023]. Contrary peak seasons of the crops could provide farmers with economic security over the full year. However, if environmental parameters during off-season require farmers to maintain seaweeds biomass on land, locally available aquaculture effluents of snail *Babylonia areolata* could be used for the fertilization of the cultivar. The edible snail is cultivated in ponds next to sea grapes, which have already been successfully cultivated in the process water of *B. areolata* [Chaitanawisuti et al., 2011, Dobson et al., 2020].

To our knowledge we made the first attempt to implement a two-layer cultivation of economically important *K. alvarezii* and *C. lentillifera* using Van Phong Bay as a case location. *K. alvarezii* thalli provide shading for the benthic sea grapes, whereas *C. lentillifera* could act as an additional high value income for farmers. The congregation of established processing and transport chains, as well as buyers for both species in Van Phong Bay would enable local farmer to diversify their respective cultivation system and generate additional income, by using their space and resources more efficiently. Hence, the study investigated the potential for this two-layer from several perspectives: (*1*) The cultivation in the pond, as well as in the inshore environment was investigated *in vivo* using the different cultivation methods implemented already for both species. (*2*) The nutrient comparability and the possibility to use locally available aquaculture effluents of *B. areolata* as a source of fertilization was investigated in an *in vitro* laboratory experiment.

6.2 Material and methods

6.2.1 Experimental location

The Khánh Hòa province is located in the Central South of Viet Nam and encompasses four main bays (Fig.6.1B). The semi-open Van Phong Bay is with 510 km^2 and a mean depth of 15 m the largest one (Barthel et al., 2009; Phu et al., 2022) (Fig.6.1C). The region has a weather regime mainly driven by the monsoon, with a wet season lasting from July/August to December and a peak in October and November [Barthel et al., 2009, ISPONRE, 2009]. The yearly rainfall sums up to ∼1300 mm year^{-1} and the rainy season is characterized by a decrease in temperature, whereas the hottest months (March, April, May) fall in the dry season [ISPONRE, 2009]. During summer, coastal upwelling can appear, which is beneficial for aquaculture activities [Barthel et al., 2009].

In the Khánh Hòa province, aquaculture covers an area of >5000 ha, from which ∼2230 ha are used for marine aquaculture and the other part for pond cultivation. The total aquaculture production of 16798 tonnes in 2015 consisted of whiteleg shrimp (5925 tonnes), finfish from cage farming (4242 tonnes), molluscs (2973 tonnes) and seaweeds (1286 tonnes Dry Weight (DW)) [Hasan et al., 2020]. *Kappaphycus* spp. (6660 tonnes DW in 2015) and *Gracilaria* spp. (240 tonnes DW in 2015) accounted for most of the produced seaweed biomass, whereas *Sargassum* spp. was harvested mostly from the wild [Fricke et al., 2021, Hong and Ha, 2022]. Furthermore, the cultivation of various high value species, including e.g. spiny lobster, snails *Babylonia areolata*, green mussels (*Perna viridis*), fin fish (sea bass, pompano) and seaweed *C. lentillifera* is developing increasingly [Hoang et al., 2009, Nghia et al., 2009, Hasan et al., 2020, Phu et al., 2022]. However, the release of nutrients from fish and lobster cages in the Khánh Hòa province was already reported to cause nutrient accumulations in the sediment [Phu et al., 2022] and to negatively affect local organisms, like corals [Du et al., 2022]. Furthermore, the wild harvest of *Sargassum* spp. seemed to have reduced the natural population in some areas [Fricke et al., 2021]. Similarly to the province, aquaculture is also in Van Phong Bay one major human activity taking place, besides tourism, fisheries, agriculture and harbor related industries (Fig.6.1D, E, F) [Phung Nguyen and Dung, 2010, Phu et al., 2022].

6.2.2 *Kappahycus alvarezii* cultivation in Van Phong Bay

Various raft cultures of *K. alvarezii* (Fig.6.1E) are located in the northern part of Van Phong Bay, west of the island *Hon Sang* (Fig.6.1C). The surrounding land protects the area from heavy rains and winds and therefore farmers chose this spot for seaweed cultivation. *K. alvarezii* was introduced in the Khánh Hòa province in 2003 and, due to the success, it is expanding ever since [Hurtado et al., 2017]. Thalli are usually cultivated on longlines using the *tie-tie* technique.
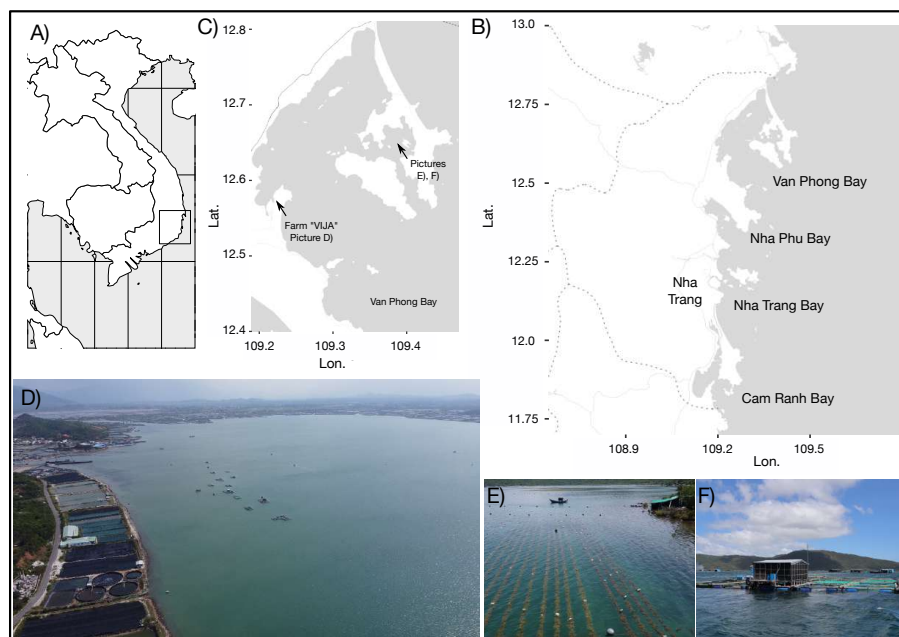
Figure 6.1: Map of **A)** Viet Nam with **B)** the Khánh Hòa province and the respective bays, including **C)** Van Phong Bay, where aquaculture takes places **D)** along the coastline in tidal ponds, as well as in the bay area, like **E)** *Kappaphycus alvarezii* on longlines and **F)** lobster farming in cages.

6.2.3 Sea grape cultivation in Van Phong Bay

In the southern part (Fig.6.1C), pond cultivation of shrimp, *B. areolata* and seaweed *C. lentillifera*, among others, takes place (Fig.6.1D). *C. lentillifera* was introduced to Viet Nam about a decade ago from Japan for cultivation [Terada et al., 2016]. The sea grape farm VIJA is located in the south of the Bay (location 12.5866, 109.2255). The ponds of VIJA are connected through adjustable channels to the nearby coast. The ponds are covered with gauze to provide a light environment of $\sim$50 µmol photons m^{-2} s^{-1} in average over the day (see data presented in [Stuthmann et al., 2022], chapter 5).

6.2.4 Experimental design

This study consisted of two experimental parts, namely *(1)* a series of field experiments investigating different set-ups of co-cultivation of *C. lentillifera* with *K. alvarezii* (hereafter called *Field experiment*) and *(2)* a laboratory-based study to examine the bioremediation properties of the species in mono- and co-cultivation (thereafter called *Laboratory experiment*). The experiments were run simultaneously during a field stay in Viet Nam in May/June 2022 at the facilities of the Institute of Oceanography (IO) in Nha Trang and at the sea grape farm VIJA located in Van Phong Bay (Fig.6.1B).

6.2.5 Measurments

6.2.5.1 Environmental parameters

The environmental parameters salinity (Absolute Salinity (S_A)), temperature (°C), oxygen (% saturation) and pH were quantified using a Manta 2 multiparameter probe (Eureka, Texas, USA). Light irradiances of PAR were measured using a LI-1400 data logger with a 2-Π and/or 4-Π sensor (LICOR Biosciences, USA).

6.2.5.2 Nutrient analysis

Water samples (20 mL) for analysis of NO_3^-, Nitrite (NO_2^-), NH_4^+ and Phosphate (PO_4^{3-}) were filtered using a 0.45 μm syringe filter and stored in plastic bottles at -20°C until analysis. The analysis of the dissolved inorganic NO_3^- and NO_2^- (NOx) was determined following the procedures of Garcia-Robledo et al. [García-Robledo et al., 2014], whereas quantification of PO_4^{3-} was following the procedures of Ringuet et al. [Ringuet et al., 2011]. NH_4^+ was quantified following the procedures of Ringuet et al. and Yu et al. [Yu et al., 1994, Ringuet et al., 2011]. For the measurements of the absorbance an infinite 200 PRO microplate reader (TECAN, Austria) was used in all cases.

6.2.5.3 Biomass

The biomass of *K. alvarezii* and *C. lentillifera* used in these experiments was retrieved from a farm in the north of Van Phong Bay and from the sea grape farm VIJA on the 13.05.22, respectively. The red and green algae were cultivated in nets in the bay at IO and in cultivation tanks, before the start of the experiments. Section E.1 in the Appendix provides an overview of the different sub-experiments, locations and sampling days, respectively.

6.2.5.4 Chlorophyll *a* fluorescence measurements

The photosynthetic efficiency (F_v/F_m) was quantified using a portable Diving-Pulse-Amplitude Modulated (PAM) Chlorophyll (Chl) fluorometer (Walz, Effeltrich, Germany). The seaweeds were 7 min dark adapted for measurement of F_v/F_m, whereas the parameter F_v'/F_m' was quantified in seaweeds, which were not dark-adapted, but at steady state light conditions in the respective cultivation set-up.

6.2.5.5 Relative growth rates

The Relative Growth Rate (RGR) was quantified using the following formula:

$$RGR\,(\%\,\text{day}^{-1}) = \frac{ln(N_t) - ln(N_0)}{t} \times 100, \tag{6.1}$$

with N_t and N_0 being the Fresh Weight (FW) at time t and 0, respectively.

6.2.5.6   Antioxidant activity and total phenolic content

The seaweed samples were rinsed with distilled water and stored at -80°C. Subsequently, the biomass was freeze dried (Beta 1-8 LSCbasic, Christ GmbH, Germany) and pulverized (FastPrep-24, MP Biochemicals, Germany). Approximately 0.04 g of powder were weighed in and 1 mL Ethanol (70%) was added for the extraction of the target compounds in a water bath (47°C, 4 hours, vortex hourly). The samples were centrifuged (2500 g, 20°C, 10 min) and the supernatant for the respective assay was transferred in an Eppendorf tube and frozen at -80°C until analysis within the next two days. The analysis of the Antioxidant Activity (AOA) was conducted using the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)$^{.+}$ assay following the procedure of Re et al. with slight modifications [Re et al., 1999, Sommer et al., 2022], using a UV/VIS-Spectrophotometer (Thermo Scientific Genesys 140/150, Fisher Scientific GmbH, Germany). The AOA was expressed as Trolox Equivalents (TE). For analysis of the Total Phenolic Content (TPC) the Folin-Ciocalteu (FC) method was used [Ainsworth and Gillespie, 2007] with slight modifications [Stuthmann et al., 2022]. The TPC was expressed as Gallic Acid Equivalents (GAE).

6.2.5.7   Carbon and nitrogen tissue content

Approximately 1-2 mg sea grape powder and $\sim$1 mg birch leaf as standard were weighted in tin cups (10x10 mm) and total elemental Carbon (C) and N contents were analyzed by combustion (Eurovector EA3000, Pavia, Italy).

6.2.5.8   Colour analysis

K. alvarezii organisms were placed in a styrofoam box equipped with two lamps in order to take pictures using a Canon EOS M50 camera (Canon Zoom Lens EF-M 14–45 mm). A grey reference scale (B.I.G, photo equipment – Brenner Import and Handels GmbH, Weiden i.d. OPf., Germany) was placed next to the organisms. The pictures were calibrated using the grey reference scale in order to correct for differences in illumination.

Subsequently, the colour of each replicate was analyzed following the description of Winters et al. [Winters et al., 2009], adapted by Stuthmann et al. [Stuthmann et al., 2022], using the software octave [Eaton et al., 2021]. The colour was expressed as value between 0-255 in one of the respective colour channels of Red (R), Green (G) or Blue (B).

6.2.6   Field experiment: Set-up and measurement of response variables

The co-cultivation potential of *C. lentillifera* and *K. alvarezii* was tested in three different set-ups. In a first approach sea grape cultivation was integrated in the conventional *K. alvarezii* longline cultivation set-up, as primarily conducted in Van Phong Bay. However, due to logistic reasons the set-up was duplicated at the facilities at IO. A number of 13 longlines were stretched between two mean ropes of an approximate length of 50 m (Fig.6.2A, B).

At each line 14 *K. alvarezii* fragments (initial weight of 64.1±35.8 g) were attached in a distance of 20 cm using the *tie-tie* technique (Fig.6.2A.II, F). For the integration of *C. lentillifera* twelve two-level plastic-cages (height: 50 cm, diameter: 27.5 cm) were constructed from perforated plastic mesh (mesh width: 1.3x0.8 cm, Fig.6.2A.III, C). Due to reports stating that strong currents prevent the successful sea grape cultivation in the open waters [Largo et al., 2016] gauze wrapping was applied to six of the twelve plastic cages (Fig.6.2D). Two plastic cages, one with and one without gauze wrapping, were attached to six longlines, respectively (initial sea grape stocking 250.7±1.5 g). A stone and a plastic bottle (functioning as buoy) were used to stabilize the cages in the currents (Fig.6.2B). For experimental measurements, the cages and the longlines were detached and brought to shore (Fig.6.2G, H).

In a second approach *K. alvarezii* longlines, prepared in a similar manner than for the set-up described above, were integrated in a sea grape pond. The lines were hung between bamboo sticks in a sea grape pond at farm VIJA. However, after two weeks, the *K. alvarezii* fragments showed strong signs of die off (see Appendix E.1, Fig.E.1) and the experiment was stopped.

In a third approach, *C. lentillifera* were integrated in the net cage cultivation of the red algae. The net cages were purchased at a local fishing shop (height: 90 cm, diameter: 35 cm). The mesh size of the net (2x1.2 cm) allowed *C. lentillifera* thalli to slip through and therefore the lower part was tied of and wrapped with gauze material (Fig.6.2A.I, E). Net-cages used for the *K. alvarezii* mono-cultivation were not tied off (Fig.6.2A.I, H), leaving the fragments with more space, compared to the co-cultivation with sea grapes (Fig.6.2A.I, E). The net cages were stocked with the initial biomass (*C. lentillifera*: 350.3±0.3 g, *K. alvarezii*: 695.8±33.9 g), before attaching them to a rope in close proximity to the longlines (Fig.6.2A). The day of initialization and measurements of response variables, as well as the experimental run differed between experimental set-ups, due to logistic reasons. An overview of the exact dates is provided in Appendix E.1.

In this study the response parameters of F_v/F_m, RGR, C and N tissue content and antioxidant analysis (AOA, TPC) were quantified and data from the end of the experiment are presented. Environmental water parameters (salinity, temperature, pH, oxygen), measured at different depths (0-3 m) and times over the tidal cycle, light irradiances of PAR at different experimental set-ups and sea water samples for nutrient measurements at IO Viet Nam and the experimental pond at VIJA were quantified at different sampling days, respectively (Appendix E.1).

The diurnal change of the photosynthetic activity (F_v'/F_m') was quantified for *K. alvarezii* and *C. lentillifera* in the different cultivation set-ups at IO. Measurements with the diving-PAM were conducted every second hour between 6 a.m. and 6 p.m. (information on dates, i.e. measurement days and times: E.1). A number of 4-6 replicated measurements were quantified for each cultivation set-up, depending on the prevailing environmental conditions. Simultaneously, the light irradiances of PAR in the air were quantified using a 2-Π and 4-Π sensor, respectively.

The cost of material was inquired at the local market, where farmers would likely also obtain their equipment. The monetary value was converted from Vietnamese Dong (VND) to United States Dollar (USD) using the rate 1000 VND=0.043 USD.
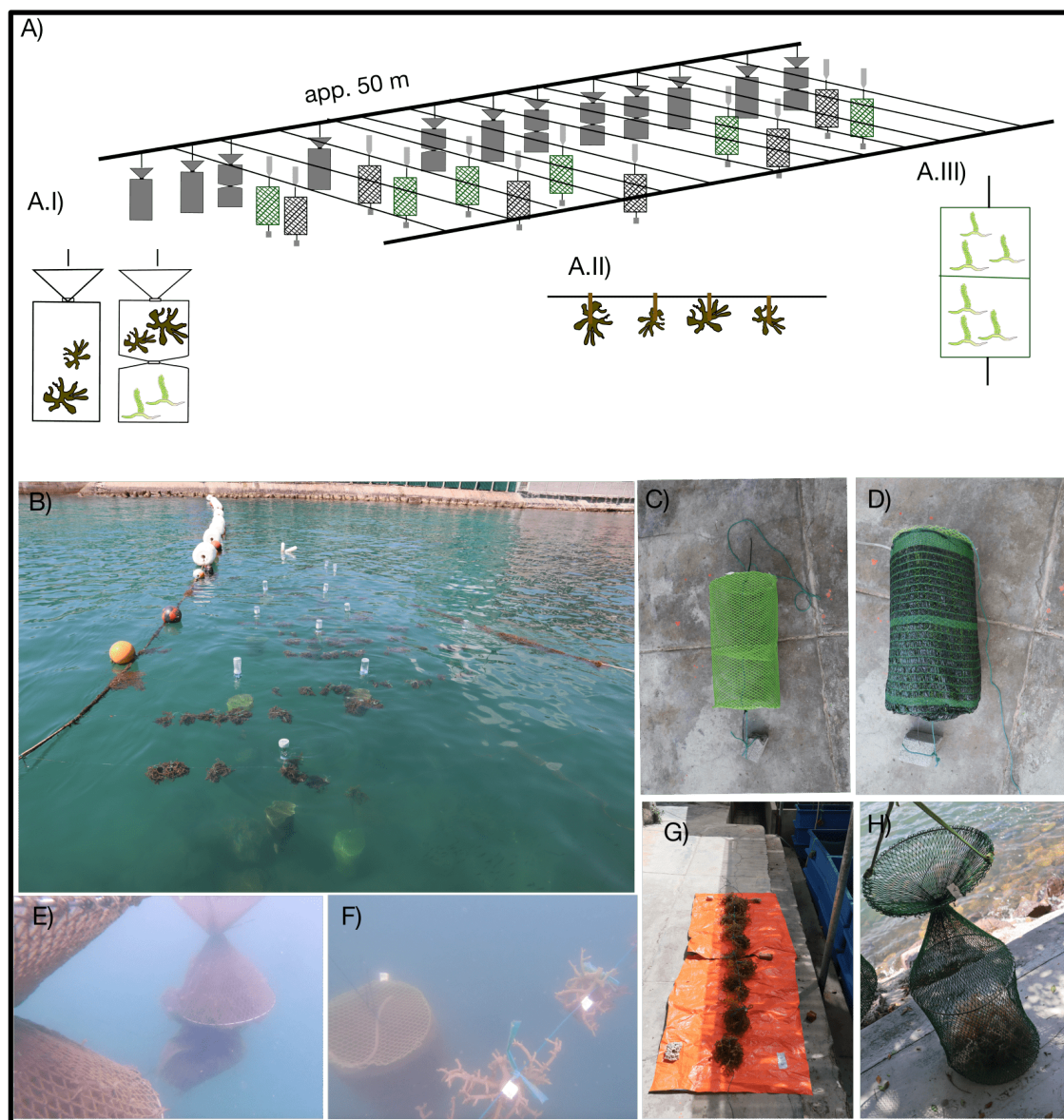
Figure 6.2: Set-up of the field experiment in Nha Trang, Viet Nam, with the **A.I)** net cages for *Kappaphycus alvarezii* mono- and co-cultivation with *Caulerpa lentillifera* being attached to main rope and **A.II)** longlines with *Kappaphycus alvarezii* fragments attached using the *tie-tie* technique. **A.III)** Plastic cages with *C. lentillifera* biomass were attached on longlines. Pictures of the **B)** set-up, the plastic cages **C)** without and **D)** with gauze wrapping, **E)** net cages and **F)** longlines and plastic cages in the water, **G)** *K. alvarezii* longlines during measurements on land and **H)** net-cages on land are shown.

6.2.7 Laboratory experiment: Set-up and measurement of response variables

A total of 45 beakers (capacity of 1 L treatment water) were placed in a water bath (25.0±1.0°C) at the laboratory facilities of IO. The beakers were either stocked with a target biomass of 20 g of one of the species or with 10 g of each for the mono- or co-cultivation treatment, respectively (initial biomass mono-cultivation: 19.95±0.41 g; co-cultivation: 10.09±0.36 g). Four Light Emitting Diode (LED) lamps were placed above the experimental units providing an irradiance of 50±10 µmol photons m^{-2} s^{-1} (12:12 h light:dark rhythm). In order to adjust for differences in the light treatment, the beakers were shifted between positions regularly and stirred daily.

B. areolata effluents were used for the fertilization of the seaweed. A snail cultivation, mimicking the situation in a pond cultivation in terms of the size of the pond in relation to the water volume and the stocking densities, was implemented in a 400 L fiberglass tank. Snails were fed every two days with sardines and a ∼40%-water exchange was conducted subsequently. The NO_3^- content was estimated using aquarium droplet tests (Salifert, Duiven, Netherlands). The NO_3^- concentration changed between days and in order to keep the conditions as constant as possible over the run of the experiment, the water was diluted with natural seawater targeting a NO_3^- concentration of 250 µmol L^{-1}. Water of the beakers was exchanged every three days. The control treatment received natural sea water from the bay at IO (in the following named *Control*), the continuous treatment received diluted snail effluents (in the following named *Continuous*) and the alternating treatment received snail effluents and natural sea water alternating with each water exchange (in the following named *Alternating*).

Water samples for nutrient analysis were taken before every water exchange and treated as described in section 6.2.5.2. Measurements of F_v/F_m and seaweeds FW were taken weekly, following the descriptions of section 6.2.5.4. Biomass samples for the analysis of C and N tissue content were taken after 21 days of experimental run. The biomass was rinsed with distilled water prior freezing at -80°C. In this study, only the data quantified after 21 days are presented. The RGRs were quantified based on the initial biomass and the measurements after 21 days. Pictures of *C. lentillifera* and *K. alvarezii* organisms were taken after the experimental run of 21 days. The colour of *K. alvarezii* fragments was estimated from the pictures, as described in section 6.2.5.8. Ten sample points (25 pixel) were randomly chosen on the algae thalli and the values of each colour channel were averaged for the respective replicate. Presented are the mean±Standard Deviation (SD) of all averaged replicates. Colour values were not analyzed for *C. lentillifera*, since the differences within each picture were very large.

6.2.8 Data analysis

The statistical analysis, as well as the graphical outputs were conducted using the software R in combination with R-studio [R Core Team, 2019, RStudio Team, 2018] and the meta package tidyverse [Wickham et al., 2019]. Outliers were identified using Grubb's test through the webpage GraphPad (https:// www.graphpad.com/quickcalcs/Grubbs1.cfm, accessed on 15.03.2023;

p<0.05). Levene's test (homogeneity of variance, p>0.05) and Shapiro-Wilk test (normal distribution, p>0.05) were conducted for each data set. Depending on the outcome of the test a parametric or non-parametric test was used to identify the effects on the response variables. A two-way Analysis of Variance (ANOVA) was used to test the effect of two main factors on the mean of different response variables, whereas a one-way ANOVA was used to test the effect of between-subject effects. Tukey's Honest Significant Difference (HSD) post-hoc test was run afterwads. In case the requirements were not met, a Kruskal-Wallis test with a Dunn-Bonferroni post-hoc test was used. A Wilcoxon pairwise test was used to test for differences between *K. alvarezii* net harvest of various cultivation methods.

The term *cultivation method* refers to *K. alvarezii* cultivation on longlines and net cages, and *C. lentillifera* cultivation in plastic cages and net cages, respectively. The term *cultivation approach* refers to the mono- or co-cultivation of the species. *Fertilization treatment* refers to the different fertilization regimes during the laboratory experiment. All statistical outputs are presented in Appendix (E.2).

6.3   Results and discussion

6.3.1   Field experiment

6.3.1.1   Environmental parameters

The salinity, pH and temperature at the experimental site at IO (Table 6.1) were in the range of measurements conducted in Nha Trang Bay during the dry season [Ba Xuan and Phuoc, 2000, Fricke et al., 2021]. However, salinity and temperature varied over the tidal cycle and throughout the water column (Fig.6.3A, B). The parameters were inversely correlated: At high tide and at the largest depth the temperature values were lowest ($\sim$27°C, Fig.6.3A), whereas the salinity showed highest values (Fig.6.3B).

The Cai and Be rivers discharge in the north and south of Nha Trang Bay, respectively, and influence the distribution of salinity and temperature, especially in the first two meters of the water column [Ba Xuan and Phuoc, 2000]. Diurnal changes of up to S_A 10.22 and 1.07°C were quantified during the dry season in the north of Nha Trang Bay [Ba Xuan and Phuoc, 2000]. The snapshot of the nutrient load showed rather low levels (Table 6.2), which were comparable to findings from Cam Ranh Bay [Hung et al., 2019]. However, the strong water fluxes provide a constant renewal of nutrients.

The irradiances of PAR decreased from an irradiance of PAR >900 µmol photons m^{-2} s^{-1} measured at the water surface to a level of 25% (<250 µmol photons m^{-2} s^{-1}, Table 6.3) at a water depth of two meters. The *K. alvarezii* longlines shaded the area below, with additional shading of *C. lentillifera* by the plastic cages and gauze material. However, the irradiances fluctuated strongly between microhabitats, reflected by high SDs (Table 6.3). Even though these measurements are only a snapshot and are likely subject to diurnal and weather-related changes, they display an assessment of the irradiances available for the seaweeds' photosynthesis.

The environmental parameters at the experimental pond of VIJA in Van Phong Bay were
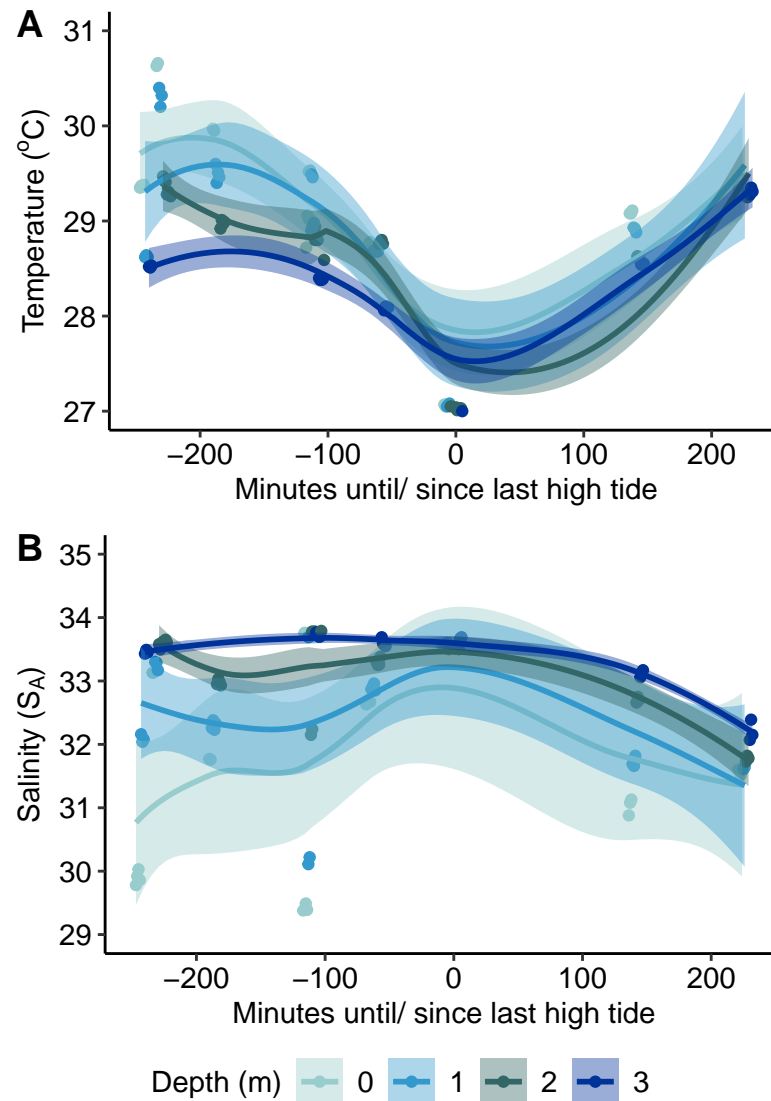
Figure 6.3: **A)** Temperature (°C) and **B)** Salinity (S_A) data collected over eight sampling days in May and June 2022 at the Institute of Oceanography (IO), Nha Trang, Viet Nam at the experimental cultivation side of *Kappaphycus alvarezii* and *Caulerpa lentillifera*. The confidence interval was received using the locally estimated scatterplot smoothing (LOESS) function. The data were plotted over the minutes before (-) or after (+) the closest high tide (0) and the groups present categorized measurement depth (m) below the surface with categories 0 ($\leq$0.35 m), 1 (0.87–1.34 m), 2 (1.51–1.96 m), 3 (>2.5 m). The information on the tidal ranges were received from https://tides4fishing.com/vn/khanh-hoa/nha-trang.

Table 6.1: Environmental parameters absolute salinity (S_A), oxygen saturation (% satura-
tion), pH, temperature (°C) presented as mean±standard deviation (SD) quantified at the
Institute of Oceanography (IO), Nha Trang, Viet Nam and at the pond VIJA, where the cul-
tivation experiment took place, respectively. The measurements at the IO and VIJA were
conducted on day one and eight, respectively.

Location	Salinity (SA)	Oxygen (% Saturation)	pH	Temperature (°C)	Replicates (n)
IO	32.65 ± 1.16	109.03 ± 7.83	8.49 ± 0.04	28.80 ± 0.82	106
VIJA	33.73 ± 0.04	79.2 ± 4.70	8.41 ± 0.02	30.59 ± 0.10	10

Table 6.2: Nutrient content of seawater at the experimental cultivation site at the Institute
of Oceanography (IO), Nha Trang, Viet Nam quantified on eight different sampling days.
Values presented as mean±standard deviation (SD).

Location	Nitrate + Nitrite	Ammonium (µmol L^{-1})	Phosphate	Replicates (n)
IO	2.92 ± 2.10	4.77 ± 3.6	0.83 ± 0.96	8

only quantified during one measurement day (Table 6.2), however the values corresponded
to previous measurements conducted at the same farm facilities [Stuthmann et al., 2023].
The pond water seemed in comparison to the site at IO warmer and with a lower oxygen
saturation (Table 6.1).

6.3.1.2 *K. alvarezii*: Growth, photosynthesis and biochemical composition

The RGRs of *Kappaphycus alvarezii* were significantly affected by the cultivation approach
(mono- vs. co-cultivation; $F(91,1)=123.36$, $p<0.001$), as well as the cultivation method (long-
line vs. net cages; $F(91,1)=8.36$, $p<0.01$, Fig.6.4A). The fragments at the longline showed the
highest RGRs (mean $4.42\pm0.84\%$ day^{-1}), compared to the cultivation in net cages in mono-
and co-cultivation with means of 2.12 ± 0.64 and $0.62\pm0.54\%$ day^{-1}, respectively. The RGRs of

Table 6.3: Irradiances of photosynthetically active radiation (PAR) quantified at the experi-
mental cultivation site at the Institute of Oceanography (IO), Nha Trang, Viet Nam using a
2-π sensor and a LICOR data logger on 21.05.2022 in the time between 10:00 and 10:30 a.m.
Data are presented as mean±standard deviation.

Location	Irradiances (µmol photons m^{-2} s^{-1})	Replicates (n)
Experimental site at surface	921 ± 513	17
Experimental site at 1 m depth	826 ± 384	11
Experimental site at 2 m depth	234 ± 27	9
Below *Kappaphycus alvarezii* longline	551 ± 323	4
Caulerpa lentillifera plastic cages without gauze	349 ± 245	6
C. lentillifera plastic cages with gauze	79 ± 68	6
K. alvarezii net cages	278 ± 173	4
C. lentillifera net cages	9 ± 7	6

the longline cultivated fragments ranged from -0.11–6.21% day^{-1}, which corresponds to values quantified for *K. alvarezii* in Nha Trang and Cam Ranh Bay [Hung et al., 2009, Diem Hong et al., 2010, Hung et al., 2019].

The *K. alvarezii* fragments implemented in the experimental pond of farm VIJA were considered dead after 14 days. The thalli became white and soft (Appendix E.1), which are well-documented signs for *ice-ice*. The syndrome is driven by unfavorable environmental conditions, like particularly increased temperatures and bacterial infection and can lead to decreased biomass and carrageenan yields [Ward et al., 2022]. Hence, considerably warmer temperatures in the experimental pond (Table 6.1), as well as hardly any water movement, which is known to be important for successful *Kappaphycus*' growth [Ask and Azanza, 2002], might have caused the outbreak of the *ice-ice* syndrome. The optimal temperature range of *Kappaphycus* spp. for outdoor cultivation was found between ∼25 and 30°C [Ask and Azanza, 2002]. Cultivation temperatures of ≥32°C, however, caused a significant decline in growth rates, compared to 28°C [Kumari et al., 2013]. The cultivation experiment took place during the warmest period of the year in the Khánh Hòa province [ISPONRE, 2009], where growth rates are usually strongly declined, compared to the cold season [Hung et al., 2009, Diem Hong et al., 2010, Hung et al., 2019]. Hence, during the cold season even higher RGRs of *K. alvarezii* are to be expected.

The AOA and TPC of the carragenophyte were in the range of values reported from Malaysia [Chew et al., 2008, Mohamed and Abdullah, 2016]. Both variables were similar between the longline and net cage cultivation treatment (Table 6.5). Non-enzymatic antioxidants play a role in the response of seaweeds to oxidative stress [Rezayian et al., 2019], e.g. triggered by excess irradiances. Damaging effects for *K. alvarezii*, due to high light have been observed at irradiances of 500 µmol photons m^{-2} s^{-1} [Barros et al., 2006] and the saturation irradiance of *K. alvarezii* was quantified as ∼150 µmol photons m^{-2} s^{-1} using PAM fluorometry with photosynthesis vs. irradiance curves (P-E curves) at 26°C [Terada et al., 2016].

The F_v/F_m values of the fragments were similar between treatments with values of ≥0.5 (Table 6.5), indicating no signs of photoinhibition [Terada et al., 2016, Borlongan et al., 2017]. Hence, the light conditions, even though different between cultivation set-ups and status (Table 6.3) were arguably in average in a suitable range for *K. alvarezii*.

However, during high irradiances at midday, *K. alvarezii* still showed typical decreases of F_v'/F_m' values, which were especially pronounced for fragments at the longlines (Fig.6.7C). Terada et al. observed a similar midday depression and argues that this sign of dynamic photoinhibition or -adaptation might be a protective mechanism for the photosynthetic apparatus from the excessive PAR [Terada et al., 2016]. *K. alvarezii* fragments in net cages showed the depression as well, however the pattern was distinctive, possibly due to the lower irradiances they were exposed to (Table 6.3). Since F_v/F_m values were similar between cultivation methods (Table 6.5), the significant differences between *K. alvarezii*s RGRs (Fig.6.4) were likely not a result of the differences in the light environment.

The significantly smaller RGRs of the net cage cultivated fragments in co-cultivation with *C. lentillifera*, compared to the mono-cultivation (Fig.6.4A) rather suggest that space restrictions were limiting the seaweeds growth. However, high losses of *K. alvarezii* fragments were recorded: Only a count of 168 (80% of initial) and 82 (39% of initial) fragments from
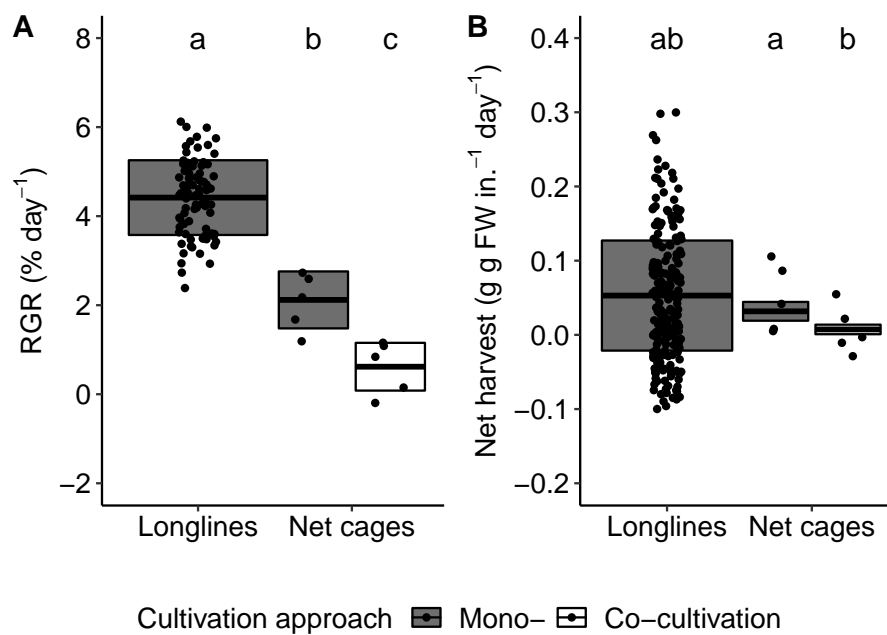
Figure 6.4: Relative growth rate, (RGR % day^{-1}) of **A)** *Kappaphycus alvarezii* cultivated at the Institute of Oceanography (IO), Nha Trang, Viet Nam on longlines and in net cages with and without *C. lentillifera* after 41 and 33 days of cultivation and n=82 and n=5, respectively. Only the fragments that were still present after 41 days were considered. **B)** Net harvest per day (g g fresh weight, FW initial (in.) day^{-1}) of *K. alvarezii* cultivated on longlines (n=210) and in net cages with (n=5) and without (n=5) *C. lentillifera*. Also missing fragments were included and their net harvest was set to zero. Data are presented as mean±standard deviation, indicated by the middle line and the box, respectively. Black dots indicate individual data points. Different letters indicate statistical differences between the treatments (One-Way ANOVA with Tukeys HSD post hoc test or Wilcox pairwise test).

the initially implemented 210 were still present after 21 and 41 days, respectively. Grazing by herbivory is a common phenomenon for the loss of *Kappaphycus* spp. harvest [Ask and Azanza, 2002], that has also been reported for the Khánh Hòa province ([Ohno et al., 1996], personal observation). However, for this experiment the fragments were presumably lost due to the high wave and tidal action in the cultivation area. The RGRs were only based on the fragments that were present at the end of the experiment. Hence, the net harvest (initial g g FW^{-1} day^{-1}) shows the farmers' potential crop of *K. alvarezii* per day per g of initial seeded biomass, with lost fragments included in the calculation (Fig.6.4B). The high RGRs of *K. alvarezii* fragments (Fig.6.4A) remaining at the longlines caused, even though not statistically significant, still a trend of a higher mean net harvest when considering also the lost fragments (6.4B).

6.3.1.3 *C. lentillifera*: Growth, photosynthesis and biochemical composition

The RGRs of *C. lentillifera* were negative throughout the cultivation experiment, ranging between means of -1.29 and -9.76% day^{-1} (Fig.6.5A). Hence, the RGR of *K. alvarezii* was overall significantly higher compared to *C. lentillifera*, when analyzed regardless of the respective cultivation method (Chi square=38.46, p<0.001, df=1). The RGRs of *C. lentillifera* were affected by the cultivation method (F(1,10)=8.71, p<0.05), as well as the status of gauze wrapping (F(1,10)=288.76, p<0.001). Sea grapes cultivated in the plastic cages without gauze wrapping lost the most weight (-9.76±0.56% day^{-1}), followed by net cage cultivated alga with gauze wrapping (-6.37±0.78% day^{-1}, Fig.6.5A). However, the values for the cultivation in the plastic cages with gauze wrapping were with a mean of -1.29±0.78% day^{-1} significantly higher (Fig.6.5A). Sea grapes are a siphonous species [Zubia et al., 2020] and their fragile thalli can be sensitive to strong physical forces, like water currents and wave action.

Largo et al. integrated *C. lentillifera* as an extractive species in an open water Integrated Multi-Tropic Aquaculture (IMTA) system in The Philippines using baskets [Largo et al., 2016]. Even though slight growth was observed during the first three weeks, the seaweeds disintegrated during stormy waters and waves [Largo et al., 2016]. The high loss of biomass in the plastic cages without gauze wrapping, compared to the significantly smaller loss in cages with gauze were likely caused by the higher exposure to the strong waves during stormy weather and high tidal ranges in the bay. This is also indicated by observed differences in the sea grapes morphologies between cultivation methods and set-ups.

Caulerpa spp. are known to be morphologically plastic as a response to different environmental parameters, among others temperature, salinity [Estrada et al., 2020] as well as light and water movement [Calvert, 1976]. The presence of gauze wrapping led to a significantly higher proportion of fronds (46.35±8.96% of total of Fronds), compared to the algae without gauze as protection (26.20±11.63% of total, Fig.6.5B). Additionally, thalli with lower wave exposure showed rather delicate stolons with considerably long fronds, compared to seemingly more sturdy thalli of sea grapes with higher wave exposure (Appendix E.1, Fig.E.1). Sea grapes that were cultivated as a pilot (without quantitative data) in tray cultivation at a sheltered place at the pier very close to the experimental cultivation site showed thalli morphologies that reminded of those from the pond cultivation (Appendix Fig.E.1). However,

these information were not quantified and hence the results should be interpreted with care and investigated further.
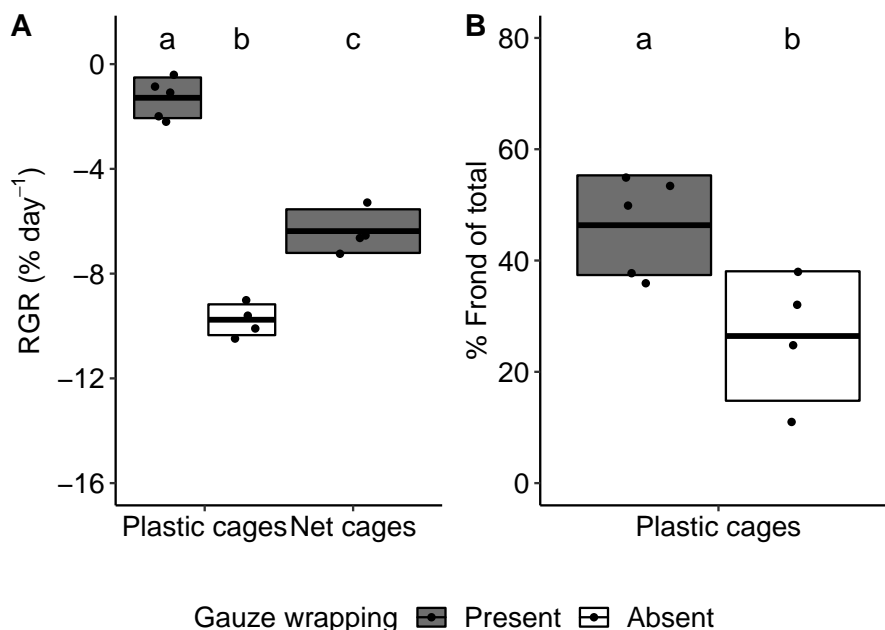
Figure 6.5: Relative growth rate (RGR, % day^{-1}) of **A)** *Caulerpa lentillifera* cultivated at the Institute of Oceanography (IO), Nha Trang, Viet Nam in plastic cages with and without gauze wrapping and in net cages after 24 and 33 days of cultivation (n=4-5), respectively and **B)** the% Frond of total of *C. lentillifera* in plastic cages with and without gauze wrapping. Data are presented as mean±standard deviation (SD), indicated by the middle line and the box, respectively. Black dots indicate individual data points. Different letters indicate statistical differences between the treatments (One-Way ANOVA, Kruskal-Wallis with respective post-hoc test).

Besides the water movement, the light regimes were different between the cultivation set-ups of *C. lentillifera* (Table 6.3). Sea grapes are known to be sensitive to light irradiances of ≥ 100 µmol photons m^{-2} s^{-1} at photoperiods of 12:12 (light:dark rhythm; [Guo et al., 2015a, Stuthmann et al., 2020]). Exposure to irradiances that exceed the limit for Carbon Dioxide (CO$_2$) – assimilation can lead to photoinhibition as an acclimation strategy of the seaweeds to avoid damage of the photosystem [Murchie and Niyogi, 2011]. A decrease in the maximum quantum yield of Photosystem II (PSII) (F$_v$/F$_m$) is often used to characterize photoinhibition [Demmig-Adams and Adams, 1992, Maxwell and Johnson, 2000]. Additionally, the AOA and the TPC can act as a protective response to photooxidative stress of *C. lentillifera* [Stuthmann et al., 2022]. The F$_v$/F$_m$ values in this experiment were not affected by the set-up (p>0.05), but showed declined values when gauze wrapping was not present (F(1,11)=13.531, p<0.01, Table 6.5).

Similarly, the values of AOA and TPC are with means of 155.88±19.29 mmol TE 100 g^{-1} DW and mg GAE 100 g^{-1} DW significantly enriched, compared to the treatments with gauze wrapping (Table 6.5). Increased C tissue contents of sea grapes cultivated in plastic cages without gauze wrapping, caused a significantly higher C:N ratio (19.20±1.34), compared to

those with gauze wrapping in net (9.13±1.23) and plastic cages (11.31±1.50, Table 6.5). Increased C:N ratios could indicate a N-limitation [Hanisak, 1990], however even though the nutrient loads in the water were low (Table 6.2), high water exchange rates, contradict a N-limitation.

The exposure to light, as well as wave action could have led to the algae's investment in structural and antioxidative compounds, like polyphenols. Seaweeds phenolic compounds are present in the soluble form, acting e.g. as protection under stress conditions, or in the insoluble, cell-wall bound form as structural elements [Cotas et al., 2020, Lomartire et al., 2021]. The strong correlation of TPC and C tissue content (r_P=0.87, p<0.001, Fig.6.6), and hence C:N ratio (Appendix E.2, r_S=0.64, p<0.5) supports this hypothesis and has been found for brown seaweed as well [Arnold et al., 1995].
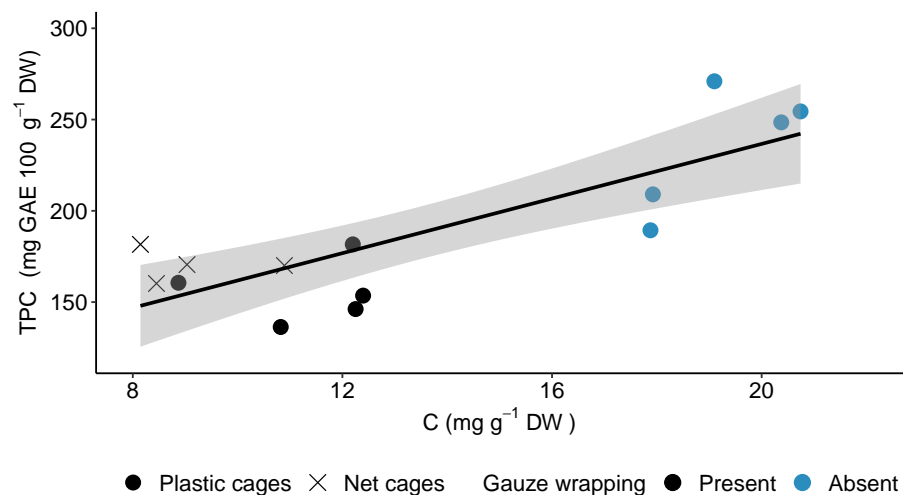
Figure 6.6: Pearson correlation (p<0.001, r_P=0.87) of carbon (C, mg g^{-1} dry weight, DW) and Total Phenolic Content (TPC, mg Gallic Acid Equivalents, GAE 100 g^{-1} DW) of *Caulerpa lentillifera* cultivated at the Institute of Oceanography (IO), Nha Trang, Viet Nam with shape indicating the cultivation set-up and colour the presence (black) or absence (blue) of gauze wrapping. The black line indicates a linear regression with 95% confidence interval.

Hence, the gauze arguably acted as a protection against strong water movement and from high irradiances. The exposure of *C. lentillifera* to irradiances of 349±245 µmol photons m^{-2} s^{-1} in the plastic cage without protection (Table 6.3), caused significantly depleted F_v/F_m values of 0.46±0.09, compared to means of ≥0.65 with protection (Table 6.5). However, the solar irradiances follow a typical diurnal fluctuations with highest values around midday [Terada et al., 2016, Stuthmann et al., 2022], similarly found at this experimental site (Fig.6.7). This pattern leads typically to a midday depression of F_v'/F_m' values of *Caulerpa* [Raniello et al., 2006], which was observed for sea grapes in plastic cages without gauze wrapping, but missing for *C. lentillifera* with gauze wrapping (6.7B).

Table 6.4: F_v/F_m values, antioxidant activity (AOA, expressed as mmol Trolox equivalents, TE 100 g^{-1} dry weight, DW), total phenolic content (TPC, expressed as mg gallic acid equivalent, GAE 100 g^{-1} DW), carbon (C) to nitrogen (N) tissue content ratio, C and N tissue content (expressed as mg g^{-1} DW) of *Kappaphycus alvarezii* and *Caulerpa lentillifera* in different cultivation methods at the Institute of Oceanography (IO), Nha Trang, Viet Nam, calculated after the total experimental run (days) and in replicate numbers (n). The data are expressed as mean±standard deviation. Different letters indicate significant differences between the means. Tested with a one-way ANOVA or Kruskal-Wallis test with a Post-hoc test.

Species	Set-up	Run days	F_v/F_m	AOA mmol TE 100 g^{-1} DW	TPC mg GAE 100 g^{-1} DW	C:N	C mg g^{-1} DW	N	n
K. alvarezii	Mono-line	41	0.55 ± 0.08^a	46.66 ± 10.93^a	127.07 ± 23.17^a	25.94 ± 5.39^a	230.6 ± 15.7^a	9.2 ± 1.6^a	7
	Net cages mono-cultivation	33	0.56 ± 0.05^a	37.60 ± 24.0^a	117.50 ± 51.75^a	24.91 ± 4.0^a	235.5 ± 24.9^a	9.6 ± 1.57^a	4
	Net cages co-cultivation	33	0.59 ± 0.04^a	-	-	-	-	-	5
C. lentillifera	Plastic cages no gauze	24	0.46 ± 0.09^a	155.88 ± 19.29^a	234.45 ± 33.96^a	19.20 ± 1.34^a	199.5 ± 25.4^a	10.4 ± 0.8^a	5
	Plastic cages gauze	24	$0.66 \pm 0.05b$	105.49 ± 14.45^b	155.66 ± 17.07^b	11.31 ± 1.50^b	$136.8 \pm 4.5b$	12.3 ± 1.6^a	5
	Net cages	33	$0.65 \pm 0.11b$	80.61 ± 7.86^b	170.59 ± 8.75^b	$9.13 \pm 1.23b$	144.4 ± 21.2^b	$15.9 \pm 2.4b$	4

Table 6.5: Costs (as United States dollar, USD) of material for different co-cultivation set-ups sourced from markets in Nha Trang, Viet Nam.

Material	unit	USD/ unit	Longlines (per m)	Plastic cages without gauze	Plastic cages with gauze	Net cages
			Units needed for respective cultivation method			
Gauze material	1 m	0.87	-	1	-	-
Perforated plastic	1 m	1.28	-	1.5	1.5	-
Plastic line	1 m	0.3	1	-	-	-
Tie-tie rope	100 g	0.043	1	-	-	-
Net cages	1 piece	3.63	-	-	-	1

Table 6.6: Nitrate (NO_3^-) + nitrite (NO_2^-), ammonium (NH_4^+), and phosphate (PO_4^{3-}) concentration of experimental natural sea water (Nat. SW) and diluted experimental effluents of *Babylonia areolata* cultivated at the Institute of Oceanography (IO), Nha Trang, Viet Nam.

Water	Nitrate + Nitrite	Ammonium	Phosphate	n
	$\mu mol\ L^{-1}$			
Nat. SW	2.89 ± 1.67	4.07 ± 3.08	0.47 ± 0.43	6
Effluence	9.51 ± 2.63	37.15 ± 36.50	1.56 ± 0.85	6

6.3.1.4 Costs of material

The cultivation methods comprise different systems with varying costs of material. The material is only one of many different factors, like workforce, material durability, transportation (boating) etc., which should be considered when comparing the economic benefit of different cultivation methods [Valderrama et al., 2015]. However, the costs differ greatly between the cultivation methods. The net cages cultivation was most expensive for *K. alvarezii*, as well as *C. lentillifera* cultivation (Table 6.5). However, even though less expensive, the longline cultivation with *tie-tie* technique was reported to require a high workload [Ask and Azanza, 2002].

6.3.2 Laboratory experiment

6.3.2.1 *Babylonia areolata* effluents

The nutrient concentration of the *B. areolata* effluents was lower than on-site estimations using NO_3^- droplet-tests estimated. Based on the on-site tests, the water was diluted to reach a target NO_3^- concentration of 250 $\mu mol\ L^{-1}$. However, the experimental treatment water only showed a mean of NO_3^- and NO_2^- of 9.51 ± 2.63 $\mu mol\ L^{-1}$ over the experimental run, due to the lower N content. In contrast, the natural sea water used as control was still considerably lower in NO_3^- + NO_2^- (2.89 ± 1.67 $\mu mol\ L^{-1}$, Table 6.6).
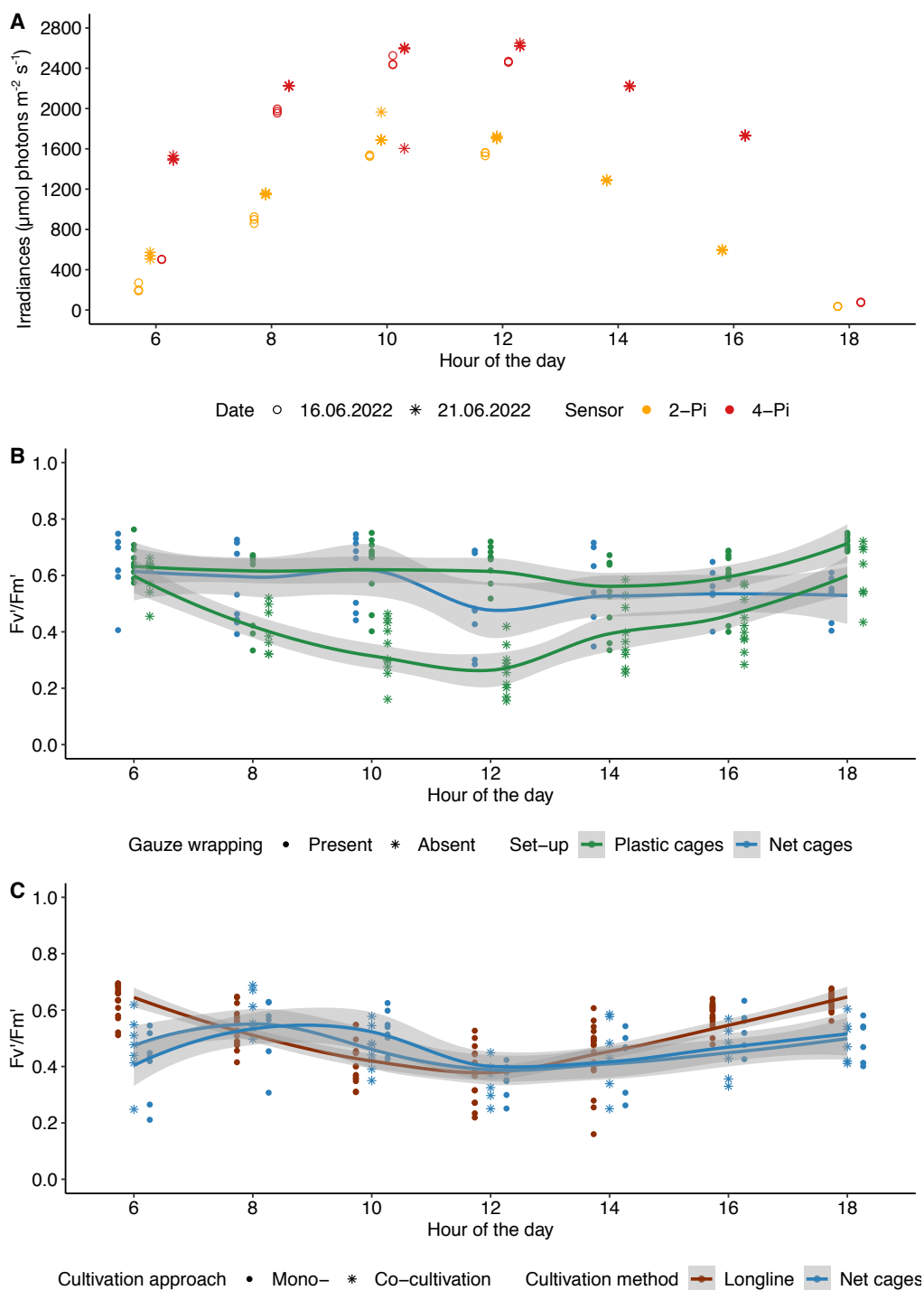
Figure 6.7: Diurnal measurements between 6 a.m. and 6 p.m. (for 12 hours of the day) of **A)** light irradiance (µmol photons m^{-2} s^{-1}) using a 2-Π and 4-Π sensor at two different dates in June 2022 at the experimental two-layer cultivation at the Institute of Oceanography (IO) in Nha Trang, Viet Nam, as well as the maximum efficiency of photosystem II (PSII) in a steady state light environment (F_v'/F_m') of **B)** *Caulerpa lentillifera* in plastic and net cages and with or without gauze wrapping and **C)** *Kappaphycus alvarezii* on longlines or in net cages.

6.3.2.2 *K. alvarezii***: Growth, photosynthesis, C, N tissue content, and colour**

K. alvarezii fragments showed RGRs around zero throughout all fertilization treatments (p=0.431) and cultivation approaches (set-up=0.507, Fig.6.8A), comparable to literature values [Dawes et al., 1993, Dawes et al., 1994, Martino et al., 2021]. In general, the in-door cultivation of *K. alvarezii* is complex [Jose de Paula et al., 2002], but ideal cultivation conditions can result in RGRs similar to or even higher than outdoor cultivation [Yong et al., 2014]. Jose de Paula observed that smaller branches with overall lower algal density of <10 g L^{-1} in the cultivation unit yielded in higher growth rates [Jose de Paula et al., 2002]. Hence, the high initial biomass in this experimental set-up (20 g L^{-1}) and the absence of permanent or alternating aeration via a pump [Jose de Paula et al., 2002] could have led to the low RGRs of the red seaweed.

*K. alvarezii*s' F_v/F_m values were similar between all treatments (p=0.22), ranging between 0.50±0.07 and 0.58±0.04 (Table 6.6). Fragments that were fertilized with effluents showed lower C:N ratios (Fig.6.9A) compared to the control, potentially caused by a trend of increased N tissue contents (Fig.6.9C). Additionally, the R and G colour channel values of fertilized fragments were lower, compared to those in natural seawater, indicating a darker colouration. The darkening of *K. alvarezii* fragments cultivated in aquaculture effluents, along with increased N tissue values [Pires et al., 2021] and overall low RGRs of <1% day^{-1} [Hayashi et al., 2008] has been observed similarly in the present experiments. The seaweeds might have stored the absorbed N in the form of Chl [Pires et al., 2021], explaining the darker colouration, as well as higher N tissue values during the absence of growth. Differences in RGRs between treatments, even though absent in laboratory cultivation, could appear in a subsequent sea cultivation [Martino et al., 2021]. The successful out-door cultivation of *K. alvarezii* is often season-dependent and the land-based cultivation with fertilization by aquaculture effluents could present an alternative to counteract potential production loss [Martino et al., 2021]. However, continuous exposure to nutrient-high cultivation media could have negative effects on *K. alvarezii* [De Paula et al., 2001]. Hence different fertilization regimes, like the alternation of nutrient-rich and -low cultivation water could improve the in-door cultivation of the red seaweed [Martino et al., 2021].

This experiment did not reveal significant differences between both fertilization regimes, but a trend of higher tissue N for the continuous fertilization (Fig.6.9C). However, this might also be caused by the overall low nutrients loads of the effluent water.

6.3.2.3 *C. lentillifera***: Growth, photosynthesis, and carbon, nitrogen tissue content**

For *C. lentillifera*, the fertilization treatment had a significant effect on the RGRs (F(2,23)=54.62, p<0.01) and share of frond (F(2,26)=24.84, p<0.01), opposite to the cultivation approach (RGRs: p=0.079, share of frond: p=0.301). The RGRs of *C. lentillifera* cultivated in natural sea water (control) were negative with means of -2.65±2.22 and -1.30±1.09% day^{-1} for the mono- and co-cultivation, respectively. However, the sea grapes exposed to an alternating (mono-cultivation: 2.15±0.42% day^{-1}, co-cultivation: 2.07±0.42% day-) and a continuous fertilization (mono-cultivation: 2.38±0.19% day^{-1}, co-cultivation: 3.29±0.81% day^{-1}) were in
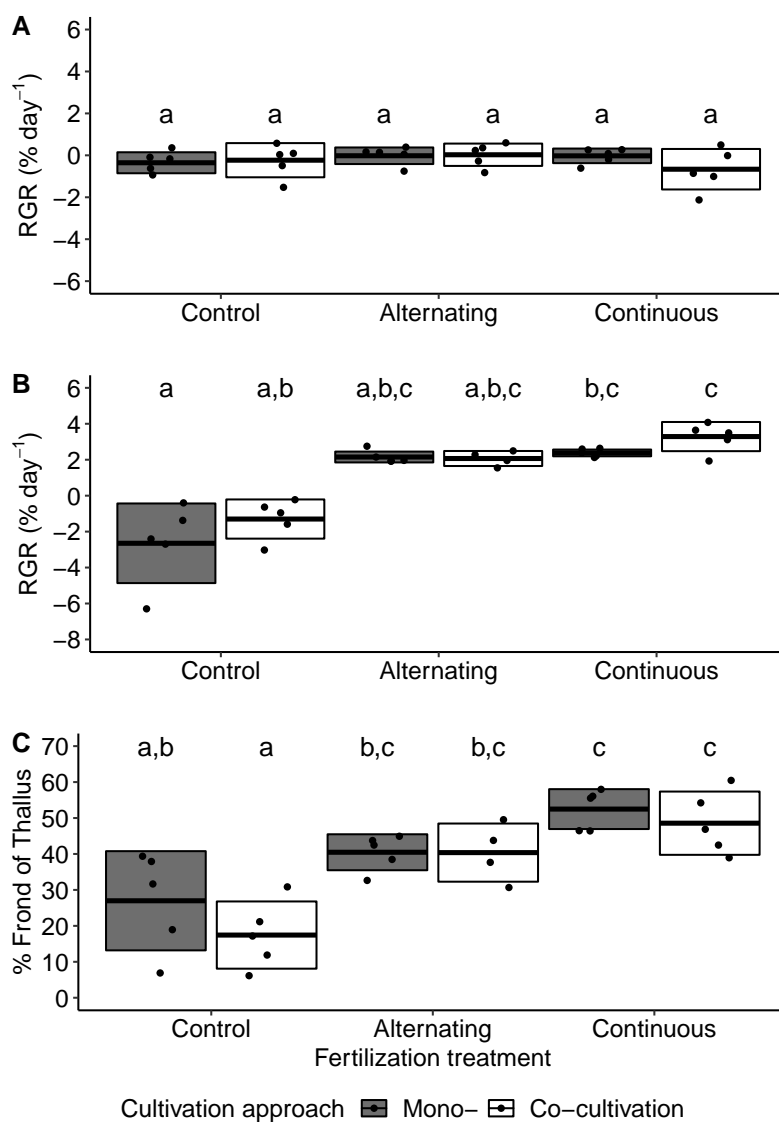
Figure 6.8: Relative growth rate (RGR, % day^{-1}) of **A)** *Kappaphycus alvarezii* and **B)** *Caulerpa lentillifera* cultivated at the Institute of Oceanography (IO), Nha Trang, Viet Nam calculated over 21 experimental days and **C)** proportion of fronds from total biomass (%) at different fertilization treatments (natural seawater, continuous pond water, alternating fertilization) and cultivation approach (mono- and co-culture) after 21 days experimental run. Different letters indicate significant differences (one-factor ANOVA). Black dots indicate individual data points. Data are presented as mean±standard deviation (n=4-5).

Table 6.7: F_v/F_m values of *Caulerpa lentillifera* and *Kappaphycus alvarezii* at the beginning (initial) and after 21 days in an experimental set-up with fertilization treatments and cultivation approaches. The experiment was conducted at the Institute of Oceanography (IO), Nha Trang, Viet Nam. Different letters indicate significant differences between treatments (one-factor ANOVA, mean $\pm$ standard deviation, n=5). The statistical analysis was conducted for the two species separately, but between all treatments within one species, respectively.

Species	Cultivation approach	Fertilization treatment		
		Control	Continuous fertilization	Alternating fertilization
Caulerpa lentillifera	Initial		0.67 ± 0.03^c	
	Mono-culture	0.51 ± 0.09^a	$0.65 \pm 0.01^{b,c}$	$0.62 \pm 0.05^{b,c}$
	Co-culture	$0.55 \pm 0.03^{a,b}$	$0.66 \pm 0.03^{b,c}$	$0.57 \pm 0.08^{a,b,c}$
Kappaphycus alvarezii	Initial		0.58 ± 0.04^a	
	Mono-culture	0.50 ± 0.07^a	0.51 ± 0.05^a	0.54 ± 0.05^a
	Co-culture	0.52 ± 0.06^a	0.53 ± 0.05^a	0.56 ± 0.02^a

a range similarly observed by other laboratory studies [Cai et al., 2021b, Liu et al., 2016] (Fig.6.8B).

The ratio of sea grape fronds to stolon seemed to increase from significantly lowest values at the control over alternating to continuous fertilization (Fig.6.8B). These results are concurrent with reports of *C. prolifera*, showing higher stolon growth at N limiting conditions, compared to increased frond when N was not limited [Malta et al., 2005]. F_v/F_m values of *C. lentillifera* were significantly affected by the fertilization treatment (F(2,26)=11.909, p<0.001), however not by the cultivation treatment (p=0.94). Similar to the RGRs, the values were lower for sea grapes cultivated at natural seawater, compared to the continuous or alternating fertilization with snail process water (Table 6.7).

The suitability of F_v/F_m as a proxy for nutrient limitation and starvation of algae is still under discussion [Parkhill et al., 2001, Tan et al., 2019]. However, for *Caulerpa* consistent F_v/F_m values were reported during nutrient limitation [Guo et al., 2015a, Guo et al., 2015b, Malta et al., 2005]. Hence, the significant decrease of *C. lentillifera* control (Table 6.7) could be a sign for a nutrient starvation, similarly reported for microalgae [Parkhill et al., 2001]. *C. lentillifera* is an oligotroph species and signs of nutrient depletion regarding the growth rates were reported during cultivation at PO_4^{3-} and NO_3^- levels of ≤ 10 µmol L^{-1} and 50 µmol L^{-1}, respectively [Guo et al., 2015a].

The C and N tissue contents of seaweeds are in general known to be highly influenced by available nutrients in the water [Harrison and Hurd, 2001], which also applied to *C. lentillifera* [Paul and de Nys, 2008, Liu et al., 2016]. Consequently, the C:N ratio of *C. lentillifera* was significantly affected by the fertilization treatment (F(2, 24)=80.06, p<0.001), as well as by the cultivation approach (F(2,24)=5.18, p<0.05). The pattern of C:N ratios showed a similar trend for both species (Fig.6.9A, B). However, in contrast to *K. alvarezii*, the C:N pattern of sea grapes was caused by differences in C tissue content (Fig.6.9D), whereas the N tissue contents were similar between treatments (Fig.6.9F). The similar N tissue values of *C. lentillifera* could have been a result of a dilution effect, due to the growth of the alga at the fertilized treatments [Teichberg et al., 2008, Liu et al., 2016] (Fig.6.8F). When *C. lentil-*

lifera was cultivated at nutrient limited seawater, the alga arguably invested the fixated C in N-free compounds, like e.g. phenols [Ilvessalo and Tuomi, 1989], resulting in significantly higher tissue C values (Fig.6.9D).

6.3.3   Potential of two-layer cultivation in Van Phong Bay

The close proximity of *K. alvarezii* and *C. lentillifera* cultivation sites in Van Phong Bay with established production chains, as well as their complementary light and nutrient demands suggest a high potential of a successful, resource-efficient two-layer cultivation. The low water movement, as well as the considerably high temperatures prevented the integration of the carragenophyte in sea grape ponds in this study (section 6.3.1.2). However, reports of successful *K. alvarezii* pond cultivation from the Ninh Thuan province in Viet Nam during the colder months of January and March [Ohno et al., 1996] suggest that especially the warm temperatures might have been detrimental for the seaweeds. Some sea grape ponds are equipped with paddle wheels (personal observation), which could be used to artificially generate water motion. Hence, *K. alvarezii* could potentially be cultivated in the sea grape ponds around the month of ∼January–March, when sea grape cultivation is not possible.

The growth of *K. alvarezii* fragments in the inshore area was more successful, even though the losses due to strong water movements were high. Growth data, as well as the economic comparison suggest that the *K. alvarezii* longline cultivation with sea grape plastic cages is more promising than the net cage two-layer cultivation. Plastic net cages were self-made, and the investment costs were low, compared to the commercially available net cages (section 6.3.1.4). Farmers could adapt the plastic cage design to their needs and schedule the maintenance according to their work on the longlines in order to use resources efficiently. Even though, *C. lentillifera* did not show positive RGRs, the net biomass loss was minimal in the plastic cages with gauze wrapping and the share of frond was high (section 6.3.1.2 and 6.3.1.3). Water movement in the north of Van Phong Bay is considerably lower than at the study site, due to the land-locked position (section 6.2.2, Fig.6.1C). Hence, in the calmer waters decreased losses of *K. alvarezii* fragments, as well positive RGRs for *C. lentillifera* are expected, suggesting a successful implementation of this *Kappaphycus-Caulerpa* two-layer set-up. The biochemical and physiological results point out, that gauze material should be used as additional shading, even in the absence of the strong currents in order to avoid photooxidative stress of *C. lentillifera* (section 6.3.1). However, considering the water depth in Van Phong Bay, sea grape plastic cages in the water column could be adjusted to the favorable irradiance environment and to potentially even obviate the gauze wrapping. Salinity levels deeper in the water column are also expected to be higher and more stable (section 6.3.1.1), potentially enabling sea grape cultivation also during the rainy season [Ly, 1999]. This could lead to a year-around supply of fresh sea grapes.

In case seaweeds need maintenance on land for transport between cultivation locations or during off-season, effluents of the edible snail *B. areolata* are a promising fertilization medium for *K. alvarezii* and *C. lentillifera*. However, timing of the growth seasons of the snail and the seaweeds should be considered [Mai et al., 2022] and in case of difficulties the effluents of other aquaculture species from the area, like *L. vannamei* could be used
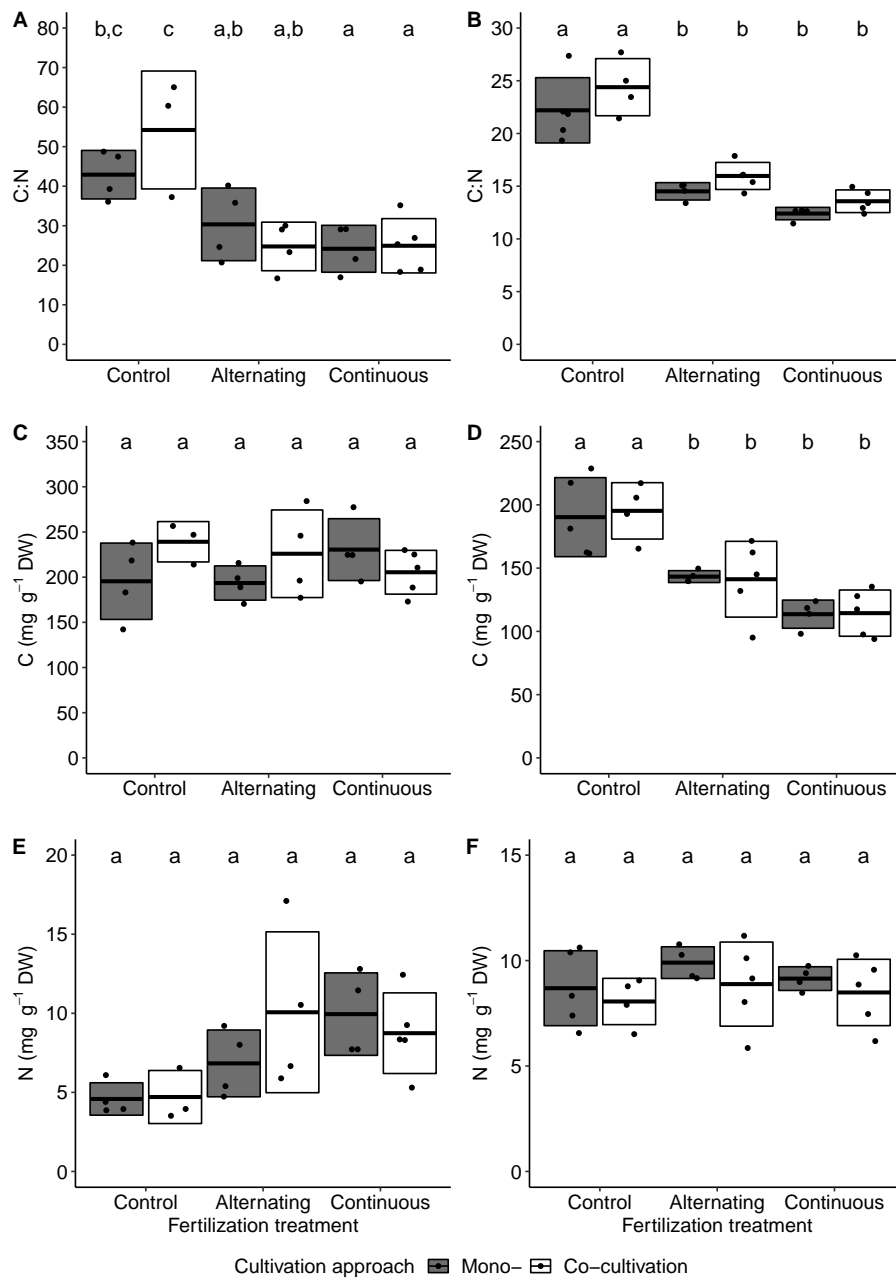
Figure 6.9: Ratio and total values of carbon (C, mg g^{-1} DW) and nitrogen (N, mg g^{-1} dry weight, DW) tissue content of **A)**, **C)**, **E)** *Kappaphycus alvarezii* and **B)**, **D)**, **F)** *Caulerpa lentillifera* after 21 experimental days at different fertilization treatments (Control, Alternating fertilization, Continuous fertilization) and cultivation approach (mono- and co-culture). The experiment was conducted at the Institute of Oceanography (IO), Nha Trang, Viet Nam. Different letters indicate significant differences (one-factor ANOVA or Kruskal-Wallis test and respective post-hoc test). Black dots indicate individual data points. Data are represented as mean±standard deviation (n=4-5).
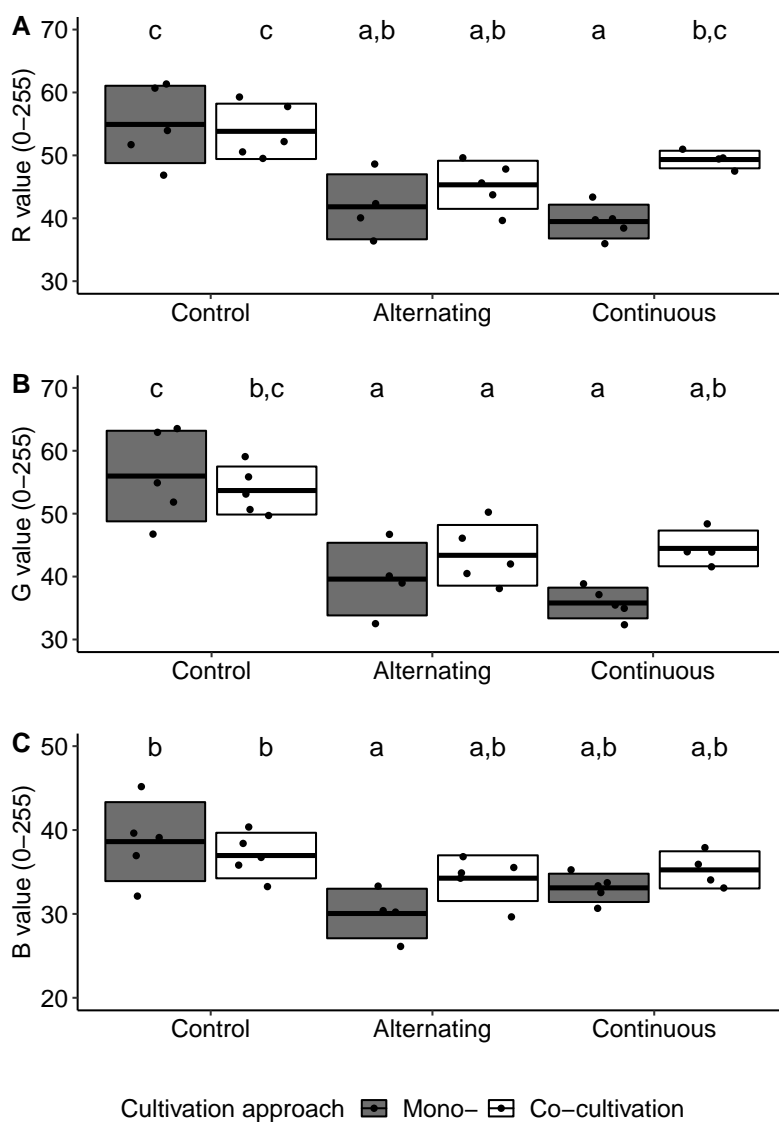
Figure 6.10: Colour values (0-255) of **A)** red (R), blue (B), green (G) and **C)** B channel of *Kappaphycus alvarezii* fragments after 21 experimental days at different water treatments (natural seawater, continuous pond water, alternating fertilization) and culture set-ups (mono- and co-culture). The experiment was conducted at the Institute of Oceanography (IO), Nha Trang, Viet Nam. Different letters indicate significant differences (one-factor ANOVA or Kruskal-Wallis test and respective post-hoc test). Black dots indicate individual data points. Data are represented as mean±standard deviation (n=4-5).

[Ly et al., 2021, Anh et al., 2022]. Even though *K. alvarezii* did not increase its biomass during laboratory cultivation, the increase in tissue N and the darker colouration suggest that nutrients were taken up and might be invested in biomass increase once deployed back in the sea (section 6.3.1.2 and 6.3.1.3).

6.4 Conclusions

The economically important carragenophyte *K. alvarezii* and the sea vegetable *C. lentillifera* could be cultivated in a resource-efficient two-layer seaweed cultivation in the calm waters of Van Phong Bay, Viet Nam. Fragments of the red seaweeds can grow on longlines, exhibiting low investment coasts and high growth rates, whereas cheap, self-made, and suitable plastic cages can be deployed at the longlines for *C. lentillifera* cultivation. Although the sea vegetable can be shaded by *K. alvarezii*, additional shading through gauze material or a depth adjustment of the sea grapes has to be considered to avoid photooxidative stress. Locally available aquaculture effluents of cultivated snails *B. areolata* provide a suitable fertilizer to maintain seaweed biomass on-land during off-season. The simultaneous cultivation method could increase the farmers income without driving the investment costs very high. Two-layer seaweed cultivations are a promising tool to resource-efficiently diversify seaweed cultivation with locally available species.

Chapter 7

Publication VII

The whiteleg shrimp *Litopenaeus vannamei*.

Potential for resource-efficient co-culture and value-adding manipulation: Fertilization of sea vegetable *Caulerpa lentillifera* with process water of *Litopenaeus vannamei*

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[1]joined senior authorship

Abstract

The green seaweed *Caulerpa lentillifera* (sea grapes, Chlorophyta) is of high demand as a sea vegetable, especially in Asia. However, in the event that demand also emerges in Europe, land-based Recirculation Aquaculture System (RAS)s for cultivation of the whiteleg shrimp (*Litopenaeus vannamei*) could provide an opportunity to resource-efficiently implement farming of the sea vegetable *Caulerpa lentillifera*. In a first approach sea grapes were cultivated for 14 days in pure shrimp process water (*CP*), artificial sea water (*CN*), process water of different Dilution Factor (DF)s (*Low*, *Medium*, *High*) with Nitrate (NO_3^-) concentrations of 48, 144, 720 µmol L^{-1}. In an additional approach, treatment water was fertilized with Phosphate (PO_4^{3-}) to reach a ratio of NO_3^-:PO_4^{3-} of 5:1 (*PF*) or the experiment was run without additional PO_4^{3-} fertilization (*NF*). Here, we show that sea grapes growth, harvestable biomass and Total Hydrolysable Amino Acids (THAA) content can be increased with fertilization of treatment *Medium PF* and *High* with shrimp process water, with a similar antioxidant concentration. The quantity of most Amino Acid (AA)s was significantly correlated with the Nitrogen (N) content of the treatment water (Spearman, p<0.05), whereas most relative contents of AAs were correlated with the Relative Growth Rate (RGR)s (Spearman, p<0.05). This finding raises the opportunity to use the process water of shrimp as a tool for targeted manipulation of the sea grapes' AA composition. Significant differences in THAA content of sea grapes between treatment *Medium NF* and *PF*, suggest a Phosphorus (P)-limitation. In conclusion, process water of the whiteleg shrimp from a land-based RAS can be used for fertilization and nutritional value manipulation of sea vegetable *Caulerpa lentillifera*. However, further upscaled experiments are required before implementation of the application.

Keywords: Amino acids, Bioremediation, Co-culture, Nutrient requirement, Sea grapes, Shrimp aquaculture

7.1 Introduction

In Europe, the algae producing industry is still in its infancy, while >99% of global seaweed production is taking place in Asia [Chopin and Tacon, 2020, FAO, 2022]. Other than in Asia, the majority of macroalgae in Europe is still harvested from the wild (68%), even though potential for aquaculture exists [Araújo et al., 2021]. Seaweeds are presented as an important component in recent bioeconomy strategies, because their biomass has versatile applications and their bioremediation potential offers various opportunities for integration in circular set-ups [Barbier et al., 2019, Aquaculture Advisory Council, 2021]. Seaweeds take up dissolved nutrients, mainly Nitrogen (N) (in form of inorganic Nitrate (NO_3^-), Ammonium (NH_4^+) and the organic form urea) and Phosphorus (P) (in form of Phosphate (PO_4^{3-})) from their surroundings to build up biomass.

In natural systems N is commonly the most limiting nutrient, followed by PO_4^{3-}. P, and especially N availability can have diverse and lasting effects on the metabolism and biochemical composition of algae [Roleda and Hurd, 2019]. Targeted farming of seaweeds to bioremediate aquaculture effluents of fed species is already implemented [Kang et al., 2021, Sarkar et al., 2021], however effective nutrient uptake requires a healthy metabolism of the extractive species [Roleda and Hurd, 2019] and hence context-dependent knowledge about specie's physiology is needed [Tanaka et al., 2020, Kang et al., 2021].

Caulerpa lentillifera is a green macroalga of high demand in certain Asian markets, especially due to the combination of its special texture and the nutritional benefits [Matanjun et al., 2009, Paul et al., 2014, Saito et al., 2010, Syakilla et al., 2022, Zubia et al., 2020]. In Europe, the sea vegetable is commonly known as sea grape and green caviar. Sea grapes are cultivated mainly in The Philippines, Viet Nam, China, and Japan in in-and outdoor cultivation systems and are eaten raw, e.g. in salads [Zubia et al., 2020]. Even though they still occupy a niche market, their economic value is high, compared to other macroalgae [Paul et al., 2013] and the demand is currently exceeding the production [de Gaillande et al., 2017].

Laboratory experiments revealed highest growth rates of sea grapes at nutrient concentrations of 500 µmol L^{-1} NO_3^- and 100 µmol L^{-1} PO_4^{3-}. With lower N supply, the nutrient seems limiting for growth, whereas concentrations of 1000 µmol L^{-1} NO_3^- and 400 µmol L^{-1} PO_4^{3-} resulted in signs of photoinhibition [Guo et al., 2015b]. However, certain N levels were found to inflict physiological stress to the sea grapes, resulting in nonlinear growth rates in relation to N content [Liu et al., 2016, Hsu et al., 2023]. P limitation was observed with PO_4^{3-} of 10 µmol L^{-1} [Guo et al., 2015a]. Accordingly, different NO_3^- loads (47, 188, 750 µmol L^{-1}) with constant PO_4^{3-} concentrations of 29 µmol L^{-1} resulted in similar growth rates [Cai et al., 2021b], indicating that P might have been limiting for sea grapes here as well.

Bryopsidales are abundant in tropical, oligotrophic waters, possibly due to their presumed capability to absorb nutrients through their extensive belowground thallus parts (stolons, rhizoids) [Williams, 1984, Alexandre and Santos, 2020]. It was hypothesized that these psammophytic forms, like *C. lentillifera* are rather P, than N limited [Littler and Littler, 1990, Lapointe et al., 1992, Hurd et al., 2014], hence supporting these results. However, growth rates based on Wet Weight (WW) without differentiation between different thallus parts of *Caulerpa* should be interpreted with care, because N availability can alter thallus

morphologies. Malta et al. observed increased stolon growth under N-limitation and under elevated N levels increased frond growth for *C. prolifera* [Malta et al., 2005]. Economically, frond growth is given preference, considering the use of the alga as sea vegetable for human nutrition.

The protein concentrations of *C. lentillifera* based on Dry Weight (DW) varied as expected between locations and studies ∼10–19% DW [Matanjun et al., 2009, Nagappan and Vairappan, 2014], but with most values around 12-14% DW [Ratana-arporn and Chirapart, 2006, Long et al., 2020, Zhang et al., 2020]. However, biochemical composition can be altered with nutrient-, and especially N-supply, as well. Chlorophyll (Chl) a, β-carotene, and soluble protein content of sea grapes significantly increased with ascending NO_3^- supply (47, 188, 750 µmol L^{-1}), whereas enzymatic antioxidants (Superoxide Dismutase (SOD), Catalase (CAT)) were not affected [Cai et al., 2021b]. For other seaweeds an increase in protein:carbohydrate ratio [Bird, 1984] and an alteration in Amino Acid (AA) pattern [Angell et al., 2014] has been observed under increased N availability. However, Total Phenolic Content (TPC) in a brown seaweed decreased under increased N supply [Arnold et al., 1995] and overall Antioxidant Activity (AOA) decreased under nutrient limitation in microalgae [Goiris et al., 2015].

Most macroalga take up NH_4^+ more readily than NO_3^-, because it is a reduced form of N which requires less energy for uptake and bypasses the enzymatic transformation to NH_4^+ later [Boyd and Hurd, 2009]. However, sea grapes were found to prefer NO_3^- over NH_4^+ as N source, in the presence of both [Liu et al., 2016]. Once NO_3^- is taken up by seaweeds, it is stored in the vacuole or cytoplasm, or it can be reduced to Nitrite (NO_2^-) (nitrate reductase), which is transported to the chloroplast for reduction to NH_4^+ (nitrite reductase, [Harrison and Hurd, 2001]). In case the uptake rate of NO_3^- is larger than the conversion rate of NO_3^- to NO_2^-, e.g. due to slow nitrate reductase activity, then NO_3^- storage might appear, potentially leading to an increase of certain AAs and pigments as storage compounds [Harrison and Hurd, 2001].

Recent studies report successful polycultures in the same tanks of *C. lentillifera* with the whiteleg shrimp *Litoenaeus vannamei* [Anh et al., 2021, Ly et al., 2021, Omont et al., 2022]. Alga organisms were held in tray culture directly in the shrimp tank and their presence increased the shrimp weight significantly and simultaneously decreased total NH_4^+-N, NO_2^-, NO_3^-, and PO_4^{3-} in the water. However, shrimp were also reported to feed on *C. lentillifera* biomass [Anh et al., 2021], which suggests a locally separated co-cultivation. Best results for nutrient uptake were gained with a sea grape density of 0.5 Kg m^{-3}, however densities of 1–2 Kg m^{-3} were suitable as well [Ly et al., 2021].

L. vannamei represents with a production of ∼4,9 million tonnes (>50% of farmed crustacean species Fresh Weight (FW)) one of the most important organisms in global aquaculture production [FAO, 2022]. Integrated approaches have (to our knowledge) taken place mostly in low-technology systems. Highly technologized Recirculation Aquaculture System (RAS)s require high initial investments and have high maintenance costs and are therefore mostly established in technologized areas, like Europe. Nevertheless, land-based RASs bear the advantage that environmental conditions are highly controlled and non-local species can be cultured as well. Additionally, use of effective biofilter technology, or rather well managed de-/nitrification microbial communities [Preena et al., 2021] can lead to extremely small water

exchange rates [Suantika et al., 2018]. However, using the process water of shrimp culture to fertilize bioremediating, economically valuable species like sea grapes, leads to a more effective use of the available resources (e.g. heat, water, space, nutrients), besides the possibility to generate an additional source of income.

Therefore, this study is designed as a pilot investigation to use process water of *L. vannamei* from a highly technologized RAS for alga/seaweed production. The N load in the water is especially high and therefore, (*1*) we tested three different dilutions for their suitability for successful sea grape growth and (*2*) potential value addition through alteration of biochemical composition, including the AA profile. Based on the literature, we hypothesize, that sea grape growth might be P-limited, therefore, we artificially fertilized the dilutions with PO_4^{3-} to test (*3*) if additional P fertilization can enhance uptake of N and growth of the alga.

7.2 Material and methods

7.2.1 Experimental set-up and biomass

The experiment was conducted at Leibniz Centre for Tropical Marine Research (ZMT) Bremen in the Marine Experimental Ecology (MAREE) unit. Sea grape biomass was cultivated at MAREE for more than 6 months at constant parameters ($\sim$25.0$\pm$1.0°C, 40-50 µmol photons m^{-2} s^{-1}, Absolute Salinity (S_A) $\sim$34.0$\pm$1.0). The experiment was designed to quantify growth, biochemical composition and photosynthetic parameters of *C. lentillifera* after 14 days at two control scenarios (Control Negative, *CN*: artificial seawater, Control Positive, *CP*: pure process water) and three different Dilution Factor (DF)s of shrimp process water. To test the effect of PO_4^{3-} fertilization the three different DF treatments were run without additional PO_4^{3-} fertilization (No PO_4^{3-} fertilization: *NF*) and with a PO_4^{3-} fertilization (PO_4^{3-} fertilization: *PF*).

Erlenmeyer flasks (1 L) containing 800 mL of the respective processed water dilutions (n=5) were placed in a water bath (27$\pm$1°C, 40-50 µmol photons m^{-2} s^{-1}). The sea grape biomass (6-7 g L^{-1}) was temperature acclimated in artificial sea water (S_A 35) for three days before start of the experiment. At the first day of the experiment, the respective treatments were applied (Table 7.1).

7.2.2 Shrimp waste water

Process water was obtained from *Hanse Garnelen AG*, Germany. A total of 60 L process water (S_A 17) was stored with aeration and at room temperature in 20 L canisters until use in the experiment. The original nutrient concentration of the process water is stated in Table 7.1. The process water was diluted with distilled water, following dilutions in Table 7.1 and Redsea salt (Redsea, Verneuil d'Avre et d'Iton, France) was used to raise the salinity of dilutions to S_A 35.

Table 7.1: Dilution factors (DF) with respective nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations used in the experiment. The phosphate fertilization indicates, if DF treatments were without (*NF*) or with an additional phosphate fertilization (*PF*) and hence different nitrate:phosphate ratios are presented. The negative (*CN*) and positive (CP) controls contained artificial and pure process water, respectively.

DF treatment	Phosphate fertilization	DF	NO_3^-	NO_2^-	NH_4^+	PO_4^{3-}	
				(μmol L^{-1})			NO_3^-/PO_4^{3-}
CN	*NF*	-	2.2	<0.1	<0.1	0.2	14.9
Low	*NF*	300	48.0	0.1	0.1	1.7	28.2
Low	*PF*		48.0	0.1	0.1	9.6	5
Medium	*NF*	100	144.0	0.4	0.2	5.0	28.8
Medium	*PF*		144.0	0.4	0.2	28.8	5
High	*NF*	20	720.1	2.0	1.2	24.9	28.9
High	*PF*		720.1	2.0	1.2	144.0	5
CP	*NF*	0	14,400	1	24.2	497.6	28.9

7.2.3 Dilution factor

The process water in the RAS shrimp facilities carried high nutrient loads, as common in intensive cultures and salinities of S_A<17. Therefore, the different DF treatments were designed based on the prevalent body of literature. The dilutions were based on a sample of the shrimp process water measured prior the start of the experiment. Nutrient measurements of the process water were conducted with each water exchange over the two weeks of the experiment, but the DFs were not adapted.

As a P-limitation was hypothesized, each DF treatment was duplicated to be fertilized with PO_4^{3-}, provided as Monopotassium Phosphate (KH_2PO_4) salt, to meet a NO_3^-:PO_4^{3-} ratio of 5:1, which was described as suitable for *C. lentillifera* [Guo et al., 2015b]. DF treatments are shown in Table 7.1.

7.2.4 Growth measurements

Increase in biomass of *C. lentillifera* was quantified by measurement of FW. The sea grapes were carefully drained from the water and dabbed with a tissue prior weighting. The Relative Growth Rate (RGR) (% day^{-1}) of sea grapes was calculated following Cai et al. based on the biomasses weight at the beginning (W_0) and at the end (W_t) of the experiment, as well as the experimental duration (t) using the formula [Cai et al., 2021b]:

$$RGR\,(\%\,\mathrm{day}^{-1}) = \frac{ln(W_t) - ln(W_0)}{t} \times 100. \tag{7.1}$$

On the last experimental day, stolons and fronds were carefully divided and FW of the respective thallus parts was quantified, after dabbing with a tissue. The percent (%) of thallus part FW was calculated based on the FW of the respective part (W_{part}) and the total weight (W_{total}), following the formula

$$\%\,FW = \frac{100}{W_{part}} \times W_{total}. \tag{7.2}$$

7.2.5 Biochemical composition

7.2.5.1 Biomass sampling

Sea grapes were washed in distilled water to remove salt and subsequently dapped with a tissue. Biomass was freeze dried (Christ, Alpha 1-4 LD plus, Germany, -80°C, 1 bar, Vacuubrand GmbH & Co KG, Germany) and pulverized with mortar and pestle.

7.2.5.2 Antioxidant activity and total phenolic content

For the extraction ∼0.05 g DW sea grape powder was kept in 1 mL ethanol (70%) for 4 hours in a water bath (47°C) and vortex hourly. The samples were centrifuged (2,500 g, 20°C, 10 min.) afterwards and the supernatant was extracted. For measurement of the AOA the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)$^{.+}$ assay [Re et al., 1999] was used with slight modifications [Stuthmann et al., 2022]. Through the oxidation of 7.0 mM ABTS with potassium disulfate for 16 hours, an ABTS$^{.+}$ stock solution of 2.45 mM was prepared. On each measurement day, a fresh working solution was prepared by dilution with ethanol absolute until an absorption of 0.70±0.02 was reached at 734 nm (UV/VIS-Spectrophotometer from Thermo Scientific Genesys 140/150, Fisher Scientific GmbH, Germany). For measurements 1 mL ABTS$^{.+}$ working solution was added to 10 µL of sample extract and the absorbance was measured after 6 minutes using the same UV/VIS-Spectrophotometer mentioned above. The AOA was expressed as Trolox Equivalents (TE) (mmol TE 100 g^{-1} DW).

For quantification of TPC the Folin-assay was used [Ainsworth and Gillespie, 2007] with slight modifications [Stuthmann et al., 2022]. Folin-Ciocalteu (FC) solution (10% Volume Fraction (v/v)) was mixed with sample extract (150 µL) and vortex. Afterwards a Na$_2$CO$_3$ solution (1200 µL, 700 mM) was added and left for 45 min in the dark at room temperature. After centrifuging (3 min, 5000 rpm, 20°C), the absorbance was read with the same UV/VIS-Spectrophotometer mentioned above. The TPC was expressed as Gallic Acid Equivalents (GAE) (100 mg GAE g^{-1} DW).

7.2.5.3 Tissue carbon and nitrogen measurements

Approximately 1-2 mg sea grape powder and ∼1 mg birch leaf as standard were weighed in tin cups (10*10 mm) and total Carbon (C) and N was analyzed by combustion (Eurovector EA3000, Pavia, Italy).

7.2.5.4 Amino acid analysis

DW of sea grape samples was weighted in order to reach an amount of 0.5 mg C$_{org}$ in the sample, based on C and N analysis (section 7.2.5.3) in pre-heated spear ampules. Samples were hydrolyzed (22 h at 110°C) after adding 4 mL Hydrochloric Acid (HCL) (6N) and welding of ampules under flow of N$_2$. Afterwards, 1 mL of the aliquot was transferred in multi-evaporator tubes and dried in a multi-evaporator Synthesis 1 (60°C, 60 mbar pressure, Heidolph, Germany), until complete evaporation of HCL. 2 mL sodium citrate buffer (pH 2.65) was added to each sample, respectively. Total Hydrolysable Amino Acids (THAA) and Total

Hydrolysable Hexosamines (THHA) (amino sugars) of *C. lentillifera* were analyzed with a BioChrom 30 ion chromatography (Biochrome, Cambridge, United Kingdom). The identification of 21 AAs and two amino sugars was carried out by comparing the retention times to those of respective standards.

The AAs were: *1.* acidic: Aspartic Acid (Asp), and Glutamic Acid (Glu); *2.* neutral: Threonine (Thr), Serine (Ser), Glycine (GLY), Alanine (Ala), Valine (Val), Isoleucine (Ile), and Leucine (Leu); *3.* basic: Histidine (His), Ornithine (Orn), Lysine (Lys), and Arginine (Arg); *4.* aromatic: Tyrosine (Tyr), and Phenylalanine (Phe); *5.* non-protein: β-alanine (β-ALA), and γ-aminobutyric acid (γ-ABA); *6.* sulfur-containing: Methionine (Met), Methionine-sulfone (Met-sulfone), Taurine (Tau), and Cysteine (Cys-OX); cysteine hexosamines (amino sugars): Glucosamine (GLUAM) and Galactosamine (GALAM).

The ratio of Essential Amino Acid (EAA) to non Essential Amino Acid (non-EAA) was calculated by division of both numbers as mg g DW^{-1} based on the requirements for humans [FAO et al., 2007].

7.2.6 Chlorophyll *a* fluorescence measurements

A portable Diving-Pulse-Amplitude Modulated (PAM) Chl fluorometer (Walz, Effeltrich, Germany) was used to determine *in vivo* photosynthetic performance. Photosynthetic efficiency, F_v/F_m [Schreiber et al., 1995, Maxwell and Johnson, 2000] was quantified in sea grapes, after 7 min dark adaptation.

7.2.7 Nutrient measurements

Water samples (20 mL) for analysis of NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-} were stored frozen (-20°C) after filtering through a 0.45 µm syringe filter (Sartorius, Germany) into plastic bottles. The spectrophotometric analysis of dissolved inorganic nutrients followed established methods for NO_3^- and NO_2^-, NH_4^+ and PO_4^{3-} [Ringuet et al., 2011, Yu et al., 1994, García-Robledo et al., 2014], using an infinite 200 PRO microplate reader (TECAN, Austria).

7.2.8 Statistical analysis

The software R [R Core Team, 2019] in combination with R-studio [RStudio Team, 2018] and the meta-package tidyverse [Wickham et al., 2019] were used to conduct the statistical analysis and the graphical outputs. For the identification of outliers Grubb's test from the website GraphPad (https:// www.graphpad.com/quickcalcs/Grubbs1.cfm, accessed on 14.07.2023; p<0.05) was used. For determination of the homogeneity of variance and the normal distribution Levene's test and Shapiro-Wilk test (p>0.05) were conducted for each data set, respectively. A one or two way (Analysis of Variance (ANOVA)) was used to test the effect of between-subject effects and the effect of two main factors on the mean of different response variables with a Tukey's Honest Significant Difference (HSD) post-hoc test, respectively. In case the requirements of ANOVA were not met, a Kruskal-Wallis test with a Dunn-Bonferroni post-hoc test was used.

The correlation of two parameters was tested using a Pearson correlation, in case the variables were both following a normal distribution. Otherwise, a Spearman correlation was conducted.

7.3 Results and discussion

7.3.1 Growth parameters

The RGRs of sea grapes ranged from positive means of 1.15 to 3.19% day^{-1} FW, matching observations of other studies [Liu et al., 2016, Cai et al., 2021b]. However, RGRs were rather at the lower end compared e.g. to Cai et al., reporting values of up to 7.85% day^{-1} [Cai et al., 2021b]. The RGR and percentage (%) FW of Fronds were significantly affected by the DF treatment (RGR: F $(4,28)$=34.776, p<0.01; % FW Frond: F $(4,1)$=20.798, p<0.01, Fig.7.1A, B), with RGRs being increased for sea grapes of DF treatment *Medium* (*NF*: 3.02±0.47% day^{-1} FW, *PF*: 3.19±0.50% day^{-1} FW), compared to *Low* (*NF*: 1.48±0.46% day^{-1} FW, *PF*: 2.03±0.66% day^{-1} FW) and *CN* (1.15±0.85% day^{-1} FW).

The observed increase with ascending NO_3^- values did not continue for treatment *High* (*NF*: 2.35±0.63% day^{-1} FW, *PF*: 2.84±0.63% day^{-1} FW). This suggested that the critical N-content, denoted as the minimum N-content required for maximum growth rates [Ulrich, 1952, Angell et al., 2014], has been reached at a NO_3^- water concentration somewhere >48 µmol L^{-1} (*Low*) and ≤144 µmol L^{-1} (*Medium*). Consequently, the growth of *C. lentillifera* in artificial sea water (*CN*) and treatment *Low* had likely been limited by N availability, whereas sea grapes cultured at treatments *High*, *CP* and, potentially even *Medium*, were exposed to excess N and had the possibility to create N storages.

Guo et al. observed strong correlations between sea grapes' growth rates and NO_3^- contents of the culture media at concentrations between 50 and 500 µmol L^{-1}, but stagnating growth with NO_3^- ≥500 µmol L^{-1} [Guo et al., 2015b], confirming the present results. However, other studies observed a significant decrease in growth rates at N levels of ∼220 µmol L^{-1} resulting in non-linear growth in relation to the N content [Liu et al., 2016, Hsu et al., 2023]. This N concentration was interpreted as a unique condition for *C. lentillifera* leading to changes in the protein regulation that suggested a stress condition [Hsu et al., 2023].

Cai et al. reported similar RGRs of *C. lentillifera* cultured at three different NO_3^- levels (47, 188, 750 µmol L^{-1}, PO_4^{3-} 29 µmol L^{-1}), arguably due to the organisms increased respiratory consumption and therefore absent net accumulation of photosynthetic products [Cai et al., 2021b]. The difference might be explained by the shorter experimental run compared to the present study (eight vs. 14 days).

Sea grape thalli are composed of creeping stolons and economically more important upright fronds [Zubia et al., 2020]. This is drawing attention not only to the growth rate, but also to the thallus properties as a parameter of interest. Interestingly, the RGR and the % FW of Fronds were positively correlated (Spearman, r_S=0.4819, p=0.0018, Fig.7.2). This indicated that an optimization of the nutrient environment during sea grape culture benefited the biomass production of the algae, as well as their biomass properties from an economic perspective. The share of Fronds was with means >58% FW at treatments *Medium* and *High*
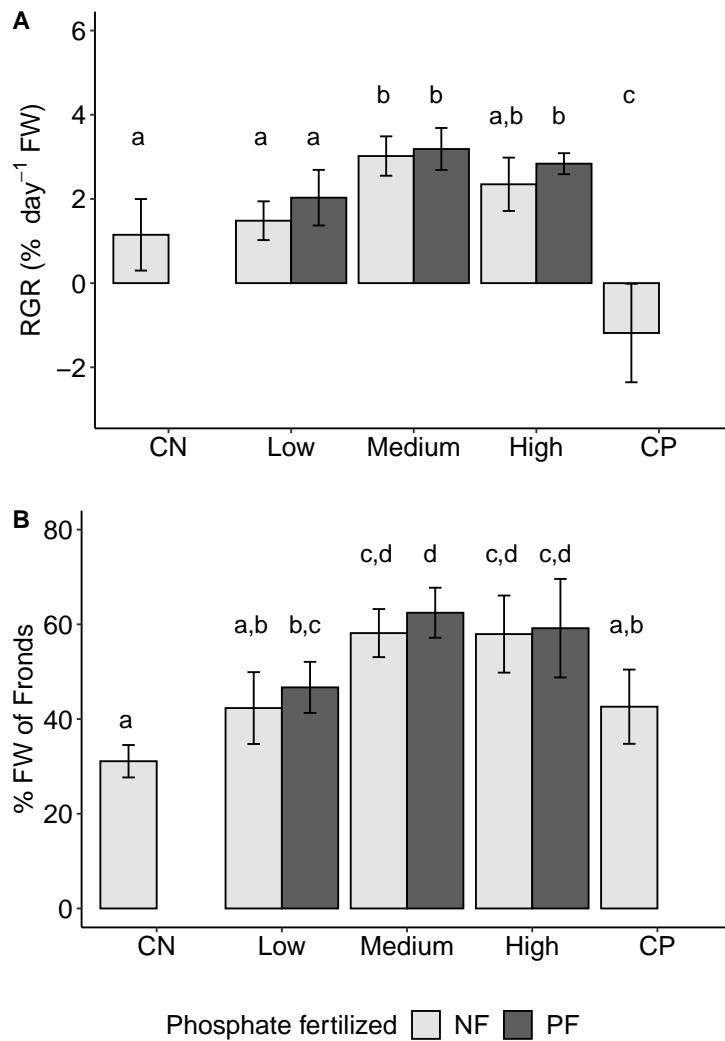
Figure 7.1: **A)** Relative growth rate (RGR, % day^{-1}) of *Caulerpa lentillifera* under artificial sea water (*Control Negative, CN*) and undiluted shrimp process water (*Control Positive, CP*), as well as under three different dilutions of the process water (*Low, Medium, High*) without (NO_3^-:PO_4^{3-} of 28:1, NF) and with (NO_3^-:PO_4^{3-} of 5:1, *PF*) fertilization, respectively. **B)** share of Fronds in regard to the whole sea grape thallus after 14 days exposure to the different treatments expressed as % of fresh weight (FW) of Fronds. Data are expressed as mean±SD (n=3-5). Letters indicate a significant difference between the treatments (One-factor ANOVA with Post-Hoc HSD, p<0.05).

the highest and <46% FW of Fronds for other treatments. Lowest values were observed when sea grapes were cultured in artificial seawater (*CN*: 31.09±3.42% FW of Fronds). A lack of N supply lead to a lower Frond:Stolon ratio in *C. prolifera* [Malta et al., 2005]. It possibly revealed the effort of the seaweed to enter areas with better nutrient supplies by investing in stolon growth, or because the stolons exhibited higher N acquisition rates than fronds [Alexandre and Santos, 2020].
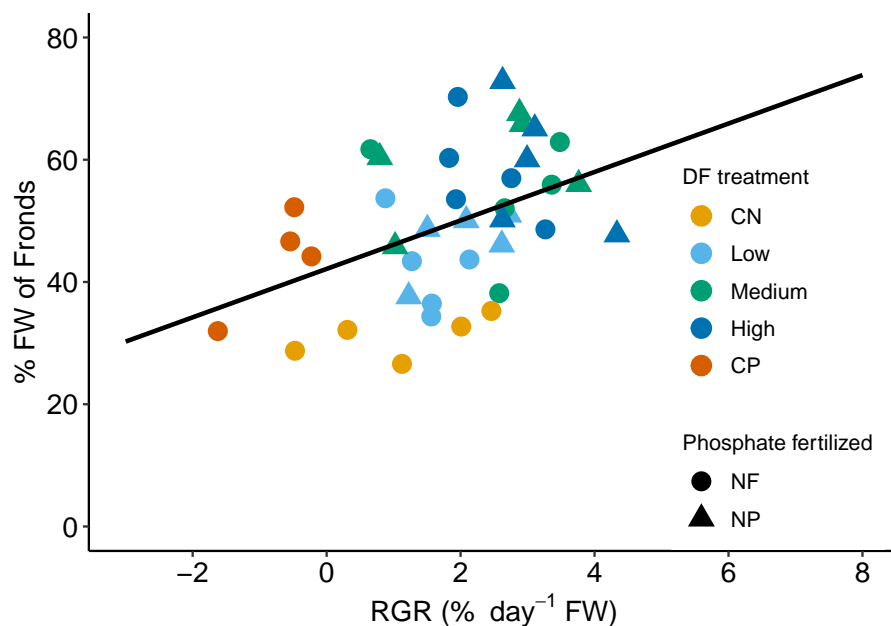
Figure 7.2: Significant correlation (Spearman, r_S=0.4819, p=0.0018) of relative growth rates (RGRs, % day^{-1}) and % fresh weight (FW) of Fronds of *Caulerpa lentillifera* at artificial sea water (*Control Negative*, *CN*) and undiluted shrimp process water (*Control Positive*, *CP*), as well as under three different dilutions of the process water (*Low*, *Medium*, *High*) without (NO$_3^-$:PO$_4^{3-}$ of 28:1, *NF*) and with (NO$_3^-$:PO$_4^{3-}$ of 5:1, *PF*) fertilization, respectively.

Supra-optimal N concentrations can also be toxic, which might explain the overall loss of sea grape biomass at treatment *CP* (-1.18±1.17% day^{-1} FW, Fig.7.1A), similarly reported for *Gracilaria lemaneiformis* [Yu and Yang, 2008]. The authors observed damages in the ultra structure of the chloroplasts, a fading of pigmentation and a drop in important antioxidative enzymes after two weeks of culture at 600 to 37.5 µmol L^{-1} N:P concentrations, suggesting that N:P values above a certain limit that might lead to senescence or death [Yu and Yang, 2008]. The depleted share of Fronds (Fig.7.1B) of *C. lentillifera* at treatment *CP* might therefore be an additional sign that senescence of the sea grapes begun, staring with the more sensitive fronds.

P is after N the second most limiting nutrient [Roleda and Hurd, 2019]. However, other than Guo et al. [Guo et al., 2015b], we did not find a clear effect of P-fertilization on growth. Thus the NO$_3^-$:PO$_4^{3-}$ ratio did not have a significant effect on the distribution of the mean of RGR and % FW of Fronds of sea grapes (p>0.05, Fig.7.1A, B). However, even though the P-fertilization did not have a significant effect, there seemed to be a trend of higher values for

Table 7.2: Maximum quantum yield of photosystem II (F_v/F_m) for *Caulerpa lentillifera* cultivated at artificial sea water (*Control Negative*, *CN*) and undiluted shrimp process water (*Control Positive*, CP), as well as under three different dilutions of the process water (*Low*, *Medium*, *High*) without (nitrate:phosphate of 28:1, *NF*) and with (nitrate:phosphate of 5:1, *PF*) fertilization, respectively. Data expressed as mean±standard deviation (SD) (n=3-5). Letters indicate a significant difference between the treatments (One way Anova with Post-Hoc HSD, p<0.05).

Nutrient	Phosphate fertilized	F_v/F_m
CN	*NF*	0.703 ± 0.015^a
Low	*NF*	0.717 ± 0.023^a
	PF	0.730 ± 0.047^a
Medium	*NF*	0.717 ± 0.023^a
	PF	0.708 ± 0.013^a
High	*NF*	0.707 ± 0.020^a
	PF	0.685 ± 0.045^a
CP	*NF*	0.723 ± 0.032^a

the *PF* treatments, compared to the respective treatment without P-fertilization (Fig.7.1B).

7.3.2 Chlorophyll *a* fluorescence

F_v/F_m values were ≥ 0.7, indicating a good physiological state of the algae [Stuthmann et al., 2020] and were not affected by DF nor by the P-fertilization (p>0.05). Microalgae were reported to maintain constantly high F_v/F_m values when nutrient limited, opposed to nutrient starvation, which triggered depletion of F_v/F_m [Parkhill et al., 2001]. Consistently, this was reported for *C. prolifera* [Malta et al., 2005]. On the other hand, *C. lentillifera* were reported to have constantly high F_v/F_m values ≥ 0.75 over NO_3^- levels of 50–4000 μmol L^{-1} (PO_4^{3-} of 10, 100 μmol L^{-1}), only slightly decreasing at high NO_3^- (≥ 1000 μmol L^{-1}) and PO_4^{3-} (400 μmol L^{-1}) levels [Guo et al., 2015b].

Overall, F_v/F_m measurements seemed to be rather inadequate to quantify the physiological response of sea grapes to changes in N and P concentrations.

7.3.3 Biochemical composition

7.3.3.1 Antioxidant activities and total phenolic content

The range of antioxidant mean values was comparably small with AOA values between 60.64 and 103.58 mmol TE 100 g^{-1} DW and TPCs between 88.43 and 105.79 mg GAE 100 g^{-1} DW, but in the range of some previous studies conducted with the same protocol [Sommer et al., 2022, Stuthmann et al., 2022].

AOA and TPC were significantly affected by the DF treatment (AOA: F(4,30)=5.632, p<0.05; TPC: F(4,31)=8.529, p<0.001), but not by the P-fertilization (p>0.05, Fig.7.3A, B). However, even though significant differences were observed, the small ranges indicated, that AOA and TPC of the seaweeds were similarly unaffected by the different nutrient treatments. The treatment *CN* had significantly lower AOA values, compared to *Medium*, *High PF* and

CP (Fig.7.3A); whereas for TPC the values of *CP* were significantly depleted compared to most other treatments (Fig.7.3B).

Seaweeds have been reported to invest their assimilated C rather in N-free C-based secondary metabolites, like phenols, than e.g. in proteins as a response to a N-limited environment [Ilvessalo and Tuomi, 1989]. On the other hand, nutrient-stress, like N-limitation, lead to an overall lower antioxidant content [Goiris et al., 2015]. From a nutritional perspective, *C. lentillifera* culture at *Medium* and *High* treatments ensured highest antioxidant values, whereas N-depletion could have caused an overall depletion in antioxidants, but with a higher share of polyphenols [Goiris et al., 2015].

7.3.3.2 Carbon and nitrogen tissue content

The tissue C and N content, as well as the C:N ratio were significantly affected by the DF (Tissue C content: $F_{(4,29)}=4.03$, $p<0.01$; Tissue N content: $F_{(4,29)}=73.83$, $p<0.001$; C:N ratio: $F_{(4,29)}=373.01$, $p<0.001$), but not by the P-fertilization ($p>0.05$, Fig.7.4). The C and N contents in the tissue of seaweeds are in general known to be highly influenced by available nutrients in the water [Harrison and Hurd, 2001], which also applied to *C. lentillifera* [Paul and de Nys, 2008, Liu et al., 2016]. Sea grapes cultivated at artificial seawater (*CN*) had significantly lower tissue N contents than all other treatments (Fig.7.4B), resulting in a high C:N ratio of 14.82 ± 0.54 (Fig.7.4C).

All other treatments depicted similar C:N ratios of ~8 (Fig.7.4C). The tissue N content of *C. lentillifera* was ~0.5% of DW higher when cultivated at high (1.4 ± 0.02 mg L^{-1} total N), compared to low N (0.017 ± 0.02 mg L^{-1} total N) conditions [Paul and de Nys, 2008]. Hence, tissue N contents and C:N ratios can be used as an indicator for nutrient uptake by seaweeds and increased C:N ratios indicated a N-limitation [Hanisak, 1990]. Thus, *C. lentillifera* might have been N-limited at treatment *CN*.

However, the pattern in RGR (Fig.7.1A) and the tissue N content (Fig.7.4B) of *C. lentillifera* were not consistent, as tissue N values stagnated at *Medium* and *High* treatments compared to *Low*, but RGR increased. A similar pattern was observed for sea grapes by Liu et al. [Liu et al., 2016] and attributed to the possibility of dilution as a result of the increased growth [Teichberg et al., 2008]. This might also explain the trend of decrease in tissue N content at treatment *Medium* (Fig.7.4B), compared to *Low* and *High*, where RGRs tended to be highest (Fig.7.1A).

7.3.3.3 Amino acid quantity and quality

The AA quantity, namely the THAA content ranged between 49.95 ± 7.76 and 84.0 ± 8.04 mg g^{-1} DW (Fig.7.5A), which was at the lower end of values compared to other studies reporting total AA contents ≥99 mg g^{-1} DW for *C. lentillifera* [Ratana-arporn and Chirapart, 2006, Matanjun et al., 2009, Long et al., 2020]. THAA quantity was affected by the DF ($F_{(4,32)}=25.985$, $p<0.001$). Treatments *Low*, *Medium* and *High NF* showed only a trend of increasing THAA with ascending NO_3^- levels without significant differences (Fig.7.5A), similarly observed for soluble protein content of *C. lentillifera* quantified with the Bradford assay (47, 188, 750 μmol L^{-1}, PO_4^{3-} 29 μmol L^{-1}, 27°C [Cai et al., 2021b]).
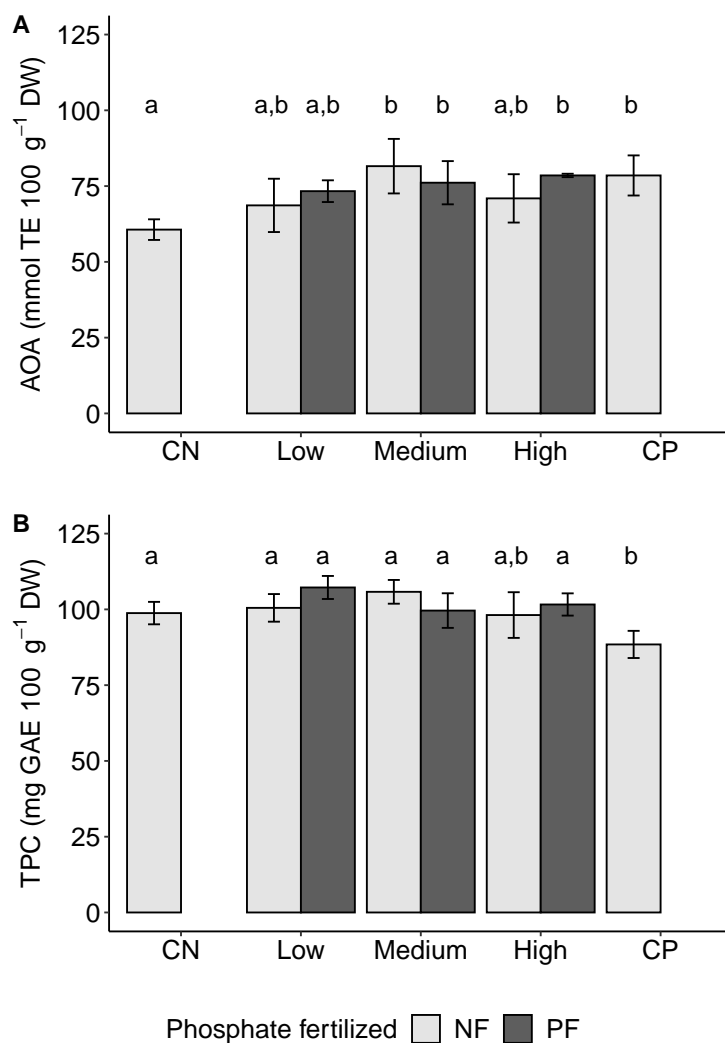
Figure 7.3: **A)** Antioxidant actvity (AOA) expressed as Trolox Equivalents (TE) (mmol 100 g^{-1} dry weight (DW) and **B)** total phenolic content (TPC) expressed as Gallic Acid Equivalents (GAE) (mg 100 g^{-1} DW) of *Caulerpa lentillifera* in artificial sea water (*Control Negative, CN*) and undiluted shrimp process water (*Control Positive, CP*), as well as under three different dilutions of the process water (*Low, Medium, High*) without (nitrate:phosphate ratio of 28:1, *NF*) and with (nitrate:phosphate ratio of 28:1 of 5:1, *PF*) fertilization, respectively. Data are expressed as mean±standard deviation (n=3-5). Letters indicate a significant difference between the treatments (One way ANOVA with Post-Hoc HSD, p<0.05).

Figure 7.4: Tissue (Tis.) **A)** carbon (C) and **B)** nitrogen (N) content (% dry weight, DW and (**C**) C:N ratio of *Caulerpa lentillifera* under artificial sea water (*Control Negative*, *CN*) and undiluted shrimp process water (*Control Positive*, *CP*), as well as under three different dilutions of the process water (*Low, Medium, High*) without (nitrate:phosphate ratio of 28:1) and with (nitrate:phosphate ratio of 5:1) fertilization, respectively. Data are expressed as mean±standard deviation (n=3-5). Letters indicate a significant difference between the treatments (One way ANOVA with Post-Hoc HSD, $p < 0.05$).

Proteins, or rather their building blocks, the AAs, are rich in N and therefore an increase in protein and AAs is expected at increasing N availability. However, the correlation of THAA quantity with the NO_3^- concentration in the experimental treatments was weak (S_rr=0.34, p<0.05, Table 7.4), whereas THAA was moderately positive correlated with the RGR (S_r=0.58, p<0.001, Table 7.4). Hence, the effect of NO_3^- on sea grapes growth rate was so strong, that this indirect effect explained more of the variation in quantity of THAA, than the direct effect on the accumulation of AAs. This pattern has been similarly observed by Angell et al. when studying the effect of salinity on *Ulva ohnoi*'s AA quantity [Angell et al., 2015].

The THAA content of sea grapes at the negative (*CN*: 49.95±7.76 mg g^{-1} DW) and positive control (*CP*: 53.12±3.71 mg g^{-1} DW) were similar and overall on the lower end (Fig.7.5A). The low THAA quantity of treatment *CN* might have been caused by the limited amount of N, whereas the low values of treatment *CP* could have resulted from the absence of growth of sea grapes due to the potential toxicity of high N-loads ([Yu and Yang, 2008], section 7.3.1).

Besides, there was a significant interaction of DF and P-fertilization (F(2,32)=10.169, p<0.001, Fig.7.6A). The interaction was disordinal, implying that the effect of P-fertilization was depending on the level of DF treatment. The effect of P-fertilization on the means of THAA quantity was exclusively significant at treatment *Medium* (*NF*: 65.99±3.68 mg g^{-1} DW, *PF*: 84.0±8.04 mg g^{-1} DW, Fig.7.6A). Hence, treatments *Medium PF* and *High* were significantly increased or showed a trend of higher values, compared to treatments *Low* and *Medium NF* (Fig.7.6A). P is required by seaweeds in average in a ratio of 30N:1P [Atkinson and Smith, 1983], roughly corresponding to the NO_3^-:PO_4^{3-} ratio in *NF* treatments (∼28:1).

However, each alga might have a different N:P optimum ratio [Roleda and Hurd, 2019]. P is essential for various compounds of the seaweed's metabolism, including phospholipids, coenzymes, the energy transfer (e.g. Adenosine Triphosphate (ATP)) and nucleic acids (e.g. Ribonucleic Acid (RNA), Deoxyribonucleic Acid (DNA)) [Hurd et al., 2014], as well as for compounds in the synthesis of AAs [Amir and Hacham, 2015]. Therefore, P could have limited the protein synthesis and hence growth, which might explain the significant difference between *Medium NF* and *PF* THAA quantity (Fig.7.5A) and the trend of different RGRs (Fig.7.1A). Conclusively, sea grapes cultivated at treatments *Medium PF* and *High* had the highest THAA content in their biomass.

The AA quality changed between the experimental treatments (Table 7.3), leading to different ratios of EAA to non-EAA (Fig.7.5B). The EAA non-EAA^{-1} was significantly decreased for biomass of treatments *CN*, *Low NF* and *Medium NF* compared to *CP* (Fig.7.5B). The content of hexosamine GALAM did not differ between sea grapes, whereas the content of GLUAM was significantly increased in *Medium*, *High* and *CP*, compared to treatments with lower N-content (Table 7.3). Asp (10.55 – 12.70% of THAA) and Glu (10.90 – 13.21% of THAA) were the most abundant AAs of *C. lentillifera*, accounting for ∼25% of all AAs (Table 7.3), coherent with previous reports [Ratana-arporn and Chirapart, 2006, Long et al., 2020].

However, contrary to findings from Malaysia, where Phe was, besides Glu, the most abundant AA [Matanjun et al., 2009], Phe accounted in this study for only ∼5% of THAA (Table 7.3). The ionic, free form of Asp and Glu are, among others, responsible for the typical seaweed taste umami [Wong and Cheung, 2000], which is arguably helping to identify protein-rich foods [Lindemann, 2000]. Seaweeds' umami taste can add *deliciousness* to foods and is
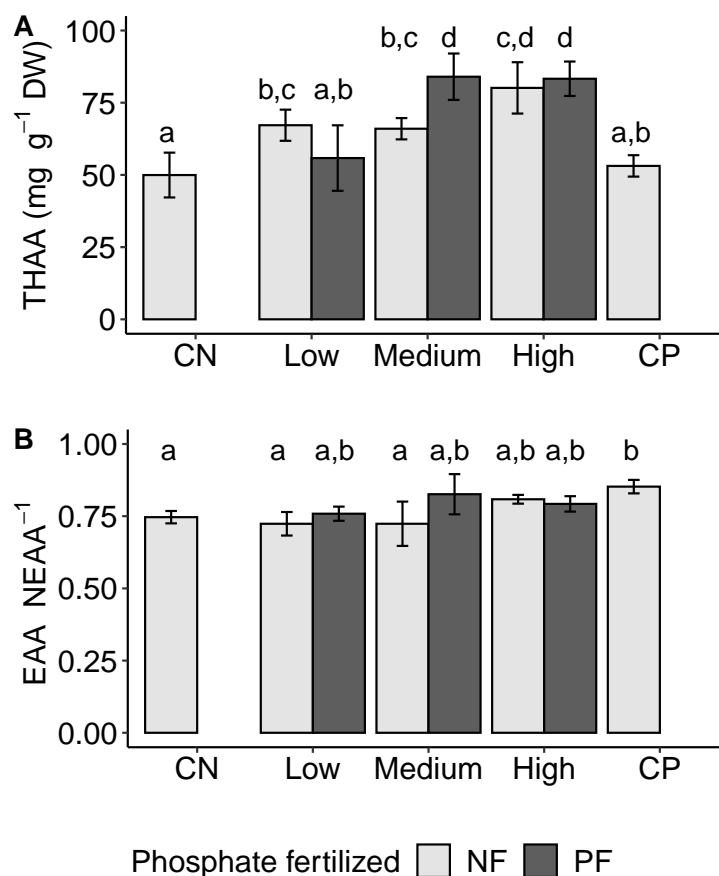
Figure 7.5: **A)** Total hydrolysable amino acids (THAA, mg g^{-1} dry weight, DW) and **B)** ratio of essemtial amino acids (EEAs) to non essential amino acids (non-EAAs) for humans of *Caulerpa lentillifera* in artificial sea water (negative control, *CN*) and undiluted shrimp process water (positive control, *CP*), as well as under three different dilutions of the process water (*Low*, *Medium*, *High*) without (nitrate:phosphate ratio of 28:1, *NF*) and with (nitrate:phosphate ratio of of 5:1, *PF*) fertilization, respectively. Data are expressed as mean±standard deviation (SD) (n=3-5). Letters indicate a significant difference between the treatments (One-factor ANOVA with Post-Hoc HSD, $p<0.05$).

responsible for the use of seaweeds also in the high-end gastronomy, while enabling a reduction of salt content and low lipid levels, compared to other foods with a strong umami taste [Fuke and Shimizu, 1993, Mouritsen et al., 2019, Milinovic et al., 2021].

The most prominent essential AAs were Leu and Lys collectively making up ~13% of THAA, whereas Met was the rarest (~0.25% of THAA, Table 7.3). Lys is of high nutritional importance for humans, as well as fish and it is likely the limiting AA in wheat and plant based fish diets [FAO et al., 2007, Li et al., 2009]. In general seaweeds tend to have lower levels of Lys, Thr, Tyr, Cys-OX and Met [Terriente-palacios and Castellari, 2022]. In this study, Lys exhibited similar means of 5.66–6.42% of THAA among all treatments (Fig.7.6F). It had been on the contrary reported as the first limiting AA in *C. lentillifera* with 1.22±0.04 mg g^{-1} DW [Matanjun et al., 2009]. As a consequence of the consistent relative Lys content, the absolute quantity increased with the THAA content (Table 7.3, Fig.7.5A). Hence Lys quality was, as only AA, neither correlated to the NO_3^- concentration of the experimental treatments, nor to the RGRs (Table 7.4).
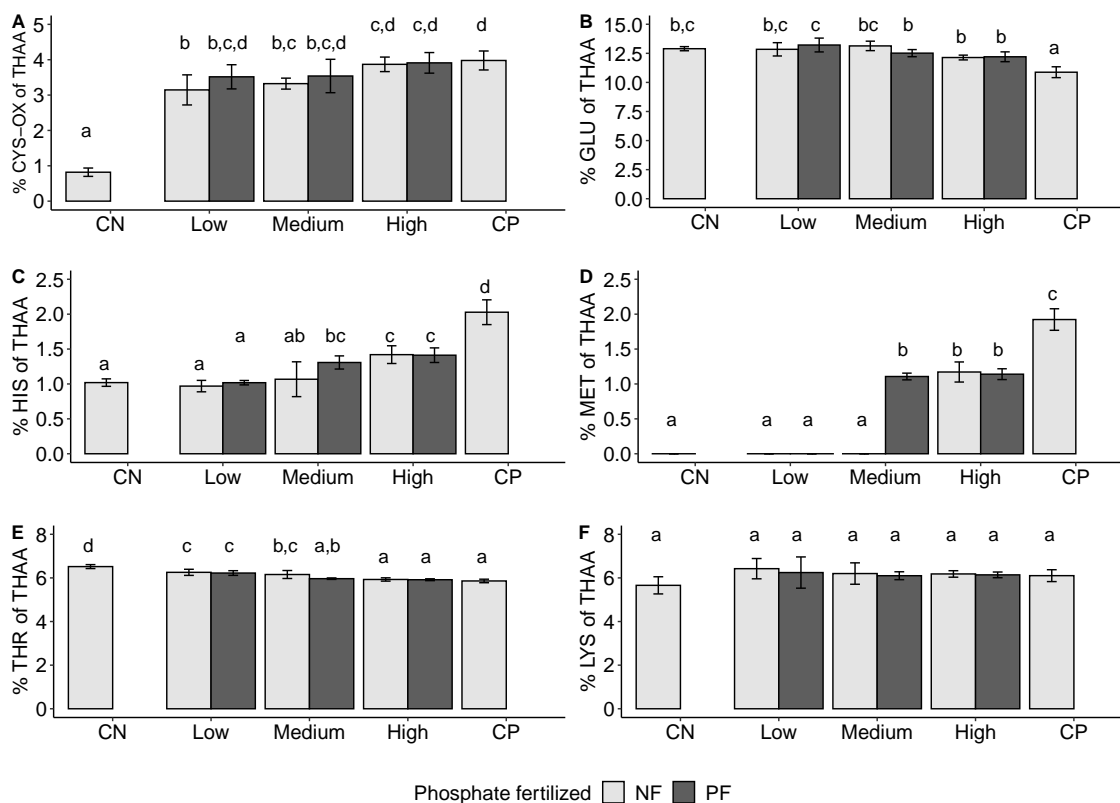
Figure 7.6: Presence of amino acids (AAs, expressed as % of total hydrolysable AAs, THAA) which are for fish and humans semi-essential (Cysteine - CYS-OX, Glutamate - GLU) and essential (Histidine – HIS, Methionine – MET, Thyrosine – THR, Lysine - LYS) in *Caulerpa lentillifera* in artificial sea water (*Control Negative*, *CN*) and undiluted shrimp process water (*Control Positive*, *CP*), as well as under three different dilutions of the process water (*Low*, *Medium*, *High*) without (nitrate:phosphate of 28:1) and with (nitrate:phosphate of 5:1) fertilization, respectively. Data are expressed as mean±standard deviation (n=3-5). Letters indicate a significant difference between the treatments (One way ANOVA with Post-Hoc HSD, p<0.05).
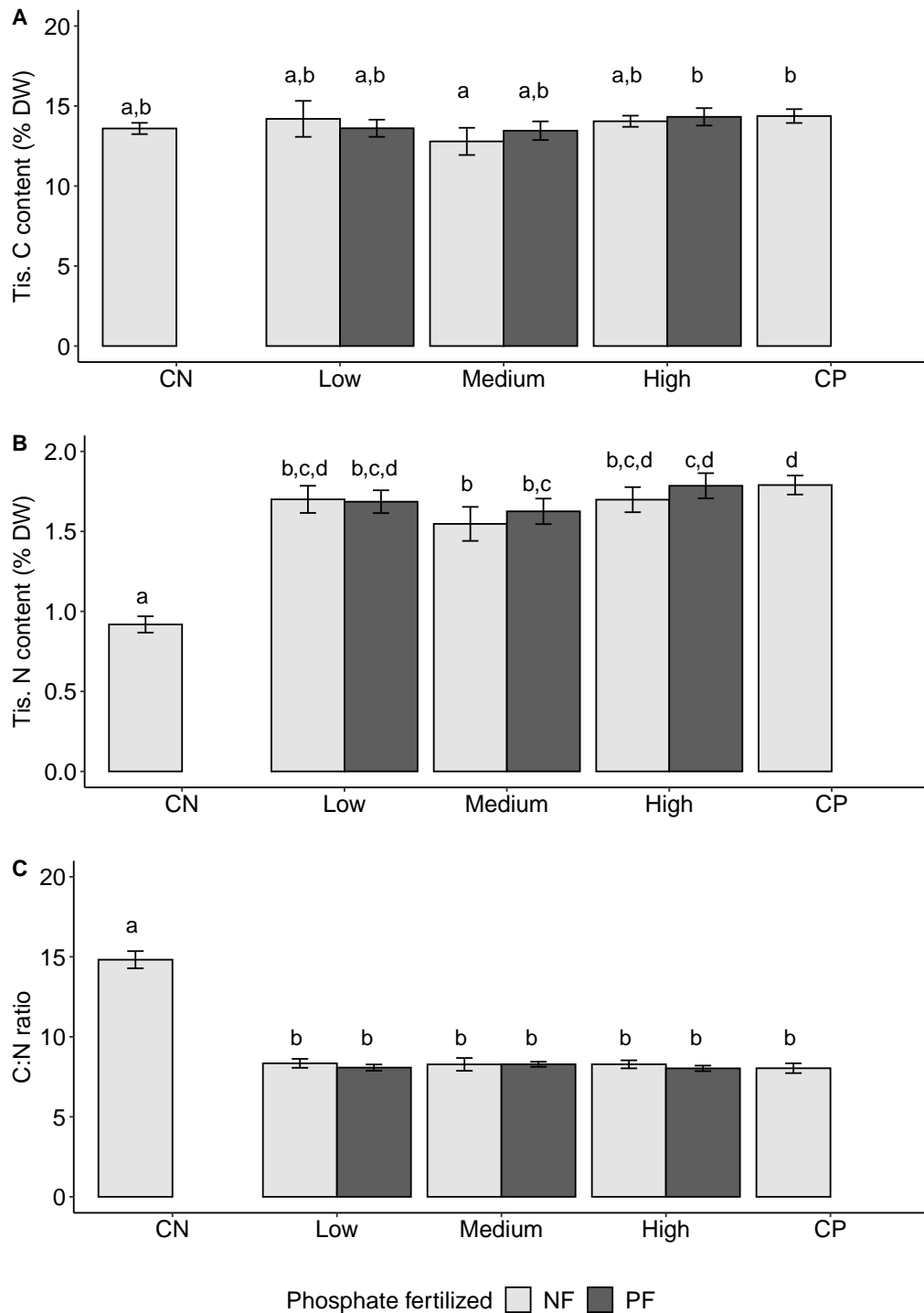
Table 7.3: Amino acids (AA) as mg g^{-1} dry weight (DW) in *Caulerpa lentillifera* in artificial sea water (*Control Negative*, *CN*) and undiluted shrimp process water (*Control Positive*, *CP*), as well as under three different dilutions of the process water (*Low*, *Medium*, *High*) without (nitrate: phosphate of 28:1) and with (nitrate: phosphate of 5:1) fertilization, respectively. Data are expressed as mean±standard deviation (SD) (n=3-5). Letters indicate a significant difference between the treatments (One way Anova with Post-Hoc HSD, p<0.05). Asterix * denote essential and (*) semi-essential AAs for humans and + essential and (+) semi-essential AA or fish [Li et al., 2009].

AA	CN	Low		Medium		High		CP
		NF	PF	NF	PF	NF	PF	
		mg g^{-1} dry weight						
ALA	3.49 ± 0.53^{a,b}	4.49 ± 0.42^{b,c}	3.71 ± 0.73^{a,b}	4.43 ± 0.29^{b,c}	5.43 ± 0.6^{c}	5.13 ± 0.61^{c}	5.44 ± 0.07^{c}	3.13 ± 0.25^{a}
ARG(*) +	3 ± 0.51^{a}	3.99 ± 0.42^{b,c}	3.3 ± 0.69^{a,b}	3.92 ± 0.26^{b,c}	5.03 ± 0.39^{d}	4.83 ± 0.58^{c,d}	5.26 ± 0.13^{d}	3.2 ± 0.21^{a,b}
ASP	6.34 ± 1^{a,b}	8.27 ± 0.79^{c,d}	6.86 ± 1.38^{a,b,c}	8.11 ± 0.56^{b,c,d}	9.85 ± 1.06^{d}	9.27 ± 1.1^{d}	10 ± 0.1^{d}	5.61 ± 0.44^{a}
b-ALA	0.12 ± 0.02^{a}	0.16 ± 0.01^{a}	0.13 ± 0.03^{a}	0.15 ± 0.02^{a}	0.44 ± 0.03^{b}	0.45 ± 0.04^{b}	0.47 ± 0.01^{b}	0.53 ± 0.06^{c}
CYS-OX(*)(+)	0.41 ± 0.06^{a}	2.12 ± 0.4^{b}	2.1 ± 0.1^{b}	2.19 ± 0.16^{b}	2.97 ± 0.44^{c}	3.09 ± 0.24^{c}	3.25 ± 0.26^{c}	2.11 ± 0.2^{b}
g-ABA	0.15 ± 0.03^{a}	0.28 ± 0.08^{a,b}	0.34 ± 0.04^{b}	0.4 ± 0.08^{b}	0.66 ± 0.11^{c}	0.66 ± 0.08^{c}	0.79 ± 0.14^{c}	0.63 ± 0.04^{c}
GALAM	0.22 ± 0.13^{a}	0.13 ± 0.18 ^{a}	0.16 ± 0.15 ^{a}	0.21 ± 0.31 ^{a}	0.69 ± 0.39 ^{a}	0.34 ± 0.47 ^{a}	0.73 ± 0.41 ^{a}	0.43 ± 0.58 ^{a}
GLU(*)(+)	6.44 ± 0.98^{a}	8.64 ± 0.84^{b,c}	7.33 ± 1.22^{a,b}	8.66 ± 0.43^{b,c,d}	10.51 ± 1.02^{d}	9.73 ± 1.16^{c,d}	10.16 ± 0.8^{d}	5.78 ± 0.5^{a}
GLUAM	0.48 ± 0.1^{a}	0.54 ± 0.06^{a}	0.45 ± 0.09^{a}	0.6 ± 0.18^{b}	1.11 ± 0.07^{b}	1.1 ± 0.1^{b}	1.16 ± 0.08^{b}	1.18 ± 0.12^{b}
GLY(*)	5.4 ± 1.17^{a,b}	7.19 ± 0.75^{b,c,d}	5.68 ± 1.37^{a,b,c}	6.51 ± 0.93^{a,b,c,d}	7.67 ± 0.18^{c,d}	7.23 ± 1.14^{b,c,d}	7.99 ± 1.06^{d}	4.74 ± 0.61^{a}
HIS*+	0.51 ± 0.07^{a}	0.65 ± 0.05^{a}	0.61 ± 0.07^{a}	0.6 ± 0.26^{a}	1.1 ± 0.09^{b}	1.13 ± 0.08^{b}	1.17 ± 0.05^{b}	1.07 ± 0.09^{b}
ILE*+	1.86 ± 0.28^{a}	2.51 ± 0.19^{b}	2.12 ± 0.45^{a,b}	2.57 ± 0.16^{b}	3.32 ± 0.27^{c}	3.19 ± 0.37^{c}	3.29 ± 0.21^{c}	2.14 ± 0.1^{a,b}
LEU*+	3.64 ± 0.58^{a}	4.85 ± 0.42^{b,c}	4.01 ± 0.79^{a,b}	4.82 ± 0.26^{b,c}	5.87 ± 0.62^{c}	5.65 ± 0.63^{c}	5.77 ± 0.42^{c}	3.4 ± 0.22^{a}
LYS*+	2.82 ± 0.42^{a}	4.31 ± 0.4^{c,d,e}	3.47 ± 0.76^{a,b,c}	4.08 ± 0.29^{b,c,d}	5.13 ± 0.57e	4.95 ± 0.5d,e	5.11 ± 0.33e	3.24 ± 0.29^{a,b}
MET*+	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.93 ± 0.07^{b}	0.93 ± 0.08^{b,c}	0.95 ± 0.03^{b,c}	1.02 ± 0.05^{c}
MET-Sulfon	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0.77 ± 0.05^{b}	0.78 ± 0.08^{b}	0.8 ± 0.01^{b}	0 ± 0^{a}
ORN	1.27 ± 0.21^{a,b}	1.84 ± 0.12^{b}	1.45 ± 0.47^{b}	1.3 ± 0.61^{a,b}	0.67 ± 0.07^{b}	0.67 ± 0.07^{a}	0.69 ± 0.03^{a}	1.45 ± 0.47^{b}
PHE*+	2.75 ± 0.42^{a}	3.66 ± 0.31^{b}	3.02 ± 0.6^{a,b}	3.66 ± 0.22^{b}	4.72 ± 0.48^{c}	4.57 ± 0.5^{c}	4.69 ± 0.31^{c}	2.96 ± 0.15^{a,b}
SER	3.25 ± 0.51^{a}	4.25 ± 0.4^{b,c,d}	3.48 ± 0.71^{a,b}	4 ± 0.06^{a,b,c}	5.18 ± 0.54d,e	4.94 ± 0.57^{c,d,e}	5.31 ± 0.08e	3.12 ± 0.24^{a}
TAU(+)	0.24 ± 0.09^{a}	0.4 ± 0.07^{a,b}	0.4 ± 0.12^{a,b}	0.45 ± 0.12^{b}	0.79 ± 0.14^{c}	0.75 ± 0.09^{c}	0.8 ± 0.07^{c}	0.75 ± 0.07^{c}
THR*+	3.25 ± 0.47^{a}	4.2 ± 0.35^{b,c,d}	3.47 ± 0.69^{a,b}	3.97 ± 0.08^{a,b,c}	5.01 ± 0.5^{d}	4.75 ± 0.58^{c,d}	4.93 ± 0.34^{c,d}	3.11 ± 0.22^{a}
TYR(*)	2.28 ± 0.28^{a,b}	2.5 ± 0.34^{a,b}	1.58 ± 0.6^{a}	2.16 ± 0.33^{a,b}	3.03 ± 0.66^{b}	3.11 ± 0.46^{b}	2.81 ± 0.66^{b}	2.39 ± 0.1^{a,b}
VAL*+	2.73 ± 0.38^{a}	3.66 ± 0.29^{b,c}	3.01 ± 0.6^{a,b}	3.36 ± 0.08^{a,b}	4.52 ± 0.35^{d}	4.3 ± 0.54^{c,d}	4.47 ± 0.3^{d}	2.72 ± 0.17^{a}

The quality of all other individual AAs, besides Orn, Tyr and Lys, presented as % AA of THAA, was significantly correlated with the NO_3^- concentration in the treatments (Table 7.4, Fig.7.6). On the other hand, there was no significant correlation for all AAs, besides Met-sulfone, Orn and Tyr with the RGR of the seaweed (Table 7.4). This is coherent with the finding of Angell et al., where AA quality of *U. ohnoi* was strongly effected by growth, whereas the AA quality was rather depending on the different experimental salinity levels [Angell et al., 2015]. Cys-OX, His and Met showed a trend of increase with ascending NO_3^- contents, whereas Glu and Thr rather decreased (Fig.7.6).

Met was not detectable for treatments *CN*, *Low* and *Medium NF*, whereas values >1% of THAA were found for the other treatments (Fig.7.6D). Interestingly, this pattern was similar to the THAA quantity (Fig.7.5A). Met is the start codon for protein synthesis and its absence in treatments *CN*, *Low*, *Medium NF* suggests that this AA may be limiting for protein synthesis [Angell et al., 2014, Cole et al., 2015]. During the metabolism of Met from Asp, P is required for several predecessors of Met, like aspartyl phosphate or O–phosphohomoserine [Amir and Hacham, 2015], therefore Met synthesis as a start codon for protein synthesis could have been P-limited in *Medium NF* (Fig.7.5A, Fig.7.6D). Hence, Met was reported as the first limiting AA for *C. lentillifera* in China [Long et al., 2020]. The relatively higher presence of Met in treatment *CP* compared to *Medium PF* and *High*, could be explained with the absence of Met-sulfone, the oxidized form of Met (Table 7.3). Met-sulfone can be enzymatically converted back to Met with involvement of enzymes of the methionine sulfoxide reductase family, which genes have been reported to be upregulated as a response to (oxidative) stress [Hsu and Lee, 2012]. Met is nutritionally indispensable for humans [FAO et al., 2007], as well as for fish [Li et al., 2009], with several metabolic functions.

Unlike for Met, the P-fertilization did not have an effect on the contribution of the other AAs to THAA (Fig.7.6). Angell et al. observed *U. ohnois* acquisition and assimilation in free AA of N above the critical point, at which growth is limited, so called *luxury uptake* ([Angell et al., 2014], whereas *C. lentillifera* has been reported to be incapable of *luxury uptake* [Paul and de Nys, 2008].

7.3.4   Implications and considerations for sea grape aquaculture

The fertilization of sea grapes with process water of a RAS cultivation with the whiteleg shrimp *L. vannamei* did affect the physiology, growth and biochemical composition of the economically important seaweed *C. lentillifera*. The cultivation at treatments *Medium* and *High* lead to highest RGRs and yields of fronds (section 7.3.1). The content of antioxidants, including phenolic compounds (section 7.3.3.1) seemed continuously high, if conditions of *CN* and *CP* were avoided (section 7.3.3.2).

It should be considered, that even though contents of certain target compounds change (e.g. per g DW^{-1}), the yield per cultivation unit is economically most important, which highly depended on the RGRs. However, it was reported that the FW:DW ratio of *Caulerpa* might change at different cultivation conditions [Paul and de Nys, 2008]. The THAA content was also highly correlated with the RGR. At treatments *Medium*, P-fertilization lead to a significant increase in THAA yield, as well as Met content (section 7.3.3.3). Met and Lys are

Table 7.4: Spearman correlation of total hydrolysable amino acid (THAA) expressed as mg g^{-1} dry weight (DW) and *% of respective amino acids (AAs)* of THAA of *Caulerpa lentillifera* with the nitrogen content (µmol L^{-1}) in the different experimental treatments and with the relative growth rate (RGR, % day^{-1}), p<0.05 *; p <0.01**; p<0.001***

Factor	Nitrogen content (µmol L^{-1}) r	t	p-value		RGR (% day^{-1}) r	t	p-value	
THAA	0.5810542	2742	0.0004012	<0.001***	0.3354491	7084	0.03434	<0.05*
ALA	-0.8579837	18356.879	2.97E-12	<0.001***	0.11627196	5784	0.5110913	-
ARG	0.34497368	5986.28552	0.03391157	<0.05*	-0.1968583	7162	0.27101017	-
ASP	-0.8637977	18414.3216	1.44E-12	<0.001***	0.05454545	6188	0.75867863	-
b-ALA	0.81001801	1877.02202	4.21E-10	<0.001***	-0.1888369	7114	0.29132218	-
CYS-OX	0.76412876	2330.40785	1.51E-08	<0.001***	0.08923797	5450	0.62018773	-
g-ABA	0.87255275	1358.58772	2.20E-13	<0.001***	-0.010848	6616	0.95185616	-
GLU	-0.7546402	18704.4647	1.86E-08	<0.001***	0.21802903	5118	0.21460884	-
GLY	-0.6775413	16574.1085	2.16E-06	<0.001***	-0.1657754	6976	0.35506685	-
HIS	0.83029275	1550.95452	1.14E-10	<0.001***	-0.1392962	6216	0.44546444	-
ILE	0.70266244	3169.61835	4.32E-07	<0.001***	-0.1016043	7210	0.56610947	-
LEU	-0.6853365	17965.6873	1.07E-06	<0.001***	0.2565317	4866	0.14278128	-
LYS	0.03898333	10244.4377	0.81124149	-	0.17127578	5424	0.33139817	-
MET	0.87999211	1185.67797	1.60E-13	<.001***	-0.1132461	6662	0.5303389	-
MET-Sulfon	0.45584711	4973.01323	0.00402535	<0.05*	0.56508613	2372	0.00075227	<0.001***
ORN	-0.2767335	13609.9791	0.08387053	-	-0.5541635	10172	0.00081768	<0.001***
PHE	0.46491227	5704.03515	0.0025068	<0.05*	0.17158136	5422	0.33052582	-
SER	-0.7910275	16368.1999	3.42E-09	<0.001***	-0.0499618	6872	0.77847833	-
TAU	0.83741962	1733.1068	1.63E-11	<0.001***	-0.0655462	6974	0.71179968	-
THR	-0.8738684	18513.8203	3.81E-13	<0.001***	-0.1132162	7286	0.52232698	-
TYR	0.07459773	9864.78825	0.6473296	-	-0.4933537	9774	0.0033806	<0.001***
VAL	-0.4285766	14114.3365	0.00648642	<0.05*	-0.0994652	7196	0.57435603	-

essential and often limiting AAs for humans [FAO et al., 2007] and fish [Li et al., 2009] and therefore their presence is desirable in the algae, when used as human food or animal feed. The relative content of Lys is similar throughout all treatments (section 7.3.3.3) and therefore higher RGRs, as well as THAA contents would increase its yield, similar e.g. for other essential AAs like Leu, Phe and Val.

Additionally, even though some essential AAs, like Glu decrease in their relative contribution to THAA, the absolute content in biomass of *C. lentillifera* cultivated at treatments *Medium PF* and *High* is still significantly increased e.g. compared to *Low*. Hence, process water of *L. vannamei* from a highly technologized RAS could be used to fertilize *C. lentillifera* with target concentrations of *Medium* or *High*, whereas a P-fertilization of DF *Medium* should be considered. The dilutions need to regularly be re-calculated based on the nutrients in the process water. Therefore, tools for nutrient analysis should be available at the farm. In case only rather inaccurate tests (e.g. droplet tests) are available, the DF should be a bit overestimated to avoid nutrient limitations. Considering that the manual P-fertilization requires additional time, it could be beneficial that treatment *High* is used for sea grape cultivation, where P-fertilization seems unnecessary. On the other hand, this might decrease the salinity further and necessitate manual salting. The individual (dis-)advantages should be weighted for each set-up. Contents of the AAs Lys and Met are not only beneficial for the human nutrition, but suggest e.g. the use of sea grape stolons or non-food grade biomass for the production of fish [Arisa et al., 2020] or shrimp feed [Putra et al., 2019]. Even the direct feeding of *L. vannamei* on sea grapes could be considered, as this species was reported to feed on fresh *C. lentillifera* [Anh et al., 2021].

The thallus morphology depended on the nutrient availability, but potentially also on the presence of bottom sediment (section 7.3.1). Additionally, the presence of bottom sediment has been reported to alter the AA quality of sea grapes [Long et al., 2020]. In this study, the seaweeds were cultivated without bottom sediment, free floating in the experimental unit. Further studies could investigate the effect of different cultivation techniques, namely sowing method or tray cultivation [Rabia, 2016] for sea grapes indoors.

Indoor RASs for shrimp production are becoming more popular in Europe, therefore the resource-saving cultivation with macroalgae might have potential. However, many seaweeds, including *C. lentillifera* need to be accepted as food according to the European Union (EU) *Novel Food Regulation* [Barbier et al., 2019, Lähteenmäki-Uutela et al., 2021], before their potential as healthy and sustainable food product can be used in the EU.

7.4   Conclusions

Economically interesting and edible seaweed *C. lentillifera* could be fertilized with process water of *L. vannamei* at NO_3^- concentrations of 144 μmol L^{-1} (*Medium*) or 720 μmol L^{-1} (*High*, NO_3^-:PO_4^{3-} of 28:1 or 5:1), whereas treatment *Medium* requires additional P-fertilization (NO_3^-:PO_4^{3-}, 5:1) to ensure higher content of THAA and essential AA Met. The cultivation of sea grapes in the same facility with shrimp could be resource-efficient and the product could be used for the human nutrition, or as feed for aquatic animals. However, hurdles, including the acceptance of sea grapes as food by the *EU Novel Food Regulation* need to be overcome, before it can be implemented in Europe.

Chapter 8

Synoptic Discussion
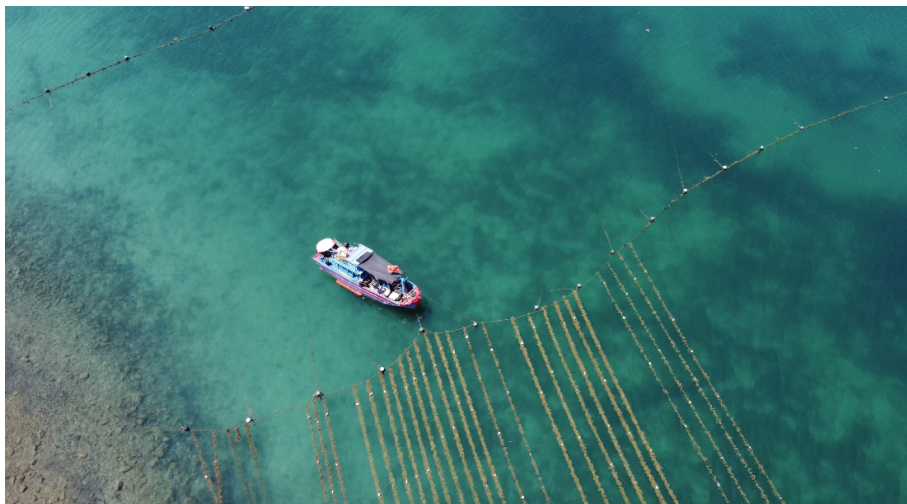
Kappaphycus farm in Van Phong Bay, Viet Nam (Foto: A. Cordes).

8.1   Research along the production cycle

In the framework of the present thesis, we aimed to investigate the ecophysiology and bio-
chemical composition of the sea vegetable *Caulerpa lentillifera* along its production cycle at
the farm VIJA in Van Phong Bay, Khánh Hòa, Viet Nam and to report on the procedures of
the production. In a second step, we strived to identify approaches with the potential to im-
prove the quality and quantity of the sea grape harvest, as well as the resource-efficiency of
its production. The structured literature review presented in **chapter 2** provides an overview
of study topics and applications, as well as a detailed summary of the state of the art on sea
grape aquaculture and apparent research gaps.

Some of these research gaps were aimed to be filled in the framework of this thesis with
laboratory and field experiments, as well as an *in-situ* field study. During the experiments a
multitude of complementary physico-chemical (e.g. salinity, temperature, light irradiances),
ecophysiological (e.g. Chlorophyll (Chl) *a* fluorescence), biochemical (e.g. Antioxidant Ac-
tivity (AOA) assay, Amino Acid (AA) content) and computer-based (colour estimations) mea-
surements and methods were applied to answer the raised research questions. The results
contribute to an understanding of three main aspects of sea grape production 8.1.

Chapter 3 provides a, to our knowledge, first report on the production cycle at a sea
grape farm in Van Phong Bay along with environmental parameters from the cultivation
pond. **Chapters 3**, **4**, **5** & **6** highlight the importance of light management and propose suit-
able irradiance levels (**publication IV**) to avoid photooxidative stress during the cultivation
of sea grapes (**publication II**, **III** & **VI**), as well as shelf-life (**publication III**). **Chapters
3**, **5** & **7** suggest the potential of targeted cultivation parameter manipulations to achieve
an accumulation of value-adding compounds, like antioxidants (**publications IV** & **V**) and
AAs (**publications VII**). In order to maintain the quality of sea grapes during this process,
a first approach in identifying important quality features from the farmers perspective and
to understand their dependence on (seasonal) environmental parameters was taken in **pub-
lication II**.

Chapters 6 & **7** propose a resource-efficient cultivation of sea grapes with the economi-
cally important carragenophyte *K. alvarezii* in a two-layer co-cultivation (**publication VI**)
and whiteleg shrimp *Litopenaeus vannamei* in a land-based high technology Recirculation
Aquaculture System (RAS) (**publication VII**). The findings can contribute to improve sea
grape production procedures in Van Phong Bay, Viet Nam and beyond.

However, besides the high potential that the applications showed in this study, they should
be experimentally scaled up before their implementation in the sea grapes' production cycle
and their socio-economic impact for the involved stakeholders should be investigated.

8.2   The importance of light management

Sea grapes are already established in the literature as a shade-adapted species ([Raniello
et al., 2004, Raniello et al., 2006, Guo et al., 2015a, Kang et al., 2020], **publication I**)
and this study confirmed their photoadaptation to a rather low light environment during
cultivation and shelf-life in experimental set-ups (**publication III** & **V**) and in the field
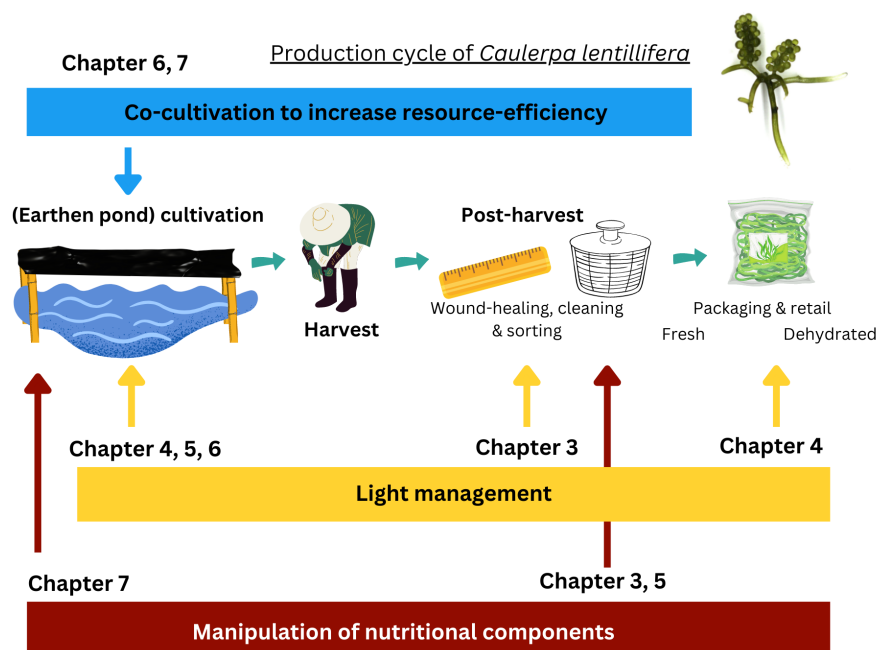
Figure 8.1: Illustration of the sea grape (*Caulerpa lentillifera*) production cycle in Van Phong Bay, Khánh Hòa, Viet Nam with different overarching aspects of the chapters discussed in the synoptic discussion.

(**publication VI**). The physiological response of seaweeds to photon fluxes exceeding their need for photosynthesis consists of different reactions and mechanisms including damage, repair, avoidance, quenching and scavenging along a continuum of adjustments [Demmig-Adams and Adams, 1992, Adams III et al., 2006, Raven, 2011].

In the frame of this thesis different photoprotective response mechanisms of sea grapes have been quantified, including photoinhibition (**publication III**, **V & VI**), acclimation and photoprotective chloroplast relocation (**publication V**), as well as antioxidant production (**publication V & VI**). Cultivated sea grapes exhibited during experiments decreased F_v/F_m values at irradiances $\geq$100 µmol photons m^{-2} s^{-1} (photoperiod 12:12 light:dark) (**publication III & V**). Dynamic photoinhibition is a protective reaction to the energy oversaturation of the Photosystem II (PSII) to dissipate energy as heat [Osmond, 1994, Häder et al., 1997, Hanelt et al., 1997]. The full recovery of sea grapes' F_v/F_m values at control irradiances after high light exposure over $\leq$2 weeks (**publication III**), as well as the typical midday depression of F_v'/F_m' at high irradiances at noon in the field (**publication VI**) suggest the absence of permanent damage of the photosystem and underline the ability of sea grapes to cope with a certain degree of high light induced stress [Demmig-Adams and Adams, 1992, Adams III et al., 2006]. This has been confirmed recently [Terada et al., 2021]. In the control treatments, where the organisms were supposedly in a state of homeostasis [Borowitzka, 2018], *C. lentillifera* exhibited mean F_v/F_m values of $\sim$0.65 to 0.74 (**publication III**, **V**, **VI**, **VII**). The values are comparable to other reports on *C. lentillifera* [Terada et al., 2018, Terada et al., 2021] and below the maximum value of 0.83, as expected for Chlorophyta [Büchel and

Wilhelm, 1993]. Hence, these values could function as a basis for future work with this species.

Additionally, adjustments in the Rapid Light Curve (RLC) parameters at ascending light irradiances (**publication V**) can be a sign of an acclimation strategy [Raniello et al., 2004, Raniello et al., 2006]. The bleaching of fronds cultivated at 100 µmol photons m^{-2} s^{-1} (**publication III**) could be caused by targeted chloroplast relocation, as a protective mechanism or due to their degradation (**publication V**). The formation of Reactive Oxygen Species (ROS) as a consequence of energy or electron transmission usually leads to the production of enzymatic and non-enzymatic antioxidants, causing increases in the AOA and the Total Phenolic Content (TPC) (**publication III**, **V & VI**). However, other environmental parameters have major physiological effects on *C. lentillifera* as well. Freshwater influxes, e.g. in the form of precipitation or through a river, leading to decreased salinities, are a decisive exclusion criteria for the cultivation location of sea grapes [Trono and Toma, 1993]. Besides, temperature and salinity restrict the growth season of sea grapes in Van Phong Bay (**publication II**). Therefore, the question remains: What causes the special role of light?

The importance of light as a parameter during the production of sea grapes is highlighted, because other than parameters like temperature or salinity, the light irradiances can be discretely managed in the out-door cultivation without high financial or technological expenses. Light is basically an infinite resource in the tropics, but the irradiance levels can be decreased fairly easy by shading of the ponds or the post-harvest facilities (*hypothesis I*). Photoprotective responses of seaweeds require energy and hence could impair mechanisms needed for optimal growth [Raven, 2011]. Therefore, farmers in Van Phong Bay acknowledge the considerably low saturation irradiances of the species by artificial gauze shading of the ponds. The shading is leading to decreases in the average light irradiances of Photosynthetically Active Radiation (PAR) by 15–50%, compared to the exposed pond environment (**publication V**) and with average values of ∼50–70 µmol photons m^{-2} s^{-1} (**publication III & V**). According to the results of this study, the shaded ponds at farm VIJA provide, on average, a suitable light environment for *C. lentillifera* and thereby avoid photooxidative stress of the seaweed (**publication III & V**). This observation is supported by measurements of sea grape fronds at the farm facilities exhibiting F_v/F_m values >0.7, indicating no signs of photoinhibition (**publication II**). Hence, even though sea grapes are exposed to high daily irradiance (**publication V**), most likely requiring short-term photoprotective responses reflected in decreasing F_v/F_m' at noon (**publication VI**), the shading seems to avoid photodamage (**publication II**, *hypothesis I.A*). The artificial gauze cover of the sea grapes was aimed to be replaced by natural shading of the red seaweed *K. alvarezii*, but the provided shade was not enough to avoid sea grapes photooxidative stress, e.g. displayed by significantly increased AOA and TPC values (**publication VI**, *hypothesis I.C*).

In general, exposure or acclimation to other stressors, like temperature [Terada et al., 2021], impairs the photoprotective mechanisms and lowers the threshold for stress responses [Demmig-Adams and Adams, 1992]. During shelf-life in the packaging environment, photosynthetically active sea grape fronds are usually exposed to desiccation conditions. These are leading to water loss and hence decreasing photosynthetic activity and oxidative stress,

which can be quantified by an increased production of various stress markers, including Malondialdehyde (MDA) (lipid peroxidation), proline, Superoxide Dismutase (SOD), Superoxide Anion (O_2^-) and Hydrogen Peroxide (H_2O_2) over the course of dehydration [Terada et al., 2018, Liang et al., 2021, Sulaimana et al., 2021]. For some seaweeds moderate desiccation is followed by an increase in photosynthesis, due to the larger substrate affinity to Carbon Dioxide (CO_2) in air compared to water (reviewed by [Davison and Pearson, 1996]). However, *C. lentillifera* showed a decrease in F_v/F_m values, Chl content and production of stress biomarkers already in the short term [Terada et al., 2018, Liang et al., 2021, Terada et al., 2021]. Therefore, the threshold of irradiances causing photoinhibition was decreased in this species and the light should be kept at room irradiances during storage in transparent packaging, while complete darkness should be avoided (**publication III**, *hypothesis I.B*).

Hypothesis I stated that "The light management is an essential tool to consider over the *Caulerpa lentillifera* production cycle."

Considering the results of **publications II**, **III**, **IV & VI** this hypothesis can be confirmed. The special role of light during the production cycle of sea grapes was demonstrated in the frame of this thesis and it enforced the need to carefully manage the light environment.

However, the results also showed that sea grapes are exposed to light fluctuations on various temporal scales, from seasonal effects, over daily fluctuations up to different microhabitats in the pond, caused by short-term shading by other seaweeds or movements of the gauze. The effect of light fluctuations on seaweeds is still considerably unclear [Comerford et al., 2021] and therefore further research is suggested in this area. Besides, the physiological reaction at light irradiances exceeding the need for photosynthesis of sea grapes has also led us to formulate the hypothesis that light stress could be used to manipulate the antioxidant production of the algae.

8.3 The potential of targeted manipulations

Manipulations of the abiotic cultivation environment of algae in order to achieve the accumulation of specific target compounds, e.g. for a higher nutritional value, is common practice for microalgae [Liyanaarachchi et al., 2021]. For seaweeds, which are often cultivated in outdoor set-ups, this practice is rather rare, but holds a huge potential [Godínez-Ortega et al., 2008, Angell et al., 2014, Angell et al., 2015, Magnusson et al., 2015, Toth et al., 2020].

In this thesis, it was shown that light irradiances, as well as fertilization with diluted process water of the whiteleg shrimp (*L. vannamei*) can be used to manipulate antioxidant production of sea grapes (**publication IV & V**), as well as the AA quantity and quality (**publication VII**), respectively. Increased amounts of these compounds could arguably contribute to an increased nutritional value of the sea vegetable for human or animal diets [Li et al., 2009, Mohamed, 2014, Syakilla et al., 2022] (*hypothesis II.B & C*). Hence, both approaches provide a promising foundation for a future application during sea grape production, especially since solar radiation and process water are easily accessible, in-expensive re-

sources and they can be applied in different cultivation scenarios from low- to high-technology set-ups.

However, the environmental conditions that are needed for accumulation of the target metabolites are not necessarily the seaweeds' optimum conditions for growth or even evoke physiological stress [Borowitzka, 2018]. This can lead to negative effects on economically important parameters of *C. lentillifera*, like growth rate or share of fronds (**publication IV**, **V & VII**), enforcing the need for a thoughtful and case-adapted application of such tools. Hence, following the *two-stage cultivation* established for microalgae [Liyanaarachchi et al., 2021], sea grapes could be first exposed to optimum growth conditions for a maximal increase in (harvestable) biomass, before applying the manipulative conditions. The cultivation cycle provides various opportunities to expose sea grapes to increased irradiances, e.g. by adapting the densities [Magnusson et al., 2015]. However, since *C. lentillifera* is continuously harvested according to the fronds' target size at VIJA, the post-harvest exposure to high light in the collection facility or even during the shelf-life (**publication III**) seems more feasible. At this stage, fronds are already separated from the stolon and the light manipulation might have to be adapted (**publication V**).

In the case of a fertilization treatment, however, the boundaries between a *growth* and a *manipulation* stage could be blurry and case-dependent. Fertilization with shrimp or snail effluences resulted in Relative Growth Rate (RGR)s ($\sim$3% day^{-1}, **publication VI & VII**) in the range of other successful laboratory cultivations (**publication I**) and a high share of fronds. AAs that showed a strong positive correlation to the water Nitrogen (N) content (e.g. Histidine (His) and Methionine (Met)) could be manipulated after a preceding growth phase. However, for the accumulation of AAs like Lysine (Lys), where the quantity was independent of the fertilization treatment, the manipulation phase would be redundant.

The N metabolism and photosynthesis are linked with the chloroplasts of Chlorophyta. The plastids are responsible for the green colouration of the algae. High light exposure and N-limitation in a plant's environment can cause a photoprotective migration of chloroplasts [Kasahara et al., 2002], as well as their degradation [Woodson, 2022]. Both can result in a bleaching of the organism. However, colour is arguably an important criterion for customers of sea grape products [Shewfelt, 2002]. Farmers in Van Phong Bay graded their harvested fronds not only according to weight and size, but also rachis colouration (**publication II**). The higher valued fronds, which are intended for export, had a significantly darker rachis colouration compared to the fronds for local retail. However, unlike our hypothesis and observations from Thailand [Chaiklahan et al., 2020], ramuli colouration and frond density were similar between the two different gradings for export and local retail (*hypothesis II.A*). In order to assess the colouration of sea grapes without the need for expensive technical equipment or invasive sampling (e.g. Chl meters or spectrophotometry) [Agarwal et al., 2021], a cost-efficient, non-destructive and accessible method was established in **publication V** and applied in **publication II & VI**. The method, originally designed for corals [Winters et al., 2009], was adapted to estimate the colouration of sea grapes in the Red Green Blue (RGB) colour space based on photographs with a reference scale. Red (R) values showed high correlations with the Chl *a* content of the respective seaweed. In agriculture, Chl meters and colour charts are commonly used to determine the N-status

of crops (e.g. [Singh et al., 2002, Mehrabi and Sepaskhah, 2022, Alkhaled et al., 2023]), since N-containing Chl is strongly affected by the nutrient status and especially decreased during N-depletion [Pinchetti et al., 1998]. The treatment-independent loss of colouration and Chl in **publication V** could be attributed to a N-limitation, similarly observed for *K. alvarezii* (**publication VI**). This N-limitation could have had an influence on *C. lentillifera*'s antioxidant anabolism, since seaweeds seemed to invest their photosynthetically fixated Carbon (C) in N-free compounds, like phenols [Ilvessalo and Tuomi, 1989] (**publication VI & VII**). Hence, in future studies the effect of the nutrient environment during high light exposure of sea grapes should be investigated.

Hypothesis II stated that "Targeted manipulations of cultivation environment of sea grapes can be used to increase the nutritional value of the seaweed."

Ramuli colouration and density were not decisive for the frond quality (**publication II**) and therefore *hypothesis II.A* had to be rejected. Nevertheless, the overall *hypothesis II* can be confirmed. The targeted manipulation of the abiotic parameters light irradiance and nutrient environment can enhance the antioxidant content, as well as the AA quantity and quality of sea grapes. However, further studies considering the nutritional properties and availabilities for humans or animals as part of their diets are required. The accumulation of nutritional target compounds is complex and requires a comprehensive understanding of the sea grapes' physiology (**publications V & VII**), as well as the quality characteristics of the product (**publication II**). The local adaptation of the tools is additionally complicated by potential biochemical variations between seasons or populations [Stengel et al., 2011, Sulaimana et al., 2021]. Therefore, *phenotyping* of seaweeds, like already established for agricultural plants [Yang et al., 2017, Araus et al., 2021], could be used to track important physiological and nutritional responses of sea grapes to manipulations in order to estimate the qualities and decide for harvest times [Demes and Pruitt, 2019, Tadmor Shalev et al., 2022] (**publication II**).

The results of this study enforce the potential to use a combination of non-invasive, inexpensive observational tools and algorithms with ecophysiological data and the experience of farmers for the development of efficient manipulation strategies for the aquaculture of sea grapes, as well as other seaweeds.

8.4 Polyculture to increase resource-efficiency and sustainability

Sustainability has become an important concept for aquaculture [Frankic and Hershner, 2003, Boyd et al., 2020] and polyculture systems offer an opportunity to move towards a more sustainable aquaculture industry (reviewed by [Thomas et al., 2021]).

In this study, two polyculture approaches of sea grapes with another seaweed (**publication VI**) and a crustacean species (**publication VII**) were investigated, respectively. Considering the framework of Thomas et al., both publications pursue a different

approach regarding (*1*) the species combination and (*2*) the farming system [Thomas et al., 2021]. Polyculture approaches require the co-farmed species to thrive in the same production system (species compatibility) [Thomas et al., 2021], as intrinsically given for *C. lentillifera* and *K. alvarezii* in a two-layer cultivation (**publication VI**). However, the species compatibility of *L. vannamei* and *C. lentillifera* has to be achieved through their local separation, in order to avoid hampering of sea grapes' growth due to predation [Anh et al., 2021] and nutrient toxicity evoked by high N-loads in the un-diluted process water of shrimps (**publication VII**).

The potential advantage of poly- over monoculture is achieved by exploiting the species' complementary [Thomas et al., 2021]. The integration of *C. lentillifera* in *K. alvarezii* longline cultures (**publication VI**) is a monotrophic approach, where the seaweeds in the two-layer cultivation show a form of basic and enhanced complementation based on commensalism in terms of their use of space and N-sources and complementarity based on the shade-provision (*hypothesis III.A*). The polyculture could enhance the socio-economic sustainability for the *Kappaphycus* farmer by addition of the high-value product sea grapes without the need for additional expensive farming technology or pond area.

Additionally, the successful integration of the system in Van Phong Bay could provide a basis to enhance the environmental sustainability of the system, by integrating fed and filter-feeding species and hence converting the mono- to a multitrophic polyculture [Hossain et al., 2022]. The single cultivation e.g. of fed spiny lobster and filter-feeding green mussel (*Perna viridis*) are already established in Van Phong Bay [Nghia et al., 2009, Phu et al., 2022]. It poses the opportunity for a large scale Integrated Multi-Tropic Aquaculture (IMTA) bay system, similarly practiced e.g. in Sanggou Bay in China [Fang et al., 2016]. In Sanggou Bay >30 aquaculture species from different trophic levels are cultivated in different IMTA-modes on an area of >100 km$_2$ [Fang et al., 2016]. The complementary preference of N-sources could benefit the overall bioremediatory capacity of the seaweeds, compared to a single species [Bracken and Stachowicz, 2006, Kang et al., 2021] (**publication VI**).

The polyculture represented in **publication VII** contained species from different trophic levels. The fertilization of sea grapes with the diluted process water of *L. vannamei* led to an increased growth of the seaweed without negative impacts for the shrimp (enhanced complementarity commensalism, *hypothesis III.B*). The experimental process water was sourced from a land-based, high-technology RAS situated in Germany. The system was equipped with biofiltration units, including a denitrifying biofilter. This bacterial dissimilation into gases is, besides the N assimilation into biomass by plants, the main approach to mitigate negative effects of aquaculture effluences on the environment [Neori et al., 2004]. Hence, due to the denitrifying biofilter, as well as the overall high N loads in the process water sea grapes could rather have a role as additional, rather than sole, bioremediators of the nutrients. Moreover, polyculture in such European land-based shrimp farms could save resources like heat, saline water, space and fertilizer. Therefore, it could potentially benefit the ecological and economic sustainability of the products, compared to the monoculture in separate facilities. Assuming sea grapes will be recognised as Novel Food in the European Union (EU), the implementation of *C. lentillifera* in shrimp farming facilities could also proportionally substitute the imported product.

hypothesis III stated that "Co-cultivation set-ups of *Caulerpa lentillifera* with other economically important seaweeds or fed-aquaculture species can enhance the resource-efficiency of the cultivation."

The hypothesis can be confirmed, since the co-cultivation of the seaweed *K. alvarezii*, as well as the shrimp *L. vannamei* with sea grapes can enhance the resource efficiency, compared to the respective mono-cultivation. However, both approaches were conducted on the experimental or pilot scale. Even though the polyculture systems pose a high potential, their practical and economic feasibility should be tested in an up-scaled experiment in cooperation with farmers of the respective crop.

The study presented in **chapter 7** was conducted in the context of a high-technology farming, but the approach is also applicable to the tropical pond cultivation in Van Phong Bay. Several polycultures with sea grapes and one or more other species have been proposed and researched (**publication I**) [Bambaranda et al., 2019b, Dobson et al., 2020, Anh et al., 2021, Omont et al., 2022, Phu et al., 2022]. In order to facilitate the fertilization of sea grapes in the pond environment in Van Phong Bay, a cage-cum-pond set-up could be considered in future research [Martínez-Porchas et al., 2010]. However, since sea grapes have a comparably high value in respect to the biomass of other seaweeds and the cultivation methods encompass benthic (sowing, trays), as well as pelagic set-ups (cages, nets, trays), different polyculture approaches with this seaweed are possible. Independent of the location of the polyculture system, the further use of co-products, like sea grape stolons or below-quality biomass [Chaiklahan et al., 2020, Srinorasing et al., 2021], is recommended [Newton et al., 2014]. In the frame of the polyculture with shrimp or other fed species, the feed of the animals could be admitted with sea grape biomass [Putra et al., 2019, Arisa et al., 2020, Nasmia et al., 2022]. This might be especially interesting, if certain AAs of importance for the specific animal would have been enhanced through adjustment of the polyculture conditions section 8.3.

In conclusion, the polyculture of *C. lentillifera* in a monotrophic two-layer cultivation with *K. alvarezii* and a ditrophic system with *L. vannamei* could provide a resource-efficient cultivation of the seaweed in Van Phong Bay and Germany, respectively. However, the approaches first require an experimental up-scaling and a social-economic analysis.

8.5 Economic and social sustainability

This thesis deals mostly with aspects of sea grape aquaculture from a biological perspective. However, it is a transdisciplinary endeavor to gain a full understanding of the sea grape farming in Van Phong Bay or elsewhere, and therefore requires the perspective of various disciplines. Different studies have shown the positive impacts of seaweed farming on coastal livelihoods, gender equality or social capital, especially in the Global South [Msuya and Hurtado, 2017, Rimmer et al., 2021, Spillias et al., 2023a]. Targeted value-adding manipulations, the diversification of the aquaculture system from one to more species, as well as the resource-

efficient use of space, work-force and equipment aim to contribute to the social and economic sustainability of the sea grape production. The thesis documents information on the production cycle at the farm VIJA, as an example for the industry in the Khánh Hòa province (**publication II**) and the *Kappaphycus* cultivation in Van Phong Bay (**publication VI**). Information on the farming environment and procedures from this area are to our knowledge still very rare and the provided information could function as a basis to plan further studies.

However, this thesis did not aim to primarily assess the economic or social viability of the proposed cultivation and post-harvest approaches (**publication III–VII**), but we recognize that these are essential factors to determine the success of such approaches (**publication I**). The global demand and research on *C. lentillifera* seems to be mainly driven by the interest in the species as a valuable sea vegetable [Cornish, 2019, Zubia et al., 2020, Moreira et al., 2021, Syakilla et al., 2022] and its pharmaceutical potential (**publication I**). *C. lentillifera* in the Khánh Hòa province is mostly produced for export and only secondarily for the local market (**publication II**). As a result, the industry is likely to depend mainly on the global market developments, rather than on local demands. Drivers like the inclusion in the European *Novel Foods Regulation* or policies towards a sustainable aquaculture sector, including nutrient effluents or CO_2 taxes, subsidies or customer perceptions could widen the target market and enforce the economic profit of ecologically viable solutions in the future [Knowler et al., 2020, Peñalosa Martinell et al., 2021]. The thesis investigated value-adding and resource-efficient approaches in the context of the cultivation in Van Phong Bay (**publication V & VI**), as well as in the context of potential future cultivation in Europe (**publication VII**). However, the contexts are exchangeable and the approaches can be adapted to different cultivation set-ups.

Stakeholders involved in the supply chain of sea grape farming and harvesting were selectively identified for some areas in The Philippines [del Rosario et al., 2020, Estrada et al., 2021] or the South Pacific Islands [Morris et al., 2014], where the industry has a long history [Trono and Toma, 1993, Chamberlain, 1998, Yap, 1999, Conte and Payri, 2006, Trono and Largo, 2019]. These studies provided valuable insights into the local situations. The industry in the Khánh Hòa province is still in its infancy and to the best of our knowledge studies exploring the local supply chains and the peoples' perspective on the industry are still missing. However, an understanding of the social-economic network could provide a basis for policy makers to help develop and ensure a sustainable growth of the industry.

8.6 Future perspective on sea grape aquaculture

8.6.1 Knowledge gaps along the production cycle at farm VIJA

This thesis provided new insights in the complex relationship between the local abiotic environment of *C. lentillifera* and the biochemical composition of the species. Simultaneously the study showed the potential that such ecophysiological data in combination with an understanding of the local farming environment can help to design new applications for seaweed aquaculture. Even though the thesis contributed to the body of knowledge about sea grape aquaculture at the farm VIJA in Van Phong Bay, several new questions remained unan-

swered and new ones were even raised.

The effect of exposure to a continuum of most important environmental cultivation parameters on *C. lentillifera* has been investigated at least once 1.3, however the interactive effects remain often still unclear. According to the farmers at VIJA the growth season is determined by the rain, and hence the salinity, and the temperature changes over the season (temperature x salinity) (**publication II**). Similarly, the fronds are exposed to desiccation during their shelf-life in plastic containers and temperature could have an effect on their physiological status (temperature x desiccation) (**publication III**). These are only two examples that require the further investigation of interactive effects of abiotic parameters on the sea grapes' physiology and composition. The data could contribute to an adaptation of the procedures along the production cycle.

8.6.2 Sea grapes as a global phycoculture crop?

The thesis laid a special focus on the farming of *C. lentillifera* in Van Phong Bay, Viet Nam. However, the increasing global utilisation of sea vegetables is part of an answer to tackle the complex and multi-dimensional challenges we are facing [Costa-Pierce and Chopin, 2021, Duarte et al., 2022]. Additionally, a diversification of the sector on the system, as well as the species level could contribute to a higher resilience for the industry and the local farmers [Harvey et al., 2017]. Sea grapes are a promising species to contribute to a growth and diversification of the global seaweed cultivation. The sea vegetable stands out due to its special texture and the resemblance with the high-end product caviar [de Gaillande et al., 2017, Zubia et al., 2020] and the nutritional value is an argument for the seaweed's place on peoples' plates [Syakilla et al., 2022]. In addition, the farming techniques are diverse with applications in high-, as well as low-technological systems without the need for expensive investment costs. However, sea grapes' global phycoculture is still considerably small, compared to the main seaweed crops [Moreira et al., 2021]. The production is on the rise and an increase in the coming decade(s) is to be expected. Despite the potential of *C. lentillifera* for an increasing global importance as a sea vegetable, different challenges should be overcome while growing the industry (**publication I**).

Sea grapes are known for their special texture, but other sensory components, including smell, taste and visuals are also important parameters for the customer's acceptance. These parameters were only rarely considered (e.g. [Tuong et al., 2016, Minh et al., 2019]) and they should be the subject of future research. They could also likely be influenced by changes in the microbial composition associated with the sea grapes, e.g. during post-harvest [Tuong et al., 2016, Tolentino et al., 2021], since it changes with the health status of the seaweed and over seasons [Liang et al., 2019, Kopprio et al., 2021, Pang et al., 2022]. Seaweed-bacteria interactions are complex [Egan et al., 2013], but their understanding in the context of aquaculture seems essential [Li et al., 2023, Wichard, 2023]. This field should be investigated further for *C. lentillifera*, especially since the seaweed is often consumed fresh and some bacteria could pose a health risk.

Regarding the aquaculture, *C. lentillifera* are reproduced mainly through fragmentation, which results in clonal growth. This practice could lead to an impoverishment of the genetic

diversity, causing a decrease of their fitness and an increase of the susceptibility to diseases, like observed for *Kappaphycus* [Loureiro et al., 2015, Charrier et al., 2017]. The red seaweed has been intensively cultivated and continuously propagated via fragmentation. Nowadays its aquaculture production is dramatically impaired by diseases, like *ice-ice*, in several countries [Ward et al., 2022]. Research focusing on the sexual reproduction of *C. lentillifera* could help to prevent such scenarios. On the other hand, the reproduction of *Caulerpa* via fragmentation gives rise to the related problem of the genus' invasion e.g. to the Mediterranean [Ceccherelli and Cinelli, 1999, Klein and Verlaque, 2008]. The case enforces the invasive potential of the genus, which should be considered before further spreading *Caulerpa* aquaculture.

To summarize, *C. lentillifera* is a macroalga, which has the potential to contribute to an increase in the global farming of Chlorophyta. This thesis aimed to propose solutions to increase the harvest in terms of quality and quantity, as well as the economic and ecological sustainability of the sea grape production cycle at the farm VIJA in Van Phong Bay, Viet Nam. However, different approaches, including the light management, the monotrophic *two-layer cultivation*, the fertilization with local aquaculture effluents or the manipulation of the nutritional value could also be adapted to other farming scenarios and set-ups.

8.7   Conclusions

This thesis has reported on the previously largely undocumented production cycle of sea grapes from the pond cultivation in Van Phong Bay at the farm VIJA to the seaweed's shelf-life and retail. The conducted experiments have demonstrated that light and nutrient fertilization are important environmental factors for *C. lentillifera* with relevant effects e.g. on photosynthesis, pigmentation, antioxidative response, growth, and the AA composition. They are therefore a strong management tool for the sea grape farmers. Shading of the cultivation ponds provides a suitable growth environment for the seaweeds, whereas targeted exposure to photooxidative stress can enhance the antioxidative content to the level of pomegranates. Farmers can manipulate the content of certain AAs by targeted fertilization with aquaculture process water. Additionally, polycultures of sea grapes with organisms from higher or a similar trophic level can increase the resource efficiency of the cultivation and arguably the economic sustainability for the farmer.

However, the manipulation methods and polyculture approaches were tested at the experimental scale and an up-scaling to farm conditions is required, taking into account potential interactive effects with other environmental parameters and social-economic considerations for the stakeholders involved.

Appendix A

Supplementary material of Publication I

A.1 Definition of categories

Definition of topical categories:

Ecophysiology: Ecophysiology describes the study on "how the environment, both physical and biological, interacts with the physiology of an organism" (nature portfolio, https://www.nature.com/subjects/ecophysiology). For seaweeds, the most important abiotic environmental factors are light, temperature, salinity, water motion, and nutrient availability. The important biotic factors are usually interactions with epiphytic bacteria, fungi, algae, sessile animals, and herbivores [Hurd et al., 2014]. This category includes all studies, where the ecophysiology of seaweeds with abiotic factors is studied. This includes studies, where the physiological reaction of sea grapes towards abiotic environmental factors in the water environment, as well as in the air (e.g. packaging environment) is quantified.

Biochemical composition: Seaweeds contain a variety of different compounds, including proteins, lipids, carbohydrates, minerals (ash), pigments and phenols [Holdt and Kraan, 2011, Olsson et al., 2020]. Some of these components exhibit bioactivities and/or are important for nutrition of humans and animals. This category includes all studies that determine the content of these compounds in sea grapes in comparison to other seaweeds, foods, or along spatial or temporal gradients and the effect these compounds have on other organisms, e.g. when integrated in their feed/food or used as pharmaceutical.

Water treatment: Wastewater is a by-product generated from any activity or process. This includes a variety of sources for organic and inorganic pollutants, e.g. but not limited to, manufacturing industries, households, textile industries, aquaculture, agriculture [Arumugam et al., 2018]. This category includes all studies, where sea grapes were used to treat the wastewater with the ultimate target to improve the water quality.

Distribution: Studies about the occurrence of organisms (e.g. articles about first occurrence or distribution of species) and about the classification of organisms. This category

includes all studies that deal with first reports or rediscovery of the alga.

Genetics/Genomics: Genetics is the study of genes and genetic variation. It explains how hereditary information is composed, how it functions, and how it is passed [Janning and Knust, 2008]. The field includes phylogenetics which studies the evolutionary relationship of species by means of gene analysis. Genomics is concerned with the structure, function, evolution, and mapping of genomes [Lesk, 2017].

Microbiome: The microbiome denotes in general a microbial community as a multi-species assemblage of microorganisms [Berg et al., 2020]. Seaweeds are known to harbour a large diversity of microorganisms, together with which they form the holobiont [Egan et al., 2013]. This category includes all studies related to the sea grapes' holobiont and potential changes of which in relation to environmental parameters.

Aquatic Ethnobiology/Ethnophycology: Humans have interacted with and depended on aquatic ecosystems for centuries and developed knowledge about the aquatic environment. Aquatic Ethnobiology describes the interaction between culture and marine biota, meaning the uses, practices, knowledge, and beliefs of a given community or culture regarding marine organisms and marine biodiversity [Arenas, 2016]. One discipline of Ethnobiology includes Ethnophycology, which focuses on the interrelationships of people with the aquatic flora [García-Quijano and Pitchon, 2010].

Definition of application categories:

Animal feed: Application of *Caulerpa lentillifera* as supplement in animal feed, e.g. in shrimp cultures.

Cosmetics: Application of *C. lentillifera* in cosmetic products e.g. creams or masks.

Cultivation: Application of *C. lentillifera* in aquaculture. Including applications in IMTAs or co-cultures.

Feedstock: Application of *C. lentillifera* as a source for biochemical compounds e.g. oils for the production of biodiesel.

Fundamental research: No direct application of research on *C. lentillifera* other than for the sake of knowledge, e.g. characterization of genome.

Industrial effluents: Application of *C. lentillifera* for biosorption in industrial effluents, e.g. removal of heavy metals or dyes.

Nutritional value: Research on *C. lentillifera* composition in regard of the use in human nutrition, e.g. for its antioxidant properties.

Pharmaceutical: Use of *C. lentillifera* or specific compounds in a medical context, e.g. anticancer or anti-inflammatory drugs.

Post-harvest: Research on *C. lentillifera* in the period of the life-cycle after harvest until retail, e.g. on increasing the shelf-life or value of waste.

A.2 Supplementary information on topic *Ecophysiology*

Table A.1: Overview of experimental parameters, whose effect on response variables was studied for publications of the topic *Ecophysiology*, grouped by applications. + denotes the presence of a certain response or experimental parameter for the respective publication. Biomass production is shown as growth rate and biomass properties shows the presence (+) of measurements to quantify the information about the thallus composition (fronds, stolon, ramuli, etc.). Experimental design described if the effect of experimental parameters was quantified in a single or a crossed-factor experimental design and *non-living* presence if the biomass of *Caulerpa lentillifera* was still alive.

Application	Source	Biomass production	Biomass properties	Chl a fluorometry	Oxygen evolution	Biochemical comp.	Enzyme activities	Gene expression	Colour/pictures	water content	Microbial analysis	Sensory	Light	Temperature	Salinity	Desiccation	Nutrients	Sediment	Desiccation	ACEF	Season	Single factor	crossed	Status	Experimental run
Cultivation	[Cai et al., 2021b]	+			+	+	+							+			+						+		8 days
	[Guo et al., 2015b]	+		+		+			+						+		+					+			7 days
	[Guo et al., 2015a]	+	+	+		+		+	+				+	+									+		7 days
	[Long et al., 2020]					+												+				+			4 weeks
	[Kang et al., 2020]	+				+		+	+				+									+			12 days
	[Tanaka et al., 2020]	+				+									+							+			13 days
	[Terada et al., 2021]			+	+								+	+		+						+			72 h, or less
	[Fakhrulddin et al., 2021]	+	+										+		+		+					+			6 weeks
	[Stuthmann et al., 2020]			+					+				+									+			3 weeks
Post-harvest	[Terada et al., 2018]			+						+					+							+			12 days
	[Stuthmann et al., 2020]			+					+	+			+		+							+			21 days
	[Anantpinijwatna et al., 2018]								+	+						+						+	+		360 min
	[Sulaimana et al., 2021]					+	+		+	+								+		+	+	+			9 days
	[Liang et al., 2021]			+		+	+											+					+		120 h
	[Tolentino et al., 2021]					+					+	+					+					+		+	10 days
Nutritional value	[Sommer et al., 2022]					+							+												14 days
	[Stuthmann et al., 2020]			+		+			+				+												14 days

A.3 Supplementary information on topic *Biochemical composition*

Table A.2: Reporting the different main methods and assays used for the investigation of different biochemical compounds in *Caulerpa lentillifera*.

Analysis of	Method	Reference
Total phenolic content	Folin-Ciocalteu method (Standard: Gallic acid or Phloroglucinol)	[Matanjun et al., 2008, Nguyen et al., 2011, Wichachucherd et al., 2019, Fakhrulddin et al., 2021]
Antioxidant activity	DPPH (Standard: Trolox)	[Matanjun et al., 2008, Nguyen et al., 2011];
	Hydrogen peroxide scavenging activity (Standard: Vitamin C)	[Nufus et al., 2019, Wichachucherd et al., 2019];
	Ferrous ion chelating activity FIC (Standard: EDTA)	[Balasubramaniam et al., 2020]; [Fakhrulddin et al., 2021, Honwichit et al., 2022]
	TEAC/ABTS (Standard: Trolox)	
	Cupric ion reducing antioxidant capacity CUPRAC (Standard: Trolox)	
	Ferric reducing-antioxidant power FRAP (Standard: Trolox)	
	Oxygen radical absorbance capacity ORAC	
Amino acid composition	HPLC Amino acid analyser	[Matanjun et al., 2009, Zhang et al., 2020, Terriente-palacios and Castellari, 2022]
Minerals	Atomic Absorption Spectrophotometry Inductively Coupled Plasma Mass Spectrometry	[Salleh and Wakid, 2008, Matanjun et al., 2009, Paul et al., 2014, Nufus et al., 2019, Fakhrulddin et al., 2021, Zhang et al., 2020]
Vitamin C	2,4 dinitrophenylhydrazine method	[Matanjun et al., 2009, Zhang et al., 2020]
	2,6 dichloroindophenol titrimetric method	
Vitamin E & A	HPLC method	[Matanjun et al., 2009]

Lipid content	Soxleth method	[Salleh and Wakid, 2008, Matanjun et al., 2009, Honwichit et al., 2022, Zhang et al., 2020]
Fatty acid composition	Gas chromatography	[Ratana-arporn and Chirapart, 2006, Matanjun et al., 2009, Saito et al., 2010, Nagappan and Vairappan, 2014, Paul et al., 2014, Zhang et al., 2020]
Crude protein	Kjeldahl method (multiplying nitrogen content with 6.25) Bradford method	[Ratana-arporn and Chirapart, 2006, Salleh and Wakid, 2008, Shevchenko et al., 2009, Nguyen et al., 2011, Zhang et al., 2020, Honwichit et al., 2022]
Ash	525°C - 900°C for a few hours or overnight (mostly 550°C)	[Matanjun et al., 2009, Nagappan and Vairappan, 2014, Zhang et al., 2020, Honwichit et al., 2022]
Moisture	Hot air-oven	[Salleh and Wakid, 2008, Matanjun et al., 2009, Nagappan and Vairappan, 2014, Honwichit et al., 2022]

Table A.3: Proximate composition of *Caulerpa lentillifera* grouped by different studies. Values are expressed as % of dry weight (DW) or in % of fresh weight (FW). The overall range of means is presented for each application, respectively.

Carbohydrate	Crude protein	Crude lipid	Crude fibre	Ash	Moisture	n	Reference	Application
		%DW			%FW			
64 ± 0.11	9.26 ± 0.03	1.57 ± 0.02	2.97 ± 0.01	22.20 ± 0.27	94.28 ± 0.24	3	[Nguyen et al., 2011]	Nutritional value
27.19	12.68	1.09	4.83 ± 0.72	47.80 ± 0.87	-	3	[Setthamongkol et al., 2015]	
44.02 ± 2.01	19.38 ± 1.48	2.87 ± 0.03	4.12 ± 0.16	29.61 ± 1.50	87.05 ± 0.50	3	[Nagappan and Vairappan, 2014]	
59.27	12.49 ± 0.3	0.86 ± 0.10	3.17 ± 0.21	24.21 ± 1.7	25.31 ± 1.15?	NA	[Ratana-arporn and Chirapart, 2006]	
72.90	15.90	0.70	8.40	2.10	92.30	NA	[Salleh and Wakid, 2008]	
	10.41 ± 0.26	1.11 ± 0.05	1.91 ± 0	37.15 ± 0.64	10.76 ± 0.80	3	[Matanjun et al., 2009]	
44.82 ± 0.98	12.50 ± 0.70	2.32 ± 0.23	12.98 ± 1.59	27.36 ± 0.13	95.95 ± 0.12	3	[Zhang et al., 2020]	
43.22 ± 1.42	14.76 ± 0.72	1.90 ± 0.05	8.87 ± 0.74	31.29 ± 0.69	95.09 ± 0.14	3	1.Hainan 2.Shandong	
-	-	3.7 ± 0.7	-	-	-	3	[Saito et al., 2010]	
-	-	1.6 ± 0.2	-	-	-	2	1.Yonaha Bay (wild)	
-	-	2.7 ± 0.2	-	-	-	2	2.& 3. Nago Bay (cultured)	
-	13.2 ± 0.04	-	-	-	-	3	[Terriente-palacios and Castellari, 2022]	
27.19 - 72.90	**9.26 - 19.38**	**0.70 - 3.7**	**1.91 - 12.98**	**2.10 - 47.80**	**87.05 - 95.95**		**Overall range of means**	
-	-	-	5.03 - 5.56	49.92 - 52.79	-	NA	[Syamsuddin et al., 2019] 1.Indoor cultivation (sowing method),	Cultivation
-	-	-	7.64 - 8.65	32.04 - 36.60	-	NA	2.Sea cultivation (tray method)	
-	**-**	**-**	**5.03 - 8.65**	**32.04 - 52.79**	**-**		**Overall range of means**	
17.76 ± 2.10	6.31 ± 0.35	2.00 ± 0.06	-	55.10 ± 1.36	95.4 ± 1.1	3	[Chaiklahan et al., 2020]	Post-harvest
20.87 ± 3.05	8.59 ± 0.50	1.98 ± 0.16	-	57.01 ± 3.09	95.8 ± 2.0	3	1.Food grade,	
26.96 ± 4.28	4.67 ± 0.31	1.84 ± 0.02	-	56.28 ± 0.70	-	3	2.& 3. Waste	
34.5	14.5	0.5	12.7	50.5	-	NA	[Honwichit et al., 2022]	
17.76 - 34.5	**4.67 - 14.5**	**0.5 - 2.0**		**50.5 - 57.01**	**95.4 - 95.8**		**Overall range of means**	

Table A.4: Fatty acid composition of *Caulerpa lentillifera*, including saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), poly-unsaturated fatty acids (PUFAs). + shows values manually calculated from mean mg g-1 dry weight (DW) to %. * The fatty acid composition was expressed as % of total lipids, the composition was quantified in wild and cultured organisms and in different lipid fractions (phosphatidylcholine, phosphatidylethanolamine, triacylglycerols, monogalactosyldiacylglycerols and digalactosyldiacylglycerols) the values present range of the means of the fractions for wild and cultured *C. lentillifera*, respectively. Values are not integrated in the range between papers, where contents are expressed as % of total fatty acids. The number of replicates (n) is indicated for each publication.

Fatty acid	Name	[Matanjun et al., 2009]	[Nagappan and Vairappan, 2014]	[Ratana-arporn and Chirapart, 2006]	[Paul et al., 2014] +	[Zhang et al., 2020]		Range of means	[Saito et al., 2010] n = 3-4 *	
		n=3	n=3	n=NA	n = 9	Hainan n = NA	Shandong n = NA		Wild	Cultured
					% of total fatty acids				% of total lipids	
C4:0	Butyric acid	-	2.3 ± 0.37	-	-	-	-		-	-
C6:0	Caproic acid	-	0.3 ± 0.06	-	-	-	-		-	-
C8:0	Caprylic	-	1.1 ± 0.12	-	-	0.17	0.26	0.17 - 1.1	-	-
C10:0	Capric	0.16	6.4 ± 0.81	-	-	0.02	0.06	0.02 - 6.4	-	-
C11:0	Undecanoic	0.85	1.1 ± 1.94	-	-	-	-	0.85 - 1.1	-	-
C12:0	Lauric	0.13	0.69 ± 1.21	-	-	0.06	0.15	0.06 - 0.69	30.5 - 51.2	23.2 - 43.9
C13:0	Tridecanoic	0.12	1.54 ± 0.99	-	-	0.02	0.03	0.02 - 1.54	0.0 - 0.5	0.2 - 0.6
C14:0	Myristic	1.65	2.92 ± 0.32	-	3.12	2.36	2.42	1.65 - 3.12	1.8 - 3.6	0.8 - 4.7
C14:1	Myristoleic	0.33	1.5 ± 0.11	-	-	0.02	0.01	0.01 - 1.5	-	-
C15:0	Pentadecanoic	0.11	2.1 ± 2.46	-	-	0.1	0.13	0.1 - 2.1	0.2 - 0.4	0.1 - 1.5

C15:1	Cis-10-pentenoic acid	-	2.54 ± 0.02	-	-	0.83	0.86	0.83 - 2.54	-	-
C16:0	Palmitic acid	33.78	8.74 ± 0.01	67.83	37.61	45.22	49.46	8.74 - 49.46	19.3 - 42.4	14.2 - 36.7
C16:1	Palmitoleic acid	1.31	3.91 ± 0.93	6.08	7.48	8.24	7.51	1.31 - 8.24	-	-
C16:1N10	-	-	-	-	-	-	-	-	0.1 - 0.9	0.0 - 1.2
C16:1N7	-	-	-	-	-	-	-	-	2.7 - 5.6	3.0 - 5.8
C16:1N5	-	-	-	-	-	-	-	-	0.2 - 3.1	0.3 - 2.6
C16:2N6	-	-	-	-	-	5.52	-	-	1.3 - 5.4	1.4 - 4.6
C16:3N3	-	-	-	-	-	12.03	-	-	3.5 - 13.6	5.1 - 19.6
C17:0	Heptadecanoic	0.16	3.36 ± 1.75	-	-	0.18	0.16	0.16 - 3.36	0.1 - 0.5	0.1 - 0.4
C17:1	Cis-10-Heptadecanoic	1.55	2.67 ± 1.02	-	-	0.68	0.88	0.68 - 2.67	-	-
C18:0	Stearic acid	7.83	3.81 ± 0.28	11.11	-	0.94	1.13	0.94 - 7.83	-	-
C18:1N9C	Oleic acid	32.49	0.93 ± 0.05	0.23	1.96	1.79	2.07	0.23 - 32.49	-	-
C18:1N9T	Elaidic	0.22	1.41 ± 0.33	-	2.49	-	-	0.22 - 2.49	-	-
C18:2N6C	Linoleic acid	7.64	4.88 ± 1.01	4.26	11.85	10.89	10.99	4.26 - 11.85	-	-
C18:2N6T	Linolelaidic	0.09	4.14 ± 1.34	-	-	-	-	0.09 - 4.14	-	-
C18:3N3	α-Linolenic	5.54	5.15 ± 1.13	2.73	14.71	13.42	7.99	2.73 - 13.42	6.2 - 19.9	5.9 - 25.1
C18:3N6	γ-Linolenic	0.31	5.99 ± 0.73	-	1.6	0.47	0.27	0.27 - 5.99	0.3 - 2.4	0.3 - 2.2
C20:0	Arachidate	0.47	1.98 ± 0.59	1.48	-	0.07	0.08	0.07 - 1.98	0.0 - 0.2	0.0 - 0.1
C20:1	Eicosanoate	0.17	1.69 ± 0.19	1.36	-	0.11	0.09	0.09 - 1.69	-	-

C20:2	Cis-11,14-Eicosadienoic	0.07	4.27 ± 0.93	-	-	0.47	0.3	0.07 - 4.27	-	-
C20:3N3	Cis-11,14,17-Eicosatrienoic	1.15	3.64 ± 0.64	-	-	0.34	0.24	0.24 - 3.64	0.1 - 1.5	0.3 - 1.4
C20:3N6	Cis-8,11,14-eicosatrienoic acid	-	3.3 ± 0.73	-	-	0.19	0.09	0.09 - 3.3	0.3 - 1.6	0.2 - 1.6
C20:4N6	Arachidonic acid	-	6.7 ± 0.53	0.84	-	-	-	0.84 - 6.7	1.1 - 8.1	1.0 - 8.0
C20:5N3			-	-	1.6	-	-	-	1.0 - 4.8	1.5 - 6.1
C20:5N6	Eicosapentaenoic acid	0.86	-	0.83	-	3.77	1.91	0.83 - 3.77	-	-
C21:0	Henocasanoic	-	1.62 ± 0.07	-	-	0.02	0.03	0.02 - 1.62	-	-
C22:0	Eicosapentaenoic acid	0.31	1.15 ± 0.21	2.28	-	0.47	0.7	0.31 - 1.15	0.2 - 0.9	0.2 - 0.3
C22:1N9	Erucate	0.27	0.85 ± 0.60	0.76	-	2.7	2.8	0.27 - 2.8	-	-
C22:2	Cis13,16-Docisadienoic	0.95	-	-	-	0.04	0.06	0.04 - 0.95	-	-
C22:6N3	Docosahexaenoic acid	-	3.64 ± 0.64	0.83	-	0.39	0.26	0.26 - 3.64	-	-
C23:0	Tricosanoi	0.14	2.05 ± 0.17	-	-	0.11	0.21	0.11 - 2.05	-	-
C24:0	Nervonic	0.7	1.55 ± 0.31	-	-	5.91	8.85	0.7 - 8.85	1.9 - 4.8	1.8 - 5.6
C24:1	Nervonic	0.66	2.79 ± 0.60	-	-	-	-	0.66 - 2.79	-	-
SFAs	Saturated fatty acids	46.41	42.71 ± 3.38	82.69	40.7	55.65	63.66	40.7 - 82.69	30.5 - 51.2	23.2 - 43.9
MUFAs	Mono-unsaturated fatty acids	36.83	18.29 ± 0.36	8.43	12	14.37	14.23	8.43 - 36.83	9.5 - 14.6	10.9 - 15.7

| PUFAs | Ploy-unsaturated fatty acids | 16.76 | 38.07 ± 4.11 | 9.49 | 47.3 | 29.98 | 22.11 | 9.49 - 38.07 | 35.9 - 54.9 | 36.7 - 63.6 |

Table A.5: Mineral composition of *Caulerpa lentillifera* from different regions and studies from the topic *Biochemical composition* and the application *Nutritional value*. Data on phosphorous (P), sodium (Na), potassium (K), calcium (Ca), iron (Fe), magnesium (Mg), copper (Cu), zinc (Zn), selenium (Se), manganese (Mn) and iodine (I) are presented as amount per dry weight (DW). The overall range of means is presented. * indicates that more minerals are available in the publication.

P	Na	K	Ca	Fe	Mg	Cu	Zn	Se	Mn	I	n	Reference	Application
					mg 100 g^{-1} DW					μg g-1 DW			
112.29 ± 14.79	9338.30 ± 183.87	661.66 ± 68.98	1186.66 ± 32.53	-	788.33 ± 22.54	11.88 ± 0.35	1.45 ± 1.41	-	-	-	NA	[Ismail et al., 2020]	
-	8917.46 ± 0.00	1142.68 ± 0.00	1874.74 ± 0.20	21.37 ± 0.00	1028.62 ± 0.58	0.11 ± 0.00	3.51 ± 0.00	1.07 ± 0.00	-	4.78 ± 0.59	3	[Matanjun et al., 2009]	
<100.00	16050.00 ± 150.000	741.00	587.50 ± 5.50	-	1665.00 ± 25.00	0.089 ± 0.04	2.755 ± 0.645	0.390 ± 0.083	0.321 ± 0.139	-	2	[Paul et al., 2014]*	Nutritional value
1030	-	970	780	9.3	630	2.2	2.6	-	7.9	1424	3	[Ratana-arporn and Chirapart, 2006]	
2.45	1229.7	141.3	3.27	14.5	17.0	0.3	0.62	-	-	-	NA	[Salleh and Wakid, 2008]	
												[Zhang et al., 2020]*	
-	9432.33 ± 146.71	3585.21 ± 51.75	3315.85 ± 127.55	510.65 ± 5.47	6715.74 ± 82.58	12.26 ± 0.40	33.90 ±0.13	-	1341.07 ± 42.43	24.06 ± 0.54	3	1. Hainan	
-	8834.85 ± 396.90	4967.34 ± 918.21	3728.35 ± 92.38	1972.97 ± 183.35	8126.59 ± 242.72	15.72 ± 1.84	11.75 ± 2.05	-	515.42 ± 35.01	7.26 ± 0.35	3	2. Shandong	
2.45 - 1030	**1229.7 - 16050**	**141.3 - 4967.34**	**3.27 - 3728.35**	**9.3 - 1972.9**	**17 - 8126.59**	**0.3 - 11.88**	**0.62 - 33.90**	**0.39 - 1.07**	**0.321 - 1341.07**	**4.78 - 1424**		**Overall range of means**	

Table A.6: Amino acid composition of *Caulerpa lentillifera* expressed as mg g^{-1} dry weight (DW). Numbers are expressed as mean $\pm$ standard deviation (SD) and the number of replicates (n) is indicated for each publication.

Amino acid (mg g^{-1} DW)	[Matanjun et al., 2009] n = 3	[Ratana-arporn and Chirapart, 2006] n = NA	[Zhang et al., 2020] Hainan n = 3	Shandong n = 3	**Range of means**
Essential					
Threonine (Thr)	5.84 ± 0.22	7.9	7.36	9.3	**5.8 - 9.3**
Valine (Val)	6.18 ± 0.19	8.7	8.26	11.16	**6.18 - 11.16**
Methionine (Met)	1.58 ± 0.08	-	1.8	2.37	**1.58 - 2.37**
Lysine (Lys)	1.22 ± 0.05	8.2	7.06	7.78	**1.22 - 8.2**
Isoleucine (Ile)	5.06 ± 0.12	6.2	5.26	6.94	**5.06 - 6.94**
Leucine (Leu)	7.79 ± 0.19	9.9	9.47	12.86	**7.79 - 12.86**
Phenylalanine (Phe)	19.95 ± 1.41	6.1	4.81	6.6	**4.81 - 19.95**
Non-essential					
Aspartic acid (Asp)	8.33 ± 0.11	14.3	12.47	14.89	**8.33 - 14.89**
Serine (Ser)	5.49 ± 0.20	7.6	7.81	9.47	**5.49 - 9.47**
Glutamic acid (Glu)	13.47 ± 0.23	17.8	13.82	14.72	**13.47 - 17.8**
Glycine (Gly)	5.14 ± 0.03	8.5	19.23	18.27	**5.14 - 19.23**
Arginine (Arg)	5.71 ± 0.22	8.7	4.81	5.75	**4.81 - 8.7**
Histidine (His)	1.44 ± 0.13	0.8	1.65	2.2	**0.8 - 2.2**
Alanine (Ala)	6.88 ± 0.19	8.5	10.82	13.36	**6.88 - 13.36**
Thyrosine (Tyr)	3.33 ± 0.08	4.8	3.61	4.74	**3.33 - 4.8**
Proline (Pro)	4.29 ± 0.11	5.7	5.56	5.75	**4.29 - 5.75**
Cystein (Cys)	-	-	1.2	0.85	**0.85 - 1.2**
Total essential amino acids	48.98 ± 2.19	47	44.02	57.01	**44.02 - 57.01**
Total non-essential amino acids	54.08	76.7	80.98	89.99	**54.08 - 89.99**
Total amino acids	101.63 ± 2.92	123.7	125	147	**101.63 - 147**

Table A.7: Quantification of antioxidants, total phenolic contents (TPC) and total flavonoid content (TFC) of *Caulerpa lentillifera*, using different assays and standards. The abbreviations in the table are: PGE (phloroglucinol equivalents), TEAC (trolox equivalent antioxidant capacity), FRAP (ferric reducing antioxidant power) assays, GAE (gallic acid equivalent), TE (trolox equivalent), QE (quercetin equivalent), ORAC (antioxidants orac value), EC50 (half maximal effective concentration), F-C (Folin-Ciocalteu assay) and AAE - ascorbic acid equivalent.

Assay	Compound	Result	Info	n	Reference
DPPH	Radical scavenging activity	$5.74 \pm 0.9\%$		3	[Balasubramaniam et al., 2020]
ORAC	Antioxidant capacity	68372 ± 1596 µmol TE 100 g^{-1}			
FRAP	Antioxidant activity	27.09 mg TE 100 g^{-1}			
DPPH	Radical scavenging activity	28.77 EC50 mg mL^{-1}		NA	[Ismail et al., 2020]
F-C	TPC	57.97 mg GAE 100g^{-1}			
Jia et al. 1999	TFC	1506.41 mg QE 100g^{-1}			
TEAC	Antioxidant activity	2.16 ± 0.04 mM mg^{-1} DW			
FRAP	Antioxidant activity	362.11 ± 15.65 µM mg^{-1} DW		3	[Matanjun et al., 2008]
F-C	TPC	42.85 ± 1.22 mg PGE g^{-1} DW			
F-C	TPC	1.30 ± 0.02 mg GAE g^{-1} DW	thermal drying	3	[Nguyen et al., 2011]
		2.04 ± 0.03 mg GAE g^{-1} DW	freeze drying		
FRAP	Antioxidant activity	109.0 ± 0.0 - 138.75 ± 0.0 µmol Fe2 g^{-1}	Different temperatures, times	NA	[Nufus et al., 2019]
CUPRAC	Antioxidant activity	14.45 ± 0.164 - 9.02 ± 0.229 µmol TE g^{-1}			

Table A.8: Pigment composition of *Caulerpa lentillifera*. Values are expressed as mean±standard deviation (SD) or as percentage.

Chl a	Chl b	β-Carotene	Lutein	Zeaxanthin	Fucoxanthin	β-Cryptoxanthin	Canthaxanthin	Astaxanthin	Carotenoids	Unit	n	Source
-	-	19.5 ± 0.0	<0.02	3.6 ± 0.0	<0.001	1.3 ± 0.0	14.6 ± 0.0	3.0 ± 0.1	-	mg g 100^{-1} DW	3	[Balasubramaniam et al., 2020]
258 ± 25	147 ± 14	15 ± 1.0	-	-	-	-	-	-	-		16	[Paul et al., 2014]
0.053	0.118	-	-	-	-	-	-	-	0.021		3	[Chaiklahan et al., 2020]
0.029	0.077	-	-	-	-	-	-	-	0.016	% DW	3	1. Food grade
0.032	0.075	-	-	-	-	-	-	-	0.022		3	2. & 3. Waste

Table A.9: Vitamin composition of *Caulerpa lentillifera*. Values are presented in mg 100g^{-1} wet weight (WW), unless indicated differently.

Vit A	Vit B1 (Thiamine)	Vit B2 (Riboflavin)	Vit C	Vit E	Vit B3 (Niacin)	n	Source
		mg 100g^{-1} WW					
15.3 mg kg^{-1}	8.8 mg kg^{-1}	2.5 mg kg^{-1}	274 mg kg^{-1}	NA	88 mg kg^{-1}	NA	[Salleh and Wakid, 2008]
-	-	-	34.7±0.02	8.41±0.12		3	[Matanjun et al., 2009]
-	0.05	0.02	1	2.22	1.09	NA	[Ratana-arporn and Chirapart, 2006]
-	-	-	41.73 ± 1.51	3.05	-	3	[Zhang et al., 2020]
-	-	-	50.33 ± 0.62	3.11	-		1. Hainan 2. Shandong
			1 - 50.33	**2.22 - 8.41**			**Overall range of means**

A.4 Supplementary information on topic *Wastewater*

Table A.10: Overview of initial stocking rate of fed species, as well as initial stocking density and growth rates of *Caulerpa lentillifera* and experimental run of the different experiments in the topic *Wastewater* and the applications *Cultivation* and *Nutritional value*.

Source	Initial stocking density co-culture organism	Initial stocking density of *C. lentillifera*	Growth rate of *C. lentillifera*	Experimental run	Type of study
[Anh et al., 2021]	1000, 2000, 3000 ind. m^{-3}	2 kg m^{-3}	0.46 - 1.05% day^{-1}	45 days	Pilot aquaculture system
[Bambaranda et al., 2019b]	40 ind. tank^{-1} $\sim$ 133 ind. m^{-3}	4.5 kg tank^{-1} $\sim$ 15 kg m^{-3}	2.5 g day^{-1}	60 days	
[Chaitanawisuti et al., 2011]	300 ind. m-2	280, 560, 840 g m^{-3}	1.70 - 2.52% day^{-1}, overall average: 2.07% day^{-1}	120 days	
[Dobson et al., 2020]	*Holothuria*: 50 ind. m^{-2}, *Babylonia*: 390 ind. m^{-2}	700 g tank^{-3}	1.86 ± 0.12% day^{-1}	84 days	
[Largo et al., 2016]	-	-	-	-	
[Ly et al., 2021]	300 ind. m^{-3}	0.5, 1, 1.5, 2 kg m^{-3}	0.71 - 1.1% day^{-1}	56 days	
[Paul and de Nys, 2008]	-	-	0 - 4% day^{-1}	42 days / 19 days	
[Paul et al., 2014]	-	4-6 kg m^{-2}	2 kg week^{-1}	42 days	
[Anh et al., 2022]	Whiteleg shrimp: 100, 200, 300, 400, 500 ind. m^{-3}	1 kg m^{-3}	1.3 - 1.55% day^{-1}	56 days	
[Omont et al., 2022]	Whiteleg shrimp: 7.62 ± 0.07 g	15.23 ± 0.10 g = 0.3 g L^{-1}	2.6 ± 0.4% day^{-1}	28 days	
Overall range; mean			**0.46 - 4% day^{-1}**	**19 - 120 days; 58 days**	
[Bambaranda et al., 2019a]	-	10, 20, 30, 40, 50 g L^{-1}	-	24 h	Laboratory study
[Liu et al., 2016]	-	2 g L^{-1}	2.9 - 3.99% day^{-1}	10 h/ 15 days	
[Lu et al., 2021]	-	-	-	-	
Overall range			**-**	**10 h - 15 days**	

Appendix B

Supplementary material of Publication II

B.1 Overview of sampling dates

Table B.1: Measurement days, count of measurement points (n) of different environmental parameters with means presented in table 1 of the publication. Salinity and temperature measurements were quantified with data loggers (HOBO, USA) and all pH, as well as salinity and temperature measurements from June 2022 were quantified using a multiparameter probe (Manta2, Eureka, USA). The list is ordered following the seasonal sequence.

Month, Year	Parameter	Measurement days	n	Device
February 2020	Temperature, Salinity	28.-29.	96	Logger
March 2020	Temperature, Salinity	1.-31.	1485	Logger
April 2020	Temperature, Salinity	1.-30.	1440	Logger
May 2019	Temperature, Salinity	6.-31.	1225	Logger
May 2020	Temperature, Salinity	1.-31.	1488	Logger
June 2019	Temperature, Salinity	1.-6., 19.-30.	814	Logger
June 2020	Temperature, Salinity	1.-29.	1364	Logger
June 2022	Temperature, Salinity	6., 23.	17	MANTA
July 2019	Temperature, Salinity	1.-31.	1486	Logger
August 2019	Temperature, Salinity	1.-13.	595	Logger
February 2020	pH	27.	27	MANTA
June 2019	pH	19.	4	MANTA
June 2022	pH	9., 23.	17	MANTA
July 2019	pH	11.	9	MANTA
August 2019	pH	13.	8	MANTA

B.2 Statistical output

Table B.2: Results of one way-ANOVA (OA) or Kruskal-Wallis test (KW) to test for in-between subject effects of sea grape frond quality (*Quality*) of *Caulerpa lentillifera* on antioxidant activity (*AOA*), total phenolic content (*TPC*), F_v/F_m values, frond weight and length, Red (R) value of rachis and ramuli and ramuli density. The asterisks indicate the significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Data	Variable	DF	Sum Sq	Mean Sq	F value/ Chi Square	P value	Shapiro	Levene	Test
TPC	Quality	1	1120	1120	3.436	0.0779	0.6654	0.4133	OA
	Residuals	21	6847	326					
AOA	Quality	1	34	34.2	0.069	0.069	0.1688	0.8274	OA
	Residuals	21	10924	496.5					
F_v/F_m	Quality	1			9.6034	0.001942	9.104e-13	0.02086 *	KW
Weight (g)	Quality	1			103.17	<2.2e-16	0.3748	9.711e-06	KW
Length (cm)	Quality	1			61.372	4.724e-15	0.3662	5.718e-08	KW
R rachis	Quality	1			46.94	7.318e-12	0.1247	0.0008933	KW
R ramuli	Quality	1			1.4289	0.2319	0.01113 *	0.647	KW
Ramuli density	Quality	1			1.0623	0.3027	0.03077 *	0.004605	KW

Table B.3: Results of Spearman and Pearson correlation test of frond weight and length, as well as antioxidant activity (AOA) and total phenolic content (TPC).

Variables	S/t	p-value	Rho/corr	Shapiro	Test
Weight (g) and length (cm)	740414	<2.2e-16	0.8178466	0.0001552, 2.255e-06	Spearman
TPC and AOA	6.6577	1.18e-08	0.661405	0.1279, 0.6654	Pearson

Table B.4: Uni- and Multivariate logistic regression models with different model evaluation parameters.

Variant	Variables		Estimate Std.	Error	Z value	P-value	Odds ratio	95% C.I.	AIC	Accuracy score	AUC
Uni-	F_v/F_m	Intercept	7.866	3.620	2.173	0.0298 *	2.606068e+03	2.664742e+00, 4.237864e+06	400.54	0.5689655	0.6053
		variable	-11.152	5.085	-2.193	0.0283 *	1.434147e-05	4.424705e-10, 2.276425e-01			
	Weight	Intercept	5.2138	0.6608	7.890	3.01e-15 ***	183.7929111	54.0856305, 726.8597791	290.1	0.7931034	0.8451
		variable	-2.6915	0.3326	-8.093	5.83e-16 ***	0.0677775	0.0338909, 0.1252809			
	Accuracy with test data set									0.7734	
	Length	Intercept	4.06891	0.63683	6.389	1.67e-10 ***	58.4932008	0.006808491, 0.07120006	345.92	0.7137931	0.7662
		variable	-0.36999	0.05664	-6.532	6.47e-11 ***	0.6907423	1.055273040, 1.10696643			
	R ramuli	Intercept	-1.38456	0.86340	-1.604	0.109	0.2504339	0.04508499, 1.343996	403.28	0.4965517	0.5406
		variable	0.01823	0.01185	1.539	0.124	1.0184016	0.99517573, 1.042641			
	R rachis	Intercept	-3.76804	0.59716	-6.310	2.79e-10 ***	0.0230973	0.006808491, 0.07120006	354.23	0.662069	0.7328
		variable	0.07667	0.01217	6.301	2.95e-10 ***	1.0796839	1.055273040, 1.10696643			
	Ramuli density	Intercept	1.1341	0.9542	1.189	0.235	3.1084132	0.4835161, 20.651969	404.05	0.5517241	0.535
		variable	-0.3542	0.2788	-1.270	0.204	0.7017677	0.4036190, 1.208396			
Multi-	Weight, R_rachis	Intercept	1.10232	0.87419	1.261	0.207	3.01115496	0.54805473; 17.16375882	242.12	0.7931034	0.892
		R_stolon	0.10124	0.01692	5.985	2.16e-09 ***	1.10654288	1.07239267; 1.14629001			
		weight	-3.07128	0.39229	-7.829	4.91e-15 ***	0.04636193	0.02035325; 0.09524036			
Multi-	Length, R_rachis	Intercept	0.22307	0.84079	0.265	0.791	1.2499128	0.2427857; 6.6247096	293.45	0.7586207	0.8374
		R_stolon	0.09044	0.01429	6.329	2.47e-10 ***	1.0946556	1.0658571; 1.1274872			
		length	-0.41755	0.06317	-6.610	3.85e-11 ***	0.6586589	0.5777315; 0.7406395			

Appendix C

Supplementary material of Publication III

Table C.1: Chronological development of maximum quantum yield of photosystem II (PSII) (F_v/F_m) of *Caulerpa lentillifera* under re-hydration conditions (10 min - 24 h) at an irradiance of 3 µmol photons m^{-2} s^{-1} after desiccation period in transparent polyethylene terephthalate (PET) containers of 2, 4, 8 and 12 days desiccation exposure under three different irradiances (0, 3, 70 µmol photons m^{-2} s^{-1}) is depicted. Initial values refer to values measured directly after desiccation period. Calculations of % of initial relate to absolute F_v/F_m values measured at the end of the desiccation and the start of the recovery period. Data represent mean values±standard deviation, SD (n=5). No significant differences were found (one-factor ANOVA followed by Tukey's HSD or Kruskal Wallis test followed by pairwise Dunn test with Bonferroni correction, $p < 0.05$)

Irradiance treatment (µmol photons m^{-2} s^{-1})	Desiccation exposure (days)	Time of Recovery	Replicates no (n)	F_v/F_m ($\pm$SD)	F_v/F_m as % of initial ($\pm$ SD)
0	2	Initial	5	0.69 ± 0.07	100.0 ± 0.0
		10 min	5	0.65 ± 0.09	94.6 ± 8.7
		3 h	5	0.62 ± 0.10	89.7 ± 8.0
		6 h	5	0.61 ± 0.13	87.4 ± 11.7
		24 h	5	0.56 ± 0.18	79.8 ± 20.8
3	2	Initial	5	0.74 ± 0.01	100.0 ± 0.0
		10 min	5	0.73 ± 0.01	98.4 ± 2.9
		3 h	5	0.72 ± 0.02	96.9 ± 4.0
		6 h	5	0.72 ± 0.01	97.5 ± 3.3
		24 h	5	0.72 ± 0.03	96.9 ± 4.4
70	2	Initial	5	0.59 ± 0.07	100.0 ± 0.0
		10 min	5	0.63 ± 0.11	108.3 ± 27.9
		3 h	5	0.65 ± 0.14	111.8 ± 29.6
		6 h	5	0.66 ± 0.16	114.3 ± 33.8
		24 h	5	0.67 ± 0.16	116.0 ± 34.0
0	4	Initial	5	0.59 ± 0.33	100.0 ± 0.0

		10 min	5	0.57 ± 0.32	97.2 ± 5.8
		3 h	5	0.59 ± 0.33	100.5 ± 2.3
		6 h	5	0.59 ± 0.33	99.7 ± 1.9
		24 h	5	0.59 ± 0.32	99.7 ± 0.6
		Initial	5	0.74 ± 0.05	100.0 ± 0.0
		10 min	5	0.73 ± 0.06	98.9 ± 5.3
3	4	3 h	5	0.72 ± 0.07	97.0 ± 4.2
		6 h	5	0.73 ± 0.06	98.9 ± 3.8
		24 h	5	0.74 ± 0.05	99.8 ± 4.9
70		Initial	5	0.60 ± 0.07	100.0 ± 0.0
		10 min	5	0.62 ± 0.1	102.7 ± 9.7
	4	3 h	5	0.64 ± 0.11	106.9 ± 16.8
		6 h	5	0.66 ± 0.12	110.7 ± 16.4
		24 h	5	0.65 ± 0.11	109.2 ± 16.8
		Initial	5	0.24 ± 0.23	100.0 ± 0.0
		10 min	5	0.18 ± 0.17	90.8 ± 27.8
0	8	3 h	5	0.19 ± 0.18	89.3 ± 23.8
		6 h	5	0.18 ± 0.17	86.8 ± 26.1
		24 h	5	0.19 ± 0.18	90.7 ± 23.0
		Initial	5	0.63 ± 0.16	100.0 ± 0.0
		10 min	5	0.61 ± 0.2	94.5 ± 11.3
3	8	3 h	5	0.61 ± 0.17	95.9 ± 4.5
		6 h	5	0.63 ± 0.19	98.9 ± 7.3
		24 h	5	0.67 ± 0.11	108.5 ± 14.7
		Initial	5	0.55 ± 0.06	100.0 ± 0.0
		10 min	5	0.54 ± 0.09	98.2 ± 16.4
70	8	3 h	5	0.59 ± 0.11	107.9 ± 22.0
		6 h	5	0.65 ± 0.10	119.7 ± 24.8
		24 h	5	0.65 ± 0.14	119.1 ± 32.8
		Initial	5	0.22 ± 0.33	100.0 ± 0.0
		10 min	5	0.24 ± 0.34	105.2 ± 13.9
0	12	3 h	5	0.21 ± 0.31	96.7 ± 5.9
		6 h	5	0.18 ± 0.27	93.7 ± 8.9
		24 h	5	0.15 ± 0.27	82.5 ± 31.6
		Initial	5	0.70 ± 0.06	100.0 ± 0.0
		10 min	5	0.67 ± 0.06	96.9 ± 6.2
3	12	3 h	5	0.71 ± 0.08	101.8 ± 10.6
		6 h	5	0.72 ± 0.08	104.0 ± 11.4
		24 h	5	0.73 ± 0.05	104.8 ± 8.5
		Initial	5	0.42 ± 0.11	100.0 ± 0.0
		10 min	5	0.48 ± 0.2	114.9 ± 36.1
70	12	3 h	5	0.49 ± 0.17	116.0 ± 25.9
		6 h	5	0.5 ± 0.22	116.7 ± 32.7
		24 h	5	0.56 ± 0.21	133.8 ± 35.2

Appendix D

Supplementary material of Publication V

D.1 Statistical output

Table D.1: Results of on-parametric Kruskal-Wallis test for in-between subject effects of logger position (*Position*) on light irradiances (*Irradiance*) quantified in ponds at sea grape farm VIJA. The asterisks indicate the significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Variable	Source	df	Chi-squared	p
Irradiance	Position	1	37.96	7.22e-10***

Table D.2: Results of two way-ANOVA for main effects of exposure time (*Day*), irradiance treatment (*Treatment*) and *thallus part*, as well as potentially significant interaction terms on antioxidant activity (AOA), total phenolic content (TPC), Chlorophyll *a* (Chl *a*) values in stolon, frond base, frond tip, respectively, in algae exposed to 50, 100, 200, 400, 600 µmol photons m^{-2} s^{-1}. The asterisks indicate the significance levels (* p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001).

Variable	Source	mean Sq.	df	F	p
F_v/F_m	Day	1.18513	4	48.62	<2.2e-16 ***
	Treatment	2.17684	4	89.30	<2.2e-16 ***
	Day:Treatment	0.42499	16	4.36	3.061e-06 ***
ABTS	Day	202678	4	34.99	<2.2e-16 ***
	Treatment	76825	4	13.26	3.04e-08 ***
	Day:Treatment	65662	16	2.83	0.001223 **
TPC	Day	115416	4	72.26	<2.2e-16 ***
	Treatment	53655	4	33.59	<2.2e-16 ***
	Day:Treatment	55271	16	8.65	2.472e-12 ***
Chl *a* Stolon	Day	0.267443	3	36.04	1.915e-15 ***
	Treatment	0.009736	4	0.98	0.4204
Chl *a* Frond base	Day	0.270196	3	105.73	<2.2e-16 ***
	Treatment	0.033645	4	9.87	1.796e-06 ***
	Day:Treatment	0.072761	12	7.12	1.809e-08 ***
Chl *a* Frond tip	Day	0.310162	3	48.40	<2.2e-16 ***
	Treatment	0.044633	4	5.22	<2.2e-16 ***
	Day:Treatment	0.05926	11	2.52	3.061e-06 ***

Table D.3: Results of one way-ANOVA or non-parametric Kruskal-Wallis test (KW) for in-between subject effects of irradiance treatment (*Treatment*) on F_v/F_m values and Rapid Light Curve parameters (RLCs) on different days of the experiment. The statistical test preformed was a one way-ANOVA. The asterisks indicate the significance levels (* p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001).

Variable	Factor	df	MS	F ratio	P value
F_v/F_m day 1	Treatment	4,20	0.12	15.83	5.47e-06 ***
F_v/F_m day 3	Treatment	4,20	KW	33.57	9.14e-07
F_v/F_m day 7	Treatment	4,31	0.21	25.86	1.72e-09 ***
F_v/F_m day 14	Treatment	4,36	0.20	26.65	2.51e-10 ***
alpha	Treatment	4,17	0.02	33.71	7.07e-08 ***
ETRmax	Treatment	4,17	418.5	1.504	0.245
Ek	Treatment	4,17	13.65	3.15	0.0414 *

Table D.4: Results of one way-ANOVA for in between subject effects of irradiance treatment (*Treatment*) on antioxidant activity (AOA) and total phenolic content (TPC) values on different days of the experiment. The asterisks indicate the significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Variable	Factor	df	MS	F ratio	P value
AOA day 1	Treatment	4,19	3549	1.88	0.163
AOA day 3	Treatment	4,11	8383	5.47	0.0113 *
AOA day 7	Treatment	4,13	12010	10.76	0.000449 ***
AOA day 14	Treatment	4,16	11680	5.26	0.00674 **
TPC day 1	Treatment	4,15	75	0.17	0.95
TPC day 3	Treatment	4,19	2911	1.49	0.244
TPC day 7	Treatment	4,18	8035	15.64	1.11e-05 ***
TPC day 14	Treatment	4,19	18393	37.93	8.26e-09 ***

Table D.5: Results of one way-ANOVA or non-parametric Kruskal-Wallis test (KW) for in between subject effects of irradiance treatment (*Treatment*) and thallus part (*Part*) on Chl *a* content, % of sum Chl *a* and Red Colour Channel. The asterisks indicate the significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Variable	Factor	df	MS	F/ chi squared	P value
Total Chl *a* , day 3	Treatment	4, 57	0.023375	2.72	0.03849 *
Total Chl *a* , day 7	Treatment	4, 55	0.018824	3.73	0.009373 **
Total Chl *a* , day 14	Treatment	4,60	KW	22.93	0.0001309
Stolon Chl *a* , day 3	Treatment	4,17	KW	4.16	0.3845
Stolon Chl *a* , day 7	Treatment	4,18	0.0073345	1.36	0.2863
Stolon Chl *a* , day 14	Treatment	4,19	0.019601	1.31	03018
Frond base Chl *a* , day 3	Treatment	4,15	0.0089975	2.15	0.1247
Frond base Chl *a* , day 7	Treatment	4,14	0.009514	2.51	0.08897
Frond base Chl *a* , day 14	Treatment	4,16	0.087895	19.61	5.184e-06 ***
Frond tip Chl *a* , day 3	Treatment	4,15	0.009572	1.69	0.2035
Frond tip Chl *a* , day 14	Treatment	4,15	KW	15.27	0.004168
%Chl *a* - day 3 - 50 μmol photons m^{-2} s^{-1}	Part	2,9	KW	8	0.01832*
%Chl *a* - day 3 - 100 μmol photons m^{-2} s^{-1}	Part	2,6	27.71	0.59	0.5854
%Chl *a* - day 3 - 200 μmol photons m^{-2} s^{-1}	Part	2,9	45.956	1.25	0.3328
%Chl *a* - day 3 - 400 μmol photons m^{-2} s^{-1}	Part	2,12	162.58	2.42	0.1305
%Chl *a* - day 3 - 600 μmol photons m^{-2} s^{-1}	Part	2,9	KW	0.35	0.84
%Chl *a* - day 7 - 50 μmol photons m^{-2} s^{-1}	Part	2,9	87.141	13.97	0.00174 **
%Chl *a* - day 7 - 100 μmol photons m^{-2} s^{-1}	Part	2,12	66.897	2.90	0.09386
%Chl *a* - day 7 - 400 μmol photons m^{-2} s^{-1}	Part	2,6	53.823	0.80	0.493
%Chl *a* - day 7 - 600 μmol photons m^{-2} s^{-1}	Part	2,6	631.86	3.84	0.08421
%Chl *a* - day 14 - 50 μmol photons m^{-2} s^{-1}	Part	2,9	82.713	2.91	0.106
%Chl *a* - day 14 - 100 μmol photons m^{-2} s^{-1}	Part	2,12	209.65	1.21	0.333
%Chl *a* - day 14 - 200 μmol photons m^{-2} s^{-1}	Part	2,9	394.06	7.69	0.01129 *
%Chl *a* - day 14 - 400 μmol photons m^{-2} s^{-1}	Part	2,6	11247.6	48.95	0.0001926 ***
%Chl *a* - day 14 - 600 μmol photons m^{-2} s^{-1}	Part	2,6	11006.9	38.41	0.0003803 ***
Red - day 3 - 50 μmol photons m^{-2} s^{-1}	Part	2,11	852.7	2.53	0.1248
Red - day 3 - 100 μmol photons m^{-2} s^{-1}	Part	2,9	767.1	0.97	0.4155
Red - day 3 - 200 μmol photons m^{-2} s^{-1}	Part	2,9	2270.2	1.60	0.2543
Red - day 3 - 400 μmol photons m^{-2} s^{-1}	Part	2,11	14310	7.62	0.008375**
Red - day 3 - 600 μmol photons m^{-2} s^{-1}	Part	2,11	13254	3.67	0.06026
Red - day 7 - 50 μmol photons m^{-2} s^{-1}	Part	2,12	624.1	0.40	0.6825
Red - day 7 - 100 μmol photons m^{-2} s^{-1}	Part	2,11	213.73	0.37	0.6961
Red - day 7 - 200 μmol photons m^{-2} s^{-1}	Part	2,9	514.4	0.28	0.7645
Red - day 7 - 400 μmol photons m^{-2} s^{-1}	Part	2,12	17502	4.83	0.02899
Red - day 7 - 600 μmol photons m^{-2} s^{-1}	Part	2,8	KW	6.73	0.03461
Red - day 14 - 50 μmol photons m^{-2} s^{-1}	Part	2,12	1024.7	1.46	0.2712
Red - day 14 - 100 μmol photons m^{-2} s^{-1}	Part	2,12	1989.4	0.61	0.5583
Red - day 14 - 200 μmol photons m^{-2} s^{-1}	Part	2,12	KW	4.28	0.1172
Red - day 14 - 400 μmol photons m^{-2} s^{-1}	Part	2,12	33400	4.64	0.03224
Red - day 14 - 600 μmol photons m^{-2} s^{-1}	Part	2,11	KW	8.38	0.01511

Table D.6: Results of one way-ANOVA for in-between subject effects of sea grapes AOA at different irradiance treatments and fruits Pomegranate, Aronia berry and Goji (*Category*). The asterisks indicate the significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Variable	Factor	df	MS	F ratio	P value
AOA	Category	7.28	21986	20.47	1.9e-09 ***

Table D.7: Correlation results of Chl *a* with Chl *b*, Red, Green and Blue colour channel and colour channels between each other for *Caulerpa lentillifera* samples, as well as correlation results of antioxidant activity (AOA), total phenolic content (TPC) and F_v/F_m using Spearman correlation. The asterisks indicate the significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Variable	r_S	S	p
AOA - TPC	0.5986116	21972	1.096e-07 ***
F_v/F_m - AOA	-0.5731531	115076	6.269e-08 ***
F_v/F_m - TPC	-0.5436259	193849	2.57e-08 ***
Chl *a* - Chl *b*	0.9794791	257028	<2.2e-16 ***
Chl *a* - Red	-0.7859198	1915301	<2.2e-16 ***
Chl *a* - Green	-0.7805456	1909537	<2.2e-16 ***
Chl *a* - Blue	-0.7373865	1863251	<2.2e-16 ***
Red - Green	0.9971604	35063	<2.2e-16 ***
Red - Blue	0.8759197	1532135	<2.2e-16 ***
Green - Blue	0.8641391	1677601	<2.2e-16 ***

D.2 Experimental light spectrum
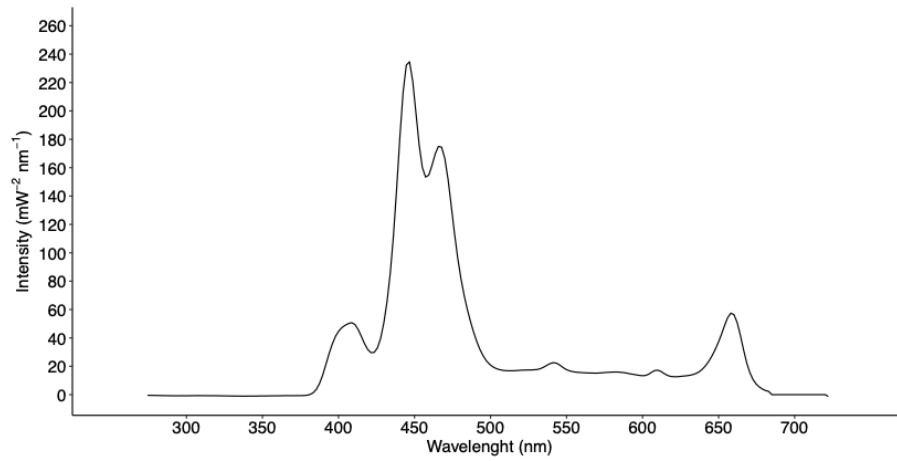
Figure D.1: Light spectrum of light-emitting diodes (LED, Aquaillumination, Hydra, Germany) adjusted to 50 µmol photons m^{-2} s^{-1} (12K), measured with an integrated hyperspectral radiometer Ramses ACC UV/ VIS (Trios, Rastede, Germany).

Appendix E

Supplementary Material for Publication VI

E.1 Supplemental material on the *Field Experiment*

Table E.1: Overview of *Field Experiments* and *Lab(oratory) Experiments* presented in this study for the species *Kappaphycus alvarezii* (KA) or *Caulerpa lentillifera* (CL) with the respective location of sampling, as well as sampling dates and days (based on the respective date of the initial measurement).

	Experiments	Location	start	in-between	end
Field Experiment	KA longlines	IO	17.05.22 initial	07.06.22 Day 21	26.06.22 Day 41
	KA longlines	VIJA	26.05.22 initial		09.06.22 Day 15
	CL cages below longlines	IO	28.05.22 initial	04.06.22 Day 7	21.06.22 Day 24
	Sea grapes in nets	IO	20.05.22	28.05.22, 04.06.22 Day 8, Day 15	22.06.22, Day 33
	KA in nets with CL	IO	20.05.22 initial	28.05.22, 04.06.22 Day 8, Day 15	22.06.22, Day 33
	KA in nets without CL	IO	20.05.22 initial	28.05.22, 04.06.22 Day 8, Day 14	22.06.22, Day 33
Lab	Nutrient growth	Lab	06.06.22 initial		27.06.22 Day 21
	Nutrient uptake	Lab			
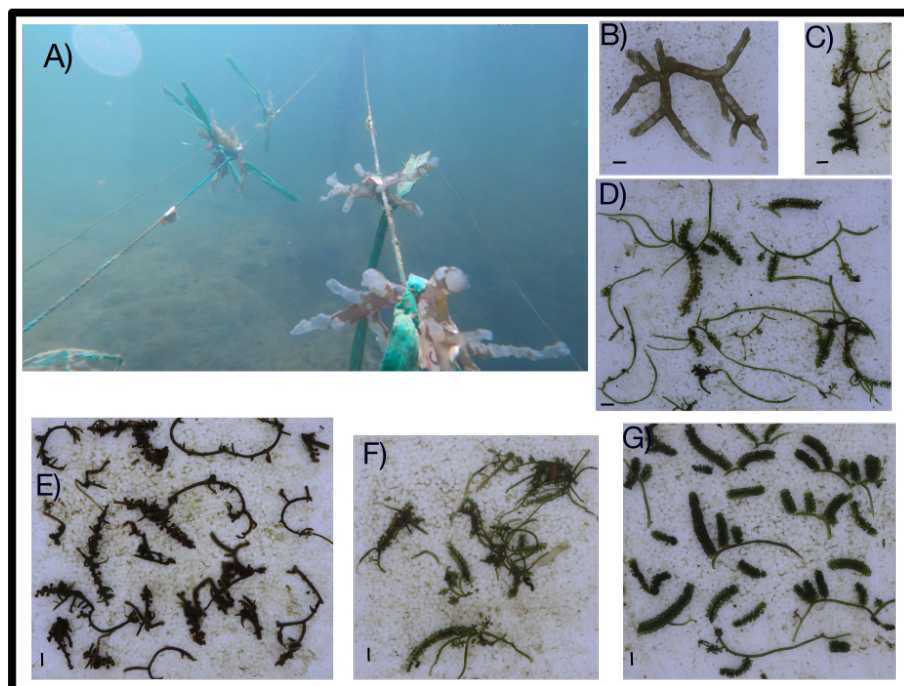
Figure E.1: Pictures of *Kappaphycus alvarezii* **A)** on longlines and **B)** in close-up after 15 days in the sea grape pond and *Caulerpa lentillifera* at the end of the Experiment when cultivated in plastic cages **D)** with and **E)** without gauze wrapping and **F)** net cages. Sea grapes showed some special small fronds **C)** like after 15 days in net cage cultivation. **G)** Sea grapes after tray cultivation at a sheltered place.

Table E.2: Overview of measurement days and times of different parameters for the species *Kappaphycus alvarezii* (KA) or *Caulerpa lentillifera* (CL) at the experimental sites of the *Field Experiment* at the Institute of Oceanography (IO) in Nha Trang, Vietnam and an experimental pond at sea grape farm VIJA.

Parameter	Location	Dates of measurement	Time of day
salinity, temperature, pH, oxygen of field Experiments	IO	21., 23., 27., 30. May 2022; 04., 16., 20., 24. June 2022	-
	VIJA	09. June 2022	-
Water samples for nutrient analysis of field Experiments	IO	27. May 2022; 08., 13., 15., 18., 20., 24., 25. June 2022	-
Irradiances of PAR at different experimental set-ups	IO	21. May 2022	10 - 10:30 a.m.
Diurnal change of photosynthetic activity (F_v/F_m') of CL in plastic cages and KA at longlines	IO	16. June 2022	6 a.m. - 6 p.m., every 2 h
Diurnal change of photosynthetic activity (F_v/F_m') of CL and KA in net cages	IO	21. June 2022	6 a.m. - 6 p.m., every 2 h

E.2 Statistical output

Table E.3: Statistical test results of Two-Way ANOVA for *Field Experiment* for the species *Kappaphycus alvarezii* (KA) or *Caulerpa lentillifera* (CL), respectively. The response variables tested were relative growth rate (RGR, % day^{-1}). The treatments *Cultivation method* refers to the *longline* and *net cage* cultivation of KA and the *plastic cage* and *net cage* cultivation of CL. The treatment *cultivation status* refers to the *Mono-*, vs *Co-cultivation* of KA with CL or the *presence* vs. *absence* of gauze wrapping for CL cultivation. The asterisks indicate the significance levels (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Species and response variable	Treatments	DF	Sum Squ	Mean Squ	F value/ chi-squared	P-value	Levene	Shapiro
KA RGR	Cultivation method	1	83.04	83.04	123.362	<2e-16 ***	0.3198	0.6652
	Cultivation status	1	5.63	5.63	8.364	0.00479 **		
	Residuals	91	61.26	0.67				
CL RGR	Cultivation method	1	4.85	4.85	8.781	0.0142 *	0.9226	0.8131
	Cultivation status	1	159.62	159.62	288.760	1.05e-08 ***		
	Residuals	10	5.53	0.55				

Table E.4: Statistical test results of One-Way ANOVA (no indication) or Kruskal-Wallis test (indicated as KW) for *Field Experiment* for the species *Kappaphycus alvarezii* (KA) or *Caulerpa lentillifera* (CL), respectively. The response variables tested were relative growth rate (RGR, % day^{-1}), net harvest (g g initial FW^{-1} day^{-1}), Share of frond from total thallus (Frond share, % of thallus), antioxidant activity (AOA, expressed as mmol Trolox equivalents, TE 100 g^{-1} dry weight, DW), total phenolic content (TPC, expressed as mg gallic acid equivalent, GAE 100 g^{-1} DW), carbon (C) to nitrogen (N) tissue content ratio (C:N), C and N tissue content (C, N expressed as mg g^{-1} DW), F_v/F_m. The treatment *Set-up* relates to the groups *net cage mono-*, and *net cage co-cultivation*; *longline cultivation* for KA and *plastic cage with gauze wrapping*, *plastic cage without gauze wrapping* and *net cage* for CL. The treatment *absence gauze* tests the absence vs. presence of gauze wrapping around plastic cages of CL. The asterisks indicate the significance levels (* p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001).

Species and response variable	Treatment	DF	Sum Squ	Mean Squ	F value/ chi-squared	P-value	Levene	Shapiro
KA RGR	Set-up	2	88.67	44.34	65.86	<2e-16 ***	0.635	0.6652
	Residuals	91	61.26	0.67				
KA Net harvest	Set-up							
	Residuals							
CL RGR	Set-up	2	164.48	82.24	148.8	3.63e-08 ***	0.9502	0.586
	Residuals	10	5.53	0.55				
CL+KA RGR	Species	1			38.455	5.604e-10	1.06e-05	KW
	Residuals							
CL Frond share	Absence gauze	1	1015.1	1015	11.16	0.0102 *	0.9763	0.5843
	Residuals	8	727.9	91				
KA AOA	Set-up	1	164.4	164.4	0.657	0.444	0.3752	0.6826
	Residuals	7	1750.3	250.0				
KA TPC	Set-up	1	183	183.1	0.159	0.702	0.3	0.7525
	Residuals	7	8040	1148.6				
CL AOA	Set-up	2	13385	6692	29.09	6.79e-05 ***	0.1911	0.4249
	Residuals	1	2301	230				
CL TPC	Set-up	2	17230	8615	15.78	0.000587 ***	0.2148	0.9986
	Residuals	11	6007	546				
KA C:N	Set-up	1	2.71	2.71	0.11	0.748	0.8536	0.3089
	Residuals	9	222.45	24.72				
KA C	Set-up	1	0.60	0.598	0.161	0.697	0.3105	0.4181
	Residuals	9	33.36	3.707				
KA N	Set-up	1	0.00517	0.005171	0.208	0.659	0.6753	0.3977
	Residuals	9	0.22408	0.024898				
CL C:N	Set-up	2	263.01	131.50	69.8	5.62e-07 ***	0.9465	0.5295
	Residuals	11	20.73	1.88				
CL C	Set-up	2	114.37	57.18	15.7	0.000599 ***	0.2009	0.2123
	Residuals	11	40.07	3.64				
CL N	Set-up	2	0.6967	0.3483	12.28	0.00158 **	0.3478	0.4998
	Residuals	11	0.3120	0.0284				
KA F_v/F_m	Set-up	2			1.4812	0.4768	0.0690	KW
CL F_v/F_m	Set-up	2	0.12164	0.06082	8.448	0.00599 **	0.465	0.331
	Residuals	1	0.07919	0.00720				

Table E.5: Wilcoxon pairwise to test for differences of response variable *net harvest* (g g initial^{-1} day^{-1}) between different set-ups of *Kappaphycus alvarezii* (KA) cultivations, namely longline cultivation, cage net cultivation.

Species and cultivation	Variables	Tested pair	p-value	p.adj.	p.format
KA Net harvest	Set-up	Longline vs Net_cage_co	0.894383843	0.890	0.8944
		Longline vs Net_cage_mono	0.391500083	0.780	0.3915
		Net_cage_co vs Net_cage_mono	0.007936508**	0.024	0.0079

Table E.6: Pearson (P) and Spearman (S) Correlation for antioxidant activity (AOA), total phenolic content (TPC), carbon (C) and nitrogen (N) tissue content and the C:N ratio of *Caulerpa lentillifera* samples from the *field Experiment*.

Correlated variables	df	t/S	p-value	Pearson/ Spearman (rho) correlation coefficient
TPC - AOA	12	3.5318	0.004133	0.7139117 (P)
TPC - C	12	5.9841	6.371e-05	0.8654482 (P)
TPC - N	12	-1.6413	0.1267	-0.428177 (P)
AOA - C	12	3.1574	0.008261	0.6736278 (P)
AOA - N	12	-1.5417	0.1491	-0.4065924 (P)
C - N	12	-1.3112	0.2143	-0.3539905 (P)
TPC - C:N	12	164	0.01627	0.6395604 (S)
AOA - C:N	12	142	0.008383	0.6879121 (S)

Table E.7: Statistical test results of Two-Way ANOVA for *Laboratory Experiment* for the species *Kappaphycus alvarezii* (KA) or *Caulerpa lentillifera* (CL), respectively. The response variables tested were relative growth rate (RGR, % day^{-1}), share of frond from total thallus (Frond share, % of thallus), carbon (C) to nitrogen (N) tissue content ratio (C:N), C and N tissue content (C, N expressed as mg g^{-1} DW), F_v/F_m. The *treatment water* refers to the treatments *natural seawater (nat. SW)*, *alternating*, *continuously*, whereas the *cultivation set-up* relates to *mono-* and *co-cultivation* of the respective species. The asterisks indicate the significance levels (* p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001).

Species and response variable	Treatments	DF	Sum Squ	Mean Squ	F value/ chi-squared	P-value	Levene	Shapiro
CL (RGR)	Treatment water	2	133.20	66.60	54.511	7.67e-10 ***	0.1322	0.0008945
	Cultivation set-up	1	4.13	4.13	3.379	0.078		
	Residuals	25	30.54	1.22				
KA (RGR)	Treatment water	2	0.703	0.3516	0.869	0.431	0.1322	0.1431
	Cultivation set-up	1	0.183	0.1829	0.452	0.507		
	Residuals	26	10.516	0.4044				
CL (Frond share)	Treatment water	2	4212	2106.0	24.836	9.3e-07 ***	0.5156	0.7905
	Cultivation set-up	1	94	94.2	1.111	0.301		
	Residuals	26	2205	84.8				
CL (F_v/F_m)	Treatment water	2	0.07862	0.03931	11.909	0.000213 ***	0.3378	0.002014
	Cultivation set-up	1	0.00002	0.00002	0.005	0.942296		
	Residuals	26	0.08583	0.00330				
KA (F_v/F_m)	Treatment water	2	0.00807	0.004033	1.625	0.216	0.7512	0.3973
	Cultivation set-up	1	0.00374	0.003741	1.508	0.231		
	Residuals	26	0.06452	0.002481				
KA (C:N)	Treatment water	2	2386.9	1193.5	16.083	6.86e-05 ***	0.7535	0.3639
	Cultivation set-up	1	16.5	16.5	0.222	0.642		
	Residuals	20	1484.1	74.2	92.8			

		Df	Sum Sq	Mean Sq	F value	Pr(>F)		
KA (C)	Treatment water	2	203	101.4	0.078	0.925	0.2974	0.9271
	Cultivation set-up	1	1190	1189.7	0.918	0.349		
	Residuals	20	25910	1295.5				
KA (N)	Treatment water	2	92.55	46.28	5.523	0.0123 *	0.2256	0.05896
	Cultivation set-up	1	2.69	2.69	0.321	0.5770		
	Residuals	20	167.58	8.38				
CL (C:N)	Treatment water	2	510.9	255.43	80.06	2.4e-11 ***	0.2194	0.06608
	Cultivation set-up	1	16.5	16.53	5.18	0.0321 *		
	Residuals	24	76.6	3.19				
CL (C)	Treatment water	2	29115	14557	29.115	3.82e-07 ***	0.479	0.6186
	Cultivation set-up	1	106	106	0.212	0.649		
	Residuals	24	12000	500				
CL (N)	Treatment water	2	2.15	1.077	0.493	0.617	0.6193	0.5115
	Cultivation set-up	1	2.15	2.146	0.982	0.332		
	Residuals	24	52.45	2.186				
KA (R)	Treatment water	2	716.4	358.2	19.61	1.29e-05 ***	0.2991	0.6616
	Cultivation set-up	1	104.4	104.4	5.717	0.0258 *		
	Interaction	2	141.8	70.9	3.882	0.0360*		
	Residuals	22	401.9	18.3				
KA (G)	Treatment water	2	1306.2	653.1	24.119	1.81e-06 ***	0.3342	0.2191
	Cultivation set-up	1	69.3	69.3	2.558	0.123		
	Residuals	24	649.9	27.1				
KA (B)	Treatment water	2	146.6	73.28	7.258	0.00343 **	0.7487	0.4826
	Cultivation set-up	1	14.3	14.30	1.416	0.24573		
	Residuals	24	242.3	10.10				

Table E.8: Statistical test results of One-Way ANOVA for *Laboratory Experiment* for the species *Kappaphycus alvarezii* (KA) or *Caulerpa lentillifera* (CL), respectively. The response variables tested were relative growth rate (RGR, % day^{-1}), share of frond from total thallus (Frond share, % of thallus), carbon (C) to nitrogen (N) tissue content ratio (C:N), C and N tissue content (C, N expressed as mg g^{-1} DW), F_v/F_m, uptake rates of both seaweed species in the uptake-experiment. The *set-up* refers to the treatments *natural seawater (nat. SW) mono-cultivation, natural seawater (nat. SW) co-cultivation, alternating mono-cultivation, alternating co-cultivation, continuously mono-cultivation* and *continuously co-cultivation*. The asterisks indicate the significance levels (* p $\leq$ 0.05, ** p $\leq$ 0.01, *** p $\leq$ 0.001).

Species and response variable	Treatments	DF	Sum Squ	Mean Squ	F value/ chi-squared	P-value	Levene	Shapiro
KA (RGR)	Set up	5	1.764	0.3529	0.879	0.51	0.5647	0.299
	Residuals	24	9.637	0.4016				
CL (RGR)	Set up	5			22.6	0.0004025	0.1113	KW
	Residuals							
CL (Share fronds)	Set up	5	4377	875.5	10.94	1.77e-05 ***	0.5156	0.4334
	Residuals	23	1841	80.1				
KA (F_v/F_m)	Set up	6	0.02531	0.004219	1.729	0.15	0.3413	0.07517
	Residuals	28	0.07078	0.002441				
CL (F_v/F_m)	Set up	6	0.11454	0.019091	6.729	0.000171 ***	0.3413	0.07517
	Residuals	28	0.07943	0.002837				
KA (C:N)	Set up	5	2670	533.9	7.89	0.000437 ***	0.7535	0.2097
	Residuals	18	1218	67.7				
KA (C)	Set up	5	6969	1394	1.234	0.334	0.2974	0.8842
	Residuals	18	20334	1130				
KA (N)	Set up	5	116.7	23.341	2.875	0.0444 *	0.2256	0.45
	Residuals	18	146.1	8.118				
CL (C:N)	Set-up	5			24.146	0.0002035	0.2194	KW
CL (C)	Set up	5	29253	5851	10.76	2.51e-05 ***	0.479	0.675
	Residuals	22	11968	544				
CL (N)	Set up	5	4.39	0.8789	0.369	0.864	0.6193	0.485
	Residuals	22	52.36	2.3800				
KA (R)	Set up	5	962.7	192.54	10.54	2.91e-05 ***	0.2991	0.6616
	Residuals	22	401.9	18.27				
KA (G)	Set up	5	1518.5	303.71	13.18	5.26e-06 ***	0.3342	0.4897
	Residuals	22	506.9	23.04				
KA (B)	Set up	5	203.0	40.6	4.463	0.00586 **	0.7487	0.7477
	Residuals	22	200.2	9.1				
(NO_x) (Uptake rates)	Set up	1			22.21	0.0004775		KW
	Residuals	5						
PO_3 (Uptake rates)	Set up	1			4.8599	0.4332		KW
	Residuals	5						
NH_4 (Uptake rates)	Set up	5	452.1	90.42	16.02	1.08e-06 ***	0.6574	0.1874
	Residuals	22	124.2	5.64				

Appendix F

Supplementary Material for Publication VII

F.1 Statistical output

Table F.1: Results of two way-ANOVA for main effects of nutrient treatment or dilution factor ("DF") and Phosphate fertilization ("P fertilization"), as well as potentially significant interaction terms on relative growth rate (RGR), F_v/F_m and antioxidant activity (AOA), total phenolic content (TPC), total phenolic content (TPC), total hydrolysable amino acids (THAA), ratio of essential to non-essential amino acids (EAA NEAA^{-1}), aspartic acid (ASP), glutamic acid (GLU), threonine (THR), serine (SER), glycine (GLY), alanine (ALA), valine (VAL), isoleucine (ILE), and leucine (LEU), histidine (HIS), ornithine (ORN), lysine (LYS), and arginine (ARG), tyrosine (TYR), phenylalanine (PHE), methionine (MET), methionine–sulfone (MET-sulfon), taurine (TAU), and cysteine (CYS-OX), glucosamine (GLUAM), and galactosamine (GALAM), tissue carbon (C) and nitrogen (N) content, C: N ratio. The asterisks indicate the significance levels (* p$\leq$ .05, ** p$\leq$0.01, *** p$\leq$0.001).

Variable	Source	mean Sq.	df	F	p	Shapiro, p	Levene, p
RGR	DF	15.680	4	34.776	1.74e-10 ***	0.1008	0.8091
	P fertilization	1.167	1	2.589	0.119		
	Residuals	0.451	28				
% Frond	DF	983.7	4	20.798	1.58e-08 ***	0.5282	0.6009
	P fertilization	73.1	1	1.544	0.223		
	Residuals	47.3	32				
F_v/F_m	DF	0.0008670	4	0.943	0.453	0.6047	0.3975
	P fertilization	0.0001838	1	0.200	0.658		
	Residuals	0.0009192	30				

AOA	DF	279.31	4	5.632	0.00167 **	0.5965	0.276
	P fertilization	18.21	1	0.367	0.54911		
	Residuals	49.60	30				
TPC	DF	227.80	4	8.529	9.2e-05 ***	0.839	0.9429
	P fertilization	5.53	1	0.207	0.652		
	Residuals	26.71	31				
THAA	DF	1378.3	4	25.985	1.18e-09 ***	0.9149	0.6504
	P fertilization	80.0	1	1.509	0.228287		
	DF:P fertilization	539.4	2	10.169	0.000381 ***		
	Residuals	53.0	32				
EAA/ NEAA	DF	0.012795	4	6.722	0.000513 ***	0.006327	0.8462
	P fertilization	0.013071	1	6.867	0.013479 *		
	DF:P fertilization	0.008350	2	4.386	0.021015 *		
	Residuals	0.001904	31				
ARG	DF	0.024782	4	3.238	0.0244 *	0.6595	0.7777
	P fertilization	0.007722	1	1.009	0.3227		
	Residuals	0.007653	32				
CYS-OX	DF	9.169	4	99.431	<2e-16 ***	0.9582	0.3746
	P fertilization	0.298	1	0.0815	0.0815		
	Residuals	0.092	33				
GLU	DF	4.726	4	26.829	8e-10 ***	0.1135	0.7111
	P fertilization	0.029	1	0.166	0.6862		
	DF:P fertilization	0.646	2	3.668	0.0368 *		
	Residuals	0.176	32				
HIS	DF	1.0306	4	57.271	3.9e-14 ***	0.6039	0.1977
	P fertilization	0.0557	1	3.096	0.088		
	Residuals	0.0180	32				
ILE	DF	0.1142	4	9.962	1.91e-05 ***	0.75	0.557
	P fertilization	0.00788	1	0.688	0.413		
	Residuals	0.01146	32				

LEU	DF	0.6689	4	18.945	2.83e-08 ***	0.2875	0.8828
	P fertilization	0.1944	1	5.505	0.0249 *		
	Residuals		34				
PHE	DF	0.07877	4	4.193	0.00725 **	0.3077	0.7117
	P fertilization	0.00124	1	0.066	0.00725		
	Residuals	0.01879	34				
THR	DF	0.4241	4	38.436	5.54e-12 ***	0.3015	0.4384
	P fertilization	0.0391	1	3.542	0.0687		
	Residuals	0.0110	33				
VAL	DF	0.10420	4	5.145	0.00247 **		
	P fertilization	0.01070	1	0.528	0.47249		
	Residuals	0.02025	33				
LYS	DF	0.3861	4	2.570	0.0555	0.01278	0.5452
	P fertilization	0.0848	1	0.564	0.4576		
	Residuals	0.1502	34				
Tissue C content	DF	1.7475	4	4.030	0.0102 *		
	P fertilization	0.1497	1	0.345	0.5614		
	Residuals	0.4336	29				
Tissue N content	DF	0.4541	4	73.834	8.87e-15 ***		
	P fertilization	0.0195	1	3.174	0.0853		
	Residuals	0.0061	29				
Tissue C:N ratio	DF	30.231	4	373.005	<2e-16 ***		
	P fertilization	0.19	1	2.403	0.132		
	Residuals	2.35	29				

F.2   Additional results on amino acid analyses

Table F.2: Results of one way-ANOVA or Kruskal-Wallis test for main effects of group (control negative, Low NF, Low PF, Medium NF, Medium PF, High NF, High PF, control positive) on growth rate (RGR), F_v/F_m and antioxidant activity (AOA), total phenolic content (TPC), total phenolic content (TPC), total hydrolysable amino acids (THAA), ratio of essential to non-essential amino acids (EAA NEAA-1), aspartic acid (ASP), glutamic acid (GLU), threonine (THR), serine (SER), glycine (GLY), alanine (ALA), valine (VAL), isoleucine (ILE), and leucine (LEU), histidine (HIS), ornithine (ORN), lysine (LYS), and arginine (ARG), tyrosine (TYR), phenylalanine (PHE), methionine (MET), methionine–sulfone (MET-sulfon), taurine (TAU), and cysteine (CYS-OX), glucosamine (GLUAM), and galactosamine (GALAM), tissue carbon (C) and nitrogen (N) content, C: N ratio. The asterisks indicate the significance levels (* p≤ .05, ** p≤0.01, *** p≤0.001).

Variable	Source	mean Sq.	df	F/ chi-squared	p	Shapiro, p	Levene, p
EAA/ NEAA	name		7	27.48	0.000273	0.0002535	0.8015
	Residuals						
	Residuals						
% FW Frond	group	574.8	7	11.51	5.27e-07 ***	0.415	0.6009
	Residuals	49.9	30				
ARG	name	0.018816	7	2.565	0.0338 *	0.9676	0.56
	Residuals	0.007334	30				
CYS-OX	Name	5.300	7	56.35	7.27e-16 ***	0.444	0.1513
	Residuals	0.094	31				
GLU	Name	2.8895	7	16.4	5.83e-09 ***	0.1135	0.8689
	Residuals	0.1762	32				
HIS	Name	0.6080	7	36.62	5.37e-13 ***	0.2683	0.1977
	Residuals	0.0166	30				
ILE	Name	0.06796	7	5.742	0.00023 ***	0.6232	0.7847
	Residuals	0.01184	32				
LEU	Name	0.4247	7	12.38	1.5e-07 ***	0.2192	0.88828
	Residuals	0.0343	32				
MET	Name	2.8127	7	412.6	<2e-16 ***	0.0003181	0.03213 *
	Residuals	0.0068	31				
PHE	Name	0.04937	7	2.592	0.0308 *	0.4397	0.7117
	Residuals	0.01905	32				
THR	Name	0.25429	7	24.66	5.37e-11 ***	0.5512	0.4384
	Residuals	0.01031	31				
VAL	Name	0.06907	7	3.496	0.00704 **	0.646	0.9423
	Residuals	0.01976	31				
LYS	Name	0.2361	7	1.486	0.5452	0.02108	
	Residuals	0.1589	32				
Tissue C content	Name	1.2662	7	3.151	0.0144 *	0.7077	0.8736
	Residuals	0.4019	27				
Tissue N content	Name	0.26417	7	43.24	4.77e-13 ***	0.07682	0.8736
	Residuals	0.00611	27				
Tissue C:N ratio	Name	17.318	7	208.2	<2e-16 ***	0.2335	0.7043
	Residuals	0.083	27				

Table F.3: Spearman correlation of relative growth rate and % freshweight (FW) Fronds

Variables	Test	Sr (rho)	P value	S
RGR - % FW Frond	Spearman	0.4819887	0.001845 *	5522
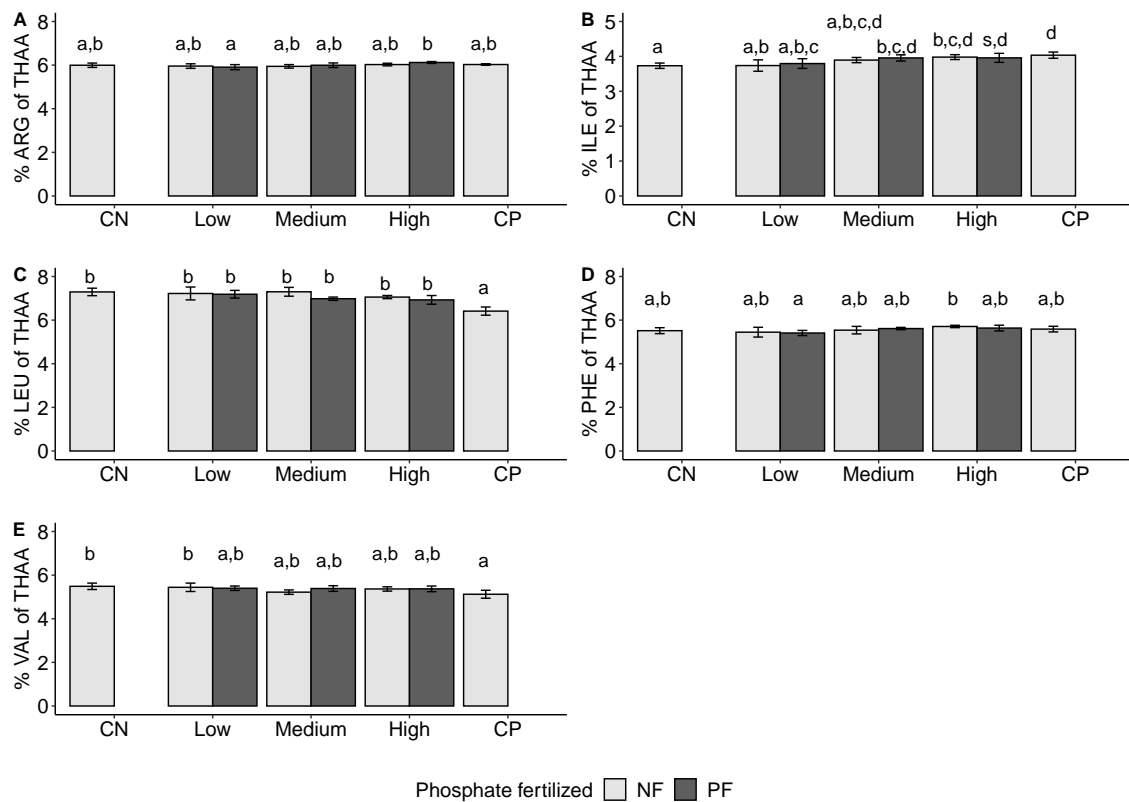
Figure F.1: Presence of amino acids (AAs, expressed as % of total hydrolysable AAs, THAA, ARG – arginine, ILE – isoleucine, LEU – leucine, PHE – phenylalanine, VAL – valine) in *Caulerpa lentillifera* at artificial sea water (Control Negative, CN) and undiluted shrimp process water (Control Positive, CP), as well as under three different dilutions of the process water (Low, Medium, High) without (nitrate: phosphate of 28:1) and with (nitrate: phosphate of 5:1) fertilization, respectively. Data are expressed as mean $\pm$ SD (n=3-5). Letters indicate a significant difference between the treatments (One-factor Anova with Post-Hoc HSD, p<0.05).

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